Periostin potently promotes metastatic growth of colon cancer by augmenting cell survival via the Akt/PKB pathway

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Summary

Molecular mechanisms associated with tumor metastasis remain poorly understood. Here we report that acquired expression of periostin by colon cancer cells greatly promoted metastatic development of colon tumors. Periostin is overexpressed in more than 80% of human colon cancers examined with highest expression in metastatic tumors. Periostin expression dramatically enhanced metastatic growth of colon cancer by both preventing stress-induced apoptosis in the cancer cells and augmenting endothelial cell survival to promote angiogenesis. At the molecular level, periostin activated the Akt/PKB signaling pathway through the $\alpha_v\beta_3$ integrins to increase cellular survival. These data demonstrated that the survival-promoting function is crucial for periostin to promote tumor metastasis of colon cancer.

Introduction

The process of malignant transformation involves the sequential acquisition of a number of genetic and epigenetic alterations as a result of increasing genomic instability caused by defects in checkpoint controls (Abraham, 2001; Hahn and Weinberg, 2002; Hanahan and Weinberg, 2000; Nowak et al., 2002). In humans, these alterations allow cancer cells to acquire the capabilities to become self-sufficient in mitogenic signals, deregulate the control of cell cycle, escape from apoptosis, and obtain unlimited replication potential via the reactivation of telomerase (Artandi and DePinho, 2000; Blasco, 2002; Hanahan and Weinberg, 2000). Within a growing tumor mass, the genetic changes during tumor progression also enable cancer cells to gain the ability to induce angiogenesis, invade neighboring tissues, and metastasize to distant organs (Cavallaro and Christofori, 2000; Chambers et al., 2002; Folkman, 2002).

The development of metastatic tumors consists of a series of complex sequential events. Neoplastic cells within the primary tumor mass have to journey through multiple steps, including intravasation into the circulation, survival in the circulation systems, arrest and extravasation into a new tissue, initiation and maintenance of growth, and reactivation of angiogenesis, in order to successfully establish metastatic colonies in distant organs (Chambers et al., 2002; MacDonald et al., 2002). Hence, few of the large number of cancer cells shed from a primary tumor ever form metastatic tumors (Chambers et al., 2002; Weiss, 1990; Wong et al., 2001). As a consequence of this complexity, the metastatic process involves genetic changes that underlie alterations in a variety of cellular functions for both cancer cells and the host environment, including the control of cell proliferation, survival, motility, cell-cell adhesion, and interactions with the extracellular matrix (ECM) (Jacks and Weinberg, 2002; Liotta and Kohn, 2001; Weaver et al., 2002). Although it is clear that those genetic changes often lead to alterations in the expression pattern of specific genes, relatively little is known about the nature of those molecular events that are directly involved in the metastatic process.

As the second leading cause of death from malignancy in the United States, colorectal cancer is the most common type of tumor to metastasize to the liver (Markowitz et al., 2002; Takeda et al., 2002). Actually, metastasis accounts for the majority of mortality associated with colorectal cancer, making an effective treatment of this disease a complicated goal. Taking advantage of the availability of both primary and metastatic tumor samples from the same patients with metastatic colon cancers, we searched for genes upregulated during metastasis and report here the identification of periostin as a candidate gene whose acquired expression by colon cancers is associated with enhanced tumor metastatic growth and angiogenesis, pri-

SIGNIFICANCE

Metastases, rather than primary tumors, are responsible for most cancer deaths. To reduce malignancy death, it is critical to identify and target proteins essential to the metastatic process. To this end, we identified periostin as a protein whose expression pattern is intimately associated with the later stage of tumor progression. Through extensive functional analyses both in vitro and in vivo, periostin was found to potently promote metastatic development of colon cancer. Given the key role periostin plays in tumor metastasis, this secreted protein could serve as a potential therapeutic target for the control of metastatic growth of colon cancers.

marily by promoting cell survival for both cancer cells and endothelial cells.

Results

Periostin is differentially expressed in human primary colon carcinomas and metastatic tumors

In order to identify genes that are potentially involved in promoting late-stage progression of colon cancers, particularly metastasis, we performed a series of differential cDNA display analyses on mRNA samples isolated from normal colon tissue, primary colon cancer, and metastatic tumor in the liver derived from the same patient. One of the genes identified to have a differential expression pattern among these three types of samples was periostin (Figure 1A). Periostin is a secreted protein that was originally isolated from osteoblasts and found to be preferentially expressed in periosteum in bone tissue (Horiuchi et al., 1999; Takeshita et al., 1993). Periostin contains an N-terminal secretory signal peptide, followed by a cysteine-rich domain, four internal homologous repeats, and a C-terminal hydrophilic domain. The four internal repeats region of periostin shares a homology with the axon guidance protein fasciclin I that is involved in the development of nervous system in invertebrates (Zinn et al., 1988).

To determine if the observed upregulation of periostin is a common feature of colon cancer progression, we performed Northern blot analysis on 29 pairs of matched normal colon tissue and colon tumor samples. As shown in Figures 1B and 1C, periostin was found to be differentially overexpressed in more than 80% of human primary colon cancer samples examined. Quantitative analysis indicated that two-thirds (20 of 29 cases) of examined primary colon carcinomas showed a tumor/normal (T/N) ratio of periostin message expression higher than 5-fold, with one-third of these primary tumors showing a T/N ratio higher than 10-fold (Figure 1D). Furthermore, we found that periostin was overexpressed in all cases of colon metastatic tumors in the liver (Figure 1C). Importantly, the expression level of periostin in the hepatic metastases derived mostly from patients with relapses of their colon cancers was noticeably higher than that in the matched primary colon tumors from the same patients in 8 out of 9 cases examined (Figures 1C and 1E). Quantitative and statistical analysis indicated that the average T/N ratio of periostin message expression in those hepatic metastases was 2.6-fold of that in the matched colon primary tumors, and the difference in periostin message expression between the hepatic metastases and their matched colon primary tumors was significant ($p < 0.02$) (Figure 1E). These results suggest that late-stage metastatic tumors express higher levels of periostin, which may play a role during the metastatic stage of colon cancer progression.

To determine if the periostin protein is overexpressed in colon cancer cells in the tumor tissues, we examined a series of matched tissue sections, including normal colon mucosae, primary colon tumors, and metastases in the liver, from the same patients by immunohistochemical staining with an antibody against human periostin. The anti-periostin polyclonal antibody was purified through an affinity column and shown to recognize specifically the periostin protein (Figure 3A). The immunohistochemical staining for periostin was performed on 25 cases of primary colon carcinomas, including 8 cases of stage II, 9 cases of stage III, and 8 cases of stage IV tumors, as well as 9 cases of matched hepatic metastases derived mostly from patients with relapses of their colon cancers. As shown in Figure 2, the immunostaining analyses indicated that high levels of periostin were present in areas containing cancer cells of the

