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ORIGINAL PAPER

Inhibition of hepatocellular carcinoma growth and angiogenesis by dual silencing of NET-1 and VEGF

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Abstract Simultaneous silencing of multiple up-regulated genes is an attractive and viable strategy to treat many incurable diseases including cancer. Herein we used dual gene targeted siRNA (DGT siRNA) conjugate composed of NET-1 and VEGF siRNA sequences in the same backbone could inhibit growth and angiogenesis HCC. DGT siRNA showed a further down regulation on VEGF mRNA and protein levels compared with NET-1 siRNA or VEGF siRNA, but not on NET-1 expression. It also exhibited greater suppression on proliferation and trigger of apoptosis in HepG2 cells than NET-1 siRNA or VEGF siRNA; this could be explained by the significant down regulation of cyclin D1 and Bcl-2. A lower level of ANG2 mRNA and protein was detected in HUVEC cultured with supernatant of HepG2 cells treated with DGT siRNA than that of VEGF siRNA or NET-1 siRNA, resulting in much more inhibited angiogenesis of HUVEC. Tumor growth was inhibited and microvessel density dropped in the xenograft tumor models compared to the untreated controls. NET-1 and VEGF silencing play a key role in inhibiting hepatocellular cell proliferation, promoting apoptosis, and reducing angiogenesis. Simultaneous silencing of NET-1 and VEGF using DGT siRNA construct may provide an advantageous alternative in development of therapeutics for Hepatocellular carcinoma.

Keywords Hepatocellular carcinoma (HCC) · NET-1 · VEGF · DGT siRNA

Introduction

Primary hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and the third cause of cancer-related deaths. Advances in molecular and cell biology have led to elucidation of the molecular mechanism underlying malignant transformation in HCC. Due to the fact that mutations and abnormal expression of various genes are involved in tumorigenesis, gene modulation is being explored as a very promising approach to correct those abnormal gene expressions (Raskopf et al. 2008). The present study aims at suppressing two upregulated genes simultaneously, NET-1 and VEGF, in HCC.

Dysregulation of the balance between proliferation and cell death represents a pro-tumorigenic principle in human hepatocarcinogenesis. NET-1 gene is a new member of the molecules of the tetraspan superfamily (TM4SF), characterized by the existence of four predicted transmembrane domains delimiting two extracellular regions of unequal size (Serru et al. 2000). The gene is implicated in signal transduction, cell adhesion, migration, proliferation, and differentiation (Maecker et al. 1997; Yauch and Hemler 2000). It was found to be overexpressed in some tumors, such as gastric cancer (Chen et al. 2008), cervical carcinoma

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(Wollscheid et al. 2002), colorectal adenocarcinoma (Chen et al. 2009) and ovarian carcinomas (Scholz et al. 2009). NET-1 overexpression was found in 96.92 % of HCC tissues and there was also a strong correlation between the level of NET-1 expression and HCC pathological grading and clinical stages (Chen et al. 2007). It suggested that the gene played a critical role in the HCC progression of tumor growth and metastasis. Therefore, NET-1 is a logical target for HCC therapy.

Additionally, HCC is a highly vascularized tumor that requires the formation of numerous blood vessels to receive sufficient blood supply to grow and proliferate. Consequently, angiogenesis is a crucial process in the development of HCC (Pang and Poon 2006). VEGF is the most potent pro-angiogenic signal and was identified as a key angiogenic stimulator in liver cancer. Bevacizumab, a humanized monoclonal antibody against VEGF, has been used in several Phase I/II trials in the treatment of advanced HCC, either as a single agent (Siegel et al. 2008) or in combination with chemotherapeutic agents (Thomas et al. 2009; Zhu et al. 2006). Although progressions had been made, the use of Bevacizumab is responsible for important toxic side effects, especially in terms of bleeding and thromboembolic events which require further evaluation. Therefore, as VEGF is an effective target for HCC therapy, a new approach to inhibit VEGF expression has to be developed.

RNA interference (RNAi) is the phenomenon in which siRNA of 21–23 nt in length silences a target gene by binding to its complementary mRNA and triggering its degradation. Potent knockdown of specific gene sequence makes siRNA a promising therapeutic strategy (Cheng and Mahato 2007; Mahato et al. 2005). In this study, we intended to evaluate the effect of dual silencing of NET-1 and VEGF genes on liver cancer cell growth and anti-angiogenic activity. We have designed and identified potent siRNA which can efficiently silence the target gene. The potent NET-1 siRNA and VEGF siRNA were used to conduct functional studies in liver cancer cells, HepG2. Cell proliferation and apoptosis were examined after the treatment with siRNAs. The effect of siRNAs on inhibiting angiogenesis ability induced by HepG2 cells was evaluated in vitro and in vivo.

Materials and methods

Cell culture

HepG2 and HUVEC cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10 % fetal bovine serum (FBS, Gibco), 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin. These cells lines were purchased from the Institute of Cell Biology, Chinese Academy of Sciences, and cell cultures were maintained at 37 °C in a humidified incubator with 5 % CO_2.

siRNA, shRNA-expression vector design, construct and shRNA-expression vector stable transfection

According to an optimization principle of siRNA, we designed a 19 nt sequence-specific siRNA targeting either VEGF (VEGF siRNA) or NET-1 (NET-1 siRNA), and a dual gene targeting siRNA (DGT siRNA, patent: Liang D., Sweedler D., Cui K., WO 2009/035539, March 19, 2009, NanTong Biomics Biotechnology Corporation) for VEGF and NET-1 studied in vitro. The NET-1 siRNA does not contain any homology with VEGF mRNA and VEGF siRNA not for NET-1 mRNA. Simultaneously, two pairs of DNA oligonucleotides shRNA targeted either NET-1 (NET-1 shRNA) or VEGF (VEGF shRNA) were cloned into the BamHI/HindIII restriction site of the pSilencer^{TM} 4.1-CMV neo vector (Invitrogen Corp., China) studied in vivo. All chemically synthesized oligonucleotides were obtained from NanTong biomics biotechnology corporation and identified by PCR and DNA sequencing. Sequences are shown in Table 1.

In brief, 1.5 × 105 cells/ml HepG2 cells were seeded in multi-well plates (Corning Costar Corp., Cambridge, MA) and grown overnight to 70–80 % confluence. The above siRNA was transfected into HepG2 cells with Lipofectamine^{TM} 2000 reagent (Invitrogen) according to the manufacturer's instructions. The effective responses of these sequences were determined and compared to untreated cells (no treatment), or to cells treated with Lipofectamine^{TM} 2000 transfection reagent (TR) alone.

