Unbiased Proteomic and Transcript Analyses Reveal that Stathmin-1 Silencing Inhibits Colorectal Cancer Metastasis and Sensitizes to 5-Fluorouracil Treatment

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Abstract

Colorectal cancer metastasis is a major cause of mortality worldwide, which may only be controlled with novel methods limiting tumor dissemination and chemoresistance. High stathmin-1 (STMN1) expression was previously established as a hallmark of colorectal cancer progression and predictor of poor survival; however, the mechanism of action is less clear. This work demonstrates that STMN1 silencing arrests tumor-disseminative cascades by inhibiting multiple metastatic drivers, and repressing oncogenic and mesenchymal transcription. Using a sensitive iTRAQ labeling proteomic approach that quantified differential abundance of 4562 proteins, targeting STMN1 expression was shown to reinstate the default cellular program of metastatic inhibition, and promote cellular adhesion via amplification of hemidesmosomal junctions and intermediate filament tethering. Silencing STMN1 also significantly improved chemoresponse to the classical colorectal cancer therapeutic agent, 5FU, via a novel caspase-6 (CASP6)–dependent mechanism. Interestingly, the prometastatic function of STMN1 was independent of p53 but required phosphorylations at S25 or S38; abrogating phosphorylative events may constitute an alternative route to achieving metastatic inhibition. These findings establish STMN1 as a potential target in antimetastatic therapy, and demonstrate the power of an approach coupling proteomics and transcript analyses in the global assessment of treatment benefits and potential side-effects.

Implications: Stathmin-1 is a potential candidate in colorectal cancer therapy that targets simultaneously the twin problems of metastatic spread and chemoresistance. Mol Cancer Res; 12(12); 1717–28. ©2014 AACR.

Introduction

Colorectal carcinoma consistently ranks among the most prevalent cancers in the world. Although early-stage colorectal cancer is largely curable by surgical resection and perioperative chemotherapy, mortality associated with distant metastases may be as high as 90% (1), suggesting that prevention of metastatic dissemination and more effective treatment against metastatic colorectal cancer, in particular, are critical to improve colorectal cancer patient survival.

Previously, stathmin-1 (STMN1) was identified as a colorectal cancer prognostic biomarker based on proteome comparison between HCT116 colon carcinoma and its hepatometastatic variant cell line (E1) derived from nude mouse after repeated intrasplenic inoculation (2). High STMN1 expression also correlated closely with clinical colorectal cancer tumor progression, and was a strong predictor of poor prognosis based on analysis of survival data (3). Although phenotypic loss of cell migration, matrix invasion, colony formation, and strengthened cellular adhesion was observable after STMN1 silencing (3), the functional involvement of STMN1 in metastatic initiation and sustenance was unclear.

In this work, we further investigate STMN1 function in the context of colorectal cancer metastatic prevention using a combination of proteomics and molecular approaches. Specifically, we aim to develop a clinical strategy to reduce colorectal cancer spread upon diagnosis of the primary lesion, through (i) dissecting molecular mechanisms of STMN1 silencing induced metastatic inhibition, and (ii) evaluating the molecular benefits of STMN1 silencing in colorectal cancer metastatic prevention.

Materials and Methods

Stable STMN1 silencing and transient overexpression

The E1 cell line was derived from liver-specific metastases of the parental cell line HCT116, as described previously (2). The HCT116 p53−/− cell line was a kind gift from Dr. Bert Vogelstein (The Johns Hopkins University Medical...
Institutes, USA). Stable STMN1 knockdown (KD) in E1, HCT116, and HCT116 p53−/− cells was performed using pRFP-shSTMN1-C-RS vector selectable by puromycin (Origene). STMN1 KD was transiently rescued by transfecting wild-type (WT) or mutant pCMV6-STMN1 (Origene), and harvested 48 hours posttransfection for Transwell migration or Matrigel invasion assays.

Proteome analysis

Comparative proteome analysis was performed using an iTRAQ labeling strategy, in which 50 μg total lysates from E1 and HCT116 STMN1 KD cells (EKD; HKD) were tagged with labels 113 and 117, whereas respective controls were tagged with 114 and 118. Threshold for significant regulation in the KD:SC ratio was set to ±1.3-fold. Label-free SWATH MS analysis was performed in quadruplicates using 30 μg total cell lysates of an independent batch. Detailed parameters used in iTRAQ and SWATH MS analyses are described in Supplementary Section SA1–SA6.

