

ORIGINAL ARTICLE

MicroRNA-192 suppresses liver metastasis of colon cancer

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Metastasis causes most deaths from colon cancer yet mechanistic understanding and therapeutic options remain limited. Here we show that expression of microRNA (miR)-192 is inversely correlated with metastatic potential of colon cancer cells. Ectopic expression of miR-192 sensitizes colon cancer cells to growth factor deprivation stress-induced apoptosis, whereas inhibition of miR-192 confers resistance. Overexpression of miR-192 inhibits metastatic colonization to the liver in an orthotopic mouse model of colon cancer. Alterations associated with the metastatic phenotype in the primary tumors include increased apoptosis, decreased proliferation and angiogenesis. Further studies indicate that miR-192 downregulates expression of Bcl-2, Zeb2 and VEGFA *in vitro* and *in vivo*, which is responsible for enhanced apoptosis, increased expression of E-cadherin and decreased angiogenesis *in vivo*, respectively. Finally, studies performed on human colonic adenocarcinoma show that expression of miR-192 is significantly reduced in neoplastic cells as compared with normal colonic epithelium. Importantly, there is a significant decrease in miR-192 expression in stage IV tumors when compared with stage I or II lesions. These findings indicate that miR-192 has an important role in colon cancer development and progression. Our studies underscore the clinical relevance and prognostic significance of miR-192 expression in colon cancer. Therefore, a major implication of our studies is that restoration of miR-192 expression or antagonism of its target genes (Bcl-2, Zeb2 or VEGFA) may have considerable therapeutic potential for anti-metastatic therapy in patients with colon cancer.

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INTRODUCTION

Colorectal cancer is the second leading cause of cancer mortality in the USA.¹ The majority of these deaths are from metastatic disease, which is one of the hallmarks of colorectal cancer.² Although 5-year survival rate for early-stage colorectal cancer is ~90%, it is only 10–15% for metastatic colorectal cancer. Thus, targeting metastasis is an important strategy for colorectal cancer treatment and to improve patient outcomes. Metastasis is a complex and multistep process.^{3,4} However, our knowledge of molecular mechanisms governing the metastatic process of colon cancer is very limited. It has been previously demonstrated that metastasis is associated with aberrant survival capacity and enhanced resistance to apoptosis, increased angiogenesis and epithelial to mesenchymal transition (EMT).^{5–7} Therefore, identification and characterization of molecules/pathways that control cell survival, angiogenesis and EMT are critical to our understanding of metastasis.

MicroRNAs (miRNAs) are a group of small non-protein coding RNAs evolutionarily conserved in the genomes of animals, plants, fungi and viruses.⁸ MiRNAs suppress expression of many different gene targets at the post-transcriptional level through sequence-specific interaction with their 3' untranslated regions (UTR), which leads to translation inhibition or mRNA degradation.⁹ In human cells, miRNAs are estimated to regulate more than one-third of human genes and the majority of genetic pathways.¹⁰ A significant number of miRNAs are located at unstable regions or genomic regions linked to cancer.¹¹ Alterations in miRNA expression are found to be associated with many human

cancers;¹² MiRNA profiling or signature of cancer is being utilized to identify potential tumor subtypes, diagnose cancer, determine treatment plan and predict patient outcome.^{13,14} In addition, miRNAs have been demonstrated to function as oncogenes or tumor suppressors depending upon their target genes.¹²

Many miRNAs have been shown to affect metastasis in different cancers.^{15–17} The pleiotropic nature of gene regulation by miRNAs suggests that certain miRNAs may function as crucial mediators of cancer metastasis. In this study, we identify an anti-metastatic miRNA, miR-192, that inhibits metastasis at multiple steps by suppressing expression of several key pro-metastatic genes. We demonstrate that miR-192 sensitizes colon cancer cells to growth factor deprivation stress (GFDS)-induced apoptosis *in vitro* and suppresses liver metastasis of colon cancer cells in an orthotopic model *in vivo*. Characterization of parameters of primary tumors indicates that miR-192-expressing tumors display increased apoptosis, reduced proliferation and angiogenesis when compared with control tumors. Further studies reveal that miR-192 increases apoptosis by repressing expression of Bcl-2, a pro-survival protein that has been implicated to have an important role in colon cancer metastasis (Wang, *et al.*, unpublished data). In addition, we identify Zeb2, a master transcriptional factor, as a direct target of miR-192. Zeb2 has been shown to promote EMT,¹⁸ an important determinant of metastasis. We show that miR-192 increases E-cadherin expression via suppression of Zeb2 expression in colon cancer cells. Furthermore, our studies indicate that miR-192 suppresses expression of VEGFA, an important pro-

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angiogenesis factor. Downregulation of VEGFA by miR-192 leads to reduced vascularization of primary tumors, which contributes to diminished liver metastasis. Finally, quantitative real-time PCR (Q-PCR) analyses performed on human colonic adenocarcinomas indicate an inverse relationship between miR-192 expression and advanced tumor stages in colon cancer patients. Our studies suggest that miR-192 could be an important determinant of colon cancer development and progression.

RESULTS

Expression of miR-192 is significantly reduced in metastatic colon cancer cell lines

It has been previously reported that transforming growth factor beta (TGF β) signaling mediates metastasis in colon cancer cells.^{19,20} To identify miRNAs that affect colon cancer metastasis, we search for miRNAs whose expression is regulated by TGF β signaling. Expression of miR-192 has been shown to be induced by TGF β in mouse glomerular mesangial cells²¹ and downregulated in colon cancer tissue samples.^{22,23} When TGF β -responsive colon cancer FET cells were treated with TGF β , expression of miR-192 was induced in a time-dependent manner (Figure 1a). Given the function of TGF β in colon cancer metastasis, these results suggest that miR-192 might have a role in colon cancer metastasis as well.

