Effects of ephrinB2 gene siRNA on the biological behavior of human colorectal cancer cells

PEIWU LI[1,2*], WANGSHENG CHEN[1,3*], YINGZHEN WANG[2], XU FU[2], KUNMING WEN[1], JIANG QIAN[1], CHUN HUANG[1] and ZHONGXUE FU[1]

[1]Department of General Surgery, First Affiliated Hospital, Chongqing Medical University, Chongqing 400010; [2]Department of Emergency, Lanzhou University Second Hospital, Lanzhou 730000; [3]Department of General Surgery, The Affiliated Hospital of Luzhou Medical College, Luzhou 646000, P.R. China

Received July 7, 2014; Accepted October 9, 2014

DOI: 10.3892/or.2014.3633

Abstract. Colorectal cancer (CRC) is a common gastrointestinal malignancy worldwide and is a lethal and aggressive malignancy with a dismal prognosis. EphrinB2 is a membrane-bound ligand and has an intracellular domain that also possesses an intrinsic signaling capacity called 'reverse signaling'. In the present study, CRC cell lines were screened for high expression of ephrinB2. Small interfering RNA (siRNA) knockdown of ephrinB2 was performed in human SW480 CRC cells. The levels of expression of ephrinB2, VEGF, CD105 and matrix metalloproteinase 9 (MMP9) protein were measured by western blotting, and messenger RNA (mRNA) levels were measured using real-time PCR. Apoptosis and cell cycle distribution were determined using flow cytometry. Cell proliferation was measured by a methyl thiazole tetrazolium (MTT) test and a scratch healing experiment was used to measure the extent of cell migration. A Transwell assay was used to detect the extent of cell invasion. The results showed that RNA interference (RNAi) of ephrinB2 effectively silenced the ephrinB2 gene at both the mRNA and protein levels in SW480 cells and inhibited the proliferation, invasion, migration and angiogenesis and induced apoptosis in SW480 cells. These effects may be attributed to VEGF and MMP9 regulation.

Introduction

Colorectal cancer (CRC) is a common gastrointestinal malignancy worldwide and is a lethal and aggressive malignancy with a dismal prognosis (1,2). It is the third most common malignant tumor and is one of the most common cancers in China (3). With the recent development of molecular biology, the treatment of CRC has made great progress but it is still difficult to treat advanced CRC, which has a poor prognosis and a 40% overall mortality rate (4-6). The recurrence rates for this type of cancer remain high and new therapeutic strategies are crucially needed. Therefore, it is of great importance to understand the molecular mechanisms of CRC progression as a prerequisite for developing more effective therapeutic strategies.

The Eph family, the largest group among the tyrosine kinase receptor families, is comprised of the EphA (EphA1-10) and EphB (EphB1-6) subclasses of receptors. They are classified according to their sequence homologies and their binding affinities for their ligands, ephrins (Eph receptor interacting protein) (7-9). The first member of the Eph family was cloned from an erythropoietin-producing hepatocellular cancer cell line in 1987 and was named EphA1. The ligands for the Eph receptors, ephrins, are divided into two subclasses, ephrinA (ephrinA1-6) and ephrinB (ephrinB1-3) (10-12).

The role of Eph-ephrin signaling has been studied in great detail in reference to the development of the nervous system and spans a wide range of functions, such as the development of neuronal networks, axon guidance, formation and remodeling of synaptic connections and nervous system repair (13). The interaction of Eph receptors with ephrins is known to mediate cell-cell repulsion, regulate axon outgrowth, restrict cell migration and maintain well-defined boundaries between different anatomical components of the developing brain (10,11,14). The Eph-ephrin interaction also regulates the remodeling of vascular network formation during embryonic development (15,16). A detailed review of research on Eph receptors and ephrin ligands in embryonic development and carcinogenesis has been reported in a number of review articles (17-19). An understanding of EphA2 and ephrinA1 expression, signaling and deregulation are important in developing strategies for cancer therapies.

EphrinB2 is a membrane-bound ligand and has an intracellular domain that also possesses an intrinsic signaling capacity called 'reverse signaling' (20). The complete deletion of the intracellular domain of ephrinB2 results in a severe defect of angiogenesis and embryonic lethality, which indicates that

Correspondence to: Dr Zhongxue Fu, Department of General Surgery, First Affiliated Hospital, Chongqing Medical University, 1 College Road, Yuzhong, Chongqing 400010, P.R. China
E-mail: fuzhongxue08@163.com

*Contributed equally

Key words: colorectal cancer, SW480 cell, ephrinB2, small interfering RNA

ephrinB2 plays a critical role in developmental blood vessel formation (21). EphrinB2 reverse signaling has been shown to regulate both developmental and tumor angiogenesis by activating vascular endothelial growth factor receptor 2 (VEGFR-2) (22,23). In a separate study, an analysis of pericyte-specific ephrinB2 deficiency indicated that ephrinB2 was essential for normal coverage of the microvasculature by pericytes (24).

More recently, RNA interference (RNAi) has been used to regulate gene expression. RNAi provides a simple, effective, specific and important mechanism to silence gene expression (25). RNAi uses a nuclease to cut double-stranded RNA into small interfering RNA (siRNA) fragments from 21 to 25 nucleotides. Based on the mechanism of base pairing, these RNA fragments recognize and cleave their homogenous target messenger RNA (mRNA) molecule, which silences the sequence-specific mRNA (26). This mechanism is sequence-specific and causes downregulation of specific protein expression (27-29). RNAi technology is currently considered an important tool for functional genomic analyses and is also an important mechanism for specific gene-silencing therapies (28,30,31). In the present study, the siRNA technique was used to silence the expression of ephrinB2 in human colon carcinoma cell line SW480 and to evaluate the biological behavior of the cells to determine the role of ephrinB2 expression in tumor proliferation, adhesion, migration and invasion.

Many scholars have conducted extensive research into gene therapy for CRC (32,33). However, several factors influence the effectiveness of these therapies, resulting in unsatisfactory effects. Recently, researchers have become aware that gene silencing can be used as an effective regulation method for gene expression because it enables the specific blocking of target gene expression. Thus, gene silencing has a key role in research fields such as gene functioning and targeted therapy (34). RNA-induced gene silencing occurs at the transcriptional and posttranscriptional levels. At the posttranscriptional level, it may be attributed to mRNA degradation and is also known as RNAi.

We designed and synthesized the first specific siRNA for ephrinB2 and evaluated its effects using experimental methods. Furthermore, we analyzed the antitumor mechanisms of ephrinB2 silencing using ephrinB2 siRNA in a human CRC cell line (SW480). We located a CRC cell line with high expression of ephrinB2. We used RNAi to target ephrinB2 gene expression and detected interference of ephrinB2, VEGF, CD105 and matrix metalloproteinase (MMP9). This enabled us to study various biological characteristics in SW480 cells after disturbance, to investigate the effects of ephrinB2's biological role in CRC and its possible mechanisms. We provide a theoretical and experimental basis for the ephrinB2 gene to become a new target for CRC molecular diagnostics and gene therapy.

Materials and methods

Cell culture. The human CRC cell lines SW480, SW620 and HT29 used in this study were provided by Professor Kun-Ming Wen from the First Affiliated Hospital of Chongqing Medical University, China. The human CRC cell line LOVO and 293T cells were purchased from the Shanghai Cell Biology Research Institute of the Chinese Academy of Sciences. All of the human CRC cell lines used in this study were screened for ephrinB2 protein and mRNA expression, detected by western blotting and real-time PCR, respectively (data not shown). SW480 cells, which expressed the highest ephrinB2 levels among the CRC cell lines examined, were used in the study. The cells were grown and maintained at 37˚C in a humidified incubator containing a 5% CO_2 atmosphere. All of the experiments were performed with cells in the logarithmic phase of growth.

Main reagents and equipment. The RNA extract reagent TRIzol and the PCR kits used in this study were purchased from Takara Biotechnology Co. Ltd. (Dalian, China). EphrinB2, VEGF, CD105 and MMP9 primers were also synthesized by Takara Biotechnology. Anti-GAPDH antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G were obtained from Beijing Zhongshan Jinqiao Biotechnology Co. Ltd. (Beijing, China). EphrinB2, VEGF, CD105 and MMP9 antibodies were purchased from Epitomics Inc. (Cambridge, MA, USA), and Transwell plates were purchased from Millipore Inc. (Billerica, MA, USA). Quantitative real-time PCR was performed in a Rotor-Gene 6000 thermal cycler (Sydney, Australia).

Construction and screening of a high efficiency interference plasmid. The coding sequence of the ephrinB2 gene in the SW480 genome was submitted to Ambion siRNA Target Finder (http://www.ambion.com/techlib/misc/siRNA_finder.html). Three 19-nucleotide siRNA target sequences corresponding to nucleotides E65 (CTGCGATTTCCAAATC GAT), E376 (CTGTGCCAAACCAGACCAA) and E730 (CAACATCCTCGGTTCCGAA) of the ephrinB2 gene were identified. The corresponding double-stranded siRNAs were synthesized *in vitro* using a Silencer™ siRNA Construction kit from Ambion (Austin, TX, USA) following the manufacturer's protocols. The annealed siRNA pSilencer-ephrinB2-siRNA65 (pE65), pSilencer-ephrinB2-siRNA730 (pE730) and pSilencer-ephrinB2-siRNA376 (pE376) were transiently transfected into 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. As a control, siRNA targeting a scrambled sequence with no known consensus to the human genome was also introduced under identical conditions. After 48 h, the cells were harvested and their mRNA and proteins were extracted. Using quantitative real-time PCR and western blotting, the siRNA targeted nucleotide E376 showed decreased ephrinB2 mRNA levels of 20% or less. The siRNA targeted nucleotide E376 was the most effective of the three siRNAs tested (data not shown). Mock transfection and the use of the control siRNA did not affect levels of ephrinB2, consistent with the specificity of the siRNA approach.

siRNA transfection and cell groups. Experimental SW480 cells were divided into several groups: transfected pSilencer2.1-ephrinB2-shRNA376 cells (RNAi), transfected siRNA negative control cells (NC) and normal SW480 cells (CON). These cells were transfection using the same methods as described above. Briefly, $2x10^5$ tumor cells were seeded in 6-well culture plates in 2 ml of antibiotic-free growth medium supplemented with FBS for 24 h. The supernatant was removed

Table I. Primer sequences used for the real-time PCR.

Gene name (accession no.)	Primer name	Primer sequences	Primer Tm	Product size (bp)
EphrinB2 (NM_004093.3)	Forward	5'-CGATTGAGCCTTACGACAC-3'	56	256
	Reverse	5'-TTTTAAGCGCTGAGCATTG-3'		
VEGF (NM_001171623)	Forward	5'-CTGCTGTCTTGGGTGCATTG-3'	60	107
	Reverse	5'-TCGTGATGATTCTGCCCTCC-3'		
CD105 (NM_000118.2)	Forward	5'-TACCCATACCCAAAACCG-3'	56	138
	Reverse	5'-ATGAGGAAGGCACCAAAG-3'		
MMP9 (NM_004994.2)	Forward	5'-AGCACGGAGACGGGTATC-3'	60	214
	Reverse	5'-CAGGCGGAGTAGGATTGG-3'		
GAPDH	Forward	5'-TGACTTCAACAGCGACACCCA-3'	55	121
	Reverse	5'-CACCCTGTTGCTGTAGCCAAA-3'		

VEGF, vascular endothelial growth factor; MMP9, matrix metalloproteinase 9.

when the cells were transfected. A total of 100 pM siRNA, 5 μl transfection reagent and 500 μl serum-free DMEM were mixed thoroughly and incubated at room temperature for 20 min. This mixture was added to the cells and incubated for another 6 h. The supernatant was then removed and replaced with fresh media containing 10% FBS. After 48 h of incubation, the cells were harvested for further analysis. Three wells were left untransfected to serve as a negative control. The efficiency of the transfection was assessed by fluorescence microscopy.

Detection of mRNA levels of ephrinB2, VEGF, CD105 and MMP9 using real-time PCR. Real-time PCR was performed to analyze the mRNA expression levels of ephrinB2, VEGF, CD105 and MMP9 in the SW480 cells. The total RNA was extracted from the SW480 cells using a commercial extract reagent RNAiso Plus obtained from Takara Biotechnology. First-strand complementary DNA was synthesized using a PrimeScript® Real-Time Master Mix kit (obtained from Takara Biotechnology). Quantitative real-time PCR was performed using a SYBR® Premix Ex Taq™ II kit (Takara Biotechnology) in a Rotor-Gene 6000 thermal cycler with continuous monitoring of fluorescence. The reactions were carried out in a 25-μl reaction volume. The primer sequences used to amplify the individual genes are shown in Table I. The real-time PCR was performed with the following program: 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec and annealing at 60˚C for 30 sec. All of the reactions were performed in triplicate. A melting curve analysis was performed to ensure the specificity of the quantitative PCR. The data were analyzed using the $2^{-\Delta\Delta CT}$ method, in which GAPDH was used as an internal standard in each experiment for the quantitative evaluation of a gene's mRNA expression.

Detection of the protein levels of ephrinB2, VEGF, CD105 and MMP9 using western blot analysis. The cells were harvested and lysed in RIPA buffer (Beyotime, Shanghai, China) with protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA) for 30 min while on ice. The cell lysates were centrifuged and the protein concentration was determined using a BCA protein assay kit (Solarbio, Beijing, China). The protein (50 μg) in the cell lysates was boiled for 10 min in a quarter volume of loading buffer and then separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis at a 30 mA constant current until the bromophenol blue shifted out of the bottom of the separating gel. The separated proteins were electroblotted onto a polyvinylidene difluoride membrane using a transfer buffer at 100 V for 60 min. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS)-0.1% Tween (TBST) for 2 h at room temperature, and then incubated with primary antibody/rabbit anti-ephrinB2 (1:2,000 dilution), rabbit anti-VEGF (1:2,000 dilution), rabbit anti-CD105 (1:2,000 dilution), rabbit anti-MMP9 (1:2,000 dilution) and rabbit anti-GAPDH (1:5,000 dilution) in TBST overnight at 4˚C. After washing 5 times in TBST, the membranes were incubated with HRP-conjugated goat anti-rabbit immunoglobulin G (1:5,000 dilution) for 2 h at room temperature. After washing 5 times in TBST, the membranes were analyzed with an enhanced chemiluminescence substrate and used to expose an X-ray film according to the manufacturer's protocol. The relative expression of the protein bands of ephrinB2 (37 kDa), VEGF (22 kDa), CD105 (37 kDa) and MMP9 (92 kDa) were quantified using densitometric scanning of the radiographic films with a GS-700 imaging densitometer and then analyzed with a computer program. The results from each experimental group were expressed as the relative integrated intensity compared to the GAPDH (35 kDa) band densities measured in the same batch.

