

ORIGINAL**Antiangiogenic agent sunitinib induces epithelial to mesenchymal transition and accelerates motility of colorectal cancer cells**

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Abstract : Although vascular endothelial growth factor receptor (VEGF-R)-targeted antiangiogenic agents are important treatment for a number of human malignancies, there is accumulating evidence that the therapies may promote disease progression, such as invasion and metastasis. How tumors become to promote their evasiveness remains fully uncertain. One of possible mechanisms for the adaptation may be a direct effect of VEGF-R inhibitors on tumor cells expressing VEGF-R. To elucidate a direct effect of VEGF-R-targeting drug (sunitinib), we established a human colorectal cancer cell model adapted to sunitinib. The sunitinib-conditioned cells showed a significant increase in cellular motility and migration activities, compared to the vehicle-treated control cells. Consistent with the phenotype, the sunitinib-conditioned cells decreased the expression levels of E-cadherin (an epithelial marker), while significantly increased the levels of Slug and Zeb1 (mesenchymal markers). Expression profiles of VEGF-R in the sunitinib-conditioned cells showed that only neuropilin-1 (NRP1) expression was significantly increased among all VEGF-R tested. Blockade of NRP1 using its antagonist clearly repressed the migration activation in sunitinib-conditioned cells, but not in the control cells. These results suggest that inhibition of VEGF-R on colorectal cancer cells can drive the epithelial-mesenchymal transition, leading to activation of cell motility in an NRP1-dependent manner. *J. Med. Invest.* 64 : 250-254, August, 2017

Keywords : VEGF receptor tyrosine kinase inhibitor, sunitinib, evasive adaptation

INTRODUCTION

Vascular endothelial growth factors (VEGF) and their tyrosine kinase receptors (VEGF-R) are the central regulators of tumor angiogenesis. Therefore, several angiogenesis inhibitors targeting the VEGF/VEGF-R pathway have been developed and have become an important option for management of a number of human malignancies (1, 2). However, there is increasing evidence that the clinical results of VEGF/VEGF-R-targeted therapy are very modest, resulting in a moderate improvement in the overall survival (3, 4). Additionally, the clinical outcome is associated with the development of resistance to the therapies and the increased risk for invasion and metastasis (5, 6).

Several mechanisms of resistance and escape from VEGF/VEGF-R-targeted antiangiogenesis therapy have been identified (1,7). VEGF/VEGF-R inhibitors target vascular endothelial cells and inhibit tumor angiogenesis, leading to hypoxia within tumor. Therefore, it has been widely accepted that the hypoxic stress is the central mechanism of the aggressive malignant progression of tumor cells, including the selection of malignant cells or hypoxia-resistant cells and the activation of malignant programs, leading to distant metastasis (6). In fact, several preclinical studies have shown that several VEGF pathway-targeting agents facilitate tumor cell invasion and metastasis that required for hypoxia (8, 9).

In addition to hypoxia, there is increasing evidence that the VEGF-R inhibitors act directly not only on endothelial cells (anti-angiogenic function) but also on tumor cells that express functional VEGF-R (anti-angiogenesis-independent function) (10-13). However, the direct effects of the VEGF-R blockers on tumor cells are not fully understood.

In this study, we examined the direct effects of VEGF-R tyrosine kinase inhibitor, sunitinib, on evasive adaptation of colorectal cancer cells. We found that chronic treatment with sunitinib at pharmacologically relevant dose (14) induced the epithelial-mesenchymal transition (EMT) and activated cellular motility. Furthermore, the cells switched from the VEGF-R-dependent mechanism to the neuropilin-1 (NRP1)-dependent one to activate their evasive adaptation.

MATERIALS AND METHODS*Reagents, cell culture and treatment*

Sunitinib (a clinical used VEGF-R tyrosine kinase inhibitor) and V1 (an NRP-binding heptapeptide) were obtained from Selleckchem. Human colorectal cancer cell lines (HCT116) were maintained in RPMI1640 medium with 5% fetal bovine serum and antibiotics.

To develop the sunitinib-conditioned cells, HCT116 cells were chronically treated with sunitinib for 5 weeks at pharmacologically relevant concentration (0.1 μ M) (14). The vehicle-treated control cells were obtained by chronically treated with dimethyl sulfoxide (DMSO) (0.01%) for 50 days.

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Cell migration assay

Equal numbers (50,000 cells per well) of cells were suspended in 0.25 ml of 1% RPMI1640–FBS without or with bevacizumab or sunitinib and placed in the top compartment of an uncoated 8 µm pore membrane chambers (BD Biosciences) ; 0.75 ml of 10% RPMI 1640–FBS was added to the bottom compartment. Following 24–48 h incubation under standard conditions (37°C/5% CO₂), non-migrating cells were scraped from the top compartment, and cells that had migrated to the bottom compartment were fixed and stained using the Hemacolor Rapid staining of blood smear (Merck). Membranes were excised and mounted on a standard microscope slide. The numbers of migrated cells were determined from five random high-power fields visualized at × 200 magnification.

Scratch wound-healing/migration assay

The scratch assay was used to further evaluate the migration activity. Cell lines were grown in 12well plates, and when cells were 80% confluent, scratches were made on the plates using CELL Scratcher (IWAKI). The plates were then carefully washed twice with RPMI1640. Fresh culture medium supplemented with 1% FBS without or with bevacizumab or sunitinib was gently added onto the plates. The plates were allowed to incubate over the 5 days. Photographs were taken at regular intervals to monitor closure of the gap.

Quantitative RT-PCR (qRT-PCR)

The levels of transcripts for VEGF receptors (*VEGF-R1*, *VEGF-R2*, *VEGF-R3*, *NRP1* and *NRP2*), EMT factors (*E-cadherin*, *Slug* and *Zeb1*) and *gapdh* were measured by quantitative real time (RT)-PCR using the following specific primer sets: *VEGF-R1*, 5'-AGAACCCCGATTATGTGAGAAA-3' (forward) and 5'-GATAGATTCCGGGAGCCATCC-3' (reverse); *VEGF-R2*, 5'-GAACATTTGGGAAATCTCTTGC-3' (forward) and 5'-CGGAAGAACAATGTAGTCTTTGC-3' (reverse); *VEGF-R3*, 5'-ATAGACAAGAAAGCGGCTTCA-3' (forward) and 5'-CCTCCCTTGGGAGTCAGG-3' (reverse); *NRP1*, 5'-CCCTGAGAATGGGTGGACT-3' (forward) and 5'-CGTGACAAAGCGCAGAAG-3' (reverse); *NRP-2*, 5'-GGACCCCAACTTGGATT-3' (forward) and 5'-ATGGTTAAAAAGCGCAGGTC-3' (reverse); *E-cadherin*, 5'-TGGAGGAATTCTTGCTTTGC-3' (forward) and 5'-CGTCTCCTCCGAAGAAAC-3' (reverse); *Slug*, 5'-TGTTGCTTCAAGGACACAT-3' (forward) and 5'-GCAAATGCTCTGTTGCAGTG-3' (reverse); *Zeb1*, 5'-CAGTGAAAGAGAAGGGAATGCT-3' (forward) and 5'-CTTCAGGCCCCAGGATTT-3' (reverse); *gapdh*, 5'-GCTAGGGACGGCTGAAG-3' (forward) and 5'-GCCAATACGACCAATCC-3' (reverse). Amplification and quantification of the PCR products were performed using the Applied Biosystems 7500 System (Applied Biosystems). Standards were run in the same plate and the relative standard curve method was used to calculate the relative mRNA expression. RNA amounts were normalized against the *gapdh* mRNA level.

Western blot analysis

Total cell lysates were prepared using a lysis buffer containing 100 mM Tris-HCl (pH 6.8), 300 mM NaCl, 2 mM EDTA and 4% (v/v) SDS. Protein concentrations were determined with the BCA protein assay (Pierce). Western immunoblotting was performed as described previously (15) using a rabbit monoclonal anti-human E-cadherin antibody (24E10; Cell Signaling Technology) at a 1/5,000 dilution, a rabbit monoclonal anti-human Slug antibody (C19G7; Cell Signaling Technology) at a 1/4,000 dilution, a rabbit monoclonal anti-human Zeb1 antibody (D80D3; Cell Signaling Technology) at a 1/4,000 dilution, a mouse monoclonal anti-human

β-actin antibody (8H10D10; Cell Signaling Technology) at a 1/10,000 dilution.

Statistical analysis

Results are expressed as means ± S.D. Statistical analyses of data were done using ANOVA and the Scheffé's test. P value < 0.01 was considered significant.

RESULTS

Effect of sunitinib on colorectal cancer cell motility

We assessed the effects of sunitinib on the cell migration activity using a modified Boyden chamber. The sunitinib-conditioned cells showed a marked increase in migration activity (Fig. 1A). To further confirm the results of the migration assay, the cell motility was assessed using a scratch assay (wound healing assay). In the scratch assay, the sunitinib-conditioned cells migrated inwardly and covered a greater area of the scratch than the control cells (Fig. 1B).

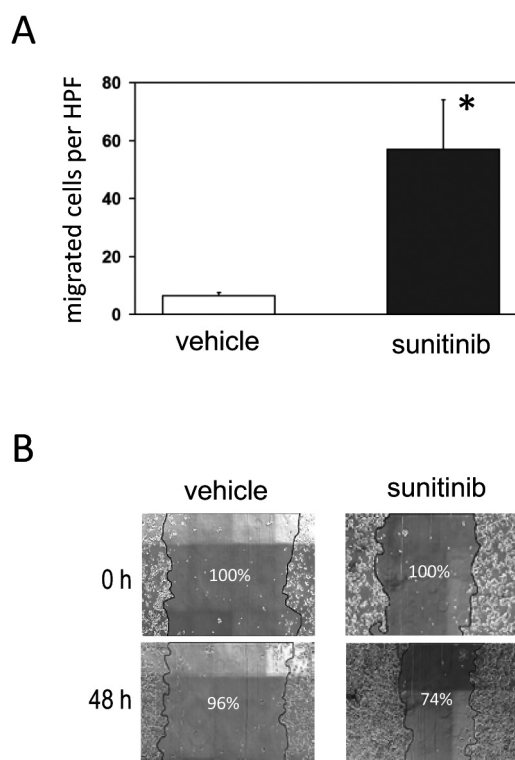


Figure 1. Effect of sunitinib on cellular migration and motility (A) Migration of the vehicle-treated control (vehicle) and the sunitinib-conditioned cells (sunitinib) was determined by an uncoated transwell migration assay (n=4-5, means ± SD). HPF, high power field. *P < 0.01, compared with the vehicle-treated control cells. (B) Cellular motility of the vehicle-treated control (vehicle) and the sunitinib-conditioned cells (sunitinib) was determined by a scratch assay. Representative photographs of the migrated cells are shown.

Effect of sunitinib on the expression of EMT-related genes

Based on the results that sunitinib activated evasive phenotype of colorectal cancer cells, there is a possibility that the sunitinib-conditioned cells may activate EMT gene program. Thus, we

examined the expression of typical EMT-related genes; an epithelial marker (E-cadherin) and mesenchymal markers (Slug and Zeb1). We found that the expression levels of E-cadherin were significantly decreased in the sunitinib-conditioned cells at both mRNA and protein levels, compared to the vehicle-treated control cells (Fig. 2A, D). In contrast, the levels of Slug and Zeb1 were elevated in the sunitinib-conditioned cells (Fig. 2B, C, E, F). These findings indicate that sunitinib treatment can drive the EMT, leading to activation of cellular motility in colorectal cancer cells.

Effect of sunitinib on gene expression profile of the VEGF-R family members

We previously reported that the chronic blockade of VEGF using anti-VEGF antibody increases the redundant expression of VEGF-R family members (12). Thus, we examined whether chronic inhibition of all of VEGF-R by sunitinib induces the redundant expression of VEGF-R family members (VEGFR-1, -2 and -3). The sunitinib-conditioned cells did not increase all of VEGF-R tested (Fig. 3A-C), suggesting that the cells do not utilize VEGF-Rs under the VEGF-R inhibited conditions, and that they may switch the VEGF-R-related receptors that can bind VEGF and transduce its signal, such as NRP1 and NRP2 (16). To test this possibility, we determined expression levels of NRP1 and NRP2 were measured using RT-qPCR. Expression levels of NRP1, but not NRP2, were

significantly increased in the sunitinib-conditioned cells, compared to the control cells (Fig. 3D, E).

Finally, we examined the effect of NRP1 blocking on evasive activity in the sunitinib-conditioned cells. Treatment with NRP1 antagonist significantly reduced the migratory activity in the sunitinib-conditioned cells, but not in the vehicle-treated control cells (Fig. 3F). These results suggest that, under all VEGF-R inhibited conditions by sunitinib, colorectal cancer cells switched from the VEGF-R-dependent mechanism to the NRP1-dependent one for adaptation to and evasion from the stress conditions.

DISCUSSION

This study focused on the direct effect of sunitinib on evasive phenotype of colorectal cancer cells. We found that the chronic blockade of all VEGF-R signaling by sunitinib induced EMT and accelerated their motility. In contrast, a short exposure (1 to 2 days) to sunitinib did not activate the activities (data not shown), indicating that the long-term inhibition of VEGF-R signaling in tumor cells is required for the evasive adaptation. This is compatible with previous preclinical studies showing that extensive treatment (1 to 3 months) with sunitinib increases migration and invasion *in vitro* and accelerates local invasion and distant metastasis *in*

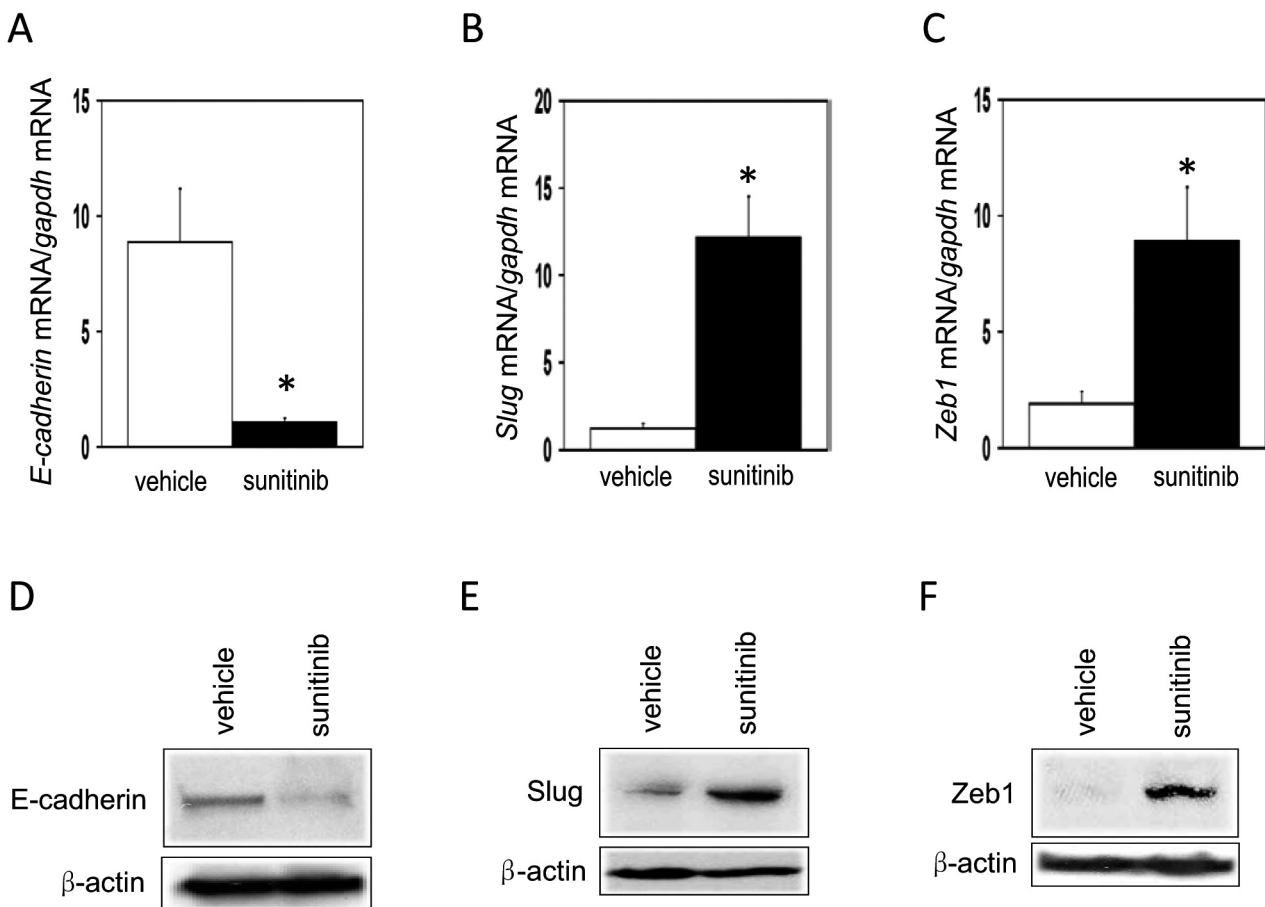


Figure 2. Effect of sunitinib on the EMT-associated gene expression (A-C) Expression levels of the transcripts of the epithelial marker (*E-cadherin*) and EMT-associated transcription factors (*Slug* and *Zeb1*) in the vehicle-treated control (vehicle) and the sunitinib-conditioned cells (sunitinib) (n=5-6, means \pm SD). **P* < 0.01, compared with the control cells. (D-F) Representative Western blot analysis of EMT-associated protein expression levels (E-cadherin, Slug and Zeb1) in the vehicle-treated control and the sunitinib-conditioned cells (n=3). The levels of β -actin are shown as a loading control (lower panel).

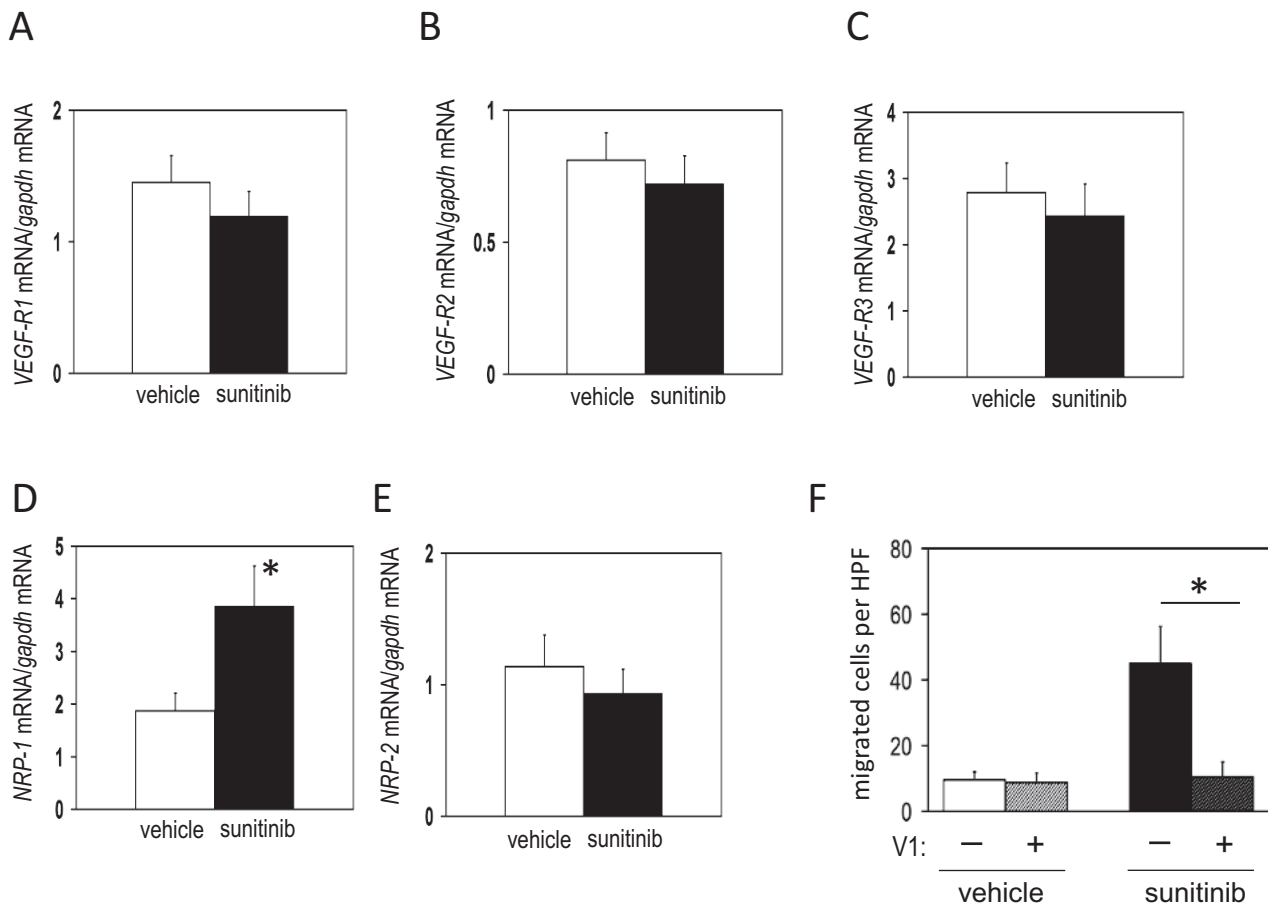


Figure 3. Effect of sunitinib on expression profile of the VEGF-R family genes (A-E) Expression levels of *VEGF-R1* (A), *VEGF-R2* (B), *VEGF-R3* (C), *NRP-1* (D) and *NRP-2* (E) mRNA in the vehicle-treated control and the sunitinib-conditioned cells were measured using quantitative RT-PCR (n=5, means \pm S.D.). * $P < 0.01$ indicates an increased expression compared with the vehicle-treated control cells.

(F) Effect of an NRP1 inhibition on the migration. The vehicle-treated control and the sunitinib-conditioned cells were pre-treated for 24 h with DMSO (-) or V1 (+), and then they were analyzed migration activity, as described above (n=5, means \pm SD). HPF, high power field. * $P < 0.01$, compared with the respective DMSO-treated cells.

in vivo, and led to a shortening of overall survival (8, 9).

During antiangiogenic therapy, antitumor effects are caused by reducing tumor microvessels and thus resulting hypoxic conditions, which lead to tumor cell death. However, the hypoxic stress selects a sub-population of tumor cells and activates malignant phenotypes, including apoptosis resistance, increased survival activity and cellular motility (6). This antiangiogenesis-dependent and hypoxia-driven malignancy has been widely accepted in cancer progression, leading to invasion and metastasis (8, 9).

In addition to hypoxic stress, we revealed that sunitinib directly acted on colorectal cancer cells and activated the EMT/migration program via a hypoxia-independent mechanism. There is increasing evidence of hypoxia-independent action of VEGF-R inhibitors on cancer cells that accelerates their evasive and acquired resistance to the therapy (10). For instance, in mouse models of glioblastoma multiforme, a direct inhibition of VEGF-R on cancer cells accelerates their invasive and metastatic activities *in vitro* and *in vivo* (17). Interestingly, Han *et al* demonstrate that simple hypoxic stress is not sufficient to trigger evasive resistance, by contrast, that targeted inhibition of VEGF-R on cancer cells induced the resistance (18).

In this study, we elucidated that the sunitinib-conditioned cells

utilized NRP1 to adapt the stress conditions in which all VEGF-R were inhibited. It seems to be reasonable, since NRP1 is a receptor for VEGF but is not the target of sunitinib (16). There is increasing evidence that NRP1 plays critical roles in the evasive phenotype of several cancer cells. Overexpression of NRP1 increased migration activities in gastric cancer cell lines and esophageal squamous cell carcinoma cell lines (19, 20). Conversely, knockdown of NRP1 reduced their abilities, and it also inhibited metastasis in gastric and lung cancer cells (19, 21). Additionally, NRP1 is preferentially expressed in metastatic MDA-MB-231 and MBA-MB-435 cells, but not in the non-metastatic MDA-MB-453 cells (22, 23).

The possible importance of NRP1 in the tumor progression of patients is supported by several clinical studies (24-27). Patients with colorectal cancers expressing high levels of NRP1 showed a significantly higher incidence of lymph node or liver metastasis than those with tumors expressing low levels of NRP1 (25). Increased expression of NRP1 occurs in gastrointestinal tumors, and this upregulation appears to parallel the tumor's invasive behavior (24). In addition, the survival time for patients with tumors expressing high NRP1 levels was significantly shorter than for those with low NRP1 levels (26-28). Therefore, NRP1 is suggested to be an important target for cancer therapy.

Collectively, we concluded that VEGF-R-targeting drug sunitinib directly induced evasive adaptation in colorectal cancer cells independently of hypoxia, and NRP1 is a critical molecule to activate the adaptation under all VEGF-R inhibited conditions.

COMPETING INTERESTS-DISCLOSURE

The authors declare no competing interests.

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REFERENCES

- Bergers G, Hanahan D : Modes of resistance to anti-angiogenic therapy. *Nat Rev Cancer* 8 : 592-603, 2008
- Ellis LM, Reardon DA : Is there really a yin and yang to VEGF-targeted therapies? *Lancet Oncol* 11 : 809-811, 2010
- Jayson GC, Kerbel R, Ellis LM, Harris AL : Antiangiogenic therapy in oncology : current status and future directions. *Lancet* 388 : 518-529, 2016
- Meadows KL, Hurwitz HI : Anti-VEGF therapies in the clinic. *Cold Spring Harb Perspect Med* 2 : a006577, 2012
- Bottsford-Miller JN, Coleman RL, Sood AK : Resistance and escape from antiangiogenesis therapy : clinical implications and future strategies. *J Clin Oncol* 30 : 4026-4034, 2012
- Ebos JM, Kerbel RS : Antiangiogenic therapy : impact on invasion, disease progression, and metastasis. *Nat Rev Clin Oncol* 8 : 210-221, 2011
- Ribatti D : Tumor refractoriness to anti-VEGF therapy. *Oncotarget* 19 : 46668-46677, 2016
- Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS : Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell* 15 : 232-239, 2009
- Pàez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Viñals F, Inoue M, Bergers G, Hanahan D, Casanovas O : Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 15 : 220-231, 2009
- Simon T, Gagliano T, Giamas G : Direct Effects of Anti-Angiogenic Therapies on Tumor Cells : VEGF Signaling. *Trends Mol Med* 23 : 282-292, 2017
- Fan F, Samuel S, Gaur P, Lu J, Dallas NA, Xia L, Bose D, Ramachandran V, Ellis LM : Chronic exposure of colorectal cancer cells to anti-VEGF mAb promotes compensatory pathways that mediate tumor cell migration. *British J Cancer* 104 : 1270-1277, 2011
- Yamagishi N, Kondo TS, Masuda K, Nishida K, Kuwano Y, Dang DT, Dang LH, Nikawa T, Rokutan K : Chronic inhibition of tumor cell-derived VEGF enhances the malignant phenotype of colorectal cancer cells. *BMC Cancer* 13 : 229, 2013
- Tomida C, Yamagishi N, Aibara K, Yano C, Uchida T, Abe T, Ohno A, Hirasaka K, Nikawa T, Teshima-Kondo S : The malignant progression effects of regorafenib in human colon cancer cells. *J Med Invest* 62 : 195-198, 2015
- Huang D, Ding Y, Li Y, Luo WM, Zhang ZF, Snider J, Vandenbeldt K, Qian CN, The BT : Sunitinib acts primarily on tumor endothelium rather than tumor cells to inhibit the growth of renal cell carcinoma. *Cancer Res* 70 : 1053-1062, 2010
- Masuda K, Kondo TS, Mukaijo M, Yamagishi N, Nishikawa Y, Nishida K, Kawai T, Rokutan K : A novel tumor-promoting function residing in the 5' non-coding region of vascular endothelial growth factor mRNA. *PLoS Med* 5 : e94, 2008
- Rizzolio S, Tamagnone L : Multifaceted role of neuropilins in cancer. *Curr Med Chem* 18 : 3563-3575, 2011
- Lu KV, Chang JP, Parachoniak MM, Aghi MK, Meyronet D, Isachenko N, Fouse SD, Philips JJ, Cheresch DA, Park M, Bergers G : VEGF inhibits Tumor Cell Invasion and Mesenchymal Transition through a MET/VEGFR2 Complex. *Cancer Cell* 22 : 21-35, 2012
- Han KS, Raven PA, Frees S, Gust K, Fazli L, Ettinger S, Hong SJ, Kollmannsberger C, Gleave ME, So AI : Cellular Adaptation to VEGF-Targeted Antiangiogenic Therapy Induces Evasive Resistance by Overproduction of Alternative Endothelial Cell Growth Factors in Renal Cell Carcinoma. *Neoplasia* 17 : 805-816, 2015
- Peng Y, Liu YM, Li LC, Wang LL, Wu XL : MicroRNA-338 inhibits growth, invasion and metastasis of gastric cancer by targeting NRP1 expression. *PLoS One* 9 : e94422, 2014
- Shi F, Shang L, Pan BQ, Wang XM, Jiang YY, Hao JJ, Zhang Y, Cai Y, Xu X, Zhan QM, Wang MR : Calreticulin promotes migration and invasion of esophageal cancer cells by upregulating neuropilin-1 expression via STAT5A. *Clin Cancer Res* 20 : 6153-62, 2014
- Hong TM, Chen YL, Wu YY, Yuan A, Chao YC, Chung YC, Wu MH, Yang SC, Pan SH, Shih JY, Chan WK, Yang PC : Targeting neuropilin 1 as an antitumor strategy in lung cancer. *Clin Cancer Res* 13 : 4759-4768, 2007
- Bachelder RE, Chung J, Wendt MA, Shaw LM, Robinson G, Mercurio AM : Vascular endothelial growth factor is an autocrine survival factor for neuropilin-expressing breast carcinoma cells. *Cancer Res* 61 : 5736-5740, 2001
- Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M : Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* 92 : 735-745, 1998
- Hansel DE, Wilentz RE, Yeo CJ, Schulick RD, Montgomery E, Maitra A : Expression of neuropilin-1 in high-grade dysplasia, invasive cancer, and metastases of the human gastrointestinal tract. *Am J Surg Pathol* 28 : 347-356, 2004
- Ochiumi T, Kitadai Y, Tanaka S, Akagi M, Yoshihara M, Chayama K : Neuropilin-1 is involved in regulation of apoptosis and migration of human colon cancer. *Int J Oncol* 29 : 105-116, 2006
- Ben Q, Zheng J, Fei J, An W, Li P, Li Z, Yuan Y : High neuropilin 1 expression was associated with angiogenesis and poor overall survival in resected pancreatic ductal adenocarcinoma. *Pancreas* 43 : 744-749, 2014
- Lee SW, Lee JE, Yