Netrin-4 overexpression suppresses primary and metastatic colorectal tumor progression

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Abstract. Tumor angiogenesis is closely associated with clinical staging and has been proposed to correlate with clinical response in terms of subsequent metastases following primary resection. Netrin-4 (NT-4) regulates angiogenic responses. Therefore, we sought to examine the effects of NT-4 on the primary tumor growth of colon cancer cells, liver and lung metastases of colon cancer cells, and responses following primary tumor resection. We used 3 different mouse models of orthotopic primary tumor and liver and lung metastases, comparing 2 human colon cancer cells lines: wild-type (low expression of NT-4) and NT-4 (overexpression of NT-4) LS174 cells. NT-4 overexpression inhibited the primary tumor growth of colorectal LS174 xenografts in nude mice (144.3 ± 12.9 vs. 62.4 ± 4.5 mm^3; $P<0.0001$) as well as its related local and systemic recurrence (38 vs. 0%; $P<0.01$). NT-4 overexpression also markedly decreased colorectal cancer progression in terms of tumor number and volume of liver metastases in the NT-4 group of the orthotopic liver metastasis model (25 vs. 90% and 4 ± 1 vs. 709 ± 190 mm^3, $P<0.001$ and $P<0.05$). Collectively, our findings indicate that NT-4 overexpression decreases colorectal lung metastasis and its associated lymph node involvement. NT-4 overexpression decreases tumor recurrence and metastasis after surgical resection, likely via an anti-angiogenic effect. These observations suggest that NT-4 may hold therapeutic potential in the treatment of colorectal cancer growth and major metastatic sites.

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer deaths in North America and Western Europe, comprising about 10% of new cancer cases every year (1,2). Approximately 20% of patients have detectable liver metastases at diagnosis, which is the predominant cause of death in patients with CRC (3-5). Only patients with a limited number of isolated, organ-confined metastases exhibit prolonged survival or the possibility of complete response following surgical resection (6-8). The development of new therapeutic strategies is critical for improving pre-existing therapeutic approaches to increase survival rates, particularly in patients with colorectal liver metastasis. Adhesion, invasion, angiogenesis, inflammation, tumor microenvironment and tumor cell growth are all mechanistic factors that facilitate liver metastasis in CRC. The growth and metastasis of solid tumors depends critically on tumor angiogenesis (9). This concept has promoted the development of angiogenesis inhibitors targeting the vascular endothelial growth factor (VEGF) pathway, which has been validated by clinical trials. Bevacizumab, a humanized monoclonal antibody targeting VEGF, is the first anti-angiogenic drug that, when administered in combination with conventional chemotherapy, has demonstrated improvements in tumor response rates and progression-free survival among patients with liver metastatic in CRC (10,11). However, the adverse side effects and the limited efficacy of the anti-VEGF inhibitors due to intrinsic and/or acquired resistance have hampered their clinical benefits and precluded their further inclusion as an adjuvant treatment (12). For these reasons, the evaluation of new anti-angiogenic targets is crucial, and other targets, such as placenta growth factor (PlGF), have been proposed (13).

Netrins comprise a family of diffusible molecules including at least five ligands (netrins 1, 2, 4, G1a and G1b) and seven receptors (neogenin, DCC, Unc5A, Unc5B, Unc5C, Unc5D and A2b) that have regulatory roles in vascular development (14,15). Although the significance of the netrin-dependent pathway in tumor angiogenesis remains unclear, emerging evidence indicates that this pathway has an important role in the development of different cancers in animal models (14). NT-4 may therefore represent a potential therapeutic target for cancer treatment. A previous study showed that netrin-4 (NT-4) overexpression delayed tumor angiogenesis through binding to neogenin and recruitment of Unc5B in a subcutaneous (s.c.) xenograft model (16). In addition, a recent study demonstrated that NT-4 regulated angiogenic responses and inhibited the growth of CRC cells (17).

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We previously identified NT-4 as a protein expressed in LS174 human colon cancer cells grown *in vitro* and further demonstrated that NT-4 inhibits tumor progression by exerting inhibitory effects on tumor angiogenesis, without altering tumor cell proliferation or apoptosis. Furthermore, we found that NT-4 exerts antiangiogenic activity, likely by binding to neogenin and decreasing CRC carcinomatosis and ascites formation, in animal models (18).