To establish whether miR-192 is differentially expressed in colon cancer cells, metastatic and non-metastatic human colon cancer cell lines were examined for miR-192 expression. Q-PCR assays showed that metastatic HCT116, RKO, RCA and GEO cells^{20,24} exhibited around 100-fold reduced miR-192 expression as compared with non-metastatic or low-metastatic FET and HCT116b cells¹⁹ (Figure 1b), indicating an inverse correlation between miR-192 expression and metastatic potential in colon cancer cells.

miR-192 has an important role in stress-induced apoptosis *in vitro*
We next characterized the biological function of miR-192 in colon cancer cells *in vitro*. First, we assessed the role of miR-192 in stress-induced apoptosis. miR-192 precursor was stably expressed in metastatic HCT116 cells using a retroviral vector. As a result, expression of mature miR-192 is comparable to those in non-metastatic FET cells determined by Q-PCR analysis (Supplementary Figure S1A). Luciferase assays revealed that the reporter activity of the plasmid containing the miR-192 recognition element in the 3'-UTR of the luciferase gene was decreased in miR-192-expressing cells as compared with the vector-expressing control cells (Supplementary Figure S1B), indicating the suppressive activity of ectopically expressed miR-192 in HCT116 cells.

Although the percentages of cells in G1 and S phase were slightly increased and decreased, respectively, in miR-192-expressing cells relative to the control cells (Supplementary Figure S2),

the differences were not statistically significant. However, miR-192-expressing cells displayed significantly increased sensitivity to GFDS-induced apoptosis as reflected by enhanced caspase 3 cleavage as compared with the control cells (Figure 2a). These observations were further confirmed by DNA fragmentation enzyme-linked immunosorbent assay assays, which showed 2.5-fold or 80% increase in apoptosis in miR-192-expressing cells when compared with the control cells after exposing to GFDS for 1 or 2 days, respectively, (Figure 2b, * $P < 0.05$, ** $P < 0.01$). To determine whether miR-192 has similar function in other colon cancer cells, miR-192 was ectopically expressed in RCA cells that show low endogenous miR-192 expression (Figure 1b). The suppressive activity of exogenous miR-192 was indicated by the decrease in luciferase activity of the miR-192 reporter (pMiRluc-192) (Supplementary Figure S3A). Similar to HCT116 cells, there was an increase in cleaved caspase 3 in miR-192-expressing RCA cells under GFDS as compared with the control cells (Figure 2c), which was confirmed by DNA fragmentation assays showing 45% increase in apoptosis (Figures 2d, * $P < 0.05$). Of note, miR-192 mimic showed same effect as stably expressed miR-192 in both cell lines (data not shown). These results indicate that ectopic expression of miR-192 sensitizes colon cancer cells to GFDS-induced apoptosis.

To further define the role of miR-192 in cell survival, we used a chemically synthesized miR-192 inhibitor to inhibit its activity. HCT116b cells expressing high levels of endogenous miR-192 were transfected with a miR-192 inhibitor. The inhibitor reduced miR-192 activity in HCT116b cells as indicated by the increase in luciferase activity of the miR-192 reporter (pMiRluc-192) as compared with the control cells (Supplementary Figure S3B). Inhibition of miR-192 effectively reduced cleaved caspase 3 and poly ADP ribose polymerase under GFDS (Figure 2e), which was confirmed by DNA fragmentation assays showing 39% decrease in apoptosis (Figure 2f, * $P < 0.05$). These results demonstrate that inhibition of miR-192 confers resistance to GFDS-induced apoptosis.

Expression of miR-192 suppresses liver metastasis *in vivo*

As cell survival capacity of cancer cells is an important determinant of metastasis,^{5,25} we next determined the role of miR-192 in metastatic potential of colon cancer cells in an orthotopic model *in vivo*. miR-192-expressing (miR-192) or control (vector) HCT116 cells were stably transfected with green fluorescent protein and characterized *in vivo*.

In vivo studies showed that animals implanted with HCT116 control or miR-192-expressing cells demonstrated 100% primary tumor growth at the site of implantation (Table 1 and Figure 3a, left panel). Although the expression of miR-192 resulted in a modest decrease (22%) in primary tumor weight (Figure 3a, right panel, * $P < 0.001$), it significantly reduced liver metastasis in the orthotopic model by more than sixfold as reflected by a reduction

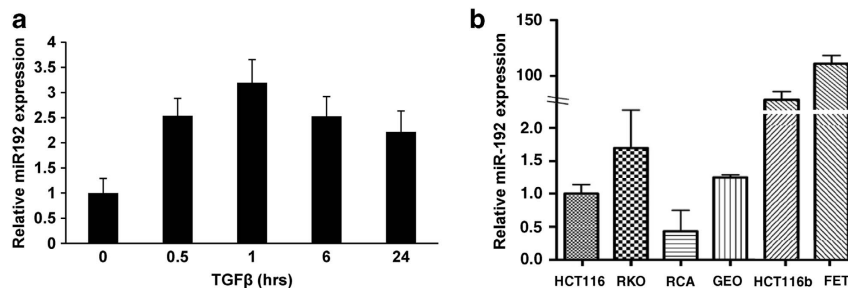


Figure 1. Expression of miR-192 in human colon cancer cell lines determined by real-time Q-PCR. (a) Expression of miR-192 in FET cells treated with TGF β for indicated time periods. (b) Expression of miR-192 in colon cancer cell lines with different metastatic potential. Values represent the means \pm s.e.m. of triplicate Q-PCR values from a representative experiment performed at least three times.