Stably transfected HepG2 cell lines expressing NET-1 shRNA or VEGF shRNA were selected using G418 due to the neomycin resistance of pSilencer^{TM} 4.1-CMV neo vector for preparing nude mice xenografts models. Stably transfected HepG2 cell line expressing both NET-1 shRNA and VEGF shRNA were obtained by transfection with NET-1 shRNA and VEGF shRNA in sequence. The empty vector was also stably transfected and used for control group.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from HepG2 cells after transfection for 48 h using Trizol reagent (Invitrogen), and then was submitted to a 25 μl PCR reaction in the presence of 12.5 μl of 2 × Master Mix, 1 μl of each primer mix (10 μM/ml), 0.5 μl of 50 × SYBR-Green I and 4 μl RNA according to one step Quantace kit (Australia). The PCR mixtures were first subjected to 30 min at 42 °C for reverse

Table 1 Sequences of siRNAs and shRNA

Name	Sequences
NET-1	5′-CCACAAUGGCUGAGCACUUdTdT-3′ (sense)
siRNA	3′-TdTdGGUGUUACCGUCACGUGAA-5′ (antisense)
VEGF	5′-GGAGUACCCUGAUGAGAUCdTdT-3′ (sense)
siRNA	3′-TdTdCCUCAUGGGACUACUCUAG-5′ (antisense)
DGT	5′-GGAGUACCCUGAUGAGAUCUGACCACAAUGGCUGAGCACUUdTdT-3′ (sense)
siRNA	3′-TdTdCCUCAUGGGACUACUCUAG-5′ 3′-TdTdGGUGUUACCGUCACGUGAA-5′ (antisense)
NET-1	5′-**GATCC**CCACAATGGCTGAGCACTTTTCAAGAGAAAGTGCTCAGCCATTGTGGTG**A**-3′ (sense)
shRNA	3′-**G**GGUGUUACCGUCACGUGAAAAGTTCTCTTTCACGTGACGGTAACACCAC**TTCGA**-5′ (antisense)
VEGF	5′-**GATCC**GGAGTACCCTGATGAGATCTTCAAGAGAGATCTCATCAGGGTACTCCTG**A**-3′ (sense)
shRNA	3′-**G**CCTCATGGGACTACTCTAGAAGTTCTCTCTAGAGTAGTCCCATGAGGAC**TTCGA**-5′ (antisense)

The underline sequences are loop areas of hairpin structure. The bold sequences are BamHI and HindIII restriction sites of the vector

transcription and initially denatured for 10 min at 94 °C and then to 45 cycles of amplification with the following cycling parameters: 20 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C. The primer pairs for each gene were designed with Primer Premier 5.0 software, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control for PCR. Sequences of all the primers were seen in Table 2.

Western blot analysis

After siRNA transfection for 48 h, HepG2 cells in 6-well plates (Corning Costar Corp., Cambridge, MA) were lysed in a RIPA buffer (Beyotime Institute of Biotechnology, China). For HUVECs, proteins were collected after 24 h incubation with HepG2 supernatant transfected with VEGF siRNA, NET-1 siRNA, DGT siRNA or with untreated HepG2 supernatant for detection ANG2, and the negative control was cultured with DMEM with 10 % FBS. The amount of total cell proteins was determined by BCA kit (Beyotime, China). Twenty-five micrograms of protein were separated on SDS-Page (sodium dodecyl sulfate–polyacrylamide) separation gel and electroblotted onto PVDF membranes (Pharrnacai, USA). For HepG2 proteins, the membrane was blocked with 5 % (wt/vol) milk and probed with rabbit-anti-NET-1 polyclonal antibody (1:400 dilution), mouse-anti-Bcl-2 monoclonal antibody (1:1,000 dilution, Santa Cruz) and rabbit-anti-cyclin D1 polyclonal antibody (1:1,000 dilution, Santa Cruz). For HUVEC proteins, the membrane was probed with mouse-anti-angiopoietin 2 (ANG2) monoclonal antibody (1:1,000 dilution, Santa Cruz). All the membranes were probed with a monoclonal mouse anti-β-actin antibody (1:2,000 dilution, Boster, China). In addition, VEGFR2 was detected in cultured HepG2 and HUVEC cells with a monoclonal mouse anti-VEGFR antibody (1:1,000 dilution, Santa Cruz). An ECL reagent was used to incubate the immune complex and signals were collected by Bio-Rad ChemiDoc XRS. Developed membranes were semi-quantitatively analyzed by scanning volume density using an Image J densitometer (National Institutes of Health). Results were expressed as optical volume density corrected by β-actin for loading. The size of target protein was measured by a comparison with protein molecular weight markers (Bio-Rad Laboratories Ltd.).

Table 2 Sequences of primers for RT-qPCR

Name	Sequences
VEGF primers	5′-GACATCTTCCAGGAGTACC-3′ (forward)
	5′-TGCTGTAGGAAGCTCATCTC-3′ (reverse)
NET-1 primers	5′-GTGGCTTCACCAACTATACG-3′ (forward)
	5′-GACTGCATTAGTTCGGATGT-3′ (reverse)
CyclinD1 primers	5′-GCCCGAGGAGCTGCTGCAAA-3′ (forward)
	5′-GCAACGAAGGTCTGCGCGTG-3′ (reverse)
Bcl-2 primers	5′-GGTCATGTGTGTGGAGAGC-3′ (forward)
	5′-GATCCAGGTGTGCAGGTG-3′ (reverse)
ANG2 primers	5′-TGGGATTTGGTAACCCTTCA-3′ (forward)
	5′-GTAAGCCTCATTCCCTTCCC-3′ (reverse)
GAPDH primers	5′-GAAGGTGAAGGTCGGAGTC-3′ (forward)
	5′-GAAGATGGTGATGGGATTTC-3′ (reverse)

Enzyme linked immunosorbent assay (ELISA)

After siRNA transfection for 48 h, cell-conditioned media were collected and centrifuged to discard all cellular fragments. VEGF protein levels were determined by ELISA, using a mouse anti-human VEGF antibody according to the manufacturer's protocol (Boster, China). Data were expressed in pg/ml as mean values from three independent experiments in triplicates.

Cell proliferation assay

Proliferation of HepG2 cell line was measured using a CCK-8 detection kit (Dojindo, Japan). CCK-8 was applied at 10 μl/well on the 1st to 5th days after transfection

followed by 2 h incubation at 37 °C. The absorbance at 450 nm was determined in Microplate Reader (Bio-Rad 680). All samples were tested in triplicates and differences among the controls and test groups were analyzed.

Annexin V-FITC apoptosis assay and flow cytometry

Briefly, after incubated for 48 h upon transfection, the cells were harvested and washed with PBS. The extent of apoptosis was measured by an annexinV-FITC apoptosis detection kit (Beyotime Institute of Biotechnology, China) as described by the manufacture's instruction. The cells were washed with PBS twice, gently re-suspended in an annexin-V binding buffer and incubated with annexinV-FITC/PI in dark for 15 min and analyzed by flow cytometry using cell quest software (BD Biosciences, USA). The fraction of cell population in different quadrants was analyzed using quadrant statistics.

Immunoprecipitation assay

HepG2 cells in logarithmic growth phase were harvested with trypsin and lysed in a prechilled RIPA buffer (1 ml/107 cells, Beyotime, China) for 1 h rocking at 4 °C.

Protein G agarose beads (Sigma) were prepared by washing twice with PBS and restoring to a 50 % slurry bead suspension with a RIPA buffer. Prior to immunoprecipitation, the cell lysate was pre-cleared by adding 50 μl of bead slurry per ml, incubated at 4 °C rocking for 10 min and centrifuged for 10 min at 10,000*g* at 4 °C. The supernatant was transfered to a new tube. Then four micrograms of a mouse anti-human VEGF antibody was added to 0.5 ml pre-cleared cell lysate followed by incubation with rocking for 3 h on ice. Normal rabbit IgG and no IgG were used as controls. 50 μl of 50 % slurry beads were added and rocked for 1 h at 4 °C. After the samples were centrifuged at 10,000*g* for 15 s, the beads were washed twice with 1 ml RIPA buffer and then 3 times with 1 ml PBS to remove detergents. Finally, the beads were re-suspended in a 60 μl Sample buffer, and boiled at 95 °C for 5 min. Western blot was performed with mouse anti-human VEGF and rabbit anti-human NET-1 antibodies to examine whether VEGF was combined with beads and NET-1 was precipitated with VEGF, respectively.