The aim of this study was to examine the effects of NT-4 overexpression on tumor angiogenesis and growth *in vivo* using 3 mouse models that mimic clinical situations: orthotopic primary tumor resection as a treatment of a local tumor stage, liver and lung metastasis as a treatment of an advanced tumor stage. For these purposes, we designed a model of CRC primary tumor and liver and lung metastases in which control or NT-4-transfected LS174 tumor cells were subsequently transplanted. Our study showed that NT-4 overexpression delayed primary tumor growth, lymph node involvement and liver and lung metastases.

Materials and methods

In the present study, we successively studied the link between NT-4 and primary and metastatic tumor development in three orthotopic murine models: i) primitive tumor (n=20 per group), ii) liver metastasis (n=10 per group), and iii) and lung metastasis (n=8 per group).

Cell culture. LS174 human colon cancer cells obtained from the American Type Culture Collection were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS)/penicillin (50 U/ml)/streptomycin (50 μg/ml) (Gibco-BRL Life Technologies Inc., Grand Island, NY, USA) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Synthesis of NT-4 cDNA. Total RNA was extracted from cultures of human umbilical artery endothelial cells (HUAEC), and reverse transcription was performed using the AMV kit (Roche Applied Science, Indianapolis, IN, USA). Two sets of primers (1-1039 and 914-1884) were used to amplify the NT-4 sequence with Taq DNA polymerase (Roche Applied Science). Products from the two PCR reactions were mixed 1:1 and used as the template to obtain a NT-4 cDNA. The construct was subcloned into a pcDNA3.1(-)/His-myc C vector (Invitrogen, Carlsbad, CA, USA).

LS174 cell transfection. The NT-4-containing vector was transfected into LS174 cells with Lipofectin (Invitrogen), according to the manufacturer's protocol. Cells were cultured in a selective media (10% FBS-DMEM) containing 200, 300, 400 and 500 μg/ml of G418 (Geneticin; Gibco-BRL Life Technologies Inc.). Cell clones were subsequently screened by enzyme-linked immunosorbent assay (ELISA) to detect increases in NT-4 expression relative to that in the pcDNA-transfected cells that were collected after 3 days in conditioned medium (data no shown).

For the *in vivo* experiments, wild-type or NT-4-transfected LS174 cells were harvested, washed with 10% FBS-DMEM, counted and resuspended in DMEM prior to injection. Cell viability was assessed by trypan blue exclusion, which verified that the cell viability was >95% in both cell lines.

Animal tumor xenograft models. Each experimental protocol satisfied all of the standard requirements of the European community guidelines for the care and use of the laboratory animals. Five-week-old female nude or nod scid mice (Charles River Laboratories International Inc., Wilmington, MA, USA) were acclimated for 1-2 weeks before tumor transplantation (10 animals per experimental group). For all *in vivo* models, the animals were anesthetized with xylazine (2 mg/ml) and ketamine (20 mg/ml) (Vibrac, Carros, France).

Animal models of primary tumor resection. We first analyzed the effect of NT-4 overexpression in a primary tumor model (19) by orthotopic xenograft on the colon of nude mice. For this purpose, either NT-4-transfected (NT-4 LS174) or wild-type (WT-LS174) LS174 cells ($1x10^6$ cells) were subcutaneously injected into 5-week-old female nude mice. The tumor volume was recorded using a caliper twice weekly. Animals were sacrificed 30 days after engraftment. After resection of the subcutaneous tumor (Fig. 1A), 1 mm³ tumor fragments were prepared (Fig. 1B) and a surgical orthotopic implantation was performed on the colon of nude mice. The colon was exposed by a small midline incision (Fig. 1C) to allow for the implantation of the tumor fragment on the colon by 5-0 surgical sutures under the serosa of the ascending colon (Fig. 1D). The application of biological glue followed by peritoneal and skin closures completed the procedure (Fig. 1E and F).

On Day 8, to reproduce human clinical care, a midline laparotomy was performed to expose the colon (Fig. 1G), and a clip was applied to provide hemostasis and digestive integrity. The colonic tumor was resected (Fig. 1I) and the tumor volume (cm³) was calculated by using the following equation: V= length x width² x p/6 (Fig. 1J). Mice were monitored to analyze recurrence patterns and were sacrificed on Day 70.

