Inhibitor of growth 4 suppresses colorectal cancer growth and invasion by inducing G1 arrest, inhibiting tumor angiogenesis and reversing epithelial-mesenchymal transition

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Key words: colorectal cancer, inhibitor of growth 4, tumor angiogenesis, cancer metastasis, epithelial-mesenchymal transition, cyclin E, interleukin (IL)-6, IL-8, vascular endothelial growth factor (VEGF), Snai1, N-cadherin and vimentin in the LoVo CRC cells. Our data provide compelling evidence that i) ING4 suppresses CRC growth possibly via induction of G1 phase arrest through upregulation of P21 cyclin-dependent kinase (CDK) inhibitor and downregulation of cyclin E as well as inhibition of tumor angiogenesis through reduction of IL-6, IL-8 and VEGF proangiogenic factors; ii) ING4 inhibits CRC invasion and metastasis probably via a switch from mesenchymal marker N-cadherin to epithelial marker E-cadherin through downregulation of Snai1 epithelial-mesenchymal transition (EMT)-inducing transcription factor (EMT-TF).

Introduction

Colorectal cancer (CRC) is the second most common malignancy of the digestive system in women and the third in men worldwide, and is the third (women) and fourth (men) leading cause of cancer-related mortality (1). Conventional treatments for CRC currently include various combinations of surgery, chemotherapy, radiotherapy and targeted therapy. Due to early screening, reduced prevalence of risk factors and/or improved treatment, the recurrence and the mortality rates of CRC have largely decreased in the past decade (1). However, the prognosis of advanced or distant-stage CRC remains poor, and the 5-year overall survival rate is less than 13% in the US (2). Accumulating alteration of oncogenes and tumor-suppressor genes is crucial for the pathogenesis and progression of CRC. To improve the therapeutic efficacy for CRC particularly metastatic CRC and its prognosis, it is therefore urgently needed to better understand the molecular mechanisms involved in CRC progression and metastasis, and correspondingly identify potential CRC-associated therapeutic genes/targets.

The inhibitor of growth 4 (ING4) has been recognized as a powerful tumor suppressor (3). ING4 frequently exhibits alterations in human cancers such as deletion, mutation, splicing variant and downregulation, contributing to cancer initiation and progression as well as poor prognosis (4). ING4 can repress the loss of contact inhibition that is induced by myelocytoma-tosis viral related oncogene, neuroblastoma derived (MYCN) and myelocytomatosis viral oncogene homolog (MYC) (5). Growing evidence has further shown that forced expression of
ING4 can trigger tumor growth suppression via induction of cell cycle alteration, apoptosis and toxic autophagy in a large variety of cancers (3,6-8). Additionally, ING4 can augment the therapeutic efficacy of chemotherapy and intracavitary or external beam radiotherapy (3,9-13). Adenoviral-mediated ING4 and interleukin (IL)-24 double tumor-suppressor gene therapy also was found to exhibit enhanced antitumor activity (3,14,15). Moreover, ING4 can suppress cancer metastasis via interaction with liprin α1 and downregulation of matrix metalloproteinases (MMPs) (7,16,17). ING4 can also inhibit tumor angiogenesis by suppressing production of proangiogenic factors through attenuating transcription activity of nuclear factor xB (NF-xB) and hypoxia-inducible factor-1α (HIF-1α) (18-20). Notably, ING4 can impair prooncogene c-myc translation via interacting with AUFI (21).

Clinical data have shown that ING4 is commonly downregulated in human CRC tissues, and is closely associated with higher clinical stage, histological grade, microvessel density (MVD) and lymph node metastasis (22,23). However, the possible role and related mechanism of ING4 in the progression of human CRC remain largely elusive. In the present study, we assessed the expression of ING4 in low and high metastatic human CRC cells, investigated the effect of ING4 on growth, cell cycle distribution and invasion of high metastatic human CRC cells following lentiviral-directed ING4 stable expression, and delineated the potential mechanisms.