Capillary tube assay

The ability of VEGF or NET-1 to induce endothelial cells to proliferate and organize into capillary-like sprouts was examined by HUVECs. Briefly, 6.0 × 104 HUVEC cells were collected, re-suspended in a conditioned medium, which was the supernatant of HepG2 transfected with VEGF siRNA, NET-1 siRNA, DGT siRNA or TR for 48 h, and seeded in 48-well plates that were coated with a 100 μl of gelled solution of matrigel. The usually used complete DMEM medium was taken as negative control. At daily intervals, the number of cells in 10 random high-power fields (200×) as well as the number of capillary-like sprouts (100×) was observed. The data were obtained from triplicate wells under each experimental condition at each time point.

Xenograft tumor models

Four to 6-week old specific pathogen-free athymic (T cell deficient) nude mice (BALB/c Nude) were provided by Animal Experiment Center of Nantong University. HepG2 cells stably transfected with NET-1 shRNA, VEGF shRNA, both shRNAs (NET-1 shRNA and VEGF shRNA or vector) were used to generate subcutaneous flank tumors. Mice were divided into 5 groups with 3 mice in each group: NET-1 shRNA, VEGF shRNA, both shRNA, vector and untreated groups. Suspensions of the upper stably expressing cells and untreated cells (1×10^7 cells in 0.2 ml basic culture medium) were injected s.c. into the nape of the mice by sterile syringes. The animals were euthanized after 30 days. Tumor volume was calculated by the following formula: $V = ab^2/2\ mm^3$, where a and b represent the two maximum diameters.

Histological sections and staining

Hematoxylin and eosin (H–E) stain was performed after the sections from paraffin embedded tumors. The subcutaneous tumor sample sections were immuno-stained with rabbit anti-human NET-1, rabbit anti-mouse VEGF (Santa Cruz) and rabbit anti-mouse CD34 (Santa Cruz). NET-1 and VEGF positive expression in tumor cells and CD34 positive expression in endothetium of vessels were judged with distinct brown staining.

Statistical analysis

All experiments were performed independently at least three times. One-way ANOVA and *t* test analyses were utilized to identify differences between groups. Statistical analysis was performed with SPSS software 16.0. $p < 0.05$ was considered to be significant.

Results

Effects of VEGF siRNA, NET-1 siRNA and DGT siRNA on VEGF and NET-1 expression in HepG2 cells

The levels of mRNA of VEGF and NET-1 genes were determined using RT-qPCR technique and protein

expression was detected by ELISA and western blot (Fig. 1). As shown in Fig. 1a, VEGF siRNA inhibited VEGF expression at the mRNA level up to 47.4 % in comparison with that of the TR cell. A silencing effect of VEGF siRNA was observed at the protein level up to 61.7 % (Fig. 1c), indicating the effective silencing at both mRNA and protein levels. As shown in Fig. 1b, d, VEGF siRNA did not produce any effect on the NET-1 expression at the mRNA and protein levels.

Similarly, NET-1 expression was effectively inhibited by NET-1 siRNA at both mRNA and protein levels by 44.5 and 63.0 % (Fig. 1b, d), respectively. Interestingly, we also found that the NET-1 siRNA significantly inhibited VEGF expression at the mRNA level up to 42.4 % (Fig. 1a, $p < 0.05$) and protein level up to 62.3 % (Fig. 1c, $p < 0.05$) compared to TR cells, which was similar with that of VEGF siRNA (both $p > 0.05$). Subsequently we examined DGT siRNA on NET-1 and VEGF expressions respectively. The knockdown efficiency of DGT siRNA on NET-1 expression was observed. The results showed that NET-1 mRNA and protein levels were down-regulated by 49.6 % (Fig. 1a, $p < 0.05$) and 69.6 % (Fig. 1d, $p < 0.05$) compared to TR cells, respectively, which was in line with that of NET-1 siRNA. When compared to NET-1 siRNA or VEGF siRNA alone, the DGT siRNA showed higher inhibition on VEGF mRNA expression up to 76.0 % (Fig. 1a) and protein level up to 78.1 % (Fig. 1c), indicating a dramatic effect of dual gene targeted siRNA on VEGF protein expression.