Animal models of liver metastasis. Under sterile conditions, either NT-4 or WT-LS174 cells ($2x10^6$ cells) were intrasplenically injected into 5-week-old female nod scid mice. First, a 10-mm left subcostal incision was made to expose the spleen over the peritoneum. Then, the cell suspension was injected into the spleen using a 27 G needle. Following light splenic compression, the spleen was repositioned back in the abdominal cavity, peritoneum and muscles were sutured closed with one stitch and the wound was closed with a clip. Thirty days after inoculation with tumor cells, the mice were sacrificed, and the liver weight was recorded to evaluate the tumor metastases. Microspecimens of the spleen and liver were harvested for pathological confirmation of liver metastasis by HES stain.

Animal models of lung metastasis. In the LS174 lung metastasis model, either NT-4 or WT LS174 cells ($2x10^6$) were injected via the tail vein of 5-week-old female nude mice. Thirty days after inoculation, the mice were sacrificed, and the lung weight was recorded for evaluation of tumor metastasis. Microspecimens of the lung, mediastinal and cervical lymph nodes were harvested for pathological confirmation of metas-
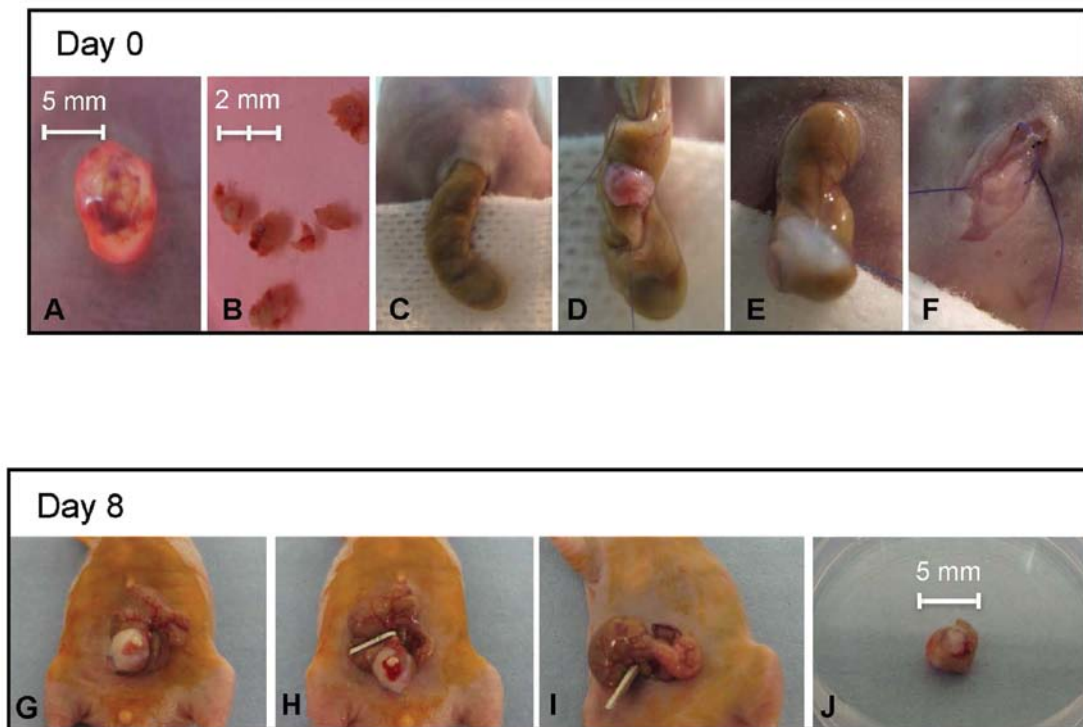
Figure 1. Intraoperative view of the primary tumor xenograft. (A) Subcutaneous tumor ablation; (B) procurement of 1 mm^3 fragment; (C) exposure of the colon by a small midline incision; (D) implantation of the tumor fragment by suture; (E) application of biological glue; (F) peritoneal and skin closure. Intraoperative view of the primary tumor xenograft resection on Day 8. (G) Midline laparotomy, colon exposure; (H) clip application providing hemostasis and digestive integrity; (I) resection of colonic tumor; (J) specimen.
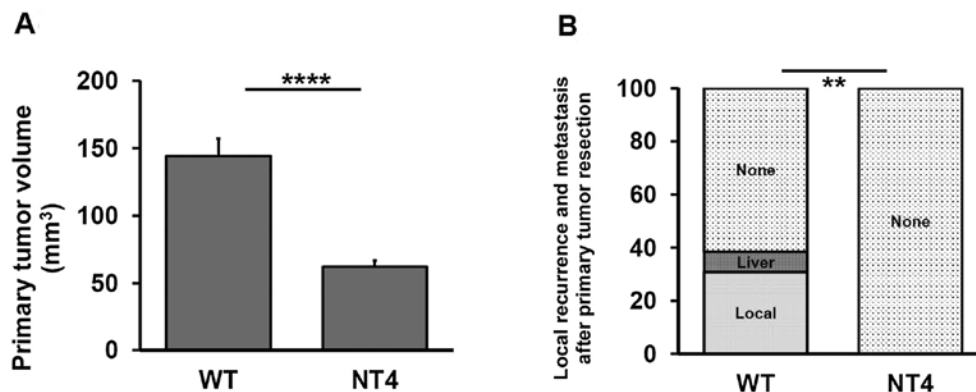