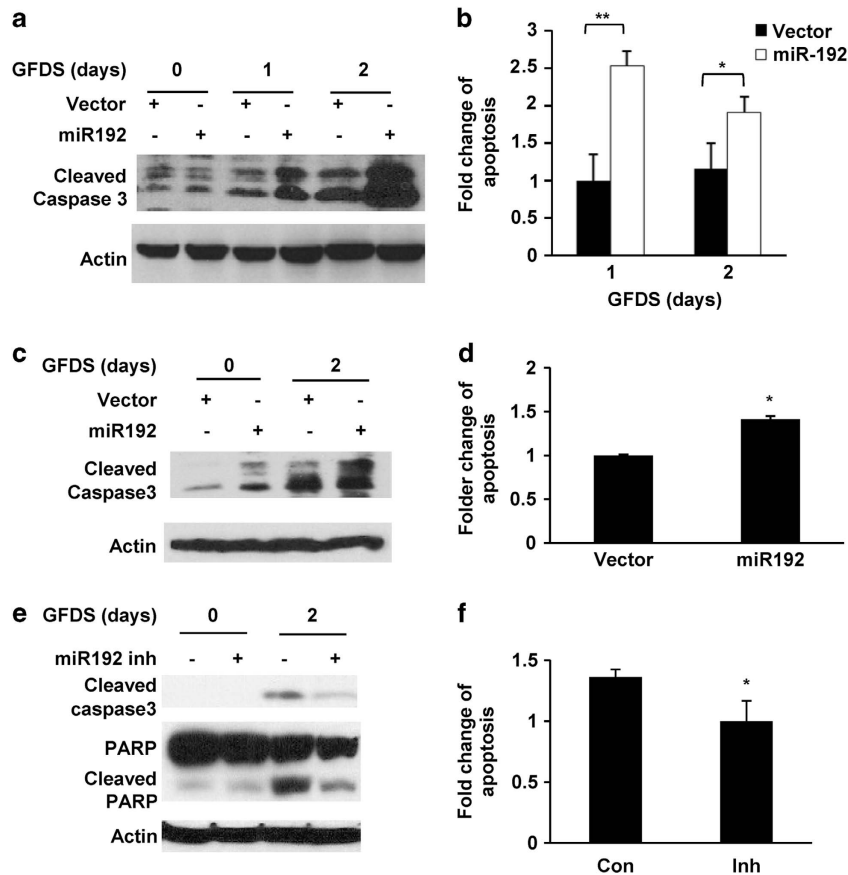


Figure 2. miRNA-192 contributes to GFDS-induced apoptosis. The miR-192 precursor was overexpressed in HCT116 (a, b) and RCA cells (c, d) and chemically synthesized miR-192 inhibitor (Inh) or negative control (Con) was transfected into FET cells (e, f). Western blot analysis of cleaved caspase 3 or poly ADP ribose polymerase (PARP) was performed in HCT116 (a), RCA (c) and FET cells (e) under GFDS for indicated time periods. DNA fragmentation assays were performed in HCT116 cells under GFDS for 1 or 2 day (b, $*P < 0.01$, $**P < 0.05$), in RCA cells under GFDS for 2 days (d, $*P < 0.1$) and in FET cells under GFDS for 2 days (f, $*P < 0.05$). The data are presented as the mean \pm s.e.m. of triplicate experiments.

	Primary tumors	Liver metastasis
Vector	100% (19/19)	53% (10/19)
miR-192	100% (26/26)	8% (2/26)

of metastatic incidence from 53 to 8% (Table 1). The presence of metastatic disease was confirmed by histological analysis (Figure 3b, lower left panel). Moreover, fluorescence imaging of explanted liver showed that, compared with vector cells, orthotopic implantation of miR-192 cells led to diminished liver metastasis (Figure 3b, upper panel). Quantification of liver metastatic loci indicated a more than 10-fold decrease in numbers of liver metastases (Figure 3b, lower right panel, $*P < 0.002$). These results demonstrate that miR-192 inhibits metastatic colonization in the liver. To determine whether miR-192-mediated apoptosis was associated with metastatic potential *in vivo*, Terminal nucleotidyltransferase-mediated nick end labeling (TUNEL) assays were performed. TUNEL staining of primary tumors showed that there were significantly more apoptotic cells in the tumors of miR-192 cells than in those of vector cells (53 vs 17%, Figure 3c, $*P < 0.001$). Meanwhile, Ki67 staining showed that tumors of miR-192 cells had fewer proliferative cells than those of vector cells (56 vs 75%, Figure 3d, $*P < 0.001$). Furthermore, when we examined

angiogenesis in the primary tumors, we found that vascular formation in the primary tumors of miR-192 cells was much lower than that in the primary tumors of vector cells as reflected by CD 31 staining (Figure 3e, upper panel). Quantification of the density of CD31 staining (Figure 3e, lower left panel) and numbers of CD31-positive cells (Figure 3e, lower right panel) showed a 2.8-fold ($*P < 0.03$) and a 1.7-fold ($*P < 0.02$) difference between two groups of primary tumors, respectively. These results indicate that the inhibitory effect of miR-192 on metastasis was not simply a result of its effect on suppression of tumor cell proliferation but also a result of its inhibition of survival of tumor cells and their capacity to develop angiogenesis. Taken together, these *in vivo* results demonstrate an important suppressive role of miR-192 in metastatic formation at distant organ sites.

miR-192 directly or indirectly regulates expression of pro-metastatic genes

The ability of miR-192 to impede multiple steps of the cancer metastasis cascade might be attributed to its ability to regulate expression of genes involved in diverse aspects of metastatic dissemination. To identify effectors of miR-192, we used several algorithms that predict the mRNA targets of miRNAs—TargetScan,²⁶ PicTar²⁷ and miRanda-mirSVR.²⁸ On the basis of the representation of miR-192 sites in their 3' UTRs and their implicated role in tumor progression, we chose the top 10 target genes to perform further studies. Three of the 10 genes studied