Cytotoxicity

STMN1 KD cells were treated for 48 hours with EC50 concentrations of cytotoxic agents (Sigma-Aldrich): Paclitaxel (4 nmol/L), Vinblastine (0.8 nmol/L), Etoposide (20 μmol/L), 5-Fluorouracil (5FU; 12.5 μmol/L), butyrate (1.5 mmol/L), and tert-butyl hydroperoxide (TBHP; 20 mmol/L), 5-Fluorouracil (5FU; 12.5 mmol/L), butyrate (1.5 mmol/L), and tert-butyl hydroperoxide (TBHP; 20 mmol/L). Cell viability was quantified by crystal violet staining, or flow cytometry described in Supplementary Section SA8.

Caspase inhibition and caspase-6 activity

To screen for 5FU-activated caspases in WT E1 and HCT116 cell lines, cells were pretreated separately with specific caspase inhibitors or pan-caspase inhibitors (R&D Systems) at 100 μmol/L for 48 hours, before apoptosis was induced with 100 μmol/L 5FU for another 48 hours in the presence of caspase inhibitors. To test whether 5FU susceptibility in STMN1 KD cells was caspase-dependent, fluoromethyl ketone (fmk)-derivatized peptides that irreversibly block caspase-3 (Z-DEVD-FMK; C3I), caspase-6 (Z-VEID-FMK; C6I), or all caspases (Z-VAD-FMK; CpanI) were used. Cells were similarly pretreated with 100 μmol/L inhibitors for 48 hours before induction of apoptosis with 5FU. STMN1 KD and SC cells were treated with 0 to 150 μmol/L 5FU for 48 hours, and caspase-6 activity was measured by the Caspase-Glo 6 Assay Kit (Promega). Luminescence was recorded by Infinite M200 Luminescence Reader (Tecan) 1 hour after cell lysis.

qPCR array

Cells were seeded in duplicates and allowed 48 hours to reach 70% confluency. Total RNA was then extracted using RNeasy Plus mini spin columns (QIAGEN) and quantified by a Nanovue spectrophotometer (GE Healthcare) in triplicates. Duplicate total RNA extracts were then pooled by RNA content, and 400 ng of each pooled sample was reverse transcribed using the RT® First Strand Kit (QIAGEN). The cDNA generated was then used as template for real-time qPCR amplification of 163 genes implicated in epithelial-to-mesenchymal transition (EMT) and metastasis (QIAGEN), in 384-well PCR array format on the ViiA7 Real-Time PCR system (Applied Biosystems). Relative quantitation was performed against β-actin (ACTB) as the loading control, and only amplifications with Ct < 30 were analyzed. The experiment was repeated to obtain data from a second array set (array set 2), in which reproducibility was verified (Fig. 5B).

Site-directed mutagenesis

Residues S16 (TCA), S25 (AGC), S38 (TCC), and 63 (TCC) on STMN1 were separately modified to A16 (GCA), A25 (GCC), A38 (GCC), and A63 (GCC) using the QuickChange II Site-directed mutagenesis Kit (Stratagene). Primers used in mutagenesis are found in Supplementary Section SA8. Quadruple alanine mutant with all 4 serine residues replaced with alanine was obtained commercially (Genscript). Mutant STMN1 sequences were sub-cloned into pCMV6 mammalian expression vector, and all constructs were sequenced before use.

Cell migration and invasion assays

In quantitative measurements of cell migration, Transwell inserts with 8-μm pore size (BD Biosciences) were coated with fibronectin (Sigma-Aldrich) for 16 hours at 4°C, before 50-k cells were seeded and allowed to migrate for 48 hours. To measure matrix invasion, Matrigel inserts (BD Biosciences) were reswelled for 2 hours before 100-k cells were seeded and allowed 48 hours to invade through the membrane barrier. In both assays, cells that have migrated or invaded across the membrane were stained with crystal violet and quantified by crystal violet dissolution in 1% SDS.

Image acquisition and data presentation

Microscopy images were acquired with the LSM710 confocal microscopy system (Carl Zeiss) and exported using Axiowision Rel. 4.8 software. All bar charts were generated using GraphPad Prism software version 5.00. Data from qPCR experiments were presented using Multiple experiment Viewer (MeV) version 4.81. All statistical tests and measures of significance were performed by the GraphPad Prism software.

Results

STMN1 silencing induces expression changes in colorectal cancer cells

stable silencing of STMN1 in metastatic (E1) or primary (HCT116) colorectal cancer cell lines strongly inhibited in vitro metastatic processes (3). To understand the molecular changes culminating in loss of metastatic phenotype, the total proteomes of stable STMN1 KD cells (EKD; HKD) were analyzed together with the scrambled control cells (ESC; HSC) by an iTRAQ labeling approach (Fig. 1A–C).

Following 2D-LC separation and ESI-MS/MS analysis, a total of 4,562 proteins were identified. By sequential filtering...
for proteins significantly and consistently regulated by STMN1 silencing in both cell lines, 192 target candidates were defined with possible contribution to metastatic inhibition. Among these, 139 (3.05%) were curated and reported to exist at the protein level (Supplementary Section SC1). These include 82 upregulated (1.80%) and 57 downregulated (1.25%) candidate proteins (Fig. 1D) that function predominantly in metastatic processes, signaling and cell death/apoptosis (Fig. 1E). These proteome changes suggest that silencing STMN1 alone is sufficient to modulate key oncogenic processes in colorectal cancer.

iTRAQ validation

Expression changes detected by iTRAQ were validated (Fig. 2). Antibody availability and type determined whether Western blotting with denaturation, or immunofluorescence under native conditions was more suitable (Supplementary Sections SA7 and SB1–SB2), whereas validation of target proteins without commercially available antibodies required a quantitative label-free SWATH MS method (Supplementary Section SC2). In total, 18 protein targets were validated by at least one of three chosen methods. Among these, S100A6, MGST1, and PI-9 were validated by two independent approaches, whereas regulation of S100A10 was demonstrated using all three methods. Because statistically significant quantitation in SWATH MS depended on peptide abundance related to baseline expression in each cell line, quantitative data was not always available from both E1 and HCT116 cells (Fig. 2C). Nonetheless, these data in combination with other validation methods further increase the confidence of iTRAQ quantitation.
STMN1 silencing inhibits metastatic processes

Proteins regulated by STMN1 silencing were stratified by cellular function (Fig. 1E) and STMN1 KD appears to inhibit metastatic processes by rebalancing the expression of prometastatic proteins and metastatic inhibitors (Fig. 1F). STMN1 KD caused significant suppression on metastatic protein expression (Git1, RACK1, S100A10, and S100A6), while simultaneously reinforcing metastatic inhibition with concomitant induction of metastatic inhibitors (ADAM15, SERPINB5, CD109, Hugl1, and NCK1; Supplementary Section SC1). The coordinated downregulation of these metastatic proteins supports that STMN1 silencing blocks metastatic activity in colorectal cancer cells, whereas the loss of metastatic phenotype observed in vitro (3) may be attributed, at least in part, to enhanced metastatic inhibition. Implications of these
STMN1 silencing enhances cellular anchorage and intracellular rigidity

STMN1 KD induced the expression of numerous cell junction and intermediate filament proteins in E1 (metastatic) and HCT116 (primary) cells (Supplementary Section SD2). Hemidesmosomal proteins (ITGA6, ITGB4, and LAMA5) were significantly increased in STMN1 KD cells, suggesting that stronger cell–cell contact could have impeded detachment processes. STMN1 silencing also promoted desmosomal tethering by increasing the expression and formation of KRT1/KRT9/ITGB4/LAMA5 intermediate filaments, as well as their docking to DSP plugs at desmosomes. These changes likely alter the intracellular architecture to reduce cellular fluidity and further cripple locomotive processes. Collectively, stronger desmosomes and more rigid cellular matrix provide explanations for stronger cellular adhesive properties in stable STMN1 KD cells (3). Upregulation and localization of ITGA6, ITGB4, LAMA5, KRT1, KRT9, and DSP were verified by three-color microscopy (Fig. 3).

STMN1 silencing promotes cell-death sensitization

STMN1 silencing surprisingly modulated the expression of proteins implicated in apoptotic response. Cytotoxic protective proteins (PI-9, MGST1, and APRIL) were significantly reduced by STMN1 KD whereas potentiators of apoptotic response (caspase-6, SGPL1, and ANT3) were consistently elevated. General markers of tumor robustness like NOP2 (4) and HIP1R (5) were also downregulated by STMN1 silencing, although no apparent inhibition on cell growth was observed (Supplementary Section SD3). These expression changes allude to the possibility of cell-death sensitization, in which an imbalance in proapoptotic protein expression and antiapoptotic defense may reduce cancer cell viability during treatment stress. This hypothesis was tested subsequently by a cytotoxicity screen.

STMN1 silencing increases 5FU sensitivity

STMN1 KD cells were significantly more sensitive to 5FU compared with scrambled control cells (**, P < 0.01; Fig. 4A), whereas FACS analysis of treated STMN1 KD cells showed significantly more apoptotic nuclei (**, P < 0.01) compared with scrambled control cells similarly treated with 50 or 100 μmol/L 5FU (Fig. 4B). Mild sensitization to paclitaxel and vinblastine (*, P < 0.05) was also observed in agreement with earlier studies in other cell line models (6, 7).

5FU-induced apoptotic cell death in WT E1 and HCT116 cells depended on the activities of caspases 6 and 3 (**, P < 0.01; Fig. 4C). When STMN1 KD cells were pretreated with caspase-6 (C6I), caspase-3 (C3I), or Pan-caspase inhibitor (CpanI), cell viability after 5FU treatment was restored (Fig. 4B). This suggested that hypersensitivity of STMN1 KD cells to 5FU also depended on caspase-6 and -3 activities. Because increased caspase-6 expression was detected in iTRAQ, we focused on testing whether higher caspase-6 activity in STMN1 KD cells was reducing 5FU tolerance.

Caspase-6 activity was low in both STMN1 KD and scrambled controls at rest, but became significantly higher in STMN1 KD cells (**, P < 0.01) upon 5FU treatment (Fig. 4D), suggesting that STMN1 KD cells had a larger reserve of caspase-6 that could be readily activated upon 5FU trigger. When caspase-6 was inhibited, lower caspase-6 activity observed was again consistent with reduced apoptotic cell death (Fig. 4D). Similarly, when caspase-6 was transiently silenced in STMN1 KD cells, chemosensitization to 5FU was clearly abrogated (Supplementary Fig. S1 and Supplementary Section SD4). Moreover, caspase-6 was determined to be the apical caspase that further activates caspase-3 in 5FU-induced apoptosis (Supplementary Fig. S2 and Supplement Section SD5). These further support that STMN1 silencing improves 5FU sensitivity through increasing expression of caspase-6, which causes more apoptotic cell death when activated in the context of low antiapoptotic defense (Fig. 4F). Increased activation of caspase-6 was further verified by western
Figure 4.  STMN1 silencing sensitizes colon cancer cells to 5FU. A, cytotoxicity (n = 4; mean ± SE). STMN1 silencing induces mild sensitization to microtubule inhibitors paclitaxel and vinblastine (*, P < 0.05), but stronger susceptibility to 5FU (**, P < 0.01). Dotted line, relative cell viability of 1.0. B, induction of apoptotic cell death by 5FU (n = 4; mean ± SD). (Continued on the following page.)
blotting in which activated caspase-6 and cleavage of Lamin A were observable at a lower dose of 5FU in STMN1 KD cells (Fig. 4E).

STMN1 silencing reverses metastatic and EMT transcriptional profile in colorectal cancer

Stable STMN1 silencing also significantly altered the expression of targets driving colorectal cancer progression (Fig. 5D; Supplementary Section SC3). Transcription of proteins needed in motility, invasion, and angiogenesis was consistently reduced (MMPs, TIMP2, SERPIN1, ITGB3, and PRL1), whereas TIMP3 that inhibits invasion (8) and promotes apoptosis (9) was significantly upregulated by STMN1 KD. Increase in PNN on the other hand further cooperates with KRT1/KRT9/DSP to promote desmosomal tethering. Changes in Wnt and TGFβ networks after STMN1 KD (FZD7, TGFβ2, TGFβ3 and SMAD2, SMAD4) also suggest plausible suppression of experimental metastasis (3) through a complex myriad of signaling modulation (10). Indeed, loss of SERPIN1 expression was a downstream effect of abrogated TGFβ signal relay, with low expression of SMADs (11). A general reversal of cancer progression was also observable based on levels of colorectal cancer prognostic markers (EPHB2, SYK, KISS1, JAG1, and PTK2).