Figure 2. WT LS174 mice exhibited voluminous primary tumors (A) and local recurrence and metastases (B) whereas NT-4 LS174 mice exhibited small primary tumors and no recurrence.

tasis by HES stain. The sera of sacrificed mice were assessed for carcinoembryonic antigen (CEA).

Statistical analysis. All results are expressed as the mean ± SEM. We used StatView® and R programs (R is available at http://www.R-project.org) for the statistical analysis. We tested associations between epidemiological and prognostic variables using the Mann-Whitney test or Fisher's exact test. A Kruskall-Wallis test was applied for the analysis of variance. P-values of 0.05 (*) or less (**P<0.01, ***P<0.001) were considered to indicate statistical significance.

Results

NT-4 overexpression decreases colorectal primary tumor growth and increases response to surgical resection. The primary tumor volume was significantly reduced in animals injected with NT-4 LS174 tumor cells compared with WT LS174 cells (n=20 per group, mean ± SEM; 62.4±4.5 and 144.3±12.9 mm^3 for NT-4 and WT LS174 tumors, respectively; P<0.0001; Fig. 2A), suggesting that NT-4 expression affects the primary tumor growth of orthotopically transplanted colorectal cancer.
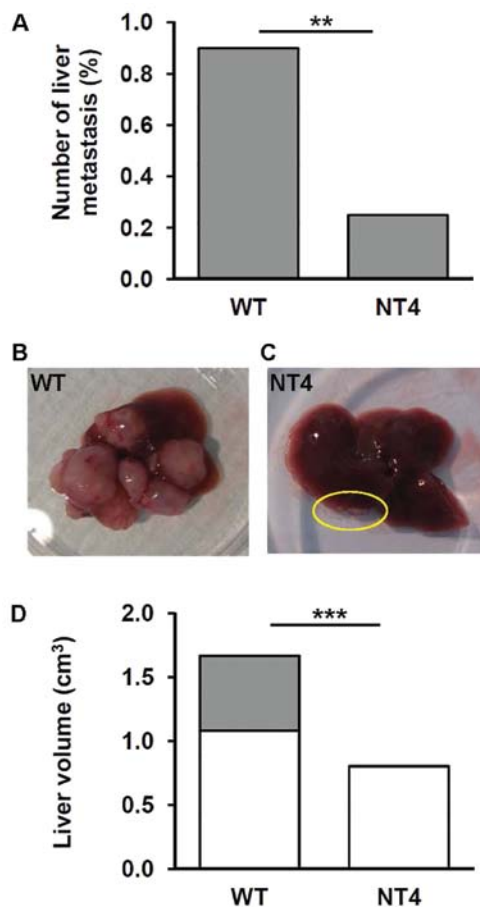
Figure 3. Macroscopic aspects of liver metastases after removal of the liver at animal sacrifice, 30 days after splenic injections. WT LS174 mice (A, B and D) exhibited numerous voluminous metastases whereas NT-4 LS174 mice (C) exhibited small metastases localized at the periphery of the liver (yellow circle). (D) Liver volume (white area) and liver metastasis (grey area) of the two groups.

Seventy days after tumor transplantation and 62 days after surgical resection, the number of local and systemic recurrences was significantly decreased in the NT-4 LS174 tumor group compared with WT LS174 group. The WT group exhibited four local and one disseminated liver tumor recurrences whereas the NT-4 group exhibited no recurrences, suggesting that NT-4 expression affects the metastatic progression of orthotopically transplanted cancer (n=13 per group, 38 and 0% for the WT and NT-4 groups, respectively; P<0.01) (Fig. 2B).

NT-4 overexpression decreases colorectal liver metastasis. Thirty days after splenic injection of WT or NT-4-transfected LS174 tumor cells, the number and tumor volume of liver metastases were assessed. The number of liver metastases was significantly lower in the NT-4 group compared with the WT group (n=10 per group, 25 vs. 90%, respectively for NT-4 and WT LS174 groups, P<0.001) (Fig. 3A-C). The liver metastasis volume was markedly reduced in the NT-4 group compared with the WT LS174 groups (n=10 per group, 4 ± 1 mm^3 and 709 ± 190 mm^3, respectively for NT-4 and WT LS174 groups, P<0.05) (Fig. 3D, grey area).

NT-4 overexpression decreases colorectal lung metastasis and its related lymph nodes involvement. The effects of NT-4 overexpression were analyzed in the lung metastasis model employing tail vein injection of WT- or NT-4-transfected LS174 tumor cells. In the WT group, tumor microemboli were detected in one mouse, and liver metastases were detected in another mouse. However, in this WT group, all mice had large, cervical, axillary, mediastinal and retroperitoneal pathological lymph nodes (Fig. 4A-F). A histological analysis of lymph nodes revealed diffuse tumor infiltration in 5 of 8 mice (62.5%). In contrast, the mice in the NT-4 group developed neither lung nor liver metastases, nor did they exhibit lymph node enlargement.

Serum levels of ACE were significantly increased in the WT mice, with lymph node involvement averaging 2.78 ± 0.43 ng/ml compared with 0.05 ng/ml (detection limit) in the control mice (P<0.05).

Discussion

Anti-angiogenic therapies are broadly applied in the treatment of several cancers, particularly CRC. Bevacizumab, the humanized monoclonal antibody targeting vascular endothelial growth factor, is a pioneering anti-angiogenic drug that demonstrates clinical benefit in combination with chemotherapy in patients with advanced metastatic CRC. No adjuvant anti-angiogenic therapy has previously exhibited success in prolonging disease-free survival of non-metastatic colon cancer patients. Recently, in adjunction of FOLFOX therapy, bevacizumab failed to significantly prolong disease-free survival in stage II and III colon cancer patients (12). In addition to the possibility of rebound effect (20), adverse and fatal effects such as hemorrhage, wound healing complications, gastrointestinal perforation, hypertension and proteinuria were elicited by anti-VEGF therapies (21-25). Furthermore, most tumors have been found to develop mechanistic resistance to anti-angiogenic agent (26).