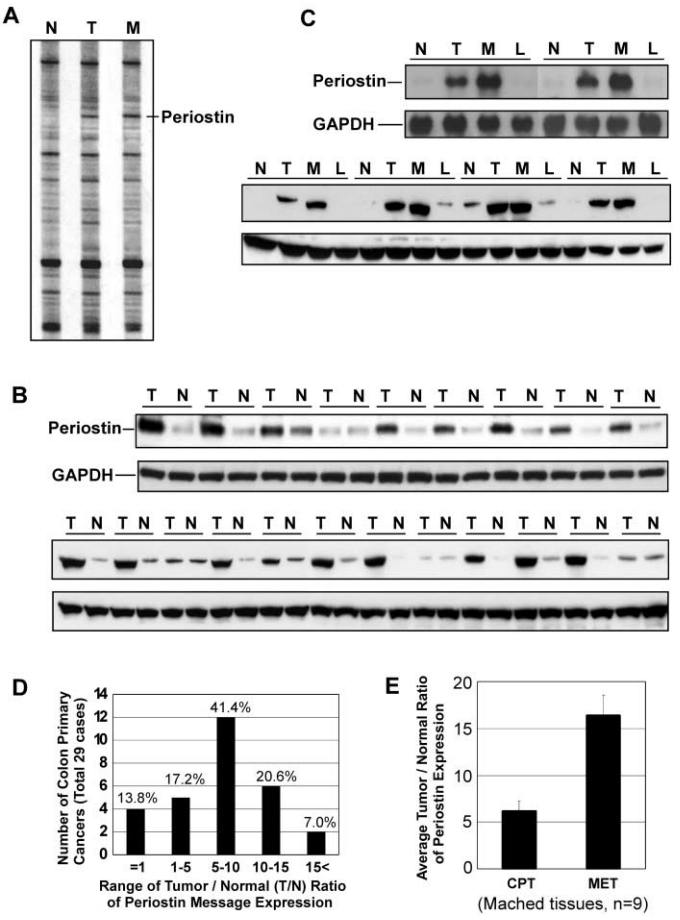

Figure 1. Differential expression of periostin in human primary and metastatic colon carcinomas

A: Identification of periostin as a differentially expressed gene in the colon metastatic tumor to the liver (M) and the matched primary colon tumor (T) versus normal colon tissue (N) from the same patient. ^{35}S-labeled PCR cDNA fragments from mRNA of these three types of samples from the same patient were displayed based on their molecular sizes.

B: Northern blot analysis of periostin message expression in paired primary colon tumor (T) and normal colon tissue (N) from the same patient. Total RNA samples from matched tissues were probed with the human periostin cDNA labeled with [α-^{32}P] dCTP. The same blot was probed with GAPDH for loading control.

C: Higher expression level of periostin mRNA was found in the metastatic tumor (M) in the liver than in the matched primary colon tumor (T) from the same patient in 5 out of 6 representative cases. Normal colon tissue (N) and normal liver tissue (L) were used as controls.

D: The number of colon primary carcinoma cases is plotted against the range of Tumor/Normal (T/N) ratios of hybridization signal for the matched samples from the Northern blot analysis in **B** and **C**. Two-thirds of colon carcinomas showed 5- to 15-fold periostin overexpression in contrast to the matched normal colon tissues.

E: The average Tumor/Normal (T/N) ratios of periostin expression between the liver metastases (MET) and the matched colon primary tumors (CPT) were quantified and plotted. Statistical analysis indicated that the periostin message expression in the hepatic metastases (MET) derived mostly from patients with relapses of their colon cancers was significantly higher ($p < 0.02$) than that in their matched colon primary tumors (CPT).

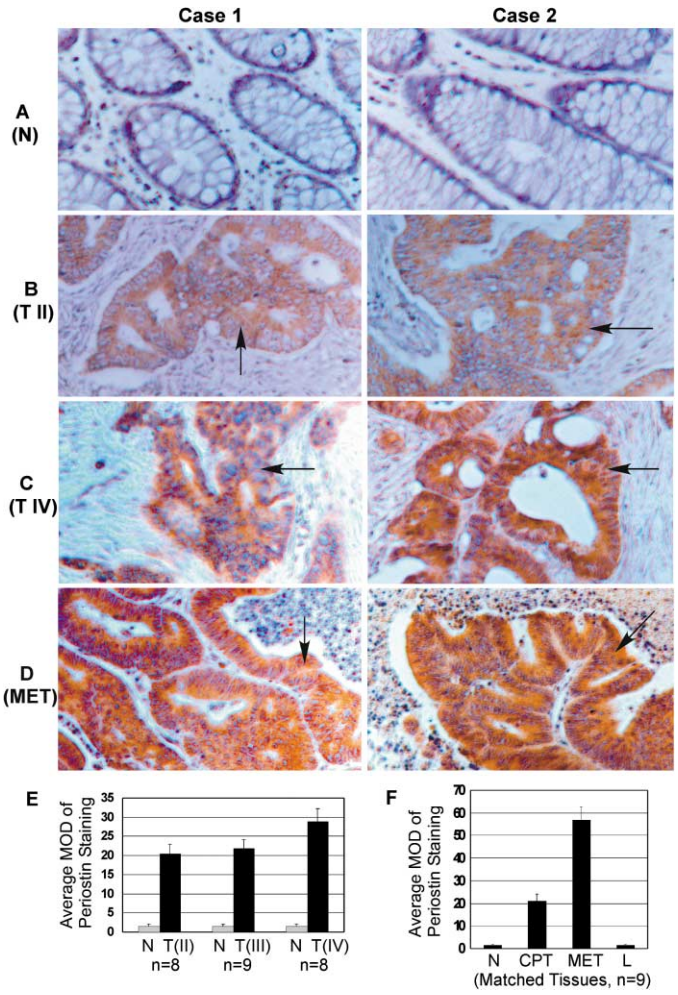

Figure 2. Immunohistochemical analysis of periostin expression in primary and metastatic colon cancers

The tissue sections of the primary colon cancers, their metastases in liver, and the matched normal colon mucosae were immunostained with an affinity-purified anti-periostin polyclonal antibody. The positive staining for periostin protein was shown in a reddish-brown color. All sections were counterstained with Hematoxylin showing a blue color.

A: Normal colon mucosae (N).

B: Stage II colon primary carcinomas (T II).

C: Stage IV colon carcinomas (T IV).

D: The matched hepatic metastases (MET) from patients with late relapses of colon cancers. Arrows indicate the carcinoma cancer cells. Two cases of matched samples as representative results are shown.

E: The average mean optical density (MOD) of periostin staining form the stage II (8 cases), stage III (9 cases), and stage IV (8 cases) primary cancers and their matched normal colon mucosae were statistically quantified and plotted according to the disease stages. The average MOD of periostin staining from the stage II, stage III, or stage IV colon carcinomas is significantly higher than that from the matched normal colon mucosae in each group, respectively ($p < 0.01$).

F: The average MOD (mean optical density) of periostin staining between the hepatic metastases (MET) and the matched colon primary tumors (CPT) were compared and plotted. Statistical analysis indicated that the average MOD of periostin staining in the hepatic metastases (MET) derived mostly from patients with relapses of their colon cancers is significantly higher ($p < 0.02$) than that in their matched colon primary tumors (CPT). N, the matched normal colon mucosae; L, the matched normal liver tissues.

primary colon tumors (Figures 2B and 2C) and metastatic tumors in the liver (Figure 2D). In contrast, periostin was undetectable in normal colon mucosae (Figure 2A). Consistent with the message expression analysis, more intense periostin staining was seen in the hepatic metastases (Figure 2D) derived from the patients with late relapses than that in the matched colon primary tumors (Figure 2B). Overall, the majority (75%) of primary colon tumors displayed prominent levels of periostin staining, whereas periostin was hardly detectable in all matched normal colon tissues. Quantitative analysis of the periostin immunohistochemistry staining results with a SAMBA 4000 computerized image analysis system with the Immuno 4.0 quantitative program indicated that the average mean optical density (MOD) of periostin staining in the stage II, stage III, or stage IV primary tumors is much higher than that in their matched normal colon mucosae in each group, respectively ($p < 0.01$, Figure 2E). Furthermore, the quantitative and statistical analysis revealed that the average MOD of periostin protein staining in the hepatic metastases was found to be 2.7-fold of that in the matched colon primary tumors, and the difference was significant ($p < 0.02$, Figure 2F). Taken together, these observations indicate that higher levels of periostin expression were associated with metastasized colon tumors.