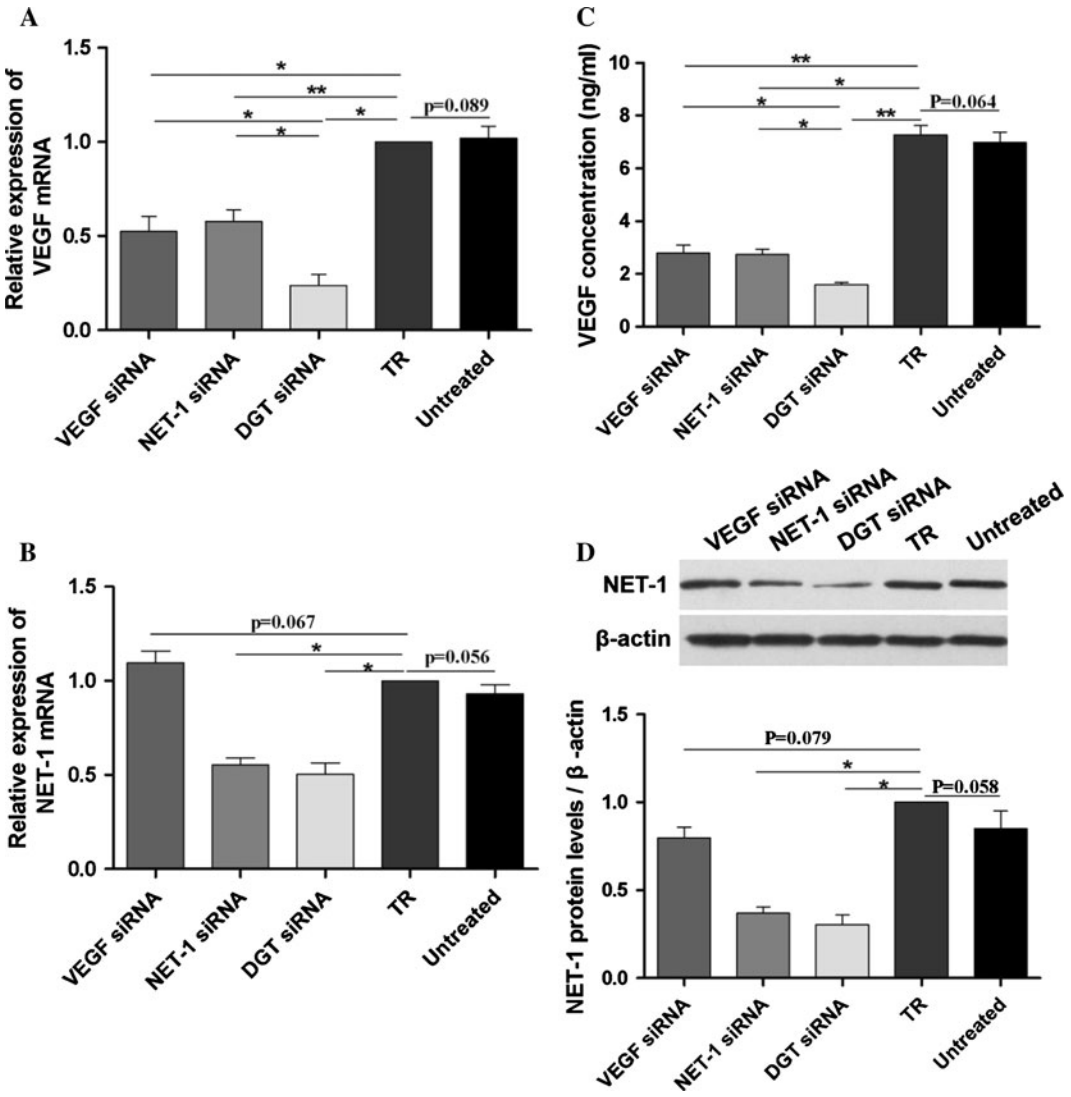

Fig. 1 Expressions of NET-1 and VEGF on mRNA and protein levels were detected. NET-1 mRNA (**a**) and VEGF mRNA (**b**) expressions in HepG2 cells were measured by RT-qPCR after siRNA transfection. **c** VEGF concentration in the supernatant measured as ng/ml by ELISA method. **d** NET-1 protein expression in HepG2 cells by Western Blot and densitometric analysis. All data are expressed as the percentage of TR control volume density (mean ± SEM). *$p < 0.05$; **$p < 0.01$

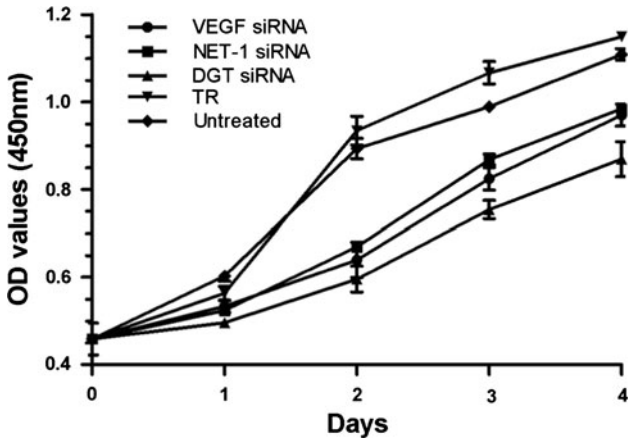

Fig. 2 The proliferation of HepG2 cells was measured using CCK-8 kits. Growth curve of HepG2 cells was shown for each group. The proliferation was assayed in triplicates at 24, 48, 72 and 96 h post-transfection of siRNAs

Effect of VEGF siRNA, NET-1 siRNA and DGT siRNA on HepG2 cell proliferation

We examined the effect of silencing of NET-1 and VEGF on cell proliferation of HepG2 cells. The absorbance values of the HepG2 cells at 48, 72 and 96 h after transfection with either VEGF siRNA or NET-1 siRNA were significantly lower than those of the TR treated cells and untreated cells, respectively. There was no significant difference between the growth of cells treated with NET-1 siRNA and that of VEGF siRNA. The absorbance value of HepG2 cells treated with DGT siRNA showed a significant decrease in cell proliferation compared with the cells treated with either NET-1 siRNA or VEGF siRNA at 48, 72 and 96 h, respectively. (Fig. 2).

Effect of VEGF siRNA, NET-1 siRNA and DGT siRNA on HepG2 cell apoptosis

HepG2 cells grown in 6-well plates were examined for apoptosis by Annexin V-FITC staining and flow cytometry analysis. As Fig. 3 illustrated, apoptotic HepG2 cells was significantly increased by VEGF siRNA treatment (34.029 ± 4.2 %) compared with the TR treated cells (12.089 ± 1.79 %, $p < 0.01$). Similarly, an increase was also identified by NET-1 siRNA transfection (36.281 ± 1.89 %, $p < 0.05$). In addition, the apoptotic cells were greatly increased by DGT siRNA treatment (49.221 ± 5.62 %, $p < 0.01$). Clearly, there were significant differences in apoptosis rates between DGT siRNA treated cells and VEGF siRNA or NET-1 siRNA treated cells (both $p < 0.05$).

The mRNA and protein expressions of cyclin D1 and Bcl-2 in HepG2 cells

The mRNA levels of cyclin D1 were down regulated by 54.1, 45.0 and 66.6 % (Fig. 4a), whereas the mRNA levels of Bcl-2 were down regulated by 69.4, 58.0 and 82.9 % (Fig. 4b) in VEGF siRNA, NET-1 siRNA and DGT siRNA transfected HepG2 cells compared with the TR treated cells, respectively. Both cyclin D1 and Bcl-2 protein expressions were measured by using western blot analysis (Fig. 4c). The protein levels of cyclin D1 were down regulated by 70.1, 65.2 and 83.2 % (Fig. 4d), and Bcl-2 protein expressions were decreased by 65.0, 53.0 and 80.9 (Fig. 4e) in VEGF siRNA, NET-1 siRNA and DGT siRNA transfected HepG2 cells compared with the TR treated cells, respectively. DGT siRNA showed greater decease of

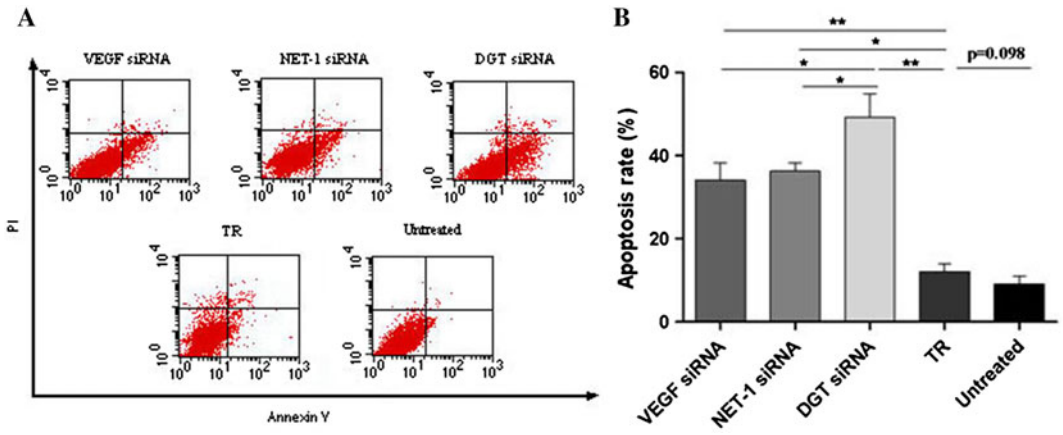

Fig. 3 HepG2 cell apoptosis was detected by flow cytometry. **a** Cells in the *lower left* (LL) quadrant represented survivals; *lower right* (LR) quadrant represented early apoptosis; the *upper right* (UR) quadrant represented necrosis or post-apoptotic and the *upper left* (UL) quadrant represented detection of error allowed. **b** Data are expressed as the percentage of TR control (mean ± SEM), $*p < 0.05$; $**p < 0.01$