Materials and methods

Vectors, cell lines, reagents and mice. The pAdTrack-CMV/ING4 plasmid containing the humanized ING4 coding sequence (CDS) was previously constructed (7). The lentiviral transfer plasmid pLenti6.3/IRES/GFP carrying a green fluorescent protein (GFP) marker gene and a Blasticidin S (BSD)-resistant gene, and the lentiviral packing plasmids including pLP1, pLP2 and VSVG were purchased from Novobio Science and Technology Inc. (Shanghai, China). The LS174T, SW480, LoVo and SW620 human CRC cell lines, the human colorectal mucous epithelial FHC cell line and the human embryonic kidney 293T cell line were purchased from the Cell Bank, Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). RPMI-1640 medium and fetal bovine serum (FBS) were purchased from HyClone (Logan, UT, USA). The MiniBEST Universal RNA Extraction kit was purchased from Takara (Dalian, Liaoning, China). The reverse transcriptase polymerase MuMLV and the primer oligo(dT)18 were purchased from Thermo Fisher Scientific (Shanghai, China). The cDNAs were then subjected to SYBR-Green I-based qPCR analysis with primers specific for human ING4: (ING4-F1, 5'-gct cat gag gga cct aga cc-3' and ING4-R1, 5'-ggc caa ttt ttc ctc gga gc-3' for amplifying 112 bp) or the housekeeping gene β-actin (β-actin-F, 5'-ctc acc atg gat gat gat atc gc-3' and β-actin-R, 5'-agg aat ctc tgc gac cca tgc-3' for amplifying 163 bp) (an internal control) using the FastStart Universal SYBR-Green Master (ROX) kit. The authenticity of the PCR products was verified by melting curve analysis and agarose gel electrophoresis. The ING4 mRNA expression was normalized to internal control β-actin and calculated using the 2ΔΔCt method as previously described (24).

Generation of a lentivirus expressing ING4. A recombinant lentivirus expressing the humanized ING4 tumor suppressor gene (LV-ING4) was generated as previously described (25). Briefly, the humanized ING4 CDS fragment was amplified by PCR using the pAdTrack-CMV/ING4 plasmid (7) as a template, and primers specific for humanized ING4 were: ING4-F2, 5'-gaa gct agc gcc acc atg gct gct ggg atg tat gct gc-3' for amplifying 163 bp). The endogenous ING4 expression in human CRC cells was determined by quantitative real-time reverse transcription (qRT)-PCR assay. In brief, the total cellular RNAs were extracted from the LS174T, SW480, LoVo and SW620 human CRC cells and the normal human colorectal mucous epithelial control FHC cells (2x106 cells/each) using the MiniBEST Universal RNA Extraction kit, respectively. The first-strand cDNAs were synthesized from RNAs using reverse transcriptase MuMLV and oligo(dT)18. The cDNAs were then subjected to SYBR-Green I-based qPCR analysis with primers specific for human ING4: (ING4-F1, 5'-gct cat gag gga cct aga cc-3' and ING4-R1, 5'-ggc caa ttt ttc ctc gga gc-3' for amplifying 112 bp) or the housekeeping gene β-actin (β-actin-F, 5'-ctc acc atg gat gat gat atc gc-3' and β-actin-R, 5'-agg aat ctc tgc gac cca tgc-3' for amplifying 163 bp) (an internal control) using the FastStart Universal SYBR-Green Master (ROX) kit. The authenticity of the PCR products was verified by melting curve analysis and agarose gel electrophoresis. The ING4 mRNA expression was normalized to internal control β-actin and calculated using the 2ΔΔCt method as previously described (24).

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Construction of the ING4-stably transgenic CRC cell line. The biological titer (transducing U/ml, i.e. TU/ml) of LV-ING4 and LV lentiviruses expressing the GFP marker gene was evaluated by calculating the number of GFP-expressing 293T cells after lentiviral infection by fluorescence microscopy following the company protocol. The ratio of infectious lentivirus (TU) to target cells is called the multiplicity of infection (MOI). For stable expression of the lentiviral-mediated ING4 transgene in CRC cells, the high metastatic human CRC LoVo cells were dispensed into 24-well plates at 1x10^5 cells/well and incubated with LV-ING4 or LV at an MOI of 10 according to the infectious dose as recommended by the manufacturer. Forty-eight hours after infection, the GFP expression and lentivirus infection efficiency were observed by fluorescence microscopy. To eliminate the uninfected cells, the transfectants were then selected with Blasticidin S (final concentration 10 µg/ml) for 1 month. Expression of the GFP marker gene and the ING4 transgene in Blasticidin S-resistant LV-ING4 or LV-infected LoVo (termed LoVo-ING4 or LoVo-Mock) tumor cells were further identified by fluorescence microscopy, RT-PCR and western blot analysis.

RT-PCR analysis. The LoVo-ING4 or LoVo-Mock human CRC and untransfected control tumor cells (2x10^6 cells/each) were collected. The lentiviral-mediated humanized ING4 transcriptional expression in the LoVo tumor cells was analyzed by RT-PCR using primers specific for humanized ING4 (ING4-F2, 5'-ctc acc atc gta gtt cga ccc att-3' and ING4-R2, 5'-ata ggc gcc ccc ttc ttc cgt tct gtc gac for amplifying 747 bp); or the housekeeping gene β-actin (β-actin-F, 5'-ctc acc atg gat gat gat atc gc-3' and β-actin-R, 5'-agg aat cct tct gac cca tgt-3' for amplifying 163 bp). The reaction products were analyzed using agarose gel electrophoresis.

Cell viability assay. The effect of lentiviral-mediated ING4 stable expression on human CRC cell growth in vitro was assessed by MTT assay. In brief, the LoVo-ING4 and LoVo-Mock tumor cells were dispensed into 96-well plates at 1x10^5 cells/well in 200 µl culture medium. At different time points of incubation (day 1-3), the amounts of IL-6, IL-8 and VEGF in the above supernatants were then determined by ELISA in vivo analysis using human IL-6, IL-8 and VEGF ELISA kits, respectively.

Flow cytometric analysis of cell cycle distribution. The effect of lentiviral-mediated ING4 stable expression on the cell cycle profile of human CRC cells in vitro was determined by flow cytometric analysis using PI staining. Briefly, the LoVo-ING4 and LoVo-Mock tumor cells were harvested, washed in cold phosphate-buffered saline (PBS) and fixed in 70% cold alcohol for 24 h. After washing, the cell pellets were stained with PI solution (50 µg/ml PI, 50 µg/ml RNase A and 0.1% Triton X-100) in the dark for 30 min and then analyzed by flow cytometry.

Transwell invasion assay. The effect of lentiviral-mediated ING4 stable expression on human CRC cell invasion in vitro was assessed by Transwell invasion assay. In brief, 12.5 µl of Matrigel (50 mg/l) was diluted in 87.5 µl serum-free RPMI-1640 medium. The 100 µl Matrigel diluted solution was added to a 24-well Transwell chamber, dried in a laminar hood overnight and reconstituted in 100 µl serum-free RPMI-1640 medium for 2 h. The LoVo-ING4 and LoVo-Mock tumor cells (2x10^5 cells/100 µl serum-free RPMI-1640 medium) were added to the upper chamber of the Transwell. The lower chamber was filled with 500 µl of culture medium. After 24 h of incubation, tumor cells on the upper surface of the insert were removed and cells invading into the bottom side of the insert were fixed with 4% paraformaldehyde, stained with crystal violet, photographed and counted by investigators that were blinded to the group allocation in 5 random x200 high-power fields. The invasive ability of the tumor cells was then analyzed.

Western blot analysis. The LoVo-ING4 and LoVo-Mock human CRC cells were collected, washed with cold PBS and lysed in lysis buffer (1x10^5 cells/ml lysis buffer) for preparation of total cellular lysates using a mammalian cell lysis kit. The protein concentration was determined by BCA protein assay. The total cellular lysates (100 µg/lane) were then subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis of human ING4, P21, cyclin E, CDK2, E-cadherin, N-cadherin, vimentin, Snail1, Snail2, ZEB1, Twist and β-actin (an internal control). After washing, the membranes were developed using the Super Enhanced chemiluminescence detection kit. The bands were then visualized after their exposure to Kodak X-ray film.

Enzyme-linked immunosorbent assay (ELISA). The LoVo-ING4 and LoVo-Mock human CRC cells were dispensed into 24-well plates at 2x10^5 cells/well/1 ml culture medium. The supernatants were collected at different time points of incubation (day 1-3). The amounts of IL-6, IL-8 and VEGF in the above supernatants were then determined by ELISA analysis using human IL-6, IL-8 and VEGF ELISA kits, respectively.

Tumor transplantation assay in vivo. Female athymic BALB/c nude mice were subcutaneously (s.c.) implanted with LoVo-ING4 or LoVo-Mock human CRC cells (1x10^6 cells/mouse) (6 mice/group). Tumor progression in vivo was monitored by investigators that were blinded to the group allocation via measurement of tumor volume and weight. Tumor volume (V) was measured with a caliper and calculated by the formula: V = tumor size = ab^2/2, where a is the larger of the two dimensions and b is the smaller. The tumor-bearing mice were sacrificed 4 weeks after tumor cell inoculation. The xenografted tumors were then removed, weighed, fixed with 10% neutral formalin and embedded in paraffin for hematoxylin & eosin (H&E) staining and immunohistochemical analysis.

CD31 immunohistochemical analysis. The expression of tumor vessel CD31 in the LoVo-ING4 and LoVo-Mock human CRC s.c. xenografted tumors was examined by immunohistochemical analysis using the UltraSensitive™ SP kit. Any endothelial cell cluster immunoreactive for CD31 clearly separated from adjacent microvessels was considered as a single countable vessel (26). The MVD was determined by investigators that were blinded to the group allocation in
ING4 expression is downregulated in CRC.

Clinical evidence indicates that the ING4 tumor suppressor is downregulated in human CRC (22,23). To further evaluate ING4 expression in human CRC cells, we quantified ING4 mRNA in a panel of human CRC cell lines including low (LS174T and SW480) and high (LoVo and SW620) metastatic cell lines by qRT-PCR analysis (Fig. 1A). Compared with normal human colorectal mucous epithelial control FHC cells, all of the tested low and high metastatic human CRC cells displayed lower expression of ING4 (p<0.05). Moreover, ING4 expression in the highly metastatic LoVo and SW620 CRC cells was less than that in the lowly metastatic LS174T and SW480 CRC cells (p<0.05).

Our cellular model data supported previously studied clinical data (22,23), suggesting that ING4 is reduced in human CRC and may facilitate the progression and metastasis of CRC.

Lentiviral-mediated ING4 stable expression. To establish ING4 stably transgenic CRC cells, we constructed a recombinant lentivirus LV-ING4-expressing humanized ING4 gene and GFP marker gene. After infection of the LoVo cells with 10 MOI LV-ING4 or LV followed by selection with Blasticidin S, we obtained the LoVo-ING4 and LoVo-Mock transfectants. Fluorescence microscopic analysis (Fig. 1B) showed that >90% of GFP expression was found in the LoVo-ING4 and LoVo-Mock tumor cells, whereas GFP expression was not noted in the uninfected LoVo control cells. To further detect the lentiviral-mediated ING4 transgene expression, the LoVo-ING4 and LoVo-Mock tumor and LoVo control cells were analyzed by RT-PCR (Fig. 1C) and western blotting (Fig. 1D). As shown in Fig. 1C and D, the lentiviral-mediated exogenous ING4 gene was highly expressed at both the transcriptional and translational levels in the LoVo-ING4 tumor cells, but not in the LoVo-Mock and LoVo control cells. These results indicated that the ING4-stably transgenic LoVo human CRC cell line directed by the lentivirus was successfully established.
ING4 suppresses CRC growth. To examine the effect of lentiviral-mediated ING4 expression on human CRC growth in vitro, we generated transgenic LoVo-ING4 and LoVo-Mock CRC cells and determined the tumor cell viability by MTT assay. As shown in Fig. 2A, lentiviral-mediated ING4 gene transfer obviously inhibited human CRC LoVo cell growth in vitro in a time-dependent manner compared to the LoVo-Mock group (p<0.05). To further assess whether the ING4-induced growth-suppressive effect on CRC in vitro could be reproduced in vivo, we monitored and compared the growth of LoVo-ING4 and LoVo-Mock human CRC s.c. xenografted tumors in athymic nude mice. As shown in Fig. 2B-E, the in vivo growth of the LoVo-ING4 CRC cells was also markedly retarded (p<0.05).