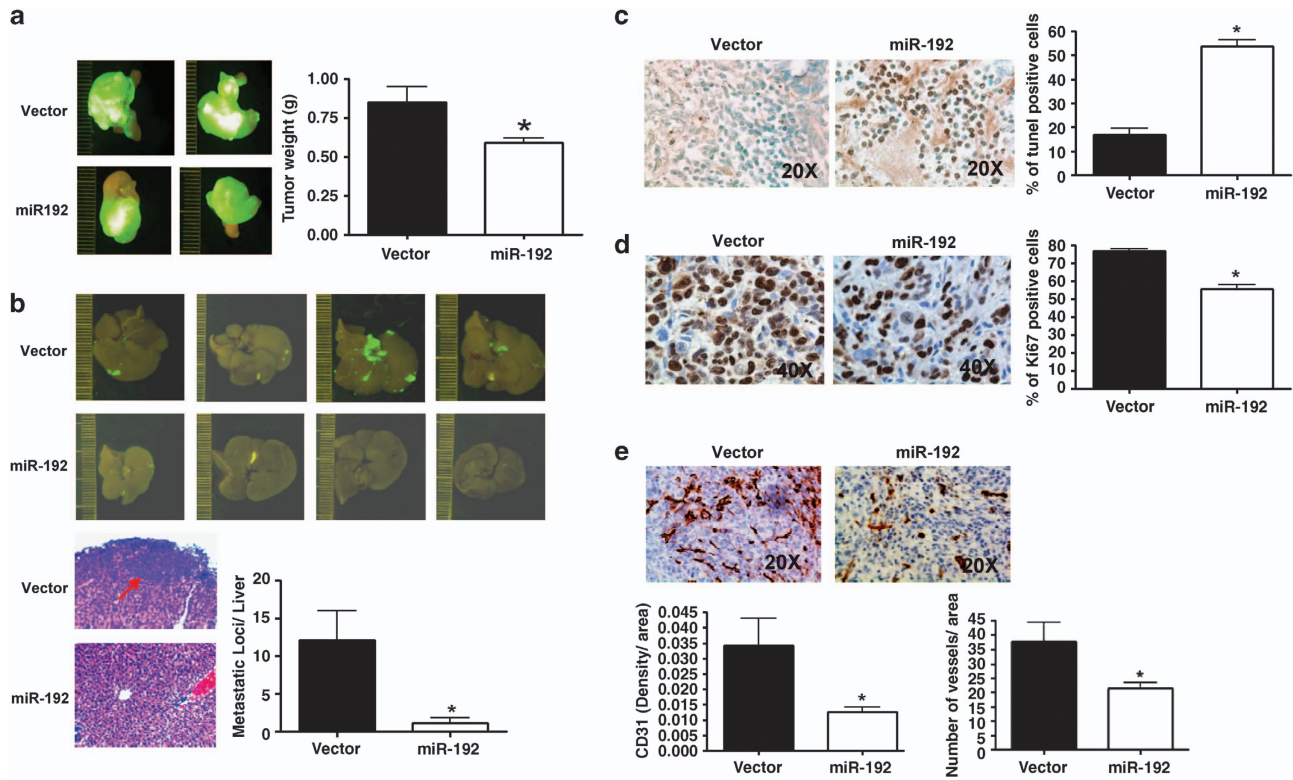


Figure 3. Expression of miR-192 suppresses liver metastasis in an orthotopic model. **(a)** Green fluorescent protein (GFP) images (left panel) and weight (right panel, $*P < 0.001$) of primary tumors are shown. **(b)** GFP images of liver metastasis (upper panel) and hematoxylin and eosin staining of liver metastasis are shown in the lower left panel. The arrow indicates cancer cells in the liver. Numbers of liver metastatic loci were counted and compared between mice bearing HCT116 vector control cells and HCT116 cells expressing miR-192. $*P < 0.002$ (lower right panel). **(c, d)** Images of TUNEL **(c)**, $\times 20$ magnification) and Ki67 **(d)**, $\times 40$ magnification) staining of primary tumors are shown in the left panels. The images are representative of multiple fields of tumor sections from six tumors per group. Numbers of positive TUNEL **(c)** and Ki67 **(d)** staining cells were determined as described in Materials and methods. The data are presented as the mean \pm s.e. $*P < 0.001$ (right panels). **(e)** Images of IHC staining of CD31 in primary tumors are shown in the upper panel. Staining density and number of vessels were measured and quantified as described in Materials and methods. The data are presented as the mean \pm s.e.m. $*P < 0.03$ (lower panel).

(Bcl-2, Zeb2 and VEGFA) showed reduced expression in HCT116 cells expressing miR-192 as compared with vector control cells (Figures 4a, c, e, left panels). To determine whether they are direct targets of miR-192, we cloned the 3' UTRs of Bcl-2, Zeb2 or VEGFA containing the potential miR-192 recognition element into a luciferase construct described above. Reporter assays revealed that miR-192 repressed the UTRs of Bcl-2 and Zeb2 but not of VEGFA (Figure 4a, $*P < 0.001$, Figure 4c, $*P < 0.01$ and Figure 4e, right panels). This indicates that expressions of Bcl-2 and Zeb2 are directly regulated by miR-192, whereas VEGFA could be an indirect target of miR-192.

Given the role of Bcl-2 in cell survival, we next determined whether miR-192-mediated induction of apoptosis under GFDS could be reversed by restoration of Bcl-2 expression. Bcl-2 complementary DNA was introduced into miR-192-expressing HCT116 cells. Ectopically expressed Bcl-2 is resistant to down-regulation mediated by miR-192 due to the lack of 3' UTR. Restoration of Bcl-2 expression partially rescued miR-192-expressing cells from GFDS-induced apoptosis as reflected by reduced caspase 3 cleavage (Figure 4b), indicating that miR-192 mediates cell survival partially through regulation of Bcl-2 expression.

Zeb2 is a master transcriptional factor involved in EMT, one of the important determinants of metastasis. Zeb2 has been shown to promote EMT through suppressing expression of E-cadherin.^{29,30} In miR-192-expressing HCT116 cells, expression of E-cadherin increased as compared with the control cells (Figure 4d, upper panel), which is consistent with reduced expression of Zeb2 in those cells (Figure 4c, left panel). When Zeb2 expression was knocked down by Zeb2-specific small hairpin

RNAs in HCT116 cells, E-cadherin expression increased (Figure 4d, lower panel). These results indicate that miR-192 upregulates E-cadherin expression through downregulating Zeb2 expression.

miR-192 inhibits expression of its target genes *in vivo*

To determine whether miR-192 represses expression of Bcl-2, Zeb2 or VEGFA *in vivo*, we examined their expression in the primary tumors of mice implanted with vector- or miR-192-expressing HCT116 cells described above. Immunohistochemistry (IHC) staining revealed that expression of Bcl-2 and VEGFA was significantly reduced in the primary tumors of miR-192-expressing cells as compared with those of the control cells (Figures 5a, b, $*P < 0.001$), which likely contributes to the differences observed in apoptosis (TUNEL, Figure 3c) and angiogenesis (CD31, Figure 3e), respectively, between these two groups. In addition, a remarkable reduction of Zeb2 expression, especially in the nucleus, was also detected in the primary tumors of miR-192 cells when compared with the control tumors (Figure 5c, $*P < 0.001$). Consequently, an increase in membrane expression of E-cadherin was observed in those tumors (Figure 5d, $*P < 0.001$). These results indicate that miR-192 inhibits the expression of Bcl-2, VEGFA and Zeb2 in the primary tumors, combination of which contributes to diminished liver metastasis in the orthotopic model.

miR-192 is differentially expressed in human colon tumors at different stages

To demonstrate the clinical relevance of miR-192 expression in human colon cancer, we extended our analyses by assaying

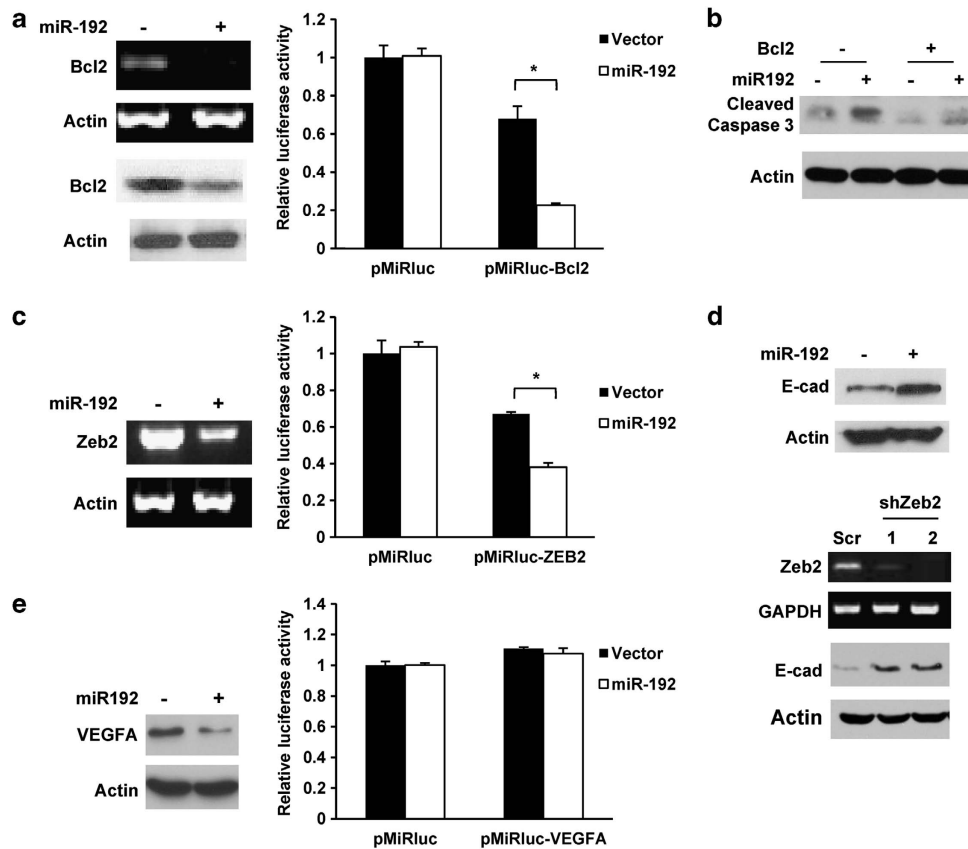


Figure 4. miR-192 directly or indirectly regulates expression of Bcl2, Zeb2 and VEGFA. (a, c, e), Bcl2 mRNA and protein (a) Zeb2 mRNA (c) and VEGFA protein (e) were determined in HCT116 control and miR-192-expressing cells (left panels). Luciferase constructs were co-transfected into HCT116 cells with β -gal expression vector. Luciferase activity was normalized to β -gal activity. The data are presented as mean \pm s.e.m. of triplicate experiments (right panels). * $P < 0.001$ (a) and * $P < 0.01$ (c). (b) Bcl2 was ectopically expressed in HCT116 control and miR-192-expressing cells. Western blot analysis of cleaved caspase 3 was performed. (d) Expression of E-cadherin was determined in HCT116 control and miR-192-expressing cells (upper panel). Expression of E-cadherin was determined in HCT116 control (Scr) and Zeb2-knocked down (shZeb2) cells (lower panel).

miR-192 expression in human colonic adenocarcinoma specimens. RNA was isolated from sections prepared from 24 normal colon and 29 individual colon cancer patients at stages I, II or IV. As shown in Figure 6a, quantitative real-time PCR assays demonstrated that expression of miR-192 in normal colonic epithelial cells was statistically higher than that in adenocarcinomas of stage I (** $P < 0.004$), II and IV (***) $P < 0.001$). Importantly, there was a significant decrease in miR-192 expression in stage IV tumors as compared with those of stage I (* $P < 0.02$) or II (***) $P < 0.001$). Expression of miR-192 in individual tissue samples is shown in Figure 6b. These results indicate that expression of miR-192 decreases early in colon cancer development and further decreases during colon cancer progression. Taken together with *in vitro* and *in vivo* results in colon cancer cells, our studies demonstrate that miR-192 has an important role in colon cancer development and progression. In addition, the stage-dependent decrease in miR-192 expression suggests that miR-192 expression could be used as a potential biomarker to predict metastasis in colon cancer patients.

Evaluate expression of Bcl-2, VEGFA and Zeb2 in human specimens

To determine whether the target genes of miR-192 show an inverse pattern of expression in human specimens, we examined expression of Bcl-2, VEGFA and Zeb2 in tissue samples from normal colon and colon cancer patients at stage I or IV.

IHC staining revealed that expression of Bcl-2, VEGFA and Zeb2 in adenocarcinomas of stages I and IV was statistically higher than that in normal colonic epithelial cells (Figure 7, * $P < 0.02$, ** $P < 0.01$, *** $P < 0.001$). In addition, their expression further increased in stage IV tumors compared with those of stage I (Figure 7, * $P < 0.02$, ** $P < 0.01$). These results indicate an inverse relationship between expression of miR-192 and its target genes in human specimens.