Overexpression of periostin in colon cancer cells potently promote metastatic tumor growth in the liver

To assess the functional significance of elevated periostin expression levels during the progression of colon cancer, we investigated whether stable overexpression of periostin in a human colon cancer cell line could alter its metastatic potential in vivo when grown as xenografts in immune-compromised mice. For this purpose, we selected a subline of the human colon cancer cell line CX-1, CX-1NS, that expresses a very low level of endogenous periostin and displays negligible metastatic potential in vivo (Figure 3A and data not shown). A C-terminally His-tagged periostin expression construct was transfected into the CX-1NS cells, and after drug selection, five individual stable clones were isolated and verified for periostin expression. Three of those stable clones (A12, B08, and B19) and three vector-transfected controls (V03, V04, and V05) were used in subsequent experiments. The expressions of the C-terminally His-tagged periostin in these three clones were verified with specific antibodies against His-tag and periostin (Figure 3A). Intriguingly, overexpression of periostin in CX-1NS cells led to a noticeable morphological change with an appearance of fibroblast-like cell shape (Figure 3B) but did not result in an alteration in the rate of cell proliferation in vitro, as measured by the ^{3}H-thymidine corporation assay (data not shown). To investigate the impact of periostin overexpression on tumor growth in vivo, we performed xenograft assays via subcutaneous injection of the tumor cells into the nude mice to assess the potential differences in tumor growth between the periostin-overexpressing and the vector control clones. As shown in Figure 3C, periostin overexpression appeared to enhance the subcutaneous tumor growth over the control with an average ratio of less than 2-fold in tumor weight between the two types of cells. Thus, although periostin could enhance tumor growth in the context of subcutaneous in vivo environment, the effect was moderate.

We next examined whether periostin overexpression could affect the metastatic potential and growth of the cancer cells in vivo. Equal numbers of cells (6×10^6/animal) of the three periostin-producing CX-1NS clones or the three vector-trans-

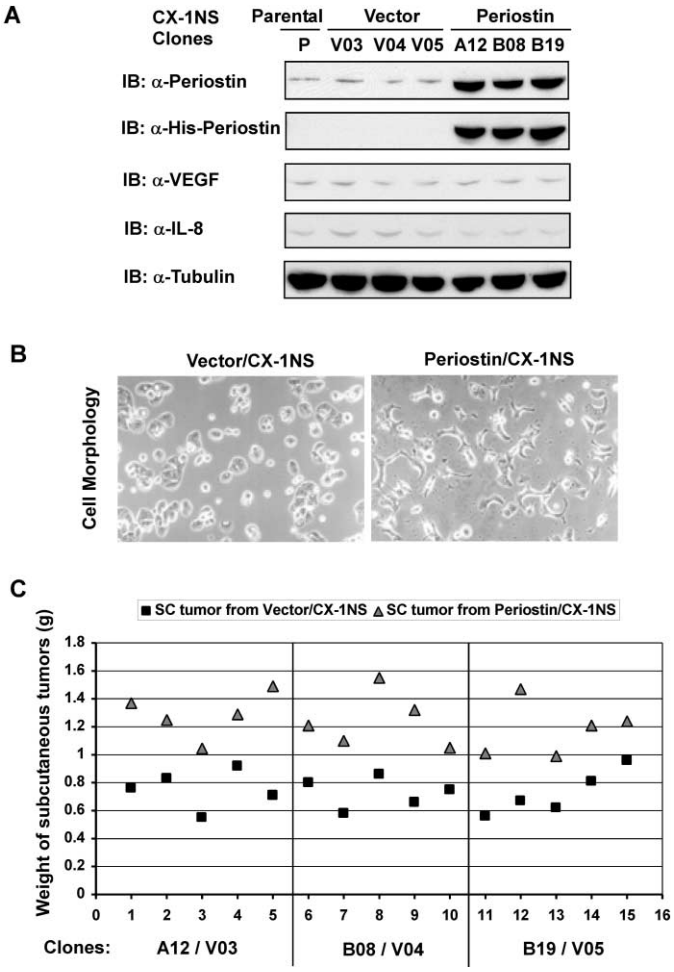

Figure 3. Characterization of periostin-overexpressing CX-1NS clones and vector control clones

A: The conditional media from parental CX-1NS cells, three vector control CX-1NS clones (V03, V04, V05), and three periostin-overexpressing CX-1NS clones (A12, B08, B19) were immunoblotted with α-periostin, α-His, α-VEGF, α-IL-8 antibodies. The expression levels of VEGF and IL-8 were shown unchanged among different clones. The whole-cell lysates from all samples were blotted with α-tubulin for loading control.
B: Overexpression of periostin in CX-1NS cells resulted in cellular morphological change with an appearance of fibroblast-like cell shape.
C: The tumor weights of subcutaneous tumors derived from three clones of periostin-overexpressing CX-1NS cells (A12, B08, B19) and three clones of vector control CX-1NS cells (V03, V04, V05). The average ratio of tumor weight between periostin-expressing CX-1NS tumor and vector control CX-1NS tumor is less than 2-fold.

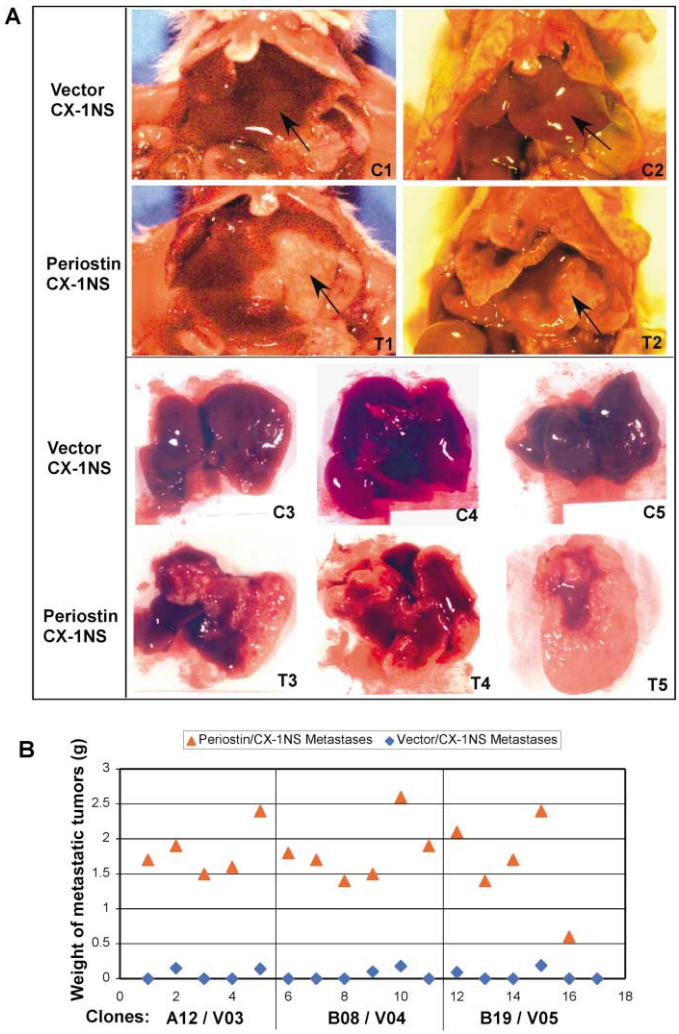

Figure 4. Overexpression of periostin in colon cancer cells dramatically promoted tumor metastasis in the liver via intrasplenic injection as xenografts in nude mice