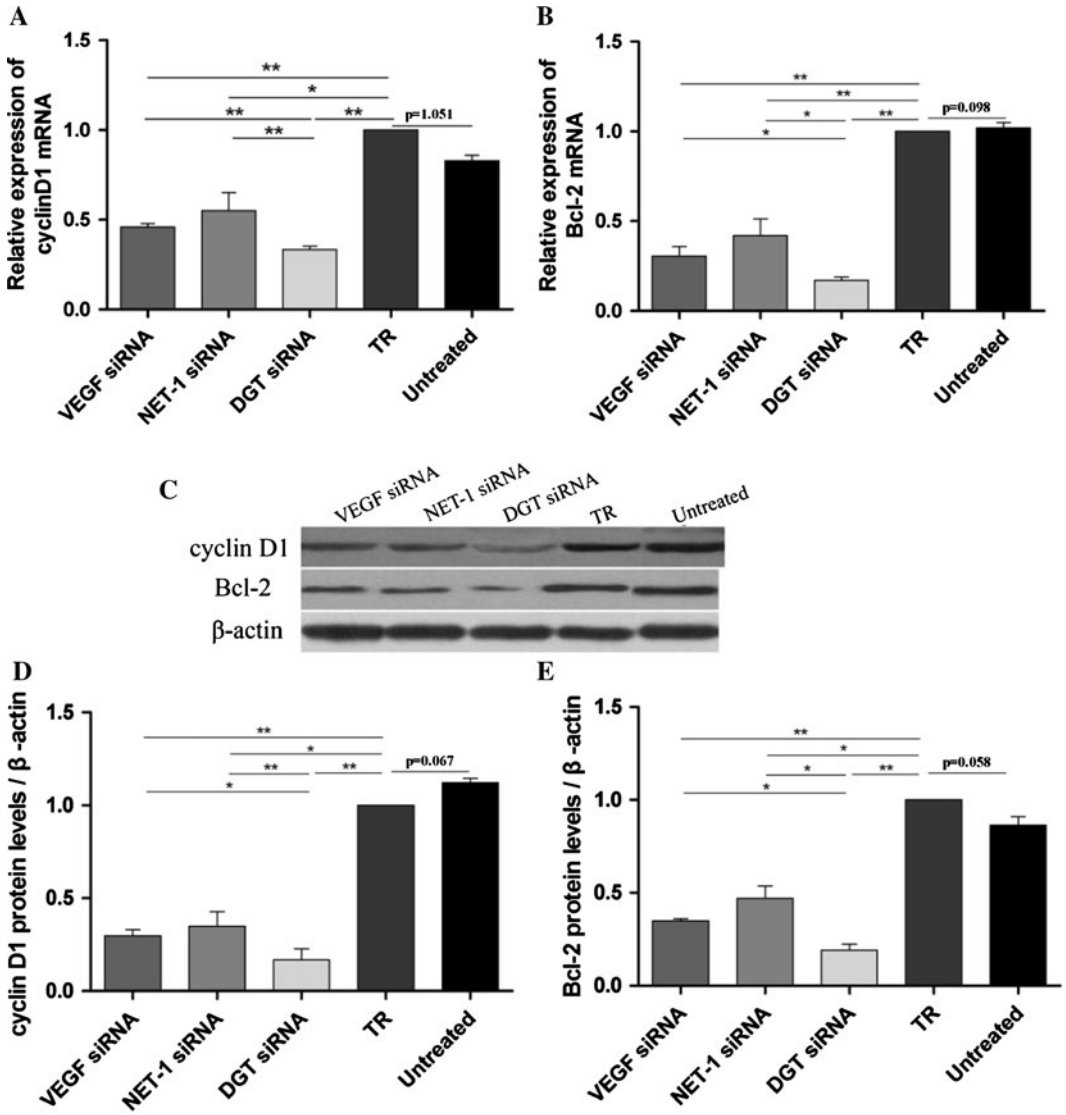

Fig. 4 Cyclin D1 and Bcl-2 expressions were detected by RT-qPCR and western blot. The mRNA levels of cyclin D1 (**a**) and Bcl-2 (**b**) in HepG2 cells determined by RT-qPCR after siRNA transfection. **c**, **d**, **e** Expressions of cyclinD1 and Bcl-2 proteins in HepG2 cells were measured by Western Blot after transfection. Densitometric analysis of the two proteins was made relative to β-actin. All data are expressed as the percentage of TR control (mean ± SEM) $^{*}p < 0.05$; $^{**}p < 0.01$

cyclin D1 and Bcl-2 expression at both mRNA and protein levels.

The relationship between VEGF and NET-1 in HepG2 cells by immunoprecipitation assay

To gain a better understanding on interactions between NET-1 and VEGF, immunoprecipitation was performed as results shown in Fig. 5. The specificity was further supported by the fact that no corresponding protein bands were observed in normal IgG and no IgG groups. Cell lysate was used as a positive control for both VEGF and NET-1 proteins. In fact, NET-1 was recognized by the NET-1 antibody in the anti-VEGF group, confirming a direct interaction between VEGF and NET-1 in HepG2 cells.

Influence of VEGF siRNA, NET-1 siRNA and DGT siRNA on capillary tube formation in vitro

An in vitro angiogenesis model was employed to evaluate the capillary tube formation of HUVECs. Firstly, VEGFR2 protein was determined in the HepG2 and HUVEC cell lines by western blot (Fig. 6a). Secondly, tube formation assay showed that VEGF siRNA, NET-1 siRNA or DGT siRNA transfected HepG2 cells inhibited HUVECs to form capillary tube-like structures on Matrigels as compared with the TR treated and untreated cells (Fig. 6b). We also determined the mRNA and protein levels of ANG2 in HUVECs. In normally cultured negative control cells, the expression of ANG2 mRNA (0.151 ± 0.02) and protein (0.215 ± 0.04) was slight, when compared to the untreated cells (mRNA:

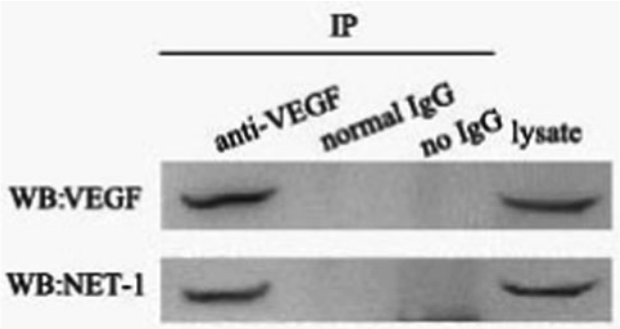

Fig. 5 The relationship between VEGF and NET-1 in HepG2 cells was detected by immunoprecipitation assay. VEGF and NET-1 proteins were detected by western blot (WB) after immunoprecipitation (IP) for VEGF, using anti-VEGF and anti-NET-1 antibodies. Normal IgG, no IgG and cell lysate were used as negative, blank and positive controls, respectively

0.920 ± 0.08; protein: 0.890 ± 0.01; both $p < 0.01$, Fig. 7a, b). TR did not cause any statistical differences compared with untreated cells. In VEGF siRNA treated cells, the expression of ANG2 mRNA (0.394 ± 0.04, $p < 0.01$, Fig. 7a) and protein (0.693 ± 0.05, $p < 0.05$, Fig. 7b) was significantly reduced compared to TR treated cells. NET-1 siRNA treated cells (ANG2 mRNA: 0.502 ± 0.05; protein: 0.665 ± 0.09) exhibited similar effect with VEGF siRNA. Furthermore, DGT siRNA treated cells showed a lower level of ANG2 mRNA at 23.6 % (Fig. 7a) as well as a lower protein level at 46.9 % (Fig. 7b) than that of the VEGF siRNA and NET-1 siRNA treated cells.

Influence of silencing NET-1 and VEGF on xenograft tumor growth

The proliferation curve of stably transfected HepG2 cells was performed and it showed similar tendency with that of siRNA transfected cells (data not shown). For the in vivo tumor model, stably transfected HepG2 cells were inoculated into the subcutaneous tissue of nude mice. 30 days after tumor cell implantation, VEGF shRNA, NET-1 shRNA, and dual-shRNAs reduced tumor growth by 69.6, 73.6, and 87.3 % (215.6 ± 36.4, 187.83 ± 48.2, 90.4 ± 10.1 vs. 710.13 ± 180.6 mm) compared to the vector treated cell (all $p < 0.05$, Fig. 8). Dual-shRNAs showed greater reduce of tumor volume compared to VEGF shRNA ($p < 0.05$) or NET-1 shRNA ($p < 0.05$).

Protein expressions and intratumoral microvessel density

As shown in Fig. 9, the expression of VEGF was mainly found in the cytoplasm, whereas the expression of NET-1 varied from membrane to cytoplasm, or mixed. VEGF and NET-1 proteins were both showed obviously expressed in vector treated and untreated cells. The expression of VEGF protein was reduced more significantly in the dual-shRNAs cells than that in the VEGF shRNA and NET-1 shRNA cells. Much lower expression of NET-1 protein was seen in the NET-1 shRNA and dual-shRNAs treated cells than that in the VEGF shRNA. Tumors derived from NET-1 shRNA, VEGF shRNA and dual-shRNAs treated HepG2 cells showed reduced microvessel density of 73.3, 75.3, 92.7 (6.7 ± 1.8, 6.2 ± 1.6, 1.8 ± 0.7 vs. 25.2 ± 4.7 tumor vessels per high power field, $n = 25$) compared to the vector treated cells (all $p < 0.05$).

Discussion

As a new member of the tetraspanins group, NET-1 is a recently discovered tumor-related gene. NET-1 is closely associated with the development and prognosis of several malignant tumors (Chen et al. 2007, 2009, 2010a, b; Danial and Korsmeyer 2004).The over-expression of NET-1 in HCC is involved in cancer cell proliferation (Chen et al. 2010a). In this study, we used a NET-1 siRNA to target NET-1 expression in HCC cells, HepG2. The mRNA and protein levels of NET-1 in HepG2 cells were down regulated by 44.5 and 63.0 %, respectively. Reports have showed that siRNA mediated down regulation of NET-1 expression resulted in decreased proliferation of colon cancer cells (Chen et al. 2010b) and skin squamous carcinoma cells (Chen et al. 2010c). In this work, we also demonstrated that NET-1 siRNA could inhibit proliferation and induce apoptosis of HepG2 cells. To elucidate its molecular mechanisms of NET-1 promoting proliferation and inhibit apoptosis, we have examined the expressions of the key regulators cyclinD1 and Bcl-2. Our results first demonstrated that the expression levels of cyclinD1 and Bcl-2 were significantly decreased in HepG2 cells upon cell transfection with of NET-1 siRNA. CyclinD1 was known to accumulate during the G1 phase of the cell cycle (Sherr 1993). Overexpression of cyclinD1 is sufficient to initiate hepatocellular carcinogenesis(Deane et al. 2001). In contrast, Bcl-2, an anti-apoptotic protein, is identified to be one of the mechanisms involved by cancer cells to evade apoptosis (Danial and Korsmeyer 2004; Hanahan and Weinberg 2000). Bcl-2 is the prominent member of a proteins family that is responsible for dysregulation of apoptosis and prevention of death in cancer cells (Gross et al. 1999; Reed 1999), which controls the pathways leading to the release of cytochrome c from the mitochondrial membrane, the activation of caspase cascade and, in the end, to execution of apoptosis. The xenografts tumor models in nude mice were established by using stably transfected HepG2 cells with plasmid pSilencer^{TM}

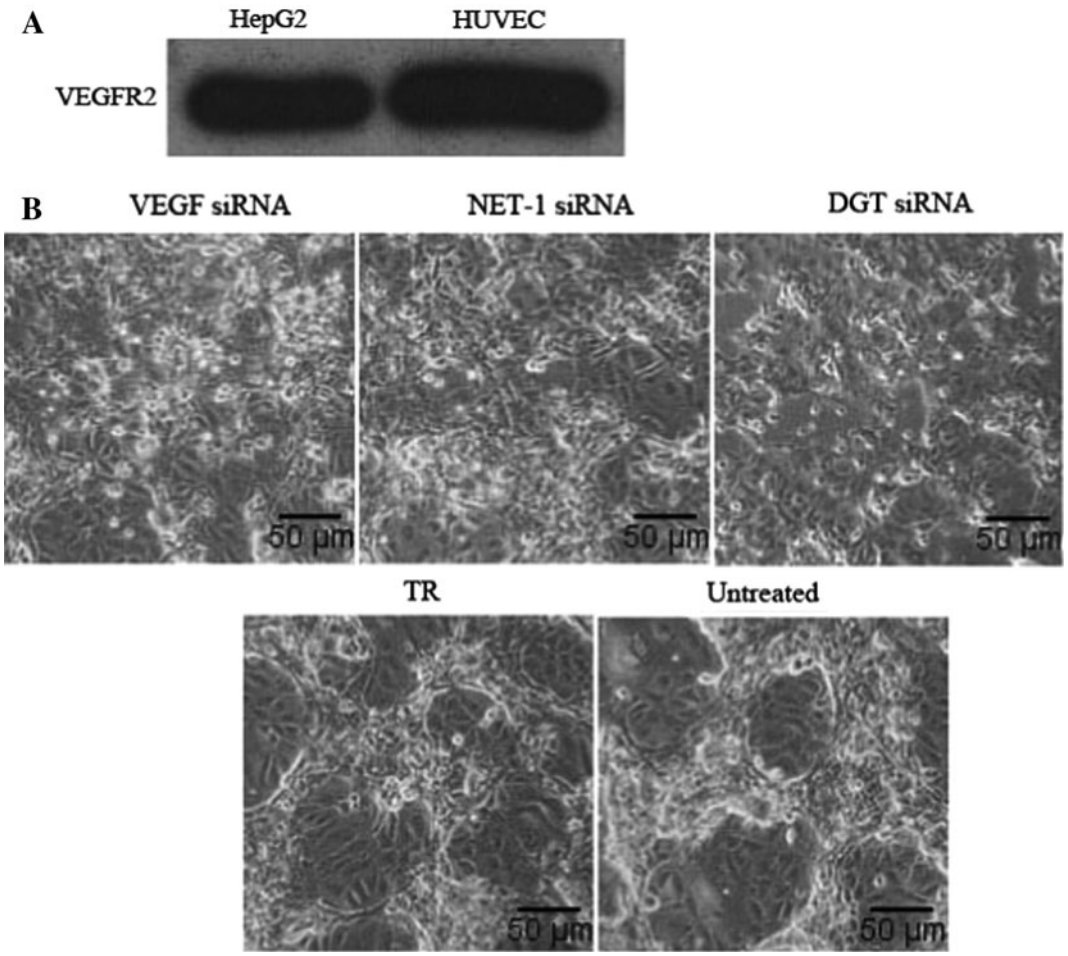

Fig. 6 **a** VEGFR2 protein was determined expressing in HepG2 cells and HUVEC cells. **b** The capillary tube formation was detected in vitro. HUVECs (6×10^4 cells per well) were seeded onto the surface of 96-well cell culture plates precoated with Matrigel and then incubated at 37 °C for 48 h in the conditioned media derived from HepG2 cells transfected with VEGF siRNA, NET-1 siRNA DGT siRNA or TR

4.1-CMV neo expressing NET-1 shRNA. The use of shRNA instead of siRNA in the in vivo study was mainly due to the consideration on better stability and longer effectiveness of shRNA is better than those of siRNA. Significant reduction in plasma NET-1 level and tumor growth in nude mice bearing HCC xenografts were observed.

Ng et al. (2001) highlighted that HCC is a hypervascularized tumor expressing extensive amounts of VEGF to form new blood vessels from pre-existing vascular beds, in order to receive an adequate supply of oxygen and nutrients. As one of the most important angiogenesis-stimulating factors, VEGF contributes to cancer progression through its action of neovascularization of tumors. In addition to these, we further demonstrated that VEGF, as a growth factor of tumor cells, can induce cell proliferation through VEGFR2 on the surface of HepG2 cells with an autocrine pathway in this study, which was supported by a positive expression of VEGFR2 in HepG2 cells (Liu et al. 2005). We were also able to observe decreased proliferation rates and increased apoptotic cells in the presence of VEGF siRNA than that in the TR treated group, suggesting the effect of VEGF siRNA on decreasing HCC cell proliferation and inducing apoptosis might be due to the autocrine role of VEGF in HepG2. The expression of gene products involved in anti-apoptosis (Bcl-2) and proliferation (cyclin D1) (Carroll and Ashcroft 2006; Oda et al. 2006) was down regulation in VEGF siRNA transfected HepG2 cells. Significant reduction in plasma and tumor cytosol VEGF levels in nude mice bearing HCC xenografts was also observed. This might be partly attributable to the reduced tumor volume, as the production of VEGF was dependent upon tumor cell mass.

Interestingly, NET-1 siRNA exhibited significant inhibition on VEGF mRNA and protein levels, while the VEGF siRNA alone did not show any effect on NET-1 expression. Immunoprecipitation assay was conducted and results showed there was a direct interaction between VEGF and NET-1 proteins in HepG2 cells. We assumed

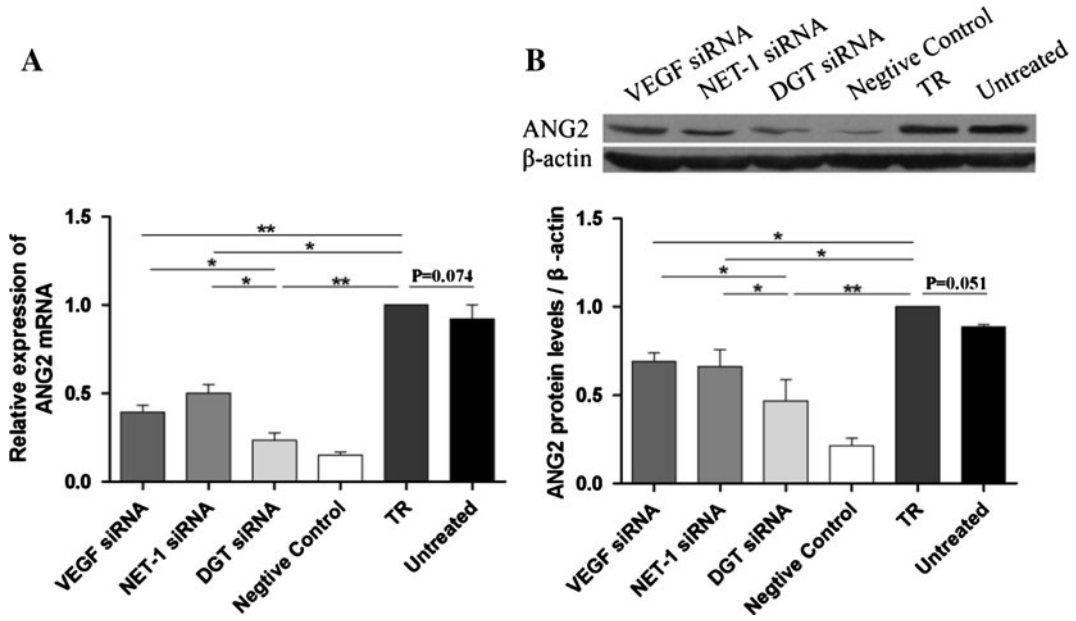

Fig. 7 ANG2 expression in HUVECs cultured with HepG2 supernatant. HUVEC complete medium was used as a negtive control. **a** ANG2 mRNA expression levels were showed by RT-qPCR method. **b** ANG2 protein in HUVECs was determined by Western Blot. Densitometric analysis of ANG2 protein was relative to β-actin. All data are expressed as the percentage of TR control volume density (mean ± SEM). $*p < 0.05$; $**p < 0.01$

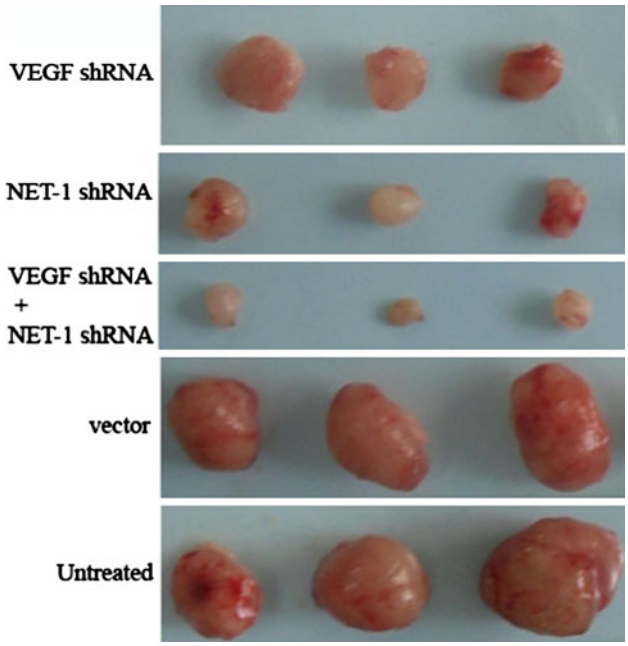

Fig. 8 Influence of silencing NET-1 and VEGF on xenograft tumor growth. HepG2 cells were stably transfected with NET-1 shRNA, VEGF shRNA or both shRNA constructs and inoculated into the subcutaneous tissue of nude mice. The tumor amasses were obtained from mice with 30 days after tumor cell implantation

that NET-1 might be as a VEGF upstream regulator, which could probably lead to down regulation of VEGF expression by NET-1 siRNA. The fact that NET-1 siRNA presented similar VEGF siRNA effects on HepG2 proliferation and apoptosis to VEGF siRNA may suggest that one of inhibiting tumor growth mechanisms of NET-1 siRNA functioned through blocking the autocrine loop of VEGF. The paracrine effect of VEGF to pro-angiogenesis was through its receptor VEGFR2 expressed on endothelial cells. VEGFR2 is regarded as a key signaling receptor required for the full spectrum of VEGF responses, including endothelial cell proliferation, migration, differentiation, and induction of vascular permeability (Meyer et al. 1999; Zeng et al. 2001). (Hongo et al. 2006) showed that HUVEC was induced to form new blood vessels with higher VEGF concentration. Yoo et al. (2007) using oncolytic viruses expressing VEGF-siRNA, showed a significant reduction of tube formation and reduced sprouting from aortic rings. In this paper, HepG2 cells treated with VEGF siRNA was found to be more difficult to induce HUVECs to rapidly organize into capillary-like spouts rapidly and thus led to an inhibiting role of angiogenesis. Furthermore, NET-1 siRNA showed a similar effect on inhibiting tube formation of HUVEC cells. These demonstrated that NET-1 acts as a VEGF upstream regulator promoting VEGF paracrine effect. VEGF could up regulates Ang2 expression (Oh et al. 1999) in endothelial cells, which expresses in the areas undergoing vascular remodeling and leads to decreased vessel maturation and enhanced vessel sprouting (Koblizek et al. 1998). Ang2 inhibition prevents the growth of new vessels by endothelial sprout formation, leading to smaller tumors in vivo

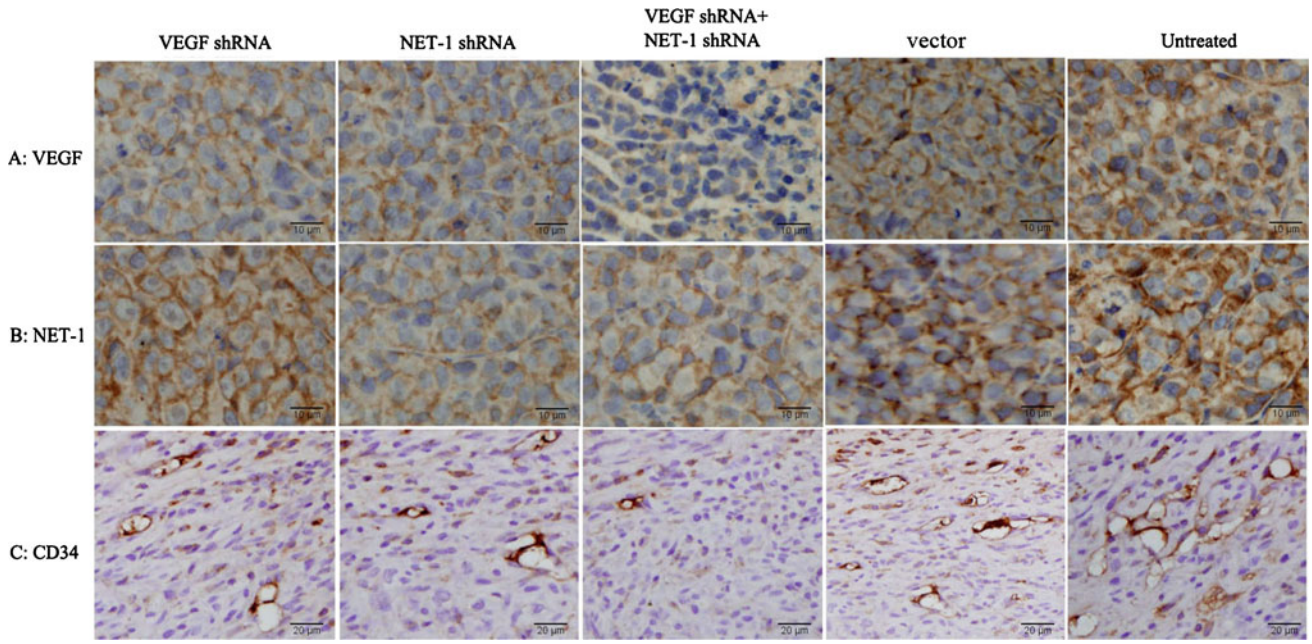

Fig. 9 **a**, **b** Expressions of VEGF and NET-1 protein in HCC xenografts, immunohistochemistry staining. **c** Blood vessels with CD34 positive in HCC xenografts, immunohistochemistry staining

(Hashizume et al. 2010). In the present study, we also observed down regulation of Ang2 in HUVECs by NET-1 siRNA and VEGF siRNA, by which formation of endothelial spouts become more difficult. The decrease in tumor cytosol VEGF level might contribute to the inhibition of tumor angiogenesis as suggested by reduced intratumoral microvessel density in the vivo studies.

It has been reported that multimerized siRNA conjugate that was composed of two different siRNA sequences showed more effective inhibition of the two corresponding target genes at one time than physically mixed multimerized siRNA conjugates (Lee et al. 2011). Therefore, dual gene siRNA targeting NET-1 and VEGF (DGT siRNA) was chemically synthesized via a cleavable linkage in this study. The result showed that the expression of VEGF protein was suppressed to a greater extent in the DGT siRNA treated group than that in the NET-1 siRNA or VEGF siRNA alone group, indicating a dramatic silencing effect of DGT siRNA on VEGF protein expression. In contrast, suppression on NET-1 expression in HepG2 cell by the DGT siRNA treated was similar to that by the NET-1 siRNA treated, which supported the observation that targeting VEGF by DGT siRNA did not influence NET-1 protein expression in HepG2 cell. Compared to NET-1 siRNA or VEGF siRNA alone, the DGT siRNA showed the highest inhibition on HepG2 cell proliferation from 48 to 96 h after transfection, and the apoptosis was increased by 5.45 folds over each of siRNAs alone. Similarly, the significant anti-angiogenesis effect of DGT siRNA was also observed (Fig. 6). Downregulation of both NET-1 and VEGF expressions simultaneously in HepG2 cells leaded to smaller tumor size and lower microvessel density in the xenografts tumor models of nude mice with the HepG2 cells stably transfected by both of the shRNAs. It was demonstrated that RNAi targeting duel genes (NET-1 and VEGF) was far more effective in the inhibitions in vitro and in vivo than that of single target siRNA on either NET-1 or VEGF gene. This co-RNAi system exhibited specific and high efficiency on silencing multi genes simultaneously and would have great potential for therapeutic siRNA applications.

In summary, this study showed that the expression of NET-1 promotes HCC cells to secrete VEGF, suggesting that NET-1 serves as an upstream regulator of VEGF gene expression. Although there were several reports using antisense oligonucleotide (Qiu et al. 2004) or siRNA (Raskopf et al. 2008) to inhibit VEGF, simultaneously inhibition of NET-1 and VEGF expression proved to be an effective approach to inhibit the growth of HCC. Duel targeting NET-1 and VEGF was capable of producing anti-angiogenic, direct anti-proliferative and pro-apoptosis effects on HCC studies in vitro and in vivo. DGT siRNA targeting NET-1 and VEGF was found to be superior to either NET-1 siRNA or VEGF siRNA used alone. To our knowledge, this is the first report providing evidence that DGT siRNA targeting NET-1 and VEGF is capable of inhibiting angiogenesis, tumor cells proliferation, inducing tumor cells apoptosis and blocking HCC growth. Our study suggested that there could be a therapeutic potential of DGT siRNA for HCC.

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Conflict of interest All the authors declared no conflict of interest.

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