ING4 induces G1 phase arrest in CRC cells possibly via upregulation of P21 and downregulation of cyclin E. To explore the cellular mechanism responsible for ING4-mediated tumor suppression in CRC cells, the cell cycle profile of LoVo-ING4 and LoVo-Mock CRC cells was analyzed using PI staining by flow cytometry. As shown in Fig. 3A and B, LoVo-ING4 transgenic CRC cells exhibited a significant increase in the cell cycle G1 phase population (G1 population in the total cell population, 67.8±5.2%) compared with LoVo-Mock control tumor cells (48.1±3.7%) (p<0.05), indicating that ING4 efficiently elicits LoVo CRC G1 arrest. To further elucidate the molecular mechanism underlying ING4-induced G1 arrest, we analyzed the expression of G1-related proteins such as Cip/Kip family cyclin-dependent kinase (CDK) inhibitor P21, cyclin E and CDK2 by western blotting (Fig. 3C and D). As expected, ING4 substantially upregulated P21 expression as well as downregulated cyclin E expression in the LoVo cells (p<0.05).

ING4 inhibits tumor angiogenesis via downregulation of IL-6, IL-8 and VEGF. To investigate the effect of lentiviral-mediated ING4 expression on tumor angiogenesis in vivo, the MVD in...
LoVo-ING4 and LoVo-Mock human CRC s.c. xenografted tumor sections was detected by CD31 immunohistochemical analysis. The positive expression of CD31 was mainly presented as brownish yellow or brownish granules in tumor vascular endothelial cells of the xenografted tumors (Fig. 4A). Compared with the LoVo-Mock group, the CD31 expression in the LoVo-ING4 group was weaker or less (Fig. 4A). In addition, the MVD assessed in the LoVo-ING4 group was significantly less than that in the LoVo-Mock group (Fig. 4B) (p<0.05). To delineate the mechanism involved in the ING4-mediated tumor angiogenesis suppression, the expression of epithelial marker E-cadherin, mesenchymal markers as N-cadherin and vimentin, and epithelial-mesenchymal transition (EMT)-inducing transcription factors (EMT-TFs) Snail1, Snail2, ZEB1 and Twist in the LoVo-ING4 and LoVo-Mock tumor cells was analyzed by western blotting (Fig. 5C and D). Compared with the LoVo-Mock group, ING4 significantly increased the E-cadherin expression as well as decreased the N-cadherin and vimentin expression in the LoVo cells (p<0.05). Notably, ING4 markedly suppressed the Snail1 expression in the LoVo cells (p<0.05). These data indicated that ING4 inhibits CRC invasion and metastasis via reversal of EMT through downregulation of Snail1 EMT-TF and a switch from N-cadherin to E-cadherin.

**Discussion**

It has been found that the expression level of the ING4 tumor suppressor in human colorectal cancer (CRC) is negatively
correlated with clinical stage, tumor angiogenesis and metastasis (22,23). This evidence promoted us to further examine the role of ING4 in human CRC progression using a high metastatic LoVo CRC tumor model. In the present study, we found that lentiviral-mediated ING4 gene transfer induced obvious tumor growth suppression, G1 phase arrest, inhibition of invasion and reduced MVD in the human CRC LoVo cells in vitro and/or in vivo in athymic BALB/c nude mice.