A: Representative results showing that injection of periostin-producing CX-1NS colon cancer cells into spleen led to the formation of large metastases in the liver (T1-T5). However, no visually observable liver metastases were seen in the mice injected with the same number of vector-transfected CX-1NS control cancer cells (C1-C5). Large metastatic tumors derived from the periostin-producing CX-1NS colon cancer cells were seen to occupy almost the entire liver in some mice (T1, T2, T5). Arrows indicate the metastatic tumors in the mouse liver (T1, T2) and the normal liver in the control mice (C1, C2). The isolated livers from control mice (C3–C5) and the livers with huge metastatic tumors (T3–T5) are shown in the lower part of the figure.
B: The weights of metastatic tumors in the liver isolated from 16 animals survived the procedure with injection of the periostin-overexpressing CX-1NS cells and from 17 mice injected with the control cells were collected and plotted together. Three periostin-overexpressing clones (A12, B08, and B19) and three vector control clones (V03, V04, and V05) were used in these experiments.

fected controls were introduced into the nude mice via intrasplenic injection. Five weeks after the inoculation of tumor cells, the animals were sacrificed for the examination of metastatic growth in the liver and other organs. Strikingly, multiple large tumor metastases were detected in the livers of 15 out of 16 mice that survived the procedure with injection of the periostin-producing CX-1NS cells (Figure 4), with a majority of the metastatic tumors grown to the extent to occupy almost the entire liver (T1, T2, T4, T5 in Figure 4A). In contrast, no visually observable metastatic tumors were found in the livers of the 17 control mice injected with the control cells (C1-C5 in Figure 4A), although 1–2 small tumor nodules were found in 6 of these mice upon examination with a dissecting microscope. The tumor weights of liver metastases isolated from each mouse of the two groups were plotted and shown in Figure 4B. Overexpression of periostin in colon cancer cells therefore strongly promoted tumor metastatic growth in vivo, supporting a critical role for periostin in the late stage of tumor progression.

Overexpression of periostin enhanced vascularization and reduced apoptosis in the growth of metastatic tumors in the liver

Since the extent of the enhanced metastatic growth due to the overexpression of periostin is far more dramatic than that of the enhanced subcutaneous growth by the same cancer cells (compare Figures 4B and 3C), we speculated that the periostin-mediated metastatic tumor development in the liver was unlikely only due to increased rate of tumor cell growth in vivo, and some other cellular activities promoted by periostin protein may also contribute to the fast metastatic development of the periostin-overexpressing colon cancer cells in the liver.

During the process of dissecting the metastatic tumors, we noticed that all metastases derived from the periostin-producing CX-1NS cancer cells displayed high levels of hemorrhaging (T1-T5 in Figure 4A), suggestive of elevated degrees of vascularization. To address this possibility, we examined the blood vessel density on the sections of those metastatic tumors by using immunohistochemical staining with the antibody MECA32 (anti-mouse panendothelial antigen), which recognizes an epitope constitutively expressed on vascular endothelium of most tissues including tumor nodules (Harmey et al., 2002). Quantitative analysis of vessel density of three metastases for each CX-1NS clone revealed that the blood vessel density of the metastatic tumors derived from the periostin-overexpressing CX-1NS cells is approximately 5-fold higher than that of the rare micrometastases derived from the control cells (Figure 5A). As shown in Figure 5B with the representative staining pattern of blood vessels, the metastatic tumors derived from the periostin-producing CX-1NS cancer cells were much more intensely vascularized than the small metastatic nodules derived from the control cancer cells. These results strongly suggest that periostin secreted by tumor cells may act in a paracrine manner to induce tumor angiogenesis during metastatic growth, a finding consistent with the notion that tumor angiogenesis is essential for the successful development of metastases (Chambers et al., 2002; Folkman, 2002; Takeda et al., 2002).

Since tumor metastatic growth is determined by the balance of cell proliferation and programmed cell death, mechanisms that promote cell survival or prevent apoptosis of cancer cells would favor the establishment of metastatic tumor colonies. To test this, we examined the extent of cellular apoptosis in those metastatic tumors. As shown in Figure 5C, very few apoptotic cells were found in the highly vascularized metastatic tumors derived from the periostin-producing CX-1NS cells. In contrast, significantly more apoptotic cells were detected in those small metastatic nodules derived from the vector-transfected CX-1NS control cells. These results indicate that periostin expression in colon cancer cells led to a higher level of cellular survival or a lesser extent of apoptosis that favors metastases development in vivo, an outcome that can at least partially be attributed to the induction of angiogenesis.

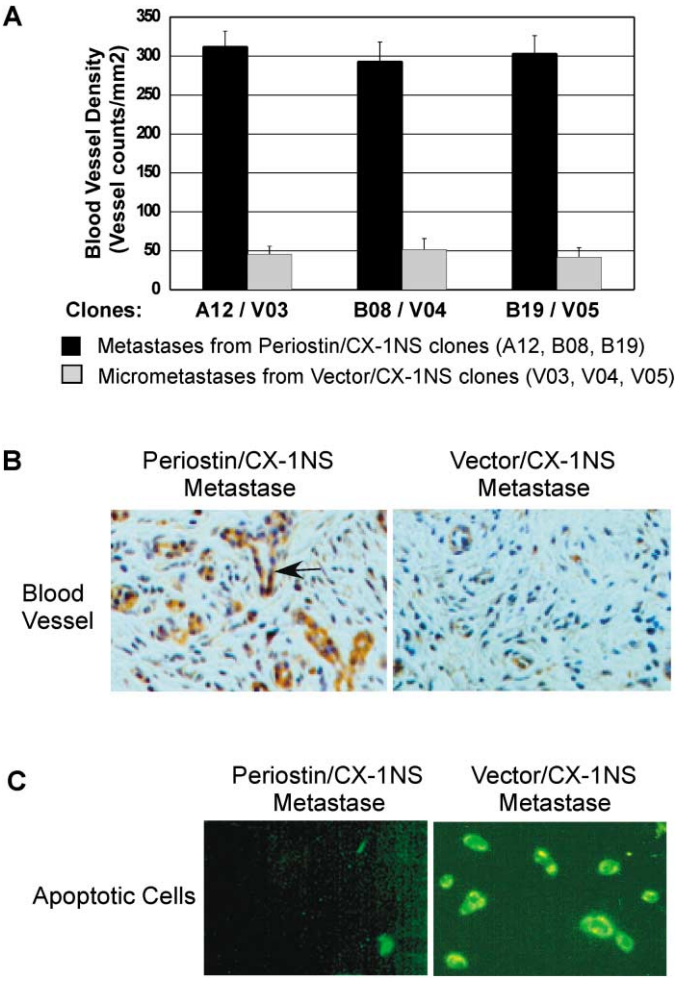

Figure 5. Analysis of blood vessel density and extent of apoptosis in the metastatic tumor sections

A: Metastatic tumor sections from macrometastases derived from three clones of periostin-overexpressing CX-1NS cells or from micrometastases derived from three clones of vector control cells were immunostained with the MECA32 antibody to examine the presence of endothelial cells and blood vessels. The blood vessel density in the metastatic tumors for each clone was analyzed and quantified statistically. The average vascular density in the metastatic tumors derived from the periostin-overexpressing CX-1NS cells was 5-fold of that in the micrometastases derived from the vector control CX-1NS cells.

