Gelsolin Induces Colorectal Tumor Cell Invasion via Modulation of the Urokinase-Type Plasminogen Activator Cascade

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Abstract

Gelsolin is a cytoskeletal protein which participates in actin filament dynamics and promotes cell motility and plasticity. Although initially regarded as a tumor suppressor, gelsolin expression in certain tumors correlates with poor prognosis and therapy-resistance. In vitro, gelsolin has anti-apoptotic and pro-migratory functions and is critical for invasion of some types of tumor cells. We found that gelsolin was highly expressed at tumor borders infiltrating into adjacent liver tissues, as examined by immunohistochemistry. Although gelsolin contributes to lamellipodia formation in migrating cells, the mechanisms by which it induces tumor invasion are unclear. Gelsolin’s influence on the invasive activity of colorectal cancer cells was investigated using overexpression and small interfering RNA knockdown. We show that gelsolin is required for invasion of colorectal cancer cells through matrigel. Microarray analysis and quantitative PCR indicate that gelsolin overexpression induces the upregulation of invasion-promoting genes in colorectal cancer cells, including the matrix-degrading urokinase-type plasminogen activator (uPA). Conversely, gelsolin knockdown reduces uPA levels, as well as uPA secretion. The enhanced invasiveness of gelsolin-overexpressing cells was attenuated by treatment with function-blocking antibodies to either uPA or its receptor uPAR, indicating that uPA/uPAR activity is crucial for gelsolin-dependent invasion. In summary, our data reveals novel functions of gelsolin in colorectal tumor cell invasion through its modulation of the uPA/uPAR cascade, with potentially important roles in colorectal tumor dissemination to metastatic sites.


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Introduction

Colorectal cancer (CRC) accounts for one of the highest mortality rates from cancer worldwide. The survival rate is highest at about 90% when diagnosed at early stages where tumor growth is localized to primary sites and about 35%–70% in invasive but regional disease. However the occurrence of distant metastasis to the liver or lungs in CRC is a major contributing factor to death, with five-year survival rate at less than 15% [1]. The pathogenesis of CRC from normal colonic epithelium to adenoma is fairly well-characterized and often involves a number of genetic alterations, including mutational activation of oncogenes such as K-ras as well as mutational inactivation of tumor suppressors such as p53 [2] and adenomatous polyposis coli (APC) gene [3]. In contrast, less is known about the molecular mechanisms which convert a non-invasive colorectal neoplasm to one with an invasive phenotype. In most solid tumors, the spread of tumor cells is facilitated by events which result in the detachment of malignant cells from the primary site and subsequent dissemination through tissues and vasculature [4]. This metastatic cascade is critically dependent on the integration of migratory and invasive signals involving cytoskeleton and extracellular matrix (ECM) remodeling [5].

Gelsolin is an actin-binding protein which severs and caps actin filaments [6], and regulates cytoskeletal turnover. Gelsolin appears to have complex roles in tumor biology, with evidence supporting its contradictory involvement in both tumor suppression as well as malignant progression. Gelsolin is reported to be down-regulated in tumors including breast [7] and lung [8] carcinomas, suggesting that loss of gelsolin promotes oncogenesis. Consistent with this, knockdown of gelsolin by small-interfering RNA (siRNA) in the
Gelsolin Promotes Migration and Invasion of Colorectal Tumor Cells

The oncogenic potential of gelsolin in colorectal tumor cells was investigated by modulating gelsolin levels using gelsolin overexpression and siRNA knockdown. Using the technique of in-vivo passaging through athymic nude mice, we had previously established the highly metastatic colon carcinoma E1 cell line from the poorly-metastatic HCT116 cell line [31]. HCT116 was observed to express intermediate levels of gelsolin in a panel of colorectal carcinoma cell lines investigated (Figure S3). Consistent with our hypothesis that there exists a correlation between metastatic potential and gelsolin levels, E1, the metastatic variant, with our hypothesis that there exists a correlation between metastatic potential and gelsolin levels, E1, the metastatic variant, expressed increased gelsolin levels compared to HCT116 (Figure 3A). To further investigate this, we overexpressed gelsolin in HCT116 cells and performed functional assays. Overexpression of gelsolin in the HCT116 was induced by stable transfection with
Figure 1. Gelsolin immunohistochemistry in human colorectal carcinoma tissues. Gelsolin is heterogeneously expressed in a number of primary tumors (A) and liver metastases (B), with islands of low (arrowed in blue) and high (arrowed in red) expression observed within a tumor. Gelsolin expression is mainly cytoplasmic but occasionally, nuclear (C) and perinuclear (D) staining are detected (arrowed). Gelsolin is strongly expressed in a mucinous adenocarcinoma (E) and in stroma (A, B).

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the pIRES2-EGFP plasmid encoding human cytoplasmic gelsolin cDNA (Figure 3B; refer to Figure S4 for the cloned sequence of gelsolin cDNA). Control HCT116 cells were generated by transfection with the empty pIRES2-EGFP plasmid. In addition gelsolin expression was reduced using siRNA knockdown in several colorectal tumor cell lines (HCT116 and its metastatic variant E1, gelsolin-overexpressing HCT116, DLD-1 and Caco-2), and compared to cells transfected with control siRNA. As shown in Figure 3C, gelsolin was greatly reduced after gelsolin siRNA treatment in these cell lines. The effects of gelsolin overexpression and knockdown in colorectal tumor cells were determined as described in the sections below.

Gelsolin overexpression in HCT116 augmented migration through uncoated transwells and invasion through matrigel-coated transwells (Figure 3D). The invasiveness of the stably-transfected gelsolin-overexpressing HCT116 cells as well as wild-type HCT116 cells was significantly decreased by siRNA knockdown of gelsolin expression, indicating a reliance on gelsolin for invasion (Figure 3E). The pro-invasive role of gelsolin was consistently demonstrated in E1 and DLD-1 cells, where reduction of gelsolin by siRNA significantly attenuated invasion. Our observations indicate that gelsolin confers invasive capacity in colorectal cancer cells, which are consistent with the reported effects of gelsolin in other types of cells [17,32].

Gelsolin Modulates the Expression of Genes Important for Tumor Dissemination

Although gelsolin appears to be necessary for invasive behavior, little is known about the mechanisms by which it enhances invasion. This may partly be attributable to its ability to enhance cellular motility through its influence on cytoskeletal dynamics such as when lamellipodia are formed[33]. However, invasion is a complex process dependent on multiple contributory factors besides motility [5]. To further elucidate the downstream mechanisms by which gelsolin induces invasion in colorectal tumor cells, microarray analysis was performed to screen for potential genes that are differentially expressed when gelsolin levels are increased. The gene expression profiles of four stable gelsolin-overexpressing HCT116 clones were compared with two vector control HCT116 clones. The gelsolin-overexpressing clones expressed a consistent pattern of differential gene expression, suggesting that gelsolin induced specific changes in gene expression of HCT116 cells (Figure S5A). In total, we identified 469 genes with an average alteration in expression level of at least two-fold. Using the Gene Ontology functional annotation under DAVID Bioinformation Resources, genes were classified into their respective biological processes, including cell differentiation, cell motility and regulation of cell adhesion (Figure S5B). The representative table of genes modulated by gelsolin is supplied in Table S1.

Figure 2. Gelsolin expression is prominent at the invasive front of human colorectal cancer tumors. Gelsolin expression is high along the tumor periphery in primary tumors and liver metastases. The tumor periphery is outlined as red dotted line. A magnified view of a region of primary tumor edge (boxed) is shown on the bottom panel. The invading liver metastases shown are confirmed by cytokeratin stain, AE1/3, on an adjacent slide (bottom middle panel). Increased gelsolin expression was also detected in less-differentiated tumor cells which appeared to be breaking away from well-formed glandular structures (arrowed, top right panel). Gelsolin expression were scored and represented in the scatter dot plot (bottom). The median scores are represented by the red horizontal bars. Mann-whitney test was to compare the gelsolin expression score between the main tumor bulk and their periphery. Bar: 50 μm.
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Figure 3. Gelsolin promotes migration and invasion of colorectal cancer cells. (A) Endogenous gelsolin level was increased in an in vivo-derived metastatic variant of HCT116, E1, which was described in [31]. (B) Gelsolin overexpression plasmid was constructed by cloning human cytoplasmic gelsolin cDNA into pIRE2-EGFP vector (left). HCT116 cells were either transfected with gelsolin-pIRE2-EGFP plasmid or empty pIRE2-EGFP plasmid to establish stable gelsolin-overexpressing cell lines and vector control cell lines respectively. Western blot analysis confirmed increased gelsolin expression in HCT116 cell lines stably-transfected with the gelsolin-overexpression plasmid compared to those transfected with control plasmid. (C) Western blot showing transient gelsolin siRNA knockdown (KD) at 3 days in wild-type HCT116 and gelsolin-overexpressing HCT116 cells, E1, as well as other colorectal cancer cell lines DLD-1 and Caco-2 cells. Control cells (Cont) were treated with control siRNA. (D) Increased gelsolin in HCT116 enhanced tumor cell migration and invasion. The migration of gelsolin-overexpressing HCT116 cells through uncoated transwells and
Gelsolin Modulates the Expression of Invasion-associated Urokinase-type Plasminogen Activator (uPA)