Originally identified as axonal guidance molecules, the netrin family has recently been shown to be involved in angiogenesis and blood vessel network formation (27). As observed in the nervous system, netrins also act as bifunctional modulators in angiogenesis. Indeed, both pro- and anti-angiogenic effects of netrin have been reported (28-30). The ability of human umbilical artery endothelial cells (HUAEC) to migrate and organize into tubular structures was inhibited by the NT-4 overexpression. Reciprocally, silencing the N4 gene in HUAEC was shown to increase both cellular branching and their ability to form tubular structures on Matrigel, when compared with non-transfected or control siRNA-transfected cells (16). Netrin 4 inhibits *in vitro* human microvascular ECs and tube formation (17). However, Netrin 4 has been shown to potentially inhibit the proliferation of various tumor cells *in vitro* with modest effects on the proliferation of endothelial cell and non-transformed cells (17). We have previously demonstrated that Netrin-4 overexpression attenuates CRC carcinomatosis by inhibiting tumor angiogenesis, with no direct effects on tumor cell proliferation or apoptosis (18).
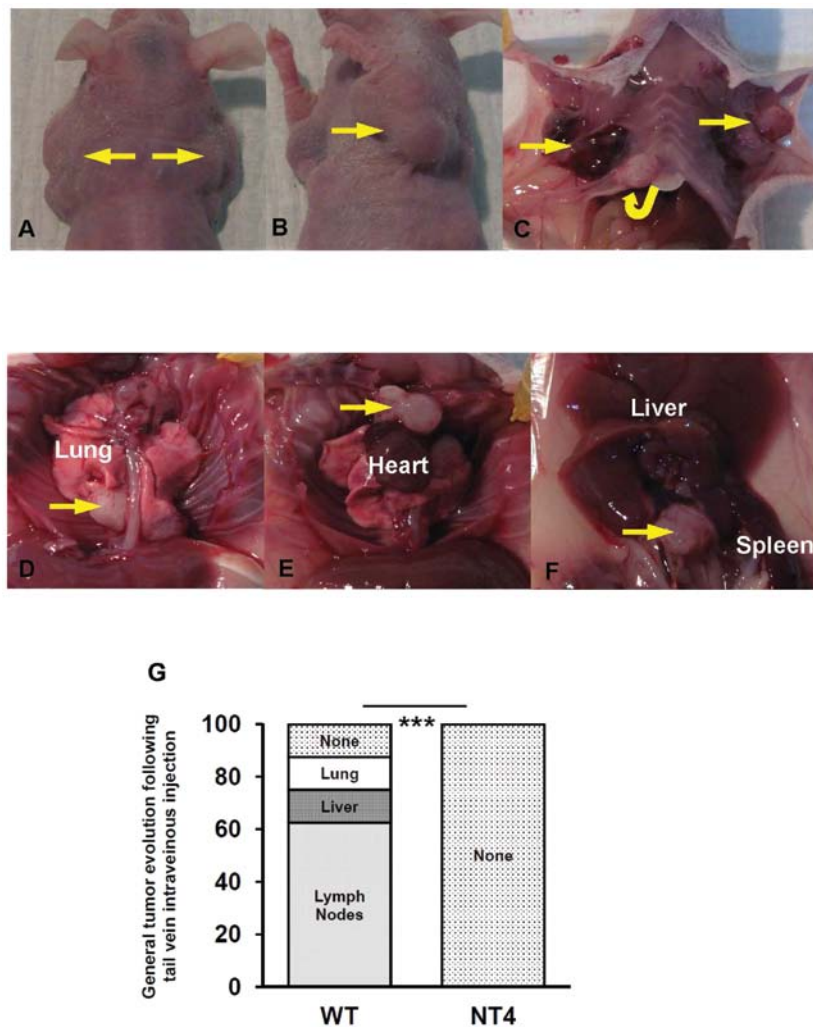
Figure 4. Macroscopic aspects of lymph node metastases, 30 days after tail vein injections. (A) Cervical lymph nodes, external view (arrow). (B) Axillary lymph nodes, external view (arrow). (C) Axillary (arrow) and parasternal (curved arrow) lymph nodes, internal view. (D and E) Mediastinal lymph nodes, internal view (arrow). (F) Retroperitoneal lymph nodes, internal view (arrow). WT LS174 mice exhibited numerous metastases (G), whereas NT-4 LS174 mice exhibited no sign of metastasis.

This investigation aimed to evaluate the *in vivo* effects of NT-4 on tumor angiogenesis and tumor growth in an orthotopic tumor and a liver and lung metastasis model of CRC. For this purpose, LS174 tumor cells were either transfected or not transfected with NT-4 before injection.

In the orthotopic CRC xenograft model, we demonstrated that NT-4 overexpression decreases the tumor volume on Day 8 after colonic CRC transplantation, indicating that NT-4 expression may delay tumor process. These findings suggest that NT-4 may exhibit therapeutic potential for the treatment of primary cancer. Furthermore, in this model, NT-4 overexpression significantly reduced the primary tumor volume and abolished local recurrence and metastasis up to 62 days after primary tumor resection.

In investigating the effect of NT-4 on distal metastasis, we found that NT-4 overexpression also reduced liver and lung metastases in the two CRC metastasis models examined.

Our previous results confirm that the differences in primary tumor and metastatic outcomes are specifically conferred by NT-4 overexpression, rather than the effects of clonal selection resulting from our technique or our usage of only one cell line. Despite the limitation of the number of CRC cell lines tested, our findings suggest that NT-4 may be exploited for anti-CRC therapy in humans. Future studies are necessary to evaluate the potential of the NT-4 as an anti-CRC target, identify the target of NT-4 in human tumors and evaluate the effect of NT-4 in a study of patients who require the resection of metastatic disease.

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