B: A representative result showing that abundant blood vessels (in reddish-brown color) were detected in the metastatic tumors derived from the periostin-producing CX-1NS cells, but not in the small metastatic tumor nodules found in 6 of 17 control mice injected with the vector-transfected CX-1NS cancer cells.

C: Metastatic tumor sections were analyzed with ApoAlert DNA fragmentation assay to detect apoptotic cells. Many more apoptotic cells (in green color) were present in the small metastatic nodules derived from the vector-transfected CX-1NS control cells, but little apoptosis was seen in the large metastases derived from the periostin-producing CX-1NS cancer cells.

Periostin expression enabled cancer cells to successfully establish metastases in the liver

To further probe if the overexpression of periostin has a direct impact on the initial stage of micrometastatic colony formation, we introduced the green fluorescent marker GFP into the periostin-producing and the control CX-1NS cells. Equal numbers of cells (6×10^6/animal) of the periostin/GFP/CX-1NS clones or the vector/GFP/CX-1NS controls were injected into nude mice by intraportal vein injection, and the formation of GFP-labeled micrometastases in the liver was examined on day 5 and day 10 after injection. On day 5, the density of micrometastases or solitary cancer cells in the liver derived from the periostin/GFP/CX-1NS cells and the vector/GFP/CX-1NS controls was not significantly different as revealed by the presence of approxi-

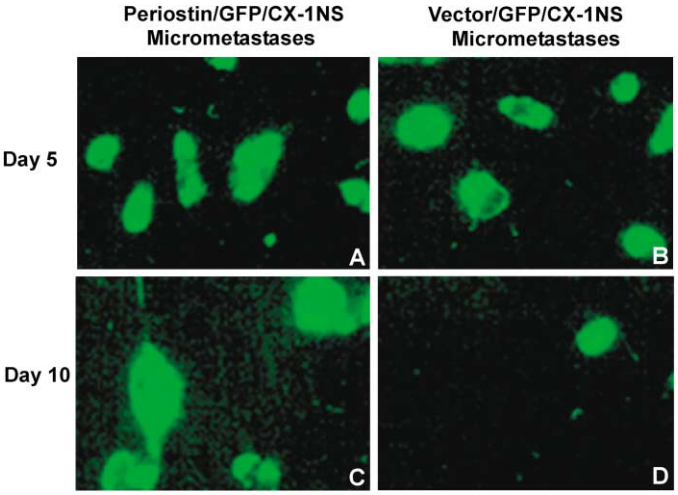

Figure 6. Significant differences in the ability to establish micrometastases by the periostin/GFP/CX-1NS cells and the vector/GFP/CX-1NS control cells in the liver of nude mice via intraportal injection

A and B: On day 5 after injection, no significant difference was observed in the number and the size of the GFP-labeled micrometastases derived from the periostin/GFP/CX-1NS cells (**A**) and from the vector/GFP/CX-1NS cells (**B**) in the liver sections.

C and D: On day 10 after injection, significant differences were seen in the number and the size of metastases derived from the periostin/GFP/CX-1NS cells (**C**) and the vector/GFP/CX-1NS cells (**D**).

mately equal numbers of GFP fluorescent-labeled colonies or single cells on liver sections (Figures 6A and 6B). On day 10, however, we found that those metastases derived from periostin/GFP/CX-1NS cells had grown to form larger metastatic colonies (Figure 6C), although the overall number of metastases was reduced. In contrast, those micrometastatic nodules derived from the vector/GFP/CX-1NS cells had failed to grow in size and the number of the micrometastases significantly decreased (Figure 6D), implicating the failure to establish metastases once seeded in the liver. These data suggest that periostin expression did not significantly affect the initial formation of micrometastases, but played a crucial role in the subsequent establishment of metastases in the new microenvironment in the liver.

Periostin augmented cellular survival for colon carcinoma cells and endothelial cells under conditions of growth stress

To explore the mechanism at the cellular level by which periostin promotes tumor metastasis and growth, we examined the biological effects of periostin on both tumor cells and the human microvessel endothelial cells (HMVECs) that are known to play an essential role in tumor angiogenesis under in vitro conditions that mimic the cellular stress inside metastatic tumors, such as hypoxia and nutrient deprivation with serum depletion. As shown in Figures 7A and 7B, results from three independent experiments with three clones (A12, B08, and B19) revealed that periostin-producing CX-1NS cells were indeed more resistant to serum starvation and hypoxia in comparison to three clones of the control cells (V03, V04, and V05). Similarly, the recombinant periostin was shown to enhance the ability of HMVECs to survive under the same serum depletion and hypoxic conditions (Fig-

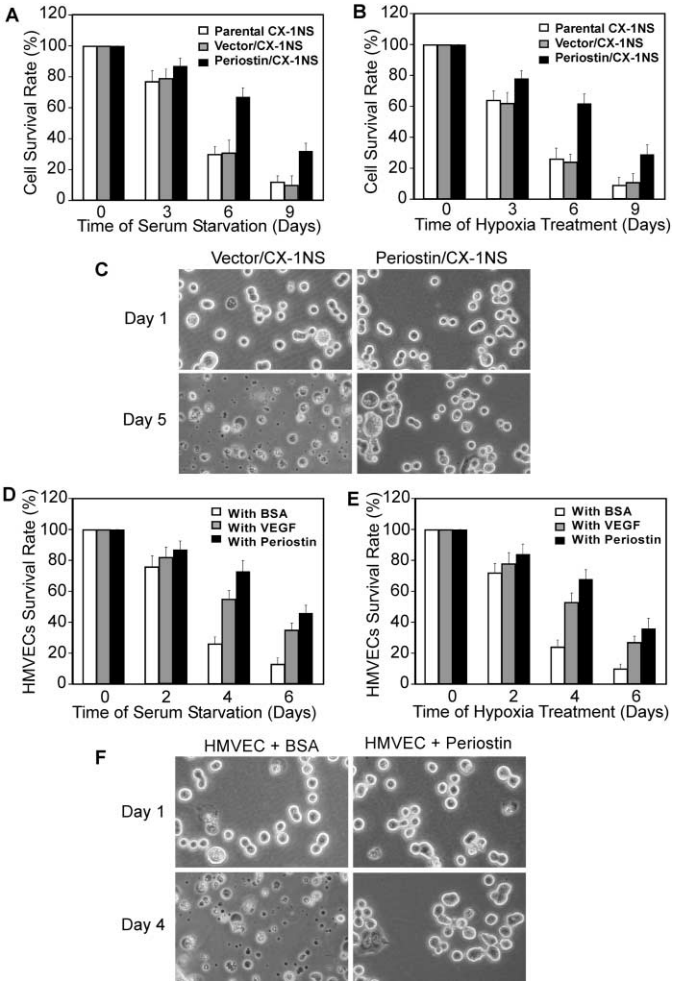

Figure 7. Periostin promoted cellular survival for both CX-1NS colon cancer cells and HMVECs under various stress conditions

A–C: Periostin expression in CX-1NS cells enhanced cellular survival under conditions of serum depletion (**A**) and hypoxia induced by DFO (Deferoxamine) treatment (**B**) and protected them from undergoing anoikis (**C**). Results in **A** and **B** were obtained from quantification of three independent experiments with three periostin-overexpressing clones and three vector control clones.