The dysregulation of cell cycle control plays an important role in cancer growth. Previous studies have demonstrated that ING4 can suppress tumor growth via induction of S phase reduction and G2/M phase arrest (6,7,10). Inconsistent with these findings, our studies showed that lentiviral-mediated ING4 expression efficiently induced G1 phase arrest in the highly metastatic human CRC LoVo cells. These results suggest that the modulatory effect of ING4 on cancer cell cycle is cell type-dependent. The cell cycle progression is subtly tuned by cell cycle regulatory proteins including cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (27). P21 as a member of the Cip/Kip family is a critical CDK inhibitor that can inhibit the activity of the cyclin E/CDK2 complex, resulting in G1 phase arrest (27). It has also been shown that ING4 upregulates P21 expression in tumor cells via a p53-dependent mechanism through enhancement of p53 acetylation and transcription activity (6). To examine the molecular mechanism involved in the ING4-induced G1 arrest, we analyzed the levels of P21, cyclin E and CDK2 by western blot analysis. We found that ING4 markedly upregulated the p53-downstream gene P21 and downregulated cyclin E in wild-type p53 LoVo cells, which may be an important mechanism responsible for ING4-mediated LoVo CRC G1 arrest and growth inhibition.

Tumor angiogenesis as a hallmark of cancer is indispensable for progressive tumor growth and metastasis, and is a potential anticancer therapeutic target (28). A great deal of data has revealed that inhibition of tumor angiogenesis and vessel normalization represents a promising and non-toxic anticancer strategy (29,30). ING4 has been found to suppress...
tumor angiogenesis via downregulation of IL-6 and IL-8 proangiogenic factors through attenuation of NF-κB and HIF-1α signaling (18,20). To investigate the effect of the lentiviral-mediated ING4 expression on the angiogenesis of human CRC s.c. xenografted tumors in vivo, the microvessel density (MVD) in LoVo xenografted tumor tissues was determined by CD31 immunohistochemical analysis. Our data showed that ING4 markedly downregulated tumor vessel CD31 expression and reduced MVD in the LoVo human CRC xenografted tumors, which may be another important mechanism accountable for ING4-induced in vivo LoVo human CRC growth inhibition in the athymic nude mice. To delineate the mechanism underlying the ING4-mediated inhibition of in vivo tumor angiogenesis, the effect of ING4 on expression of proangiogenic factors IL-6, IL-8 and VEGF which are regulated by NF-κB and HIF-1α transcription factors (18,20,31) in LoVo CRC cells was further assessed. We found that ING4 profoundly downregulated the expression of IL-6, IL-8 and VEGF in the LoVo cells. These results indicated that ING4 suppresses LoVo CRC tumor angiogenesis possibly via reduction of proangiogenic factors IL-6, IL-8 and VEGF.

Tumor invasion and metastasis are key hallmarks of cancer, resulting in as much as 90% of cancer-related deaths (28,32). Distant metastasis is also the major cause of cancer-related mortality in CRC patients. Epithelial-mesenchymal transition (EMT) as a developmental regulatory program has been shown to be prominently implicated in cancer invasion and metastasis (28,33). Downregulation of epithelial marker E-cadherin and upregulation of mesenchymal marker N-cadherin (also referred to as cadherin switch) is a hallmark...
of EMT (33). Cancer-associated EMT in epithelial cancers induces a mesenchymal phenotype with increased migration and invasion potential. The activation of EMT is orchestrated by EMT-inducing transcription factors (EMT-TFs) such as Snail1, Snail2, ZEB1 and Twist by western blotting. We demonstrated that ING4 obviously downregulated the expression of Snail1 in the LoVo cells, leading to the upregulation of E-cadherin and the reduction of N-cadherin and vimentin. Our data suggest that ING4 suppresses CRC invasion and metastasis via reversal of EMT through downregulation of Snail1 and a switch from N-cadherin to E-cadherin.

In summary, our study showed that ING4 inhibits CRC growth via induction of G1 phase arrest through regulating G1 phase checkpoint molecules, and inhibition of tumor angiogenesis by reducing proangiogenic factors. Importantly, the present study also provides the first compelling evidence that ING4 is capable of suppressing CRC invasion and metastasis via reversal of EMT through downregulation of EMT-TF Snail1.

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