DISCUSSION

Metastasis has been associated with resistance to stress-induced apoptosis that can be attributed to aberrant survival capacity of cancer cells.^{5,25} Therefore, inducing cell death has become an important therapeutic strategy to treat metastasis. We have demonstrated in this study that miR-192 decreases cell survival capacity under stress by inhibiting expression of Bcl-2 (Figures 4a, b). In addition, as environmental restriction on growth is very common in solid tumors, a mechanism that enables increased angiogenesis to provide sufficient nutrients and oxygen would be highly advantageous to malignant cells. VEGFA is a potent angiogenic factor that has an essential role in cancer. We show here that, miR-192 suppresses expression of VEGFA and as a consequence, reduces angiogenesis *in vivo* (Figure 3e). Furthermore, EMT has been shown to contribute to metastasis, and Zeb2 is an important regulator of EMT. Our studies indicate that Zeb2 is

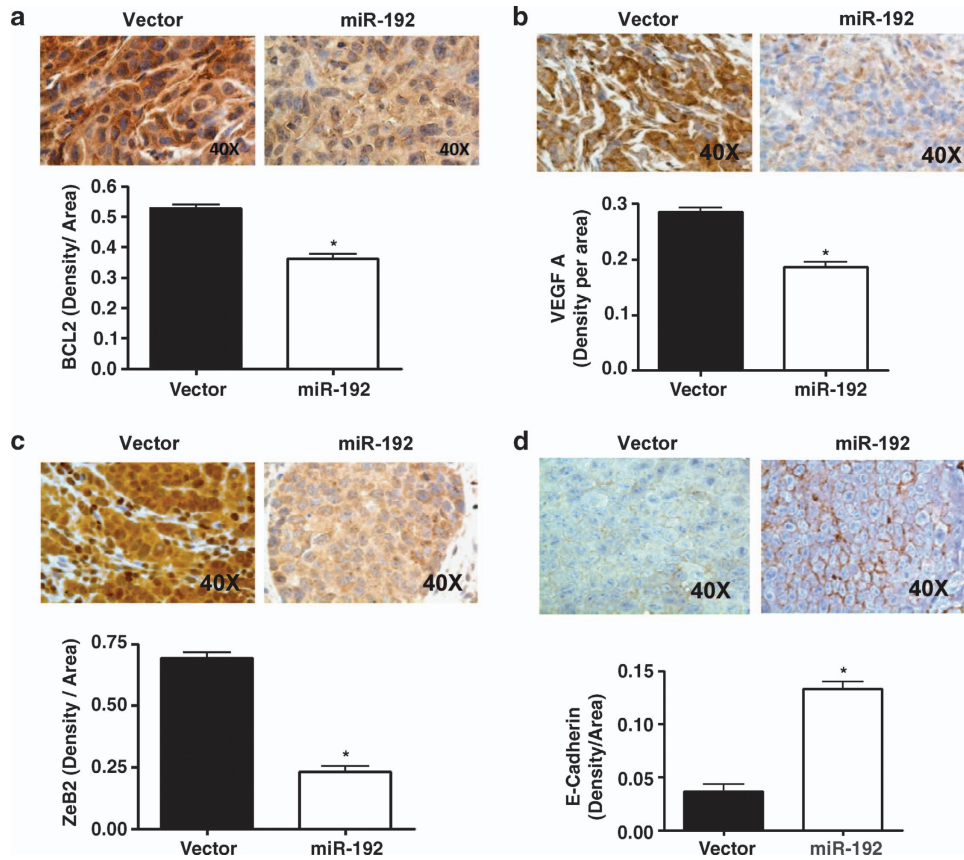


Figure 5. miR-192 inhibits expression of its target genes *in vivo*. Images of IHC staining of Bcl2 (a), VEGFA (b), Zeb2 (c) and E-cadherin (d) in primary tumors are shown in the upper panels. Staining density was measured and quantified as described in Materials and methods. The data are presented as the mean \pm s.e.m. * $P < 0.001$ (lower panels).

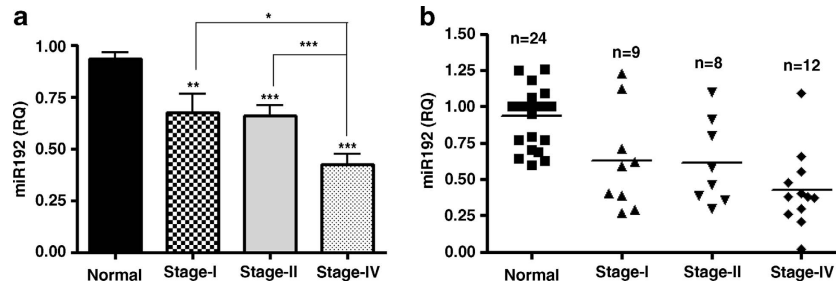


Figure 6. Expression of miR-192 in human colon cancer specimens. (a) RNA samples prepared from 24 normal colon and 29 individual colon cancer patient specimens at different stages were used to determine miR-192 expression as described in Materials and methods. The data are presented as the mean \pm s.e.m. * $P < 0.02$, ** $P < 0.004$, *** $P < 0.001$. (b) Expression of miR-192 in individual tissue samples is shown. Bars indicate mean values of each group of samples.

a direct target of miR-192 and that miR-192 increases E-cadherin expression through decreasing Zeb2 expression (Figures 4c, d). Thus, the concomitant suppression of Bcl-2, VEGFA and Zeb2 by miR-192 could decrease metastatic potential by regulating multiple steps of the metastasis cascade. In support of this view, when tested in an *in vivo* orthotopic model that recapitulates the pattern of colorectal cancer metastases in humans, miR-192 inhibits metastatic colonization in the liver (Table 1 and Figure 3b). Taken together, our studies indicate that reduced cell survival, angiogenesis and EMT conferred by miR-192 contribute to diminished metastasis of colon cancer cells *in vivo*.

TGF β has an important role in tumorigenesis and metastasis. We and others have demonstrated that TGF β suppresses tumor

initiation in a variety of cancers including colon cancer.^{31–34} However, the role of TGF β signaling in metastasis has been controversial. Although many studies show that TGF β promotes EMT and metastasis,^{35,36} others demonstrate that TGF β suppresses metastasis.^{37,38} Our previous studies indicate that loss of TGF β signaling is associated with increased metastasis, whereas enhanced TGF β signaling suppresses metastasis in an orthotopic model of colon cancer,¹⁹ indicating a metastasis suppressor function of TGF β in a subset of colon cancer cells. Given the anti-metastatic role of miR-192, TGF β might suppress metastasis through induction of miR-192 and subsequent suppression of pro-metastatic Bcl-2, VEGFA and Zeb2 in those cells. On the other hand, TGF β might promote EMT and/or metastasis by