Detection of cell apoptosis and cell cycle by flow cytometry. To assess cell apoptosis, the cells were harvested 48 h after transfection; washed in cold PBS twice; stained using propidium iodide (PI); and then detected using an ApoScreen Annexin V apoptosis detection kit (Southern Biotech, Birmingham, AL, USA), according to the manufacturer's protocol. Both the experiments and apoptosis rates were conducted using BD FACSVerse (BD Biosciences, Franklin Lakes, NJ, USA). To detect the cell cycle, the cells were harvested and incubated overnight in 75% cold alcohol. After washing with PBS, the cell suspensions were added to RNaseA at a concentration of 0.5 mg/ml, stained with PI for 30 min in a dark room and then

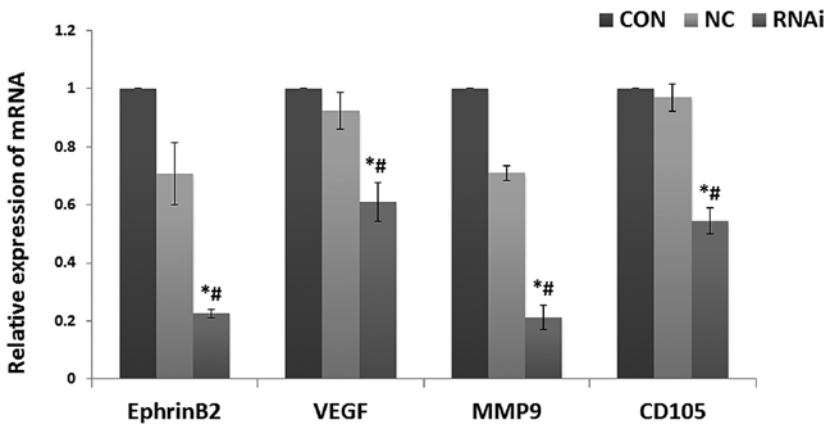

Figure 1. Effects of ephrinB2 siRNA on ephrinB2, VEGF, CD105 and MMP9 mRNA levels in SW480 cells in the normal cell group (CON), the siRNA negative control cell group (NC) and the ephrinB2 siRNA group (RNAi). The differences in the average threshold cycle values were determined and normalized to the expression of GAPDH. Values are expressed as means ± SD. *P<0.05, compared with the CON group. #P<0.05, compared with the NC group.

analyzed using a cell cycle analysis kit (Beyotime, Shanghai, China). Quantitative DNA fragments from the different cell cycles were analyzed by flow cytometry.

Measurement of cell growth. Cell proliferation was measured by a methyl thiazole tetrazolium (MTT) assay (18). The cells were seeded onto 96-well plates at a density of $2x10^4$ cells/well and incubated in a corresponding medium containing 10% serum. Cell growth was analyzed after 1, 2, 3, 4 and 5 days. Six duplicate wells were established for each group. At each time-point, 20 μl MTT (5 mg/ml, Sigma-Aldrich) was added to each well and allowed to incubate for 4 h, then 150 μl DMSO was added into each well. After complete solubilization of the dye, the absorbance of the wells was measured using a microplate spectrophotometer (from Bio-Rad) at 490 nm. Cell growth curves were drawn using SigmaPlot 12.0 software.

Detection of cell migration by scratch test. Cell migration was determined using a scratch assay (35,36). The transfected cell lines were seeded on a 24-well plate and allowed to reach confluency. After scratching the bottom of the well with a pipette tip, the monolayer of cells was washed 3 times with PBS to remove the detached cells. The remaining adherent cells were incubated in a medium (either DMEM/F12 or DMEM) containing 1% FBS for 24 h. This medium was then replaced with a medium containing 10% FBS. Space filling by cell migration was evaluated 24 h later using bright-field microscopy. This experiment was performed in triplicate.

Detection of cell migration by a Transwell assay. The Boyden chamber technique (also called Transwell analysis) was also used to analyze cell migration. Filters with 8-μm pores were coated with 100 μl of 1 mg/ml Matrigel (dissolved in serum-free RPMI-1640 medium). Then, 600 μl RPMI-1640 medium containing 10% FBS was added to the lower chambers. Forty-eight hour post-transfection, homogenous single-cell suspensions ($1x10^5$ cells/well) were added to the upper chambers and allowed to invade for 24 h at 37˚C in a CO_2 incubator. The cells that remained attached to the upper surface of the filters were carefully removed with cotton swabs. The migrated cells were stained with 0.1% crystal violet for 10 min at room temperature and then examined using light microscopy. A quantification of the migrated cells was performed according to established criteria (37).

Statistical analysis. The data were analyzed using SPSS 18.0 software. The average values are presented as mean ± standard deviation (SD). The statistical differences between the groups were analyzed using a one-way ANOVA. Differences were considered significant when the P-value was <0.05.

Results

Effects of ephrinB2 siRNA on ephrinB2, VEGF, CD105 and MMP9 mRNA levels in SW480 cells. VEGF, CD105, MMP9 and ephrinB2 mRNA was chosen for analysis based on their key roles in tumor angiogenesis. Real-time PCR was used to determine whether ephrinB2 siRNA could reduce the expression level of each of these four key substances. As shown in Fig. 1, the expression levels of VEGF, CD105, MMP9 and ephrinB2 mRNA were all significantly decreased in the siRNA group cells compared to the NC and CON cells (all P<0.05). However, there was no significant difference between the levels of expression of VEGF, CD105, MMP9 and ephrinB2 mRNA in the normal SW480 cells and the NC cells (P>0.05). These results indicate that targeted ephrinB2 gene silencing not only effectively silenced ephrinB2 expression, but also reduced VEGF, CD105 and MMP9 mRNA expression levels.

Effects of ephrinB2 siRNA on ephrinB2, VEGF, CD105 and MMP9 protein levels in SW480 cells. To investigate the effect of ephrinB2 gene silencing on colon carcinoma angiogenesis-related genes, VEGF, CD105, MMP9 and ephrinB2 protein levels were measured using western blot analysis. As shown in Fig. 2, the expression levels of VEGF, CD105, MMP9 and ephrinB2 protein were all significantly decreased in the siRNA group cells compared with the CON and NC group cells (all P<0.05). However, there were no significant differences between the CON and NC group cells.

Effects of ephrinB2 siRNA on SW480 cell apoptosis. Correlations between ephrinB2 silencing and apoptotic ratios

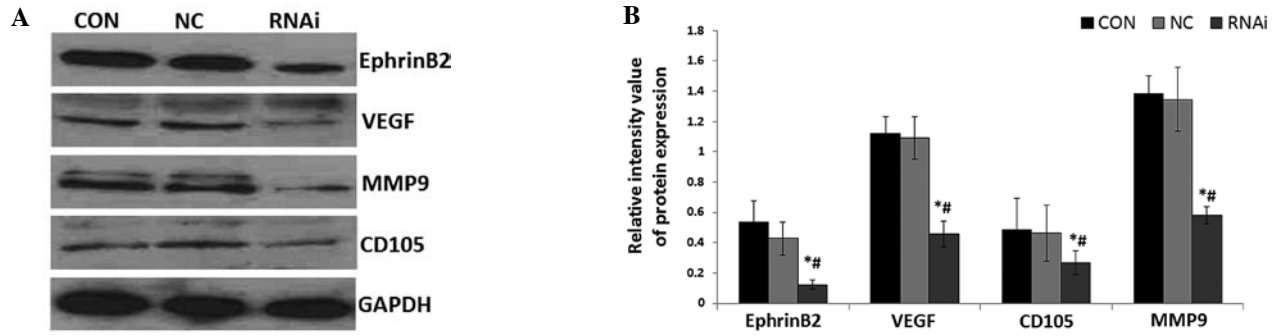

Figure 2. (A) Effects of ephrinB2 siRNA on ephrinB2, VEGF, CD105 and MMP9 protein levels in SW480 cells in the normal cell group (CON), the siRNA negative control cell group (NC) and the ephrinB2 siRNA group (RNAi). (B) Relative intensity values of the protein expression levels in the three groups of cells. The differences in the average threshold cycle values were determined and normalized to the expression of GAPDH. Values are expressed as means ± SD. *P<0.05, compared with CON group. #P<0.05, compared with NC group.

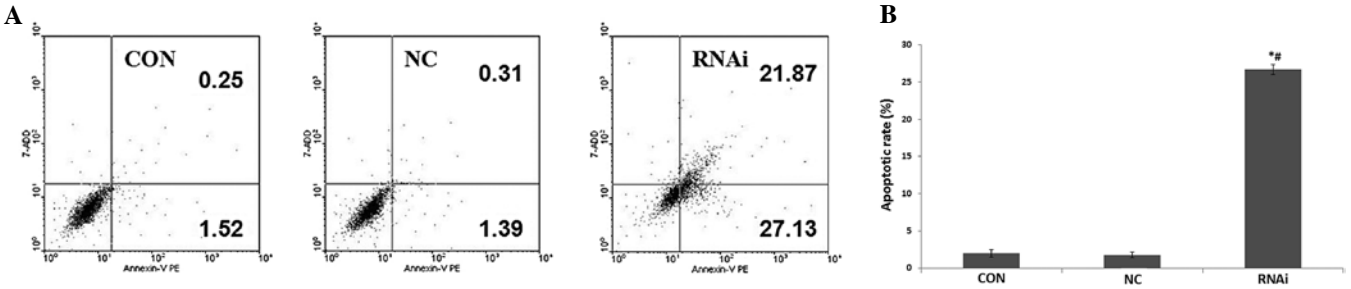

Figure 3. (A) Effects of ephrinB2 siRNA on SW480 cell apoptosis in the normal cell group (CON), the siRNA negative control cell group (NC) and the ephrinB2 siRNA group (RNAi). (B) The apoptotic rate of the different cell groups. The differences in the average threshold cycle values were determined and normalized to the expression of GAPDH. Values are expressed as means ± SD. *P<0.05, compared with the CON group. #P<0.05, compared with the NC group

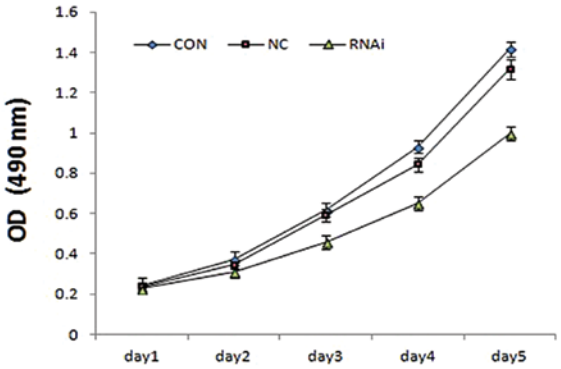

Figure 4. The growth of SW480 cells was monitored by MTT assay in the normal cell group (CON), the siRNA negative control cell group (NC) and the ephrinB2 siRNA group (RNAi).

in SW480 cells were evaluated using flow cytometry. These results showed that the apoptosis ratio in the siRNA group cells (26.69±0.67) was significantly higher (P<0.05) than this ration in both the CON group (1.97±0.47) and the NC group (1.74±0.36) (Fig. 3). The results of the apoptosis assay indicate that ephrinB2 siRNA promotes SW480 cell apoptosis.

Effects of ephrinB2 siRNA on SW480 cell proliferation. To determine the effects of ephrinB2 siRNA on cell viability, the biological effects of ephrinB2 siRNA on the proliferation of SW480 cells were monitored using MTT assays. The MTT assays showed that the proliferation of SW480 cells in the siRNA group cells was inhibited in a time-dependent manner, compared with the control cells. There were no obvious differences between the CON and NC group cells (Fig. 4). These results showed that the knockdown of ephrinB2 by siRNA can inhibit the growth of SW480 cells.

Effects of ephrinB2 siRNA on the cell cycle distribution of SW480 cells. As shown in Fig. 5, ephrinB2 siRNA was also found to have an effect on the cell cycle. The percentage of cells in the S phase in the siRNA group (18.32±0.38%) was significantly lower than that of the CON group (25.22±0.89%) and the NC group (24.34±1.26%). However, the percentage of cells in the G1 phase in the siRNA group (74.49±1.55%) was significantly higher than that in the CON group (55.98±1.66%) and the NC group (57.78±2.22%). These results suggest that ephrinB2 siRNA may interfere with cell mitosis and cell cycle progression, arresting cells in the G1 phase and inhibiting SW480 cell proliferation.

Effects of ephrinB2 siRNA on SW480 cell migration. Scratch experiments were carried out to study the effects of ephrinB2 siRNA on the migratory ability of SW480 cells. As shown in Fig. 6, the cell migration distance (mm) in the siRNA group (18.33+0.71) was significantly shorter (P<0.05) than that in the CON (32.7±1.25) and NC groups (30.57±1.36). There was no significant difference between the cell migration distances in the CON and NC groups (P>0.05).

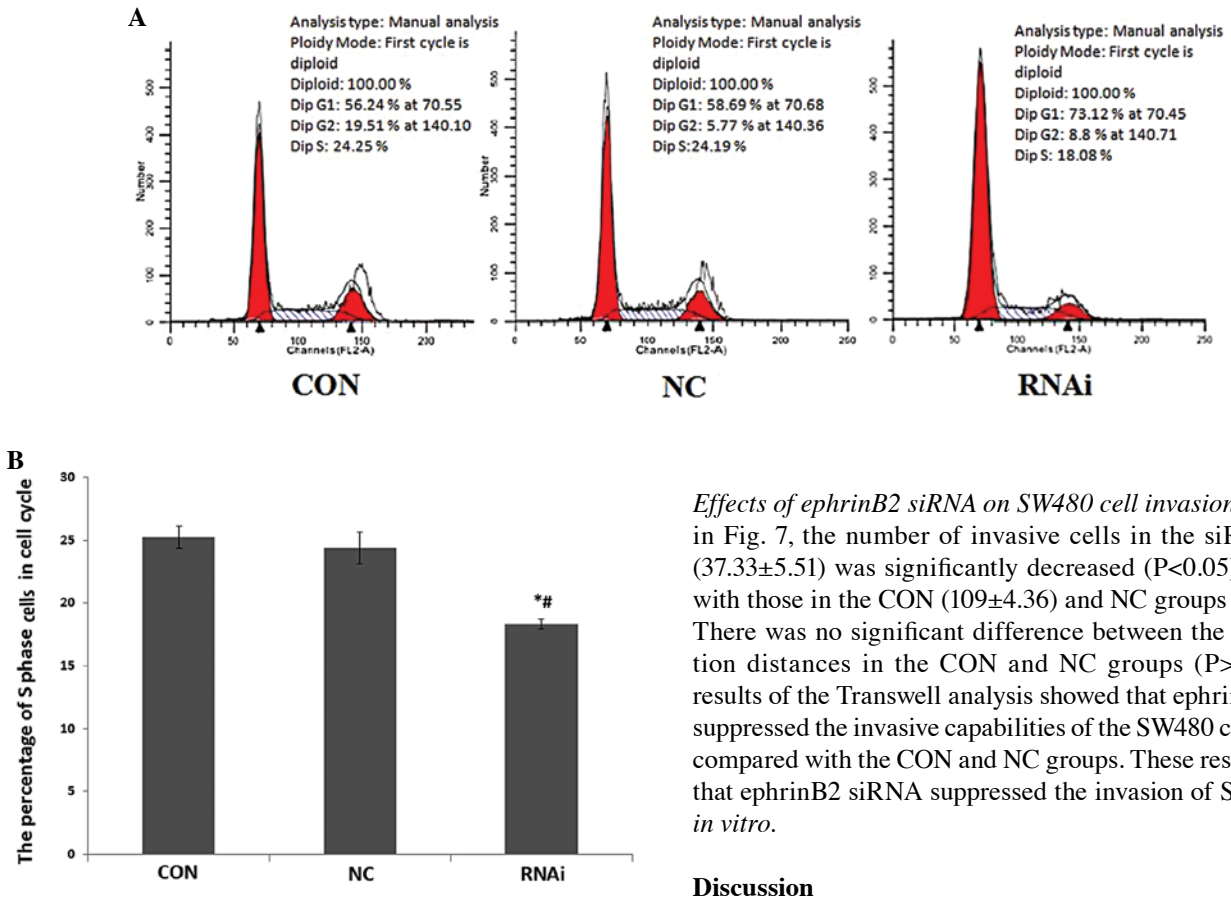

Figure 5. (A) Cell cycle distribution as detected by flow cytometry in the normal cell group (CON), the siRNA negative control cell group (NC) and the ephrinB2 siRNA group (RNAi). (B) The percentage of cells in the S phase of the cell cycle. The differences in the average threshold cycle values were determined and normalized to the expression of GAPDH. Values are expressed as means ± SD. *P<0.05, compared with the CON group. #P<0.05, compared with the NC group.

Effects of ephrinB2 siRNA on SW480 cell invasion. As shown in Fig. 7, the number of invasive cells in the siRNA group (37.33±5.51) was significantly decreased (P<0.05) compared with those in the CON (109±4.36) and NC groups (102±4.58). There was no significant difference between the cell migration distances in the CON and NC groups (P>0.05). The results of the Transwell analysis showed that ephrinB2 siRNA suppressed the invasive capabilities of the SW480 cells for 48 h compared with the CON and NC groups. These results suggest that ephrinB2 siRNA suppressed the invasion of SW480 cells *in vitro*.

Discussion

Previous studies have shown that tumor angiogenesis may provide sufficient nutrients for the growth of a tumor and also provide an effective channel for tumor invasion and metastasis. Therefore, the roles and mechanisms of tumor angiogenesis and anti-angiogenesis in tumor growth, invasion and migration

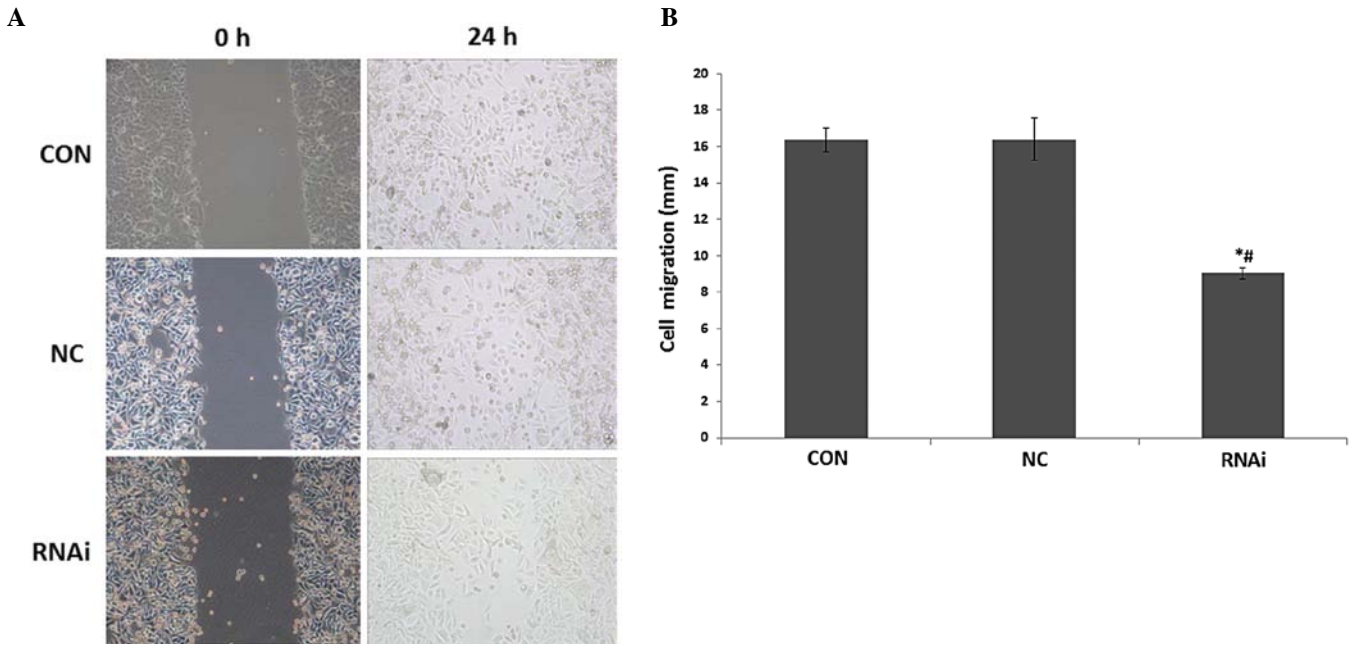

Figure 6. (A) Effects of ephrinB2 siRNA on SW480 cell migration in the normal cell group (CON), the siRNA negative control cell group (NC) and the ephrinB2 siRNA group (RNAi) (magnification, x200). (B) Quantitation of the cell migration (mm). The differences in the average threshold cycle values were determined and normalized to the expression of GAPDH. Values are expressed as means ± SD. *P<0.05, compared with the CON group. #P<0.05, compared with the NC group.

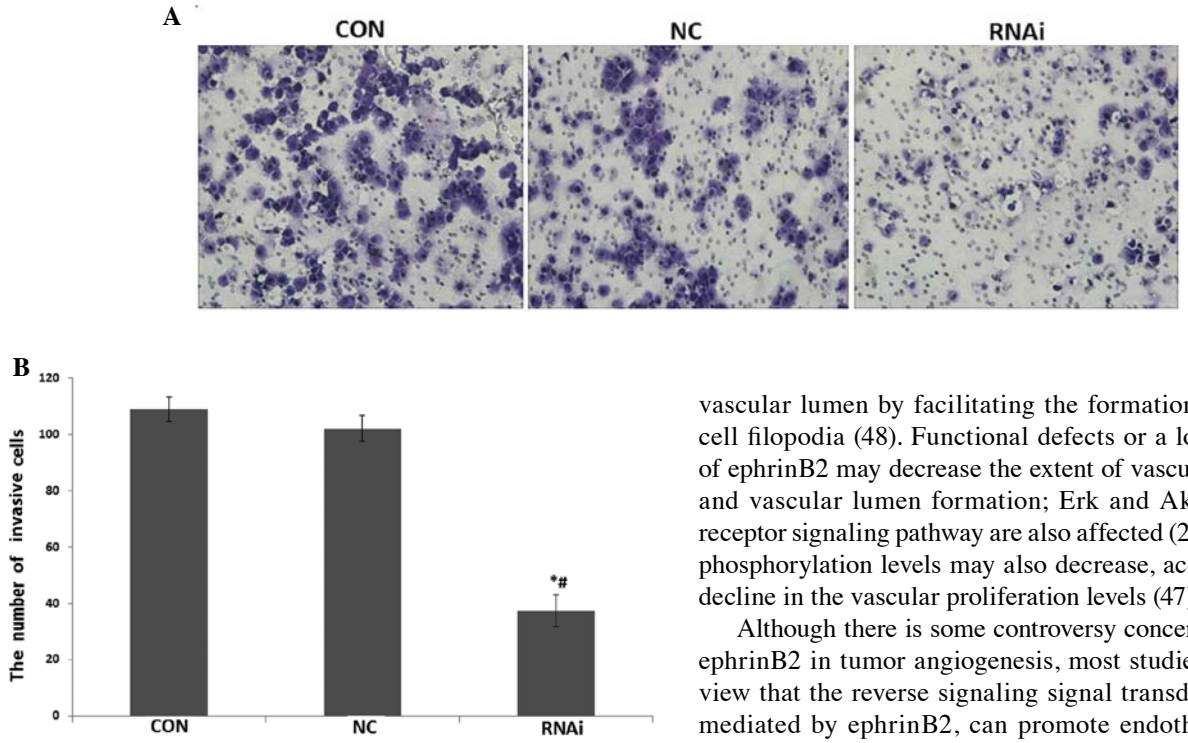

Figure 7. (A) Effects of ephrinB2 siRNA on SW480 cell invasion in the normal cell group (CON), the siRNA negative control cell group (NC) and the ephrinB2 siRNA group (RNAi) (magnification, x200). (B) The number of invasive cells. The differences in the average threshold cycle values were determined and normalized to the expression of GAPDH. Values are expressed as means ± SD. *P<0.05, compared with the CON group. #P<0.05, compared with the NC group.

have been given a great deal of attention. The inhibition of tumor angiogenesis is expected to become an important and effective means of targeted tumor therapy. Recent studies have also found that the ephrinB2/EphB4 system and its specific bidirectional signal transduction plays a particularly important role in tumor angiogenesis and is associated with the occurrence, development and prognosis of tumors (38,39). EphrinB2 is part of the Eph family, which is the largest subfamily of receptor tyrosine kinases. The Eph family includes the Eph receptor and its corresponding ligand ephrin. The Eph receptors are divided into two subfamilies, EphA (A1-A8) and EphB (B1-B6) receptors (40,41). The ligands can also be divided into the ephrinA (ephrinA1-6) and ephrinB (ephrinB1-B6) categories (42). EphrinB2 can activate downstream pathways through the combination of its extracellular portion and the Eph receptor, which is phosphorylated at the intracellular tyrosine kinase binding sites in Eph. EphrinB2 also has an intracellular domain that possesses an intrinsic signaling capacity called 'reverse signaling' (20). The complete deletion of the intracellular domain of ephrinB2 results in a severe defect of angiogenesis and embryonic lethality, indicating a critical role for ephrinB2 reverse signaling in developmental blood vessel formation (21). This bi-directional signaling pathway has an important function in angiogenesis (13). Numerous studies have shown that ephrinB2 is mainly expressed in the small arteries, especially in the neovessels in a proliferative state (43-47). EphrinB2 can also increase the migratory capability of endothelial cells and promote the formation of the vascular lumen by facilitating the formation of endothelial cell filopodia (48). Functional defects or a lower expression of ephrinB2 may decrease the extent of vascular proliferation and vascular lumen formation; Erk and Akt in the VEGF receptor signaling pathway are also affected (22,23). EphrinB2 phosphorylation levels may also decrease, accompanied by a decline in the vascular proliferation levels (47).

Although there is some controversy concerning the role of ephrinB2 in tumor angiogenesis, most studies have held the view that the reverse signaling signal transduction pathway, mediated by ephrinB2, can promote endothelial cell invasion, migration and tube formation; plays a catalytic effect on angiogenesis; and is closely associated with tumor invasion and metastasis (49-52). However, the effects of the reverse signaling signal transduction pathway mediated by ephrinB2 on CRC angiogenesis, invasion and metastasis and the specific molecular mechanism by which this may occur remain unknown.

Angiogenesis is a highly varied process (53-56) and the mechanism by which it progresses is extremely complex. Multiple bioactive factors are involved, including tumor angiogenesis promoting factors VEGF, MMP9, βGF, EGFR, TGF-β and CD105; tumor angiogenesis inhibiting factors, such as MMP inhibitors, endostatin and vascular endothelial growth factor inhibitors (57,58); and multiple signaling pathways, such as p38MAPK, Akt/Pkb, PI3K, receptor tyrosine kinases, Dll4-Notch and VEGF-VEGFR. Studies have shown that the microvessel density is a good indicator of tumor angiogenesis and has a close relationship with tumor invasion, migration and prognosis (59). The level of CD105 (Enderlin) can also reflect the microvessel density because CD105 is only expressed in proliferating vascular cells. In addition to being a marker of microvessel density, CD105 can also affect tumor angiogenesis by other factors, such as through TGF-β (56).

Research has shown that endothelial growth factor VEGF and MMP9 also play an important role in the process of tumor angiogenesis and may have a synergistic effect (60-62). VEGF is a member of the platelet derived growth factor family and several subfamilies have been found, such as VEGF-A, VEGF-B, VEGF-C and VEGF-D. VEGF-A plays a particularly important role in angiogenesis. MMP9 is a member of the MMP family and is considered the most important substance in dissolving tumor cells, endothelial extracellular matrices and the basilar membrane (63). Many authors have demonstrated that MMP expression patterns have a positive correlation with tumor invasion and metastasis (64) and that it plays an important role in the process of tumor invasion, metastasis and angiogenesis (65,66). High expression of MMP9 in gastrointestinal tumors is closely related with tumor

invasion and migration and can be used as an independent risk factor affecting prognosis (67,68).

Recently, siRNA has been shown to inhibit the expression of the corresponding target gene in mammals (69). In this process, siRNA molecules are separated into single strands and incorporated into the RNA-induced silencing complex, which then cleaves the corresponding cellular mRNA. RNAi serves as a powerful technology to block the expression of a specific target gene (70,71). Yang *et al* (72) found that RNAi against α-fetoprotein could silence the α-fetoprotein gene effectively and thus inhibit cell growth and induce apoptosis in Huh7 cells. Other studies have found that MACC1 siRNA transfection affected HeLa cell biological behavior, caused a significant decrease in cell proliferation and migration and increased the cell apoptosis rate (73). Lui *et al* (74) also showed that the downregulation of VEGFR3 expression by siRNA provided a therapeutic strategy for inhibiting tumor growth and metastasis.

Recently, RNAi technology has become more capable at specifically silencing particular genes. It can be used as a powerful tool for investigating the functions of genes and for genetic therapy for carcinoma. To explore the role of ephrinB2 in tumorigenesis and tumor progression, we silenced the expression of ephrinB2 in the CRC cell line SW480 by using RNAi. The results showed that stealth RNAi against ephrinB2 could not only silence ephrinB2 expression effectively at both the mRNA and protein levels in SW480 cells after transfection but also decreased the expression of VEGF, CD105 and MMP9 compared with the CON and NC cells. Other studies have shown that CD105 acts as a tumor marker for angiogenesis and correlates well with the tumor microvessel density. Our results found that the use of stealth RNAi against ephrinB2 inhibited tumor angiogenesis. This effect may be attributed to the regulation of VEGF and MMP9.

In addition, an MTT assay confirmed that the proliferation of SW480 cells was reduced significantly after the ephrinB2 gene was silenced by RNAi. This also illustrated, indirectly, that the ephrinB2 gene may promote tumor cell proliferation. The state of tumor cell proliferation is closely related to the cell cycle and apoptosis. Given this fact, we used flow cytometry to measure the cell cycle and apoptosis. The results showed that the percentage of cells in the S phase in the siRNA group was significantly decreased. However, the percentage of cells in the G1 phase was significantly increased compared with those in the CON and NC groups. This suggested that ephrinB2 siRNA may have inhibited the SW480 cell proliferation by interfering with cell mitosis and cell cycle progression. The results of the apoptosis assay indicated that ephrinB2 siRNA promoted SW480 cell apoptosis.

A significant feature of malignant tumors that is different from carcinoid tumors is that the ability to invade and metastasize is significantly stronger in malignant tumors. This is one of the main reasons for differences in treatment and long-term survival rates (68). To investigate the effect of ephrinB2 RNAi on SW480 cell migration and invasion capacity, a scratch test and Transwell experiments were used. The results revealed that ephrinB2 siRNA suppressed the migration and invasion of SW480 cells *in vitro*.

Taken as a whole, our study provide three important findings. Stealth RNAi against ephrinB2 can silence the ephrinB2 gene effectively at both the mRNA and protein levels in SW480 cells. Silencing the expression of ephrinB2 inhibits tumor angiogenesis, cell proliferation, invasion and migration and induces apoptosis in SW480 cells. These effects may be attributed to the regulation of VEGF and MMP9 in SW480 cells. EphrinB2 may also play a role in tumor angiogenesis, invasion and metastasis in CRC. However, more research is needed to confirm this finding. These conclusions suggest that ephrinB2 may function as a CRC growth stimulator. Thus, interference with ephrinB2 expression may be an option for suppressing tumor growth and improving the prognosis of CRC patients with high ephrinB2 levels, although further *in vivo* studies are necessary to confirm and extend these findings.

Acknowledgements

We thank Dr Li Ma for her critical review of the manuscript.

References

1. Siegel R, Ma J, Zou Z and Jemal A: Cancer statistics, 2014. CA Cancer J Clin 64: 9-29, 2014.
2. Brenner H, Kloor M and Pox CP: Colorectal cancer. Lancet 383:1490-1502, 2014.
3. Guo P, Huang ZL, Yu P and Li K: Trends in cancer mortality in China: an update. Ann Oncol 23: 2755-62, 2012.
4. Burotto M, Hartley ML, Marshall JL and Pishvaian MJ: Future of targeted agents in metastatic colorectal cancer. Colorectal Cancer 5: 433-443, 2012.
5. Wan D, He S, Xie B, *et al*: Aberrant expression of miR-199a-3p and its clinical significance in colorectal cancers. Med Oncol 30: 378, 2013.
6. Zhou J, Li P, Xue X, *et al*: Salinomycin induces apoptosis in cisplatin-resistant colorectal cancer cells by accumulation of reactive oxygen species. Toxicol Lett 222: 139-145, 2013.
7. Gale NW and Yancopoulos GD: Ephrins and their receptors: a repulsive topic? Cell Tissue Res 290: 227-241, 1997.
8. Kullander K and Klein R: Mechanisms and functions of Eph and ephrin signalling. Nat Rev Mol Cell Biol 3: 475-486, 2002.
9. Murai KK and Pasquale EB: 'Eph'ective signaling: forward, reverse and crosstalk. J Cell Sci 116: 2823-2832, 2003.
10. Pasquale EB: The Eph family of receptors. Curr Opin Cell Biol 9: 608-615, 1997.
11. Zozulya SA and Udovichenko IP: Eph family receptors as therapeutic targets. Bioorg Khim 38: 267-279, 2012 (In Russian).
12. Pasquale EB: Eph receptor signalling casts a wide net on cell behaviour. Nat Rev Mol Cell Biol 6: 462-475, 2005.
13. Pasquale EB: Eph-ephrin bidirectional signaling in physiology and disease. Cell 133: 38-52, 2008.
14. Zisch AH and Pasquale EB: The Eph family: a multitude of receptors that mediate cell recognition signals. Cell Tissue Res 290: 217-226, 1997.
15. Heroult M, Schaffner F and Augustin HG: Eph receptor and ephrin ligand-mediated interactions during angiogenesis and tumor progression. Exp Cell Res 312: 642-650, 2006.
16. Cheng N, Brantley DM and Chen J: The ephrins and Eph receptors in angiogenesis. Cytokine Growth Factor Rev 13: 75-85, 2002.
17. Surawska H, Ma PC and Salgia R: The role of ephrins and Eph receptors in cancer. Cytokine Growth Factor Rev 15: 419-433, 2004.
18. Vaught D, Brantley-Sieders DM and Chen J: Eph receptors in breast cancer: roles in tumor promotion and tumor suppression. Breast Cancer Res 10: 217, 2008.
19. Kinch MS and Carles-Kinch K: Overexpression and functional alterations of the EphA2 tyrosine kinase in cancer. Clin Exp Metastasis 20: 59-68, 2003.
20. Kuijper S, Turner CJ and Adams RH: Regulation of angiogenesis by Eph-ephrin interactions. Trends Cardiovasc Med 17: 145-51, 2007.
21. Adams RH, Diella F, Hennig S, *et al*: The cytoplasmic domain of the ligand ephrinB2 is required for vascular morphogenesis but not cranial neural crest migration. Cell 104: 57-69, 2001.

22. Sawamiphak S, Seidel S, Essmann CL, *et al*: Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis. Nature 465: 487-491, 2010.
23. Wang Y, Nakayama M, Pitulescu ME, *et al*: Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. Nature 465: 483-486, 2010.
24. Foo SS, Turner CJ, Adams S, *et al*: Ephrin-B2 controls cell motility and adhesion during blood-vessel-wall assembly. Cell 124: 161-173, 2006.
25. Fire A, Xu S, Montgomery MK, *et al*: Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391: 806-811, 1998.
26. Martinez J, Patkaniowska A, Urlaub H, *et al*: Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell 110: 563-574, 2002.
27. Shi Y: Mammalian RNAi for the masses. Trends Genet 19: 9-12, 2003.
28. Grimm D and Kay MA: Therapeutic application of RNAi: is mRNA targeting finally ready for prime time? J Clin Invest 117: 3633-3641, 2007.
29. McManus MT and Sharp PA: Gene silencing in mammals by small interfering RNAs. Nat Rev Genet 3: 737-747, 2002.
30. Aagaard L and Rossi JJ: RNAi therapeutics: principles, prospects and challenges. Adv Drug Deliv Rev 59: 75-86, 2007.
31. Lee WR, Chung CL, Hsiao CJ, *et al*: Suppression of matrix metalloproteinase-9 expression by andrographolide in human monocytic THP-1 cells via inhibition of NF-κB activation. Phytomedicine 19: 270-277, 2012.
32. Van Laar RK: An online gene expression assay for determining adjuvant therapy eligibility in patients with stage 2 or 3 colon cancer. Br J Cancer 103: 1852-1857, 2010.
33. De Gramont A, de Gramont A, Chibaudel B, *et al*: From chemotherapy to targeted therapy in adjuvant treatment for stage III colon cancer. Semin Oncol 38: 521-532, 2011.
34. Dykxhoorn DM and Lieberman J: The silent revolution: RNA interference as basic biology, research tool and therapeutic. Annu Rev Med 56: 401-423, 2005.
35. Wu B, Hu K, Li S, *et al*: Dihydroartiminisin inhibits the growth and metastasis of epithelial ovarian cancer. Oncol Rep 27: 101-108, 2012.
36. Wu B, Li S, Sheng L, *et al*: Metformin inhibits the development and metastasis of ovarian cancer. Oncol Rep 28: 903-908, 2012.
37. Nicosia RF and Ottinetti A: Growth of microvessels in serum-free matrix culture of rat aorta. A quantitative assay of angiogenesis in vitro. Lab Invest 63: 115-122, 1990.
38. Djokovic D, Trindade A, Gigante J, *et al*: Combination of Dll4/Notch and Ephrin-B2/EphB4 targeted therapy is highly effective in disrupting tumor angiogenesis. BMC Cancer 10: 641, 2010.
39. Alam SM, Fujimoto J, Jahan I, *et al*: Overexpression of ephrinB2 and EphB4 in tumor advancement of uterine endometrial cancers. Ann Oncol 18: 485-490, 2007.
40. Coulthard MG, Lickliter JD, Subanesan N, *et al*: Characterization of the Epha1 receptor tyrosine kinase: expression in epithelial tissues. Growth Factors 18: 303-317, 2001.
41. Boyd AW, Ward LD, Wicks IP, *et al*: Isolation and characterization of a novel receptor-type protein tyrosine kinase (hek) from a human pre-B cell line. J Biol Chem 267: 3262-3267, 1992.
42. Tandon M, Vemula SV and Mittal SK: Emerging strategies for EphA2 receptor targeting for cancer therapeutics. Expert Opin Ther Targets 15: 31-51, 2011.
43. Arvanitis D and Davy A: Eph/ephrin signaling: networks. Genes Dev 22: 416-429, 2008.
44. Gale NW, Baluk P, Pan L, *et al*: Ephrin-B2 selectively marks arterial vessels and neovascularization sites in the adult, with expression in both endothelial and smooth-muscle cells. Dev Biol 230: 151-160, 2001.
45. Korff T, Braun J, Pfaff D, *et al*: Role of ephrinB2 expression in endothelial cells during arteriogenesis: impact on smooth muscle cell migration and monocyte recruitment. Blood 112: 73-81, 2008.
46. Shin D, Garcia-Cardena G, Hayashi S, *et al*: Expression of ephrinB2 identifies a stable genetic difference between arterial and venous vascular smooth muscle as well as endothelial cells and marks subsets of microvessels at sites of adult neovascularization. Dev Biol 230: 139-150, 2001.
47. Salvucci O, Maric D, Economopoulou M, *et al*: EphrinB reverse signaling contributes to endothelial and mural cell assembly into vascular structures. Blood 114: 1707-1716, 2009.
48. Bentley K, Mariggi G, Gerhardt H and Bates PA: Tipping the balance: robustness of tip cell selection, migration and fusion in angiogenesis. PLoS Comput Biol 5: e1000549, 2009.
49. Xia G, Kumar SR, Masood R, *et al*: EphB4 expression and biological significance in prostate cancer. Cancer Res 65: 4623-4632, 2005.
50. Alam SM, Fujimoto J, Jahan I, *et al*: Coexpression of EphB4 and ephrinB2 in tumour advancement of ovarian cancers. Br J Cancer 98: 845-851, 2008.
51. Brantley-Sieders DM: Clinical relevance of Ephs and ephrins in cancer: lessons from breast, colorectal and lung cancer profiling. Semin Cell Dev Biol 23: 102-108, 2012.
52. Yancopoulos GD, Davis S, Gale NW, *et al*: Vascular-specific growth factors and blood vessel formation. Nature 407: 242-248, 2000.
53. Sun B, Zhang D, Zhang S, *et al*: Hypoxia influences vasculogenic mimicry channel formation and tumor invasion-related protein expression in melanoma. Cancer Lett 249: 188-197, 2007.
54. Cao Y: Tumor angiogenesis and therapy. Biomed Pharmacother 59: S340-S343, 2005.
55. Mahabeleshwar GH and Byzova TV: Angiogenesis in melanoma. Semin Oncol 34: 555-565, 2007.
56. Kerbel RS: Tumor angiogenesis. N Engl J Med 358: 2039-2049, 2008.
57. Nyberg P, Salo T and Kalluri R: Tumor microenvironment and angiogenesis. Front Biosci 13: 6537-6553, 2008.
58. Perez-Gomez E, Del Castillo G, Juan Francisco S, *et al*: The role of the TGF-β coreceptor endoglin in cancer. Sci World J 10: 2367-2384, 2010.
59. Medetoglu B, Gunluoglu MZ, Demir A, *et al*: Tumor angiogenesis in predicting the survival of patients with stage I lung cancer. J Thorac Cardiovasc Surg 140: 996-1000, 2010.
60. Kiselyov A, Balakin KV and Tkachenko SE: VEGF/VEGFR signalling as a target for inhibiting angiogenesis. Expert Opin Investig Drugs 16: 83-107, 2007.
61. Kubota Y: Tumor angiogenesis and anti-angiogenic therapy. Keio J Med 61: 47-56, 2012.
62. Bergers G, Brekken R, McMahon G, *et al*: Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2: 737-744, 2000.
63. Bellon G, Martiny L and Robinet A: Matrix metalloproteinases and matrikines in angiogenesis. Crit Rev Oncol Hematol 49: 203-220, 2004.
64. Velinov N, Poptodorov G, Gabrovski N and Gabrovksi S: The role of matrix metalloproteinases in the tumor growth and metastasis. Khirurgiia (Sofiia) 1: 44-49, 2010 (In Bulgarian).
65. Groblewska M, Siewko M, Mroczko B and Szmitkowski M: The role of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in the development of esophageal cancer. Folia Histochem Cytobiol 50: 12-19, 2012.
66. Deryugina EI and Quigley JP: Matrix metalloproteinases and tumor metastasis. Cancer Metastasis Rev 25: 9-34, 2006.
67. Kim TD, Song KS, Li G, *et al*: Activity and expression of urokinase-type plasminogen activator and matrix metalloproteinases in human colorectal cancer. BMC Cancer 6: 211, 2006.
68. Lukaszewicz-Zajac M, Mroczko B and Szmitkowski M: Gastric cancer - The role of matrix metalloproteinases in tumor progression. Clin Chim Acta 412: 1725-1730, 2011.
69. Grishok A, Tabara H and Mello CC: Genetic requirements for inheritance of RNAi in *C. elegans*. Science 287: 2494-2497, 2000.
70. Harborth J, Elbashir SM, Bechert K, *et al*: Identification of essential genes in cultured mammalian cells using small interfering RNAs. J Cell Sci 114: 4557-4565, 2001.
71. Yu JY, DeRuiter SL and Turner DL: RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. Proc Natl Acad Sci USA 99: 6047-6052, 2002.
72. Yang X, Zhang Y, Zhang L, *et al*: Silencing alpha-fetoprotein expression induces growth arrest and apoptosis in human hepatocellular cancer cell. Cancer Lett 271: 281-293, 2008.
73. Chai H and Yang Y: Effects of MACC1 siRNA on biological behaviors of HeLa. Arch Gynecol Obstet 289: 1271-1280, 2014.
74. Lui Z, Ma Q, Wang X and Zhang Y: Inhibiting tumor growth of colorectal cancer by blocking the expression of vascular endothelial growth factor receptor 3 using interference vector-based RNA interference. Int J Mol Med 25: 59-64, 2010.