D–F: Incubation of HMVECs with the recombinant periostin (100 ng/ml) increased cellular survival under conditions of serum depletion (**D**) and hypoxia induced by DFO treatment (**E**), and protected them from anoikis induced by the loss of cell attachment (**F**). To prevent adhesion, CX-1NS cells and HMVECs were cultured on Petri dishes coated with PolyHema in serum-free medium. Results in **D** and **E** were quantified from three independent experiments with purified periostin protein.

ures 7D and 7E), which is compatible with the survival-promoting effect induced by the potent angiogenic factor VEGF. In addition, we observed that periostin overexpression or the presence of the recombinant periostin protected both tumor cells and HMVECs from undergoing anoikis, the cell death induced by loss of adhesion. When the control CX-1NS cells were cultured in the Petri dish coated with PolyHema to prevent adhesion, they displayed massive anoikis in 5 days (Figure 7C, left). However, most periostin-producing CX-1NS cells survived under the same condition (Figure 7C, right). Similar results were obtained with HMVECs untreated or treated with 100 ng/ml of

recombinant periostin for 4 days (Figure 7F). Taken together, these data demonstrate that periostin could act to promote cellular survival for both tumor cells and endothelial cells under stress conditions that are commonly associated with the metastatic tumor and a fast-growing tumor mass, such as hypoxia, nutrient depletion, and loss of adhesion.

Periostin activated the Akt/PKB pathway via the $\alpha_v\beta_3$ integrins to promote cellular survival

To explore the molecular mechanism by which periostin promotes cellular survival, we examined the potential effect of periostin on the activities of several known cellular survival pathways. To this end, we found that periostin expression or treatment with the recombinant periostin in both CX-1NS cancer cells and HMVECs led to activation of the Akt/PKB signaling pathway as measured by the specific phosphorylation of Akt1/PKBα on Ser473. As shown in Figure 8A, Ser473 phosphorylation that indicates the activation of Akt/PKB pathway (Nicholson and Anderson, 2002) was readily detected in the periostin-producing CX-1NS cells, but not in the parental and vector-transfected CX-1NS cells under the serum-free condition. Similarly, treatment of HMVECs with 100 ng/ml periostin under the serum-free condition led to Akt1/PKBα phosphorylation on Ser473 (Figure 8B).

A recent report indicated that periostin could bind to the $\alpha_v\beta_3$ and the $\alpha_v\beta_5$ integrins in ovarian cancer cells (Gillan et al., 2002). To probe if the integrins are involved in the mediation of periostin action, we pre-incubated the CX-1NS cells and HMVECs with antibodies against each of these two integrins and measured the phosphorylation of Akt1/PKBα on Ser473 induced by the presence of periostin. As shown in Figures 8A and 8B, the results indicate that activation of the Akt/PKB survival pathway by periostin is mediated primarily through the $\alpha_v\beta_3$ integrins signaling pathway, since the antibody against the $\alpha_v\beta_5$ integrins did not block the phosphorylation event in the HMVECs. These results indicate that at the mechanistic level, the binding of periostin to the $\alpha_v\beta_3$ integrins leads to activation of the Akt/PKB cellular survival pathway, consequently protecting both tumor cells and endothelial cells from stress-induced cell death and promoting angiogenesis and metastasis.

To further confirm that periostin activates the Akt/PKB kinase to promote cellular survival, we used two approaches to block the Akt/PKB function and then examined the effect on the periostin-mediated enhancement in cell survival under stress condition. For this purpose, we either overexpressed PTEN, a well-established negative regulator of the Akt/PKB pathway, to suppress the phosphorylation of Akt/PKB phosphorylation and inactivate its function (Davies et al., 1999; Nicholson and Anderson, 2002), or directly blocked Akt/PKB kinase activity by treating cells with a specific Akt/PKB inhibitor, 1L-6-Hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (Hu et al., 2000). As shown in Figures 8C and 8D, overexpression of PTEN in CX-1NS colon cancer cells or HMVECs, as well as incubation of those cells with 15 μM of the specific Akt/PKB inhibitor, abrogated the survival promotion or protection effect of periostin in both cell types under the condition of serum depletion stress. These data firmly established that periostin protects cells from stress-induced death by activating the Akt/PKB survival pathway.

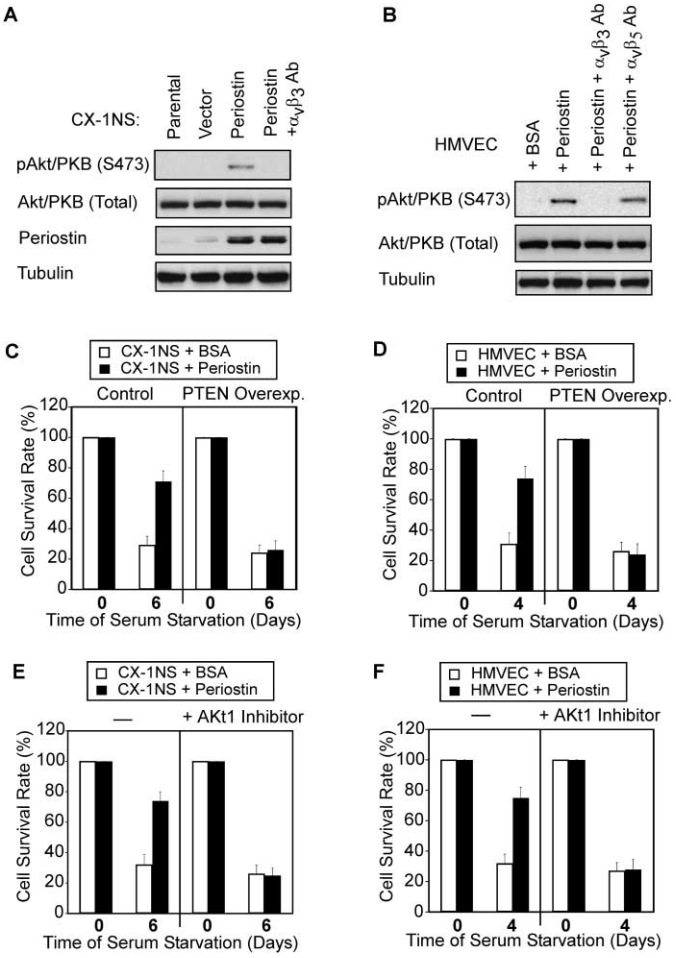

Figure 8. Periostin activated the Akt/PKB survival pathway through the $\alpha_v\beta_3$ integrins to enhance cellular survival

A: The phosphorylation of Akt1/PKBα on Ser473 was detected in periostin-producing CX-1NS cells (lane 3), but not in the parental (lane 1) and the vector-transfected CX-1NS (lane 2) cells under the serum depletion condition. This specific phosphorylation event was blocked by the presence of an antibody against the $\alpha_v\beta_3$ integrins (lane 4).

B: Phosphorylation of Akt1/PKBα on Ser473 was induced by the recombinant periostin in HMVECs in the basic medium without serum. This specific phosphorylation event induced by periostin was blocked by the antibody against the $\alpha_v\beta_3$ integrins (lane 3) but not by an antibody against the $\alpha_v\beta_5$ integrins (lane 4).

C and D: Overexpression of PTEN in CX-1NS cells (**C**) or HMVECs (**D**) abolished the survival promotion or protection effect of periostin under the condition of serum depletion stress. PTEN overexpression was mediated via the retrovirus infection. The control group was infected with the retrovirus containing backbone vector without the PTEN cDNA. The result was quantified from three independent experiments.