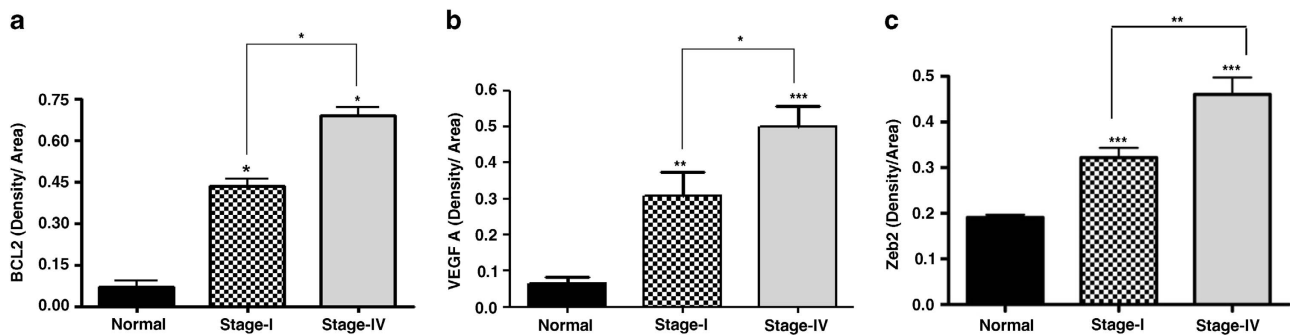


Figure 7. Expression of Bcl-2, VEGFA and Zeb2 in human colon cancer specimens. IHC staining of Bcl2 (a), VEGFA (b) and Zeb2 (c) was performed in normal colon and colon cancer patient specimens at stages I and IV. Staining density was measured and quantified as described in MATERIALS AND METHODS. The data are presented as the mean \pm s.e.m. * $P < 0.02$, ** $P < 0.01$, *** $P < 0.001$.

downregulating miR-192 expression in other cell models in which TGF β acts as a EMT promoter.^{39,40} We have previously shown that TGF β signaling suppresses tumor formation and metastasis through many different mechanisms including inhibition of cell proliferation and induction of apoptosis.^{19,31} Our current studies further highlight the importance of the TGF β regulatory pathway in colon cancer progression and may provide another important and novel mechanistic basis for TGF β -mediated suppression of metastasis. The frequent loss of TGF β signaling, which occurs in 30–50% of colon cancer patients,^{41,42} may account for one of the mechanisms underlying reduced expression of miR-192 in colon cancer patients (Figure 6).

High levels of Bcl-2 expression in primary colon cancer have been associated with over 60% of the cases at the time of diagnosis.⁴³ The regulation of Bcl-2 mRNA is critical in the development of cancer.⁴⁴ Our study suggests that miRNA-mediated modulation is an additional mechanism for Bcl-2 expression in colon cancer. Of note, in addition to miR-192, a recent study shows that Bcl-2 expression can be repressed by miR-15 and miR-16 to induce apoptosis.⁴⁵ Similar to Bcl-2, expression of VEGFA is increased in colon cancer and is associated with disease localization, stage and long-term disease-specific survival.⁴⁶ It has been shown that expression of VEGFA is mainly regulated at the transcriptional and translational levels.^{47,48} Recent studies indicate that VEGFA expression can also be regulated by miRNAs at the post-transcriptional level.⁴⁹ Our current findings that miR-192 suppresses VEGFA expression suggest that miRNA-mediated post-transcriptional regulation could be a novel mechanism for the upregulation of VEGFA expression in colon cancer. Moreover, overexpression of Zeb2 at the invasion front correlates with tumor progression and predicts cancer-specific survival in primary colorectal cancer.⁵⁰ The miR-200 family has been reported to regulate Zeb1 and Zeb2 expression.^{29,30} Zeb2 has been previously shown to be a target of miR-192.^{51,52} Our results confirm that miR-192 is another miRNA whose reduced expression could contribute to overexpression of Zeb2 in cancer. Given that miRNA targets can also be regulated by other mechanisms, it is conceivable to observe a tissue- or context-specific effect of miRNAs. miR-192 has been shown to suppress activated leukocyte cell adhesion molecule expression in gastric cancer cells.⁵³ However, we did not detect any effect of miR-192 on activated leukocyte cell adhesion molecule expression in colon cancer cells (data not shown).

Although miR-192 has been shown to be downregulated in colorectal cancer,^{22,23} it is not clear whether expression of miR-192 differs in cancer patients at different stages. We showed here that expression of miR-192 in normal colonic epithelia cells is statistically higher than that in adenocarcinomas of stages I, II and IV, and that there is a significant reduction of miR-192 expression in stage IV tumors as compared with those of stage I or

II (Figure 6). Consistently, expression of Bcl-2, VEGFA and Zeb2 shows an inverse pattern relative to miR-192 expression in human specimens (Figure 7). Therefore, our findings underscore the clinical relevance and prognostic significance of miR-192 expression in colon cancer. Bcl-2, VEGFA and Zeb2 may have a key role in colon cancer progression driven by the loss of miR-192. However, there are hundreds of genes regulated by a single miRNA. It is likely that miR-192 can target other oncogenes relevant to colon cancer.

Our studies may have therapeutic significance for treatment of colon cancer metastases. Much effort has been made to pharmacologically target Bcl-2, including development of Bcl-2 antisense phosphorothioate oligonucleotide compound, modified peptides and small-molecular inhibitors.^{54,55} Avastin, a monoclonal antibody directed against VEGFA, has been widely used with chemotherapy to treat metastatic colon cancer with improved efficacy.⁵⁶ However, some patients eventually develop resistance to the treatment.⁵⁷ The restoration of miR-192 could be theoretically more effective, due to the simultaneous inhibition of Bcl-2, VEGFA, Zeb2 and other possible targets. In our experimental models, re-expression of miR-192 in colon cancer cells was able to significantly suppress their metastatic potential. Thus, although colon cancer is extremely heterogeneous in the clinical setting, miR-192 may have considerable therapeutic potential, either as single agent or in combination with other therapeutic drugs.