Stable STMN1 silencing also evidently reversed the EMT transcriptional profile (Fig. 5D; Supplementary Section C3). E-cadherin (CDH1) expression was restored in STMN1 KD cells whereas Vimentin (VIM) and a large group of EMT transcription factors implicated in colorectal cancer were consistently reduced. These include SNAI2, SNAI3, TCF4, ZEB1, and ZEB2, which have direct affinity for the CDH1 promoter, as well as TWIST1, FOXC2, and GSC, which regulate CDH1 expression indirectly (12). Strong inhibition on CD44 expression also supports the loss of matrix invasion (13) after STMN1 silencing, and likely prevents the direct reprogramming of colorectal cancer cells (14). These transcript changes following stable STMN1 silencing strongly indicate a reversal of EMT phenotype, especially in the metastatic E1 cell line.

In another liver-specific metastatic colorectal cancer cell line SW1116-M, EphB2 expression was reportedly lost whereas MMP3 and 9 transcripts were elevated (15). In this work, STMN1 silencing in the metastatic colorectal cancer cell line E1 also reversed the levels of MMP3, 9, and EphB2, further suggesting that loss of STMN1 may reverse metastatic and EMT changes in colorectal cancer in general. Increased expressions of CDH1, TIMP3, and PNN, and downregulation of MYC, MTA1, STAT3, MMP2, MMP9, SERPIN1, SMAD2, and SMAD4 at the protein level were validated by Western blotting (Fig. 5E). Protein levels of STAT3 and CD44 were also validated by label-free SWATH MS in the E1 STMN1 KD cell line with higher baseline expression (Fig. 2C).

STMN1 S25/38 phosphorylation is required for prometastatic activity

In these and previous studies (3), only colorectal cancer cell lines with WT p53 were used. Because p53 is mutated or lost in more than 70% of all colorectal cancers (16), and also regulates the expression of STMN1 (17) and caspase-6 (18), stable STMN1 silencing was next performed in HCT116 p53−/− cells (Fig. 6A) to test whether functional p53 is required for metastatic inhibition in vitro. STMN1 KD in HCT116 p53−/− cells similarly inhibited Transwell migration and Matrigel invasion (***, P < 0.001; Fig. 6B) in a manner comparable with WT HCT116 cells (3), suggesting that functional p53 is not critically required for STMN1 silencing induced metastatic inhibition.

Next, the effect of posttranslational modification was tested. Because the STMN1 sequence lacks any functional domains or structural features directly related to prometastatic function, interactions with other proteins are likely to govern its activity instead. STMN1 may be phosphorylated at 4 serine residues located in the N-terminal regulatory and helical regions (Fig. 6D). Although earlier studies have established the effect of STMN1 phosphorylation on tubulin binding (19, 20), whether specific STMN1 phosphorylation(s) was crucial for metastatic activity remained to be investigated. To this end, STMN1 S16A, S25A, S38A, S63A, and quadruple alanine mutant at all 4 sites (4Ala) were generated and transfected into stable STMN1 KD cells to assay for rescue of in vitro migration and invasion (Fig. 6C; Supplementary Section SA10). Transient transfection of S25A or S38A STMN1 mutants failed to reconstitute Transwell migration and Matrigel invasion (**, P < 0.01), suggesting that STMN1 phosphorylation on S25 or S38 is crucial for prometastatic activity. Inhibition of all serine phosphorylations on the other hand had no cumulative effect on metastatic phenotype, because transfection of 4Ala mutant produced similar effects as S→A mutants at either S25 or S38 (Fig. 6C).

Discussion

Metastatic transition in colorectal cancer is extremely complex, because extensive changes in protein expression and signaling crosstalk are required to support the acquisition of metastatic niche. To fully interrogate the intricacies of metastatic initiation and sustenance, we adopted a comprehensive workflow encompassing proteomics discovery, (Continued)
functional validation, transcript analysis and molecular verification. Using this approach, we evaluated comprehensively the potential benefits of STMN1 silencing in colorectal cancer metastatic prevention.

Proteome analysis of STMN1 KD cells was highly descriptive and provided a high-resolution, albeit static snapshot of metastasis-related expression changes. This yielded fresh molecular insight into the mechanism of STMN1 silencing induced metastatic inhibition. Analysis of transcripts on the other hand served to complement proteomics data by capturing the dynamic process of proteome regulation through targeted measurement of the metastatic drivers implicated. Moreover, proteomics also provided a good starting point for molecular investigations, because new hypotheses may be formulated on the basis of the expression changes detected. Herein, our decision to investigate caspase-6–dependent chemosensitization after STMN1 silencing was strongly influenced by proteomics data.