E and F: The Akt/PKB specific inhibitor (15 μM 1L-6-Hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate) abrogated the survival promotion ability of periostin in both CX-1NS cancer cells (**E**) and the HMVECs (**F**) under serum depletion condition. In the absence of the Akt/PKB inhibitor, periostin protein (100 ng/ml) exhibited the survival promotion effect on both CX-1NS cells and HMVECs under serum depletion. In contrast, incubation of CX-1NS cells or HMVECs with the Akt/PKB inhibitor abolished the protection effect of periostin.

Discussion

The findings presented here demonstrated the validity and fruitfulness of our approach in identifying specific genes whose

altered expression pattern is intimately associated with later stages of tumorigenesis, particularly metastasis. Through extensive functional analyses, one of the candidate genes identified by this approach, periostin, was shown to promote later stages of tumor progression. A colon cancer cell line with a low metastatic potential engineered to overexpress periostin displayed a striking phenotype of greatly accelerated tumor metastatic growth as xenografts in the animal model system of metastasis. The underlying mechanism of periostin-mediated promotion of tumor metastasis is largely associated with the ability of periostin to enhance cellular survival for both cancer cells and endothelial cells under stress conditions. The discovery of a link between periostin and the $\alpha_v\beta_3$ integrin-Akt/PKB signaling pathway provides a molecular explanation for the prosurvival activity of periostin in the context of tumor progression through the successful development of angiogenesis and establishment of metastatic colonies, as both the $\alpha_v\beta_3$ integrins and the Akt/PKB pathway have been implicated to play important roles in promoting cell survival and tumorigenesis (Brooks et al., 1994; Nicholson and Anderson, 2002; Stupack and Cheresh, 2002).

In addition to playing a critical role in the progression of colon cancers, periostin appears to be involved in other human cancers as a recent report showed that serum levels of periostin are elevated in patients with bone metastases from breast cancer (Sasaki et al., 2003) and periostin expression is upregulated in ovarian cancers (Ismail et al., 2000). In a separate study, we have found that periostin was overexpressed by a majority of breast cancers examined by genearray analysis (data not shown). Interestingly, βig-h3, a secreted RGD sequence-containing protein that shares a significant structural homology with periostin, was also found by SAGE analysis to be differentially overexpressed in human colon cancer (Zhang et al., 1997), indicating that βig-h3 may be similarly involved in promoting tumorigenesis. However, βig-h3 does not contain the sequence homologous to the C-terminal hydrophilic domain in periostin, suggesting that functional differences may exist between the two proteins during tumor development.

Tumor metastatic process consists of multiple and complex steps, all of which must be successfully completed to give rise to the outgrowth of metastatic tumors in a new organ environment (Chambers et al., 2002; Folkman, 2002). During this process, cancer cells have to overcome many types of stresses such as hemodynamic shearing, loss of adhesion, nutrient depletion, hypoxia, and accumulation of wastes that may all induce cell death. Furthermore, it has been recognized that metastasis is a relatively inefficient process (Chambers et al., 2002; Luzzi et al., 1998; Weiss, 1990). Although early steps in the hematogenous metastatic process from the entry of cancer cells to the bloodstream until their extravasation into secondary organs are remarkably efficient, subsequent steps associated with the establishment of metastases are completed inefficiently, with only a small number of cancer cells being able to initiate the formation of micrometastases in the secondary site, and an even smaller subset of those micrometastases evolving into macroscopic metastases (Chambers et al., 2002; MacDonald et al., 2002; Naumov et al., 2002). Two critical aspects that allow micrometastases to progress into established metastatic colonies are the initiation of angiogenesis within the micrometastases and the prevention of cancer cell death that tips the balance favoring cell proliferation over apoptosis. The ability for cancer cells to survive and grow in the distant organ is critical for the successful development of metastases (Chambers et al., 2002; Hynes, 2003). Our results indicate that the presence of periostin did not significantly affect the initial formation of micrometastases in the liver in the animal models, but that it did stimulate metastatic growth and development at later stage by promoting cancer cell survival and inducing angiogenesis. Thus, periostin secreted by the tumor cells functions to promote the survival of tumor cells, in addition to playing a role in a paracrine manner to augment the survival of endothelial cells and induce neovascularization, an activity consistent with the notion that enhanced survival of endothelial cells within tumors is critical for the successful development of tumor angiogenesis (Brooks et al., 1994; Scatena and Giachelli, 2002; Stupack and Cheresh, 2002). The dual functions of periostin on the two types of cells within the tumor mass may contribute to the rapid development and establishment of metastases in the liver. Since this protein is intimately involved in the critical rate-limiting steps of tumor metastatic process, periostin could be a potential target for the development of novel treatment for metastatic colon cancers.

At the mechanistic level, it is not surprising that we found periostin to exert its effect via the integrins, as a large number of studies have demonstrated that interactions between integrins on the surface of tumor cells and adhesion molecules in the ECM microenvironment are extremely important for tumor cell migration, survival, and growth in different anatomical locations to form distant metastases (Green and Evan, 2002; Jacks and Weinberg, 2002; Liotta and Kohn, 2001; Weaver et al., 2002). The ECM-integrin interactions are not only critical for tumor cell survival, but also important to trigger intracellular signaling and activation of certain genes that lead to tumor cell proliferation during metastatic growth (Meredith and Schwartz, 1997). In addition, the interactions between cell surface integrins and ECM components are also essential for the survival of endothelial cells and the formation of blood vessels within the tumor mass (Brooks et al., 1994; Scatena and Giachelli, 2002; Stupack and Cheresh, 2002). The life and death decision at the cellular level is controlled by the proper cell-matrix interactions (Jacks and Weinberg, 2002; Stupack and Cheresh, 2002). Thus, tumor cells that are unable to adapt to the new microenvironment in distant sites lose their ability to form metastases. Without acquiring specific genetic changes, most cancer cells reaching different anatomical tissues are unlikely to survive in the new ECM environment and are unable to initiate metastatic growth, providing a plausible explanation for the low efficiency of metastatic process. In this regard, acquired expression of periostin and similar types of proteins may enable tumor cells to thrive in the new environment of distant organs and grow as successful metastatic colonies.

Experimental procedures

Differential cDNA display and Northern blot analysis

Differential cDNA display and Northern blot analysis were performed as described previously (Bao et al., 1998). Poly(A)$^+$ mRNA used for the display was purified from total RNA by using Oligotex mRNA kit (Qiagen, Valencia, CA). The total RNA was extracted from colon primary or metastatic tumors or normal colon tissues (surgical specimens) by Tri-reagent (Leedo Medical Lab., Houston, TX). Surgical specimens of metastatic tumors and primary colon carcinomas at different stages were obtained from Division of Tumor Pathology, Xiamen First Hospital. Hybridization signals from Northern blot were quantified and analyzed as previously described (Barnard et al., 1992).

Immunohistochemical staining and analysis of tumor vascular density

Paraffin-embedded colon tumor and normal tissue sections were processed for antigen retrieval by heating in 10 mM sodium citrate (pH 6.0) at 95°C for 20 min. Then, sections were immunostained with an affinity-purified polyclonal antibody raised against the recombinant human periostin protein. The immunostaining was performed with an ABC staining system (Vector Laboratories, Burlingame, CA) using avidin-biotinylated-peroxidase detection method. The immunostaining to detect the blood vessels in the metastases of mouse liver with the MECA32 antibody (rat anti-mouse panendothelial antigen; Pharmingen, San Diego, CA) was performed as described (Harmey et al., 2002). The blood vessel density in tumor was calculated and quantified as previously described (Filleur et al., 2001; Vermeulen et al., 1996).