Among the invasion-associated genes identified to be induced by more than 2-fold in gelsolin-overexpressing cells is the serine protease, urokinase-type plasminogen activator (uPA), which is involved in the plasminogen activation cascade that results in the activation of a broad-spectrum protease plasmin. Although microarray analysis detected less than 2-fold changes in the levels of other uPA cascade genes, we included these in real-time PCR analysis as these comprise important components for regulating uPA-dependent matrix degradation. Besides uPA, the following genes were screened for mRNA expression: the uPA receptor uPAR, and known inhibitors of the uPA cascade including plasminogen activator inhibitor-1 (PAI-1), plasminogen activator inhibitor-2 (PAI-2) and α2-antiplasmin (α2-AP) [34]. Real-time PCR showed that increased levels of gelsolin in HCT116 cells upregulated the expression of uPA and uPAR, and reduced the levels of the inhibitors PAI-2 and α2-AP (Figure 4A). Consistent with observations in the overexpression studies, mRNA levels of uPA were also reduced by siRNA knockdown of gelsolin in wild-type HCT116, DLD-1 and Caco-2 cells (Figure 4B). The mRNA levels of uPAR were reduced by gelsolin siRNA-treated HCT116 and Caco-2 cells but not in DLD-1 cells. Our data indicates that uPA levels are altered by changes in gelsolin expression, and the uPA cascade is a possible mechanism through which gelsolin mediates invasion.

Gelsolin Increases uPA Secretion by Colorectal Tumor Cells and Promotes Invasion through the uPA Cascade

In the plasminogen activator cascade, the secretion of uPA is required to activate the proenzyme plasminogen to active plasmin in the extracellular environment. We investigated the levels of secreted uPA protein in the conditioned media of gelsolin-overexpressing colorectal cancer cells obtained after 48 hours of serum-deprivation, using enzyme-linked immunosorbent assays (ELISA). Consistent with the increase in uPA gene expression, we also detected significantly increased uPA secretion in gelsolin-overexpressing HCT116 compared to control cells (Figure 5A). We examined the effects of gelsolin-induced uPA expression on matrix degradation using zymographic analysis. Conditioned media of the cells were loaded and electrophoresed in zymographic gels containing plasminogen and fibrinogen for analysis of MMPs (MMP7) and TIMP2 were also modulated by gelsolin overexpression in HCT116 (Table S1). Since tumor cells may secrete different proteases during the course of dissemination, we investigated whether the gelsolin-mediated invasion may involve, besides uPA, members of the MMP family. Gelsolin-overexpressing cells were subjected to similar transwell invasion assays following treatment with either the synthetic pan-MMP inhibitor GM6001 (which blocks MMP-1, -2, -3, -8 and -9 activities) or other specific inhibitors to MMP-2, MMP-2/−9, MMP-3, MMP-7 and MMP-8. Pan-MMP inhibition using GM6001, as well as MMP-2 inhibition reduced the invasiveness of cells by about 20%, although the results were not statistically significant (Figure S6). The simultaneous inhibition of MMP-2/MMP-9 showed similar effect to inhibition of MMP-2 alone, suggesting that MMP-9 played little or no role in the invasion of gelsolin-overexpressing cells. There was however no significant increase in the MMP-2 activity in gelsolin-overexpressing cells compared to vector control and HCT116 based on gelatin zymography (Data not shown). Inhibition of MMP-3, MMP-7 and MMP-8 had no effect on invasion (Figure S6). Taken together our data indicated that in addition to its pro-migratory role, gelsolin promotes invasion in the colorectal carcinoma cells predominantly via increased expression and secretion of uPA.