MATERIALS AND METHODS

Cell Lines and Reagents

The human colon carcinoma HCT116, RCA, HCT116b and FET cell lines were cultured in McCoy's 5A serum-free medium (Sigma, St Louis, MO, USA) supplemented with 10ng/ml epidermal growth factor, 20ug/ml insulin and 4ug/ml transferrin.⁵⁸ When cells were under growth factor and nutrient deprivation stress, they were cultured in the medium in the absence of growth factor or serum supplements. Recombinant human TGF β 1 and miScript miR-192 inhibitor were purchased from R&D Systems (Minneapolis, MN, USA) and Qiagen (Venlo, Netherlands), respectively. The sources of antibodies for western blot analyses are: caspase 3, poly ADP ribose polymerase and phosphorylated AKT (Ser473), Cell Signaling Technology (Beverly, MA, USA), Bcl2 and VEGFA, Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), actin, Sigma, E-cadherin, a gift from Dr Masatoshi Takeichi.

Reverse transcriptase PCR and Q-PCR- for reverse transcriptase PCR, 2 μ g of RNA was reverse transcribed with M-MLV reverse transcriptase (Promega, Madison, WI, USA) using a random primer. Two microliters complementary DNA product was used to amplify human Bcl2 and Zeb2. Primer sequences for Bcl2 were 5'-ATCAAGTGTCCGCGTGATT-3' (forward) and 5'-AGCTGGCTGGACATCTCG-3' (reverse) and for Zeb2 were 5'-CGGCATATGGTGACACACAA-3' (forward) and 5'-CACCTAAGTGCTGCATTGGA-3' (reverse). miR-192 expression was determined by Taqman microRNA assays (Applied Biosystems, Foster City, CA, USA). U6 was used as an endogenous reference gene.

Luciferase assays

The miR-192 binding site was synthesized and cloned into an Ambion pMIR-REPORT vector to generate pMiRluc-192. 3' UTRs of Bcl2, Zeb2 and VEGFA containing miR-192 binding sites were amplified and cloned into the same vector to generate pMiRluc-Bcl2, -Zeb2 and -VEGFA, respectively. The reporter was co-transfected with a cytomegalovirus β -galactosidase vector using FuGENE HD (Roche, Boulder, CO, USA). Luciferase activity was measured 4 h later using luciferase reporter assay (Promega, Madison, WI, USA). Values were normalized with β -galactosidase activity. Statistical analyses were performed using the Student's *t*-test.

Plasmid, retroviral infection and apoptosis assays

Complementary DNA encoding miR-192 precursor (~300 bp) was cloned into a retroviral expression vector and transduced into colon cancer cells. Cells were deprived of serum and growth factors for the indicated time (0–2 days). Apoptosis was detected using a DNA fragmentation ELISA kit (Roche, Indianapolis, IN, USA). Statistical analyses were performed using the Student's *t*-test.

In vivo orthotopic model

Experiments involving animals were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Orthotopic implantation was performed as previously described.⁵⁹ Briefly, green fluorescent protein-labeled cells (2×10^6) were directly injected to the subserosal layer of the cecum of male athymic nude mice (4–5 weeks old). Mice were euthanized between 4 and 5 weeks. Cecal tumors and livers were dissected out and washed with cold phosphate-buffered saline. Green fluorescent protein imaging was performed using Illumatool Light Source (Tokyo, Japan).

IHC staining

Formalin-fixed paraffin-embedded tissue blocks of primary tumors were stained for TUNEL, Ki67, CD31, Bcl2, VEGFA and Zeb2 expression. TUNEL assays were performed using ApopTag Peroxidase *In Situ* Oligo Ligation Apoptosis Detection Kit (Millipore, Billerica, MA, USA). The sources of primary antibodies used for IHC are: Ki67 (BD Pharmingen, San Diego, CA, USA), CD31 (Abcam, Cambridge, UK), Bcl2, VEGFA and Zeb2/SIP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and E-cadherin (a gift from Dr Masatoshi Takeichi). Tissue slides were subjected to antigen retrieval using Novocastra Epitope Retrieval Solutions, pH6 (Leica, Wetzlar, Germany), followed by incubation with primary antibodies at 4°C overnight. To neutralize endogenous peroxidase, Dako Dual Endogenous Enzyme Block (Dako North America, Inc., Pittsburgh, PA, USA) was used for all the markers except for Zeb2. For CD31 and VEGFA, the secondary antibody was Envision Flex horseradish peroxidase-labeled Polymer Anti-Rabbit (DAKO). A biotin goat anti-mouse secondary antibody (BD Pharmingen) was used for Ki67, Bcl2 and E-cadherin followed by incubation with Streptavidin-Horseradish-Peroxidase (SAV-HRP, BD Pharmingen). For Zeb2, Ultravision LP Detection System (Thermo Scientific, Waltham, MA, USA) was used.

Apoptosis and proliferation were determined quantitatively by counting the numbers of positively stained cells for TUNEL and Ki67 at $\times 20$ and $\times 40$ magnification, respectively. Staining density of CD31, Bcl2, VEGFA, Zeb2 and E-cadherin was quantified with ImagePro plus 7.0 Software (Media Cybernetics, Inc., Rockville, MD, USA). Six animals were analyzed for each cell type. Ten histologically similar fields were randomly selected from each slide for analysis. *P*-values were calculated using Student's *t*-test.

Detection of expression of miR-192 and its target genes in human tissue samples

Formalin-fixed paraffin-embedded blocks of normal human colon removed for reasons other than malignancy and those containing invasive colonic adenocarcinomas were obtained from files of Department of Pathology and Microbiology at University of Nebraska Medical Center (UNMC). The ages of all patients (including both men and women) were between 55 and 85 years. The cancer patients did not receive neoadjuvant therapy before surgical removal of the tumors. The study was performed with the approval of the ethics committee (Institutional Review Board) of UNMC.

The formalin-fixed paraffin-embedded slides were deparaffinized using histoclear and rehydrated following hematoxyline staining to identify the normal crypt and tumor areas under the microscope. The normal crypt or tumor areas were then scraped with a fine scalpel and collected into an

ependorf tube. RNA was extracted using the miRNeasy formalin-fixed paraffin-embedded kit (Qiagen). For Q-PCR, 50 ng of RNA was reverse transcribed using QuantiTect Reverse Transcriptase kit (Qiagen). A quantity of 2.5 μ l of complementary DNA product was used to amplify miR-192 using StepOne Real-Time PCR System (Applied Biosystems). U6 was used as an internal control. Expression of Bcl-2, VEGFA and Zeb2 was determined by IHC staining as described above.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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