This method generates working hypotheses that are neither limited by the imagination of the experimenter, nor biased toward extensively studied protein targets, but instead represents the most time efficient and cost effective way to raise a research question, which may be further addressed by molecular methods.

**Molecular benefits of STMN1 silencing**

Using this approach, we show that STMN1 silencing inhibits colorectal cancer metastatic processes by reinstating the default cellular program of metastatic inhibition. This is achieved through reinduction of a large number of metastatic inhibitors often lost with tumor progression, together with the simultaneous suppression on metastatic protein expression. Subsets of these expression changes translate into altered cell surface composition and stronger basal–lateral anchorage, as well as reduced intracellular fluidity and hindered cytoskeletal remodeling, which directly affect detachment processes and morphologic plasticity required for metastasis. These antimetastatic expression changes observed after STMN1 silencing are also highly reflective of repressed oncogenic signaling, further highlighting the complexity of STMN1-dependent metastatic regulation.

In addition, the molecular benefits of STMN1 silencing in colorectal cancer also include chemosensitization, in which 5FU gains significant potency against metastatic cells via a

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**Figure 5.** STMN1 silencing inhibits EMT and metastatic gene expression in colon cancer cells. A, p38 phosphorylation (representative image; n = 3). B, qPCR reproducibility plot. Duplicate amplifications (R1; R2) of 682 out of 768 reactions (r² = 0.9906; *, P < 0.001). C, STMN1 silencing inhibits the colorectal cancer oncogenic pathway and cytoskeletal remodeling. Fold change (KD:SC) log₂ transformed. D, STMN1 silencing regulates metastatic processes and EMT in colorectal cancer cells. Fold change (KD:SC) log₂ transformed. E, Western validation. Cell lysate prepared in parallel with total RNA extraction.
novel caspase-6-dependent mechanism that we report here. Metastatic colorectal cancers usually respond weakly to 5FU-based chemotherapy but STMN1 silencing apparently tilts the balance in favor of apoptotic events by weakening apoptotic defense while simultaneously reducing the capacity to detoxify. Such observations further suggest the utility of STMN1 silencing in the context of colorectal cancer metastatic prevention and therapy.

At the global level, STMN1 silencing also appears to ameliorate colorectal cancer metastatic progression by suppressing oncogenic expression signatures and reversing EMT transcriptional profiles. These changes indicate better colorectal cancer prognosis, but more importantly highlight the far-reaching impact of potential STMN1-based cancer therapy, because almost all the oncogenic and EMT transcription factors modulated by STMN1 silencing further control downstream amplification of other metastasis-related genes. This is contrary to the belief that STMN1 functions only at the receiving end of signal transduction, but instead suggests a surprisingly pervasive role for STMN1 in driving EMT, supporting colorectal cancer progression and initiating metastatic processes.

**STMN1 interactions and S25/38 phosphorylation determine prometastatic activity**

The benefits of STMN1 silencing, however, cannot be explained by structural features in the STMN1 sequence.
Other than a prominent tubulin-interacting alpha-helical region (Fig. 6D), no other functional domains allude to the mechanism of proteome regulation. Current knowledge of STMN1 function is mostly associated with regulation of mitotic spindle assembly. Because STMN1 sequesters dimeric tubulin (21), it is a direct modulator of microtubule dynamics and anaphase chromosomal segregation (22). Even so, the prometastatic activities of STMN1 observed here are unlikely to be dependent on microtubule regulation, because cell division should preclude locomotive or invasive processes to ensure fidelity of chromosomal division. In agreement with this, S63A does not affect metastatic phenotype despite its central location in the tubulin-interacting helical region (Fig. 6D). This further suggests that microtubule regulation and metastatic events are likely to be discrete functions of STMN1 at different times.

On the contrary, STMN1 is more likely to function in metastatic processes through interactions with other proteins. In this respect, the N-terminal structure could determine affinity and accessibility of STMN1 interactions, because the majority of phosphorylation sites reside in the N-terminus. It was also suggested that N-terminal STMN1 may be intrinsically unstructured, and the molecule could depend on phosphorylation-induced conformation changes for activation (23).

In agreement with this proposed model, we report that S25A and 38A mutants are not prometastatic, suggesting that key interactions required to initiate metastatic processes may indeed be precluded in the absence of phosphorylation-induced conformation changes in the N-terminal regulatory region. This implies that apart from STMN1 silencing, inhibiting STMN1 phosphorylation at S25/38 may also prevent metastatic initiation. To date, vast majority of investigations into STMN1 phosphorylation and activity fail to address functional differences that may exist between differently phosphorylated species of STMN1 (24–26). We show, on the other hand, that different phosphorylations on four available serine residues further distinguish STMN1 function, and that specific S25/38 phosphorylations on STMN1 critically enable prometastatic activity. This imposes an additional layer of control in the regulation of STMN1 function, in which metastatic initiation is concerned.

**STMN1 silencing regulates metastatic networks**

Although the molecular effects of STMN1 silencing described above are diverse, tipping the balance remains a recurrent theme summarizing the changes we observe. STMN1 silencing appears to offset the balance between (i) metastatic activators and suppressors, (ii) apoptotic potentiators and inhibitors, as well as (iii) mesenchymal and epithelial maintenance, such that metastatic inhibition is favored, and chemoresponse improved. These network changes suggest that STMN1 silencing regulates colorectal cancer progression at the systems level, rather than controlling single pathways.

For instance, instead of relying on one dominant mechanism to inhibit EMT, STMN1 silencing relays the signal to maintain epithelial phenotype through multiple pathways that converge at promoting CDH1 transcription. Increased E-cadherin expression was achieved in three ways: (i) down-regulating mesenchymal factors that repress CDH1 (SNAIL2, SNAI3, ZEB1, FOXC2, GSC, TWIST1, and TCF4; ref. 27), (ii) reducing the expression of STAT3 (28) and CTBP1 (29) that inhibit CDH1 transcription, as well as (iii) upregulating PNN, which interacts with CTBP1 to further relieve CTBP1-mediated repression on the CDH1 promoter (30).

In the regulation of epithelial phenotype described above, STMN1 silencing targets not only CDH1 transcription and repression, but also protein–protein interactions that promote CDH1 expression. Such a multifoci mechanism of action could probably also be more robust in clinical application, because patients present with different disease etiology, as well as activating or inhibitory mutations.

**STMN1 silencing: a potential therapy against metastatic colorectal cancer**

In summary of the data presented, STMN1 silencing appears to be a plausible form of therapy against colorectal cancer metastasis, in addition to its clinical significance in colorectal cancer prognosis (3). Tumor dissemination arises when metastatic processes become more efficient, and chemotherapy fails to eliminate malignant cells in circulation. STMN1 silencing targets these problems by inhibiting metastatic processes and simultaneously amplifying chemoresponse. The mechanism of action depends not on microtubule regulation but on phosphorylative changes that potentially alter functional interactions, and consequently oncogenic signaling and mesenchymal transition (summarized in Fig. 7).

Unlike conventional targeted therapy, blocking STMN1 expression alters not only one signaling pathway, but relies on a multifoci regulatory network to redefine the balance in metastatic signaling and cell survival. Given the increasing recognition that metastatic regulation must involve network perturbations and compensatory effects on top of linear signal relay, the potential for STMN1 silencing based colorectal cancer therapy is perceivable.

Even though STMN1 silencing tweaks global expressions, these effects are highly specific to metastatic response or chemoresistance, and condensed into alteration of only 3% of the detectable proteome (Fig. 1D). This further indicates that STMN1 silencing possibly blocks colorectal cancer metastatic machinery specifically, with low off-target effects and minimal collateral damage. STMN1 expression in the normal colon is also extremely low, suggesting that high STMN1 level is not a prerequisite to maintain normal colonic function, and that noncancerous parts of the colon would be less affected by STMN1 depletion compared with cancerous lesions. Moreover, the efficacy of STMN1 silencing should also be minimally affected by loss of p53 that accompanies colorectal cancer progression, because p53 activity is not required in this novel mechanism. Collectively, these considerations further strengthen the feasibility of STMN1 silencing in colorectal cancer metastatic inhibition.
Nevertheless, this potential antimetastatic regime is still relatively distant from clinical application without animal testing. The next phase of investigations should, thus, involve validating the efficacy of STMN1 silencing in vivo. The comprehensive in vitro data presented here will serve as crucial justifications for the use of animals in subsequent investigations. In a rapidly aging world population that is increasingly plagued by cancer, metastatic prevention may be the key to improving colorectal cancer patient survival and reducing global healthcare burden. More importantly, the implications of data presented, herein, may extend beyond the context of colorectal cancer, because STMN1 upregulation is also observed in cancers of the stomach (31), breast (32), liver (33), and prostate (34).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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