Discussion

In this study, we show that invading populations of tumor cells enriched in gelsolin are found in both primary as well as metastatic human colorectal cancers. This is consistent with earlier observations of prominent gelsolin expression along the invasive front of liver metastases, in contrast to its low expression in primary colorectal adenocarcinomas [28]. The significance of “leading cells” at the invasive front of tumors in promoting tumor spread...
has been highlighted in a number of studies. Cells at the invasive edge acquire molecular changes such as increased expression of matrix-digesting proteases and integrins for matrix-remodelling [38,39], which may be accompanied by a switch towards a mesenchymal-like, dedifferentiated phenotype. These changes in leading cells at the invasive front drive proteases- and force-mediated matrix remodeling which pave the way for collective cell invasion by non-invasive “follower cells” [39]. Notably, the increased tumor aggressiveness has been associated with increased expression or altered localization of other actin-binding proteins such as β-catenin [30], actinin-4 [40], cortactin [41] and fascin [42] at the invasive region of tumors. We have demonstrated here that gelsolin is crucial for the invasive behavior of colorectal tumor cells. Gelsolin has previously been shown to be important for migration of fibroblasts and invasion of other tumor cells, attributable partly to its actin-depolymerizing effects. However, invasion involves the coordination of multiple mechanisms, which include migration and ECM degradation. It is conceivable that the

**Figure 4. Gelsolin modulates the expression of invasion-associated urokinase-type plasminogen activator (uPA) cascade in colorectal cancer cells.** (A) uPA was detected by microarray analysis to be differentially regulated by gelsolin overexpression. The average gene expression fold changes in the gelsolin-overexpressing clones compared to vector control clones are shown. The gene expression was verified by real-time PCR, and indicated that increased gelsolin upregulated uPA and uPAR mRNA, and decreased mRNA expression of endogenous inhibitors of uPA and plasmin, PAI-2 and α2-AP, respectively. The relative mRNA levels were normalized to vector control cells. (B) siRNA knockdown of gelsolin reduced uPA levels in colorectal cancer cells. Real-time PCR showing the relative mRNA expression of uPA and uPAR in HCT116, DLD-1 and Caco-2 cells after treatment with gelsolin siRNA as compared to control siRNA. All data shown are the mean ± standard error of triplicate measurements and are representative of at least three independent experiments. *P<0.05.

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Figure 5. Gelsolin increases uPA secretion of colorectal tumor cells and enhances invasion via the urokinase-type plasminogen (uPA) cascade. (A) Increased gelsolin in HCT116 cells augmented the secretion and activity of uPA. The levels of secreted uPA by cells cultured for 48 hours in serum-free conditions were detected in the supernatant using ELISA. Gelsolin-overexpressing HCT116 cells secreted significantly higher uPA levels compared to control cells (left), which correlated with higher uPA enzymatic activity, as evident from uPA zymographic analysis using 16-
Our work shows that, in addition to previous work highlighting the ability to modulate the expression of well-known mediators of migration [43], gelsolin also confers invasive properties via its ability to modulate the expression of -well-known mediators of tumor invasion - genes involved in the uPA cascade which degrade the ECM. 

The uPA cascade is initiated when secreted uPA from cancer cells [44] or stromal components [45] binds to its receptor uPAR and converts the inactive plasminogen to active plasmin by proteolytic cleavage. Plasmin promotes remodeling of ECM by direct proteolysis, or indirectly through activation of MMPs [46,47]. The uPA system is implicated as a major factor leading to aggressive tumor behaviour, as it promotes invasion and metastasis in several tumor types including colorectal cancer [48,49]. uPA and uPAR activities contribute to proteolysis of ECM at the invasive front of tumors [50], and elevated levels in colorectal tumors correlate with tumor progression and poor survival [51,52].

Our findings revealed the ability of gelsolin to modulate uPA gene expression and secretion. The roles of cytoskeletal proteins in regulation of gene expression is an emerging field of interest, as it is now clear that the nuclear cytoskeleton participates in several transcriptional regulatory processes [53]. Indeed, actin-binding proteins, including gelsolin and its family members have prominent roles in mediating nuclear receptor-directed transcription [54]. Gelsolin was previously reported to physically interact with androgen receptor (AR) and enhance AR transcriptional activity [55]. More recently, gelsolin was found to be a key determinant for the assembly and/or stability of estrogen receptor nucleus complexes [56]. Interestingly, the nuclear import of CapG, another gelsolin family member, is suggested to interfere with chromosome condensation [53] and promote invasion into collagen [57]. It is unknown if gelsolin also regulates non-nuclear receptor-dependent transcription, though its presence in the nuclei of colorectal tumor tissues suggests wider roles for nuclear-associated gelsolin. As yet, it is unclear how gelsolin regulates uPA gene expression - this would be an interesting avenue for further exploration into gelsolin’s roles in transcriptional regulation of invasion genes.

Besides modulating the mRNA levels of uPA, we also found that gelsolin regulates uPA secretion by colorectal cancer cells. Increased circulating levels of plasma uPA have previously been associated with advanced cancers including colorectal [58] and prostate cancers [59]. Very recently gelsolin was reported to regulate insulin exocytosis through its direct interaction with syntaxin 4, a plasma membrane protein which mediates docking of transport vesicles [60]. The expression of other actin-associated proteins, such as cortactin and LIMK1, has also been reported to correlate with the secretion of matrix proteases including MMPs [25,26,61]. LIMK1 in particular, which regulates the activities of actin-depolymerizing factor (ADF/cofilin), has also been shown to interact with membrane type 1 (MT1)-MMP and regulate its vesicular trafficking [25]. It is possible that gelsolin may also participate in the transport of vesicles containing uPA or other proteases through its influence on F-actin dynamics which is essential for vesicular trafficking. Future studies on the role of gelsolin in the secretory pathway of proteases such as uPA would yield a clearer picture of the integration of the distinct but synergistic cellular processes mediated by gelsolin to promote invasion. Nevertheless, the revelation of gelsolin’s function in regulating uPA expression and secretion in colorectal cancer invasion provides further insight into the mechanisms behind gelsolin’s oncogenic role, and lends support to the multiple roles of actin cytoskeletal proteins in promoting cancer cell dissemination.

In the current study we also investigated the contribution of a number of MMPs in the gelsolin-mediated invasion in colorectal cancer cells. Inhibition of the gelatinase MMP-2 alone led to a slight decrease in invasion but not the inhibition of MMP-3, -7, -8 and -9 in gelsolin-overexpressing cells. Treatment with GM6001 which inhibits MMP-1 in addition to MMP-2, MMP-3, MMP-8 and MMP-9 reduced invasion to a small extent but the results were not statistically significant. Interestingly proMMP-2 was reported to be activated by plasmin [47,62], which may increase the pool of active MMP-2. Although plasmin can also activate the other gelatinase proMMP-9, it is pertinent to add that plasmin is not an efficient activator of proMMP-9 [46]. Nevertheless, the balance between MMPs and their endogenous inhibitors, tissue inhibitor of metalloproteinases (TIMPs), is critical in determining the net effect on matrix degradation. Our unpublished data indicated that TIMP-2 is increased in gelsolin-overexpressing HCT116 cells, with the direct implication being that the matrix-degrading effects of MMPs can be counter-balanced. Of note, literature also reveals paradoxical, pro-tumor roles of TIMPs in cancer biology [63]. Due to the complexity of the biological activities of MMPs and TIMPs, it is not within the scope of this study to look into the interactions between MMPs and TIMPs in detail. Moreover the evidence we presented in this manuscript suggests that the uPA cascade is a major downstream pathway by which gelsolin induce matrix degradation.

Together with the well-established functions of gelsolin in cytoskeletal dynamics, our findings implicate gelsolin as a regulatory determinant of the uPA cascade, with significant impact on colorectal cancer invasion. The potential contributions of gelsolin...
towards the further spread of tumor cells from liver metastases warrant further investigations into the roles of cytoskeletal proteins in metastatic disease. Further dissection of the mechanisms by which gelsolin and other cytoskeletal proteins regulate invasive pathways could contribute towards the understanding of how cancer progresses, and the development of effective strategies which counteract its spread.

**Materials and Methods**

**Cell Lines and Reagents**

HCT116, HT29, WiDr, RCM-1, RKO, Caco-2, SW837, SW480, SW403, SW620, DLD-1, LS513, COLO201 and COLO205 are human colon cancer cell lines obtained from ATCC. The in vivo-derived metastatic E1 cell line was developed from HCT116, as previously described [31]. HCT116, E1, HT29 and WiDr were cultured in McCoy’s 5A modified medium; Caco-2, SW480, SW837, SW403 in Dulbecco’s Modified Eagle’s Medium and DLD-1, RKO, LS513, RCM-1, COLO201, COLO205 in RPMI 1640 (All media from Sigma-Aldrich). All media were supplemented with 10% fetal bovine serum (FBS) (Hyclone). Stable HCT116 cell lines overexpressing gelsolin and empty vector control cell lines were grown using the McCoy’s 5A medium with addition of 500 μg/ml Geneticin (Gibco). Cells were maintained at 37°C in a humidified incubated with 5% CO2.

Antibodies used include mouse antibodies against human gelsolin (Abcam), β-actin (Sigma-Aldrich), GAPDH (Santa Cruz Biotechnology), pan-cytokeratin (AE1/3) (Dako), uPA (América Diagnostica), uPAR, mouse IgG1 and goat IgG (R&D Systems). Secondary antibody goat anti-mouse IgG conjugated with HRP (Santa Cruz Biotechnology) was used. Metalloproteinases (MMP) inhibitors include GM6001, MMP-2 Inhibitor I, MMP-2/MMP-9 Inhibitor IV, MMP-3 Inhibitor II, MMP-8 Inhibitor I (Calbiochem) as well as anti-MMP7 antibody (R&D Systems).

**Construction and Transfection of Gelsolin-overexpression Plasmids**

A coding sequence of human gelsolin was amplified from a PMW172 expression plasmid containing human cytoplasmic gelsolin cDNA, by polymerase chain reaction (PCR) using the forward primer 5’ CG GAA TTC ATG GTG GTG GAA CAC and reverse primer 5’ CG CCG CGG TCA CCG GAG TTC 3’ and reverse primer 5’ CG CCG CGG TCA GGC AGC CAG GTG AGC CAT GAC 3’, followed by cloning of gelsolin insert into pIRE2-EGFP vector (Becton Dickinson) at Eco RI and Sac II enzyme restriction sites. The resulting vectors were transformed into competent Escherichia coli XL1B MRF’ cells (Stratagene) and clones were selected based on kanamycin resistance. Sequencing of the cloned gelsolin cDNA verified that the cDNA sequence encodes the human cytoplasmic gelsolin protein (Swiss-Prot accession P06396). To generate stable cell lines, gelsolin- pIRE2-EGFP vector or empty vector were transfected into HCT116 using FuGENE 6 (Promega) and selected using 500 μg/ml G418.

**Immunohistochemistry**

24 primary colorectal tumors and 26 colorectal liver metastases tissues as well as 15 adjacent normal colonic tissues were obtained for use from the Department of Pathology, National University Hospital, Singapore, with approval from the National University of Singapore Institutional Review Board. Briefly, the 4 μm-thick tissue specimens were deparaffinized, boiled in citric acid, and treated with hydrogen peroxide, before incubation with anti-gelsolin primary antibody overnight at 4°C, followed by incubation with polymer-horse radish peroxidase-conjugated secondary antibody at room temperature. Sections were developed with diaminobenzidine (DAB) and counterstained with hematoxylin. Images were acquired using Olympus BX43F microscope equipped with a DP70 camera. Gelsolin staining in the muscularis propria and stroma [28,29] were used as internal positive controls. The intensity of staining was graded from 0 (undetectable) to 3 (intense staining), whilst the proportion of positive staining tumor cells within a tissue was scored from 0 to 3 where: 1 = <30%, 2 = 30-60%, 3 = >60% of tumor cells identified. The staining score was expressed as the product of intensity of staining and proportion of tumor positivity. Both cytoplasmic and nuclear staining were scored and summed. The maximum possible score for a sample is therefore 18, the sum of the maximum cytoplasmic (9) and nuclear scores (9).

**siRNA Transfection**

10 nM of the Stealth siRNA of the sequence 5’ AAA CGU CCA AUC UUG UUG GAG CAG G 3’ (Invitrogen), complexed with lipofectamine, was used to silence gelsolin expression in cells. Medium GC control siRNA which matched the GC content of the gelsolin siRNA used and no-siRNA treatment were included as controls. Cells were harvested at 48 or 72 hours, and treated as used in other assays as described.

**Matrigel Invasion Assay**

The transwell assay was carried out as previously described [31]. 5×104 cells were seeded for HCT116 and E1 cell lines and 5×103 cells for DLD-1, for 48 hours. 10% FBS was used as chemotaxtractant in the bottom transwell chamber. For function-blocking experiments, cells were seeded with 200 μg/mL anti-uPA or 80 μg/mL anti-uPAR (American Diagnostica). For MMP inhibition treatments, 0.15×106 gelsolin-overexpressing HCT116 cells were seeded for 24 hours with 40 μM of MMP inhibitor (either GM6001 or MMP-2, MMP-2/-9, MMP-3 inhibitor), except for MMP-8 inhibitor which was used at 1 μM. Mouse IgG1and goat IgG were included as controls for anti-uPA/anti-uPAR and anti-MMP7 respectively whereas the vehicle control dimethyl sulfoxide (DMSO) was used alongside with the remaining MMP inhibitor treatments. The same protocol was used for migration studies but without the addition of matrigel coating on the transwell membrane.

**ELISA**

For measurement of secreted uPA under standard culture condition, cells were incubated in serum-free medium for 48 hours before harvest of culture supernatant for assay. For stimulated conditions, cells were serum-starved overnight prior to incubation with either 5 or 10 ng/mL of human recombinant TNF-α (PeproTech) in fresh serum-free media for 24 hours. As a control for TNF-α stimulation, cells were incubated with serum-free media in parallel. All harvested cell culture supernatants were kept at −20°C until analysis by ELISA. For measurement of secreted uPA under gelsolin-knockdown conditions, cells were treated with siRNA before the serum-starvation step on the following day. Levels of uPA present in the neat supernatants were determined by the human DuoSet ELISA Development Kit (R&D Systems), according to the manufacturer’s protocol.

**Microarray Analysis**

Microarray analysis was performed on the wild-type HCT116 cell line as well as stably-transfected HCT116 cells including four gelsolin-overexpressing cell lines and two empty vector-transfected control cell lines using Sentrix HumanRef-8 Beadchips (Illumina),
Western Blotting

Total cell lysates were extracted using lysis buffer (6 M urea, 1% SDS, 2 M β-mercaptoethanol, 1 M Tris pH 7.4, PBS). Equivalent amounts of protein from each sample were separated on 10% SDS-PAGE gels and transferred to PVDF membranes, blocked and incubated with primary antibody overnight at 4°C followed by secondary antibody before chemiluminescence substrate detection.

Zymography

Secreted uPA enzymatic activities were examined by zymography. Briefly, cells were grown in serum-free medium for 3 to 16 hours. The conditioned media were normalized using cellular lysate and then combined with non-reducing sample loading buffer and loaded into 10% SDS-PAGE gels containing 730 μg/mL of human fibrinogen and 20 μg/mL human plasminogen (Sigma-aldrich). After electrophoresis, gels were rinsed with distilled water and incubated with wash buffer (2.5% Triton X-100 and 50 mM Tris-HCl pH 8.0) for 1 hour, followed by incubation at 37°C with incubation buffer (0.1 M glycine buffer, pH 8.0) for 16 hours. The gels were then stained with Coomassie blue (0.05% Coomassie dye, 40% methanol, and 10% acetic acid) for 1 hour at room temperature, and destained with distilled water. Areas of lysis were expected to appear as zones of clearance against the Coomassie blue-stained background of undegraded substrate.

Statistical Analysis

Statistical analysis was performed using Student’s t-test except for IHC gelsolin expression analysis in which Mann-Whitney test was used. Differences between sample means were considered statistically significant with P<0.05.
any of the MMP inhibitor treatments, although GM6001 and MMP-2, MMP-2/–9 inhibitors showed a slight reduction. All data shown are the mean ± standard error of at least duplicate measurements and are representative of at least two independent experiments.

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<th>Table S1 Gelsolin modulates the expression of genes important for various cellular processes.</th>
<th>Genes from the microarray output were classified under various biological processes using Gene Ontology annotation from DAVID bioinformation resources. Average fold change in gene expression is determined from comparison between 4 gelsolin-overexpressing HCT116 clones and 2 vector-control HCT116 clones. (PDF)</th>
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<td>Author Contributions</td>
<td>Concised and designed the experiments: JLZ EHT CTY SM SK MST. Performed the experiments: JLZ EHT KHT LT YCL. Analyzed the data: JLZ EHT MJ CTY MST. Contributed reagents/materials/analysis tools: SM SCH. Wrote the paper: JLZ CTY. Review and critique of manuscript: CTY YCL YCG APK.</td>
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