To insure the comparability of the periostin immunohistochemical staining, a common reference standard was included to serve as an internal or intra-assay control in every slide as previously described (Sompuram et al., 2002a, 2002b). The detailed method is described in the Supplemental Data (http://www.cancercell.org/cgi/content/full/5/4/329/DC1).

Quantitative and statistical analysis of periostin immunohistochemical staining

The quantitative analysis of the immunohistochemical (IHC) staining for periostin expression in tumor tissues was performed as previously described (Mady and Melhem, 2002; Ang et al., 2002). The staining of each tissue was analyzed to determine the Mean Optical Density (MOD), which represents the concentration of the stain as measured per positive pixels. The detailed method can be seen in the Supplemental Data (http://www.cancercell.org/cgi/content/full/5/4/329/DC1).

Cell culture and transfection

CX-1NS cells were cultured in DMEM growth medium (Invitrogen Corp., Carlabad, CA) supplemented with 10% FBS and antibiotics. The human microvascular endothelial cells HMVECs (Clonetics, Walkersville, MD) were maintained in endothelial growth medium (EGM-2-MV; Clonetics) supplemented with 5% FBS (Clonetics). Cell transfections were performed using FuGene 6 reagent (Roche) according to the manufacture's instruction.

Generation of periostin-producing colon cancer cells

To construct a periostin expression plasmid, the full open reading frame of human periostin cDNA was cloned into pcDNA3.1 mammalian expression vector (Invitrogen) with a hexa-histidine tag at COOH terminus of periostin protein. Then, the periostin/pcDNA3.1 plasmid or the vector alone was introduced into CX-1NS colon cancer cells, and the stable clones were obtained by G418 selection (950 μg/ml) in the culture medium. 3 of 5 periostin-expressing clones and 3 of 6 control clones were chosen for the subsequent experiments. In order to observe the initial formation of the micrometastases in the liver, the GFP plasmid (pEGFP; Clontech, Palo Alto, CA) was introduced into two of those periostin-producing CX-1NS clones and two of the pcDNA3.1 vector-transfected control clones to stably express the green fluorescent marker.

Xenograft assays in nude mice

To examine whether periostin expression in colon cancer cells affects metastatic growth in the liver, periostin-expressing CX-1NS cells (6×10^6/animal) were injected into female nude mice (Charles River, Raleigh, NC) via intrasplenic injection as previously described (Khatib et al., 1999). The control groups were injected with the same number of vector-transfected CX-1NS cancer cells. Three periostin-overexpressing clones (A12, B08, and B19) and three vector control clones (V03, V04, and V05) were used in this experiment. Seven to eight mice were injected for each clone. The mice that survived the procedure were sacrificed 5 weeks after injection and examined for metastases development. To detect the GFP-labeled micrometastases formation, periostin/GFP/CX-1NS cells (6×10^6/animal) or the same number of the vector/GFP/CX-1NSs were injected into nude mice by intraportal vein injection. On day 5 and day 10 after injection, liver sections of mice from both groups were checked to detect GFP micrometastases under a fluorescent microscope. For the subcutaneous injection, three clones of periostin-expressing CX-1NS cells (5×10^6/animal) and three clones of vector control CX-1NS cells (5×10^6/animal) were injected to SCID mice with 5 mice for each clone. 4 weeks after injection, mice were sacrificed and examined for the growth of subcutaneous tumors.

Apoptosis assay

To examine the apoptotic events in the liver metastases derived from the periostin-producing CX-1NS cancer cells or the vector-transfected CX-1NS control cells, frozen sections of the metastatic tumors were analyzed by using the ApoAlert DNA fragmentation assay kit (BD-Clontech). The assay was performed according to the user manual. Apoptotic cells appear in green color with the FITC filter under a fluorescent microscope.

Periostin protein expression and purification

The recombinant periostin protein was expressed in the insect cells using the baculovirus expression system (Invitrogen). A hexa-histidine tag was added to the C terminus of the human periostin. The detailed method can be found in the Supplemental Data (http://www.cancercell.org/cgi/content/full/5/4/329/DC1).

Anoikis assay

To determine the effect of periostin on cell survival under anoikis-inducing condition, cells were prevented from adhering to the plastic dishes by culturing them in Petri dishes coated with PolyHema (Sigma, St. Louis, MO) as described previously (Zhu et al., 2001). The detailed assay is described in the Supplemental Data (http://www.cancercell.org/cgi/content/full/5/4/329/DC1).

Cell survival assay under hypoxia and serum-depletion conditions

Periostin-producing CX-1NS cells and the vector-transfected CX-1NS cells were grown in the normal medium until 60%–70% confluency. Then, they were incubated with serum-free medium or treated with 100 μM DFO (Deferoxamine; Sigma) to mimic hypoxia (Bianchi et al., 1999) for different time points as indicated, and the numbers of survival cells at different time points were analyzed and counted. The results were quantified from three independent experiments with three periostin-expressing CX-1NS clones and three vector control clones. For endothelial cells, after HMVECs were grown in the growth medium until 60%–70% confluency, the cells were incubated with serum-free basic medium containing 100 ng/ml of periostin, VEGF, or BSA, or were treated with 100 μM DFO (Sigma) in the medium containing 100 ng/ml of periostin, VEGF, or BSA for different time points as indicated, and the surviving cells were analyzed and counted at different time points. At least three independent experiments were performed to obtain the statistical results.

Western blot analysis

For the detection of secreted periostin protein, periostin-producing cells or control cells were grown in normal medium until 80% confluency and then cultured in the serum-free medium for 24 hr. The serum-free conditional medium were collected and analyzed by immunoblot with a specific anti-periostin polyclonal antibody, anti-His antibody (H-15 or H-3; Santa Cruz Biotechnology), anti-VEGF polyclonal antibody (Ab-4, Calbiochem, La Jolla, CA), and anti-IL-8 mAb (B2, Santa Cruz). The specific anti-periostin antibody was generated by immunizing the rabbits with recombinant periostin protein and purified through an affinity column. For the detection of Akt and other proteins, cell lysates were analyzed by immunobloting with the anti-Akt1/PKBα (#9272) and anti-pS473-Akt1/PKBα (#9271) rabbit polyclonal antibodies (Cell Signaling Technology Inc., Beverly, MA). Anti-$\alpha_v\beta_3$ mAb (LM609; Chemicon Inc., Temecula, CA) and anti-$\alpha_v\beta_5$ mAb (PIF6; Chemicon) were used at 10 μg/ml for the functional blocking experiments.

PTEN retrovirus and the Akt inhibitor

The PTEN retroviral construct was generated by inserting the PTEN full-length open reading frame into the retroviral backbone vector pBabe with a puromycin selection marker. The production of the retrovirus and the infection of cells for protein overexpression were performed as previously described (Rich et al., 2003). Control cells were infected with retrovirus containing the backbone vector but without the PTEN cDNA. The Akt/PKB-specific inhibitor (1L-6-Hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate) that has been shown to potently inhibit Akt/PKB (Hu et al., 2000) was obtained from Calbiochem. 15 μM Akt/PKB inhibitor was used to treat CX-1NS cells and HMVECs for the indicated experiments according to the manufacturer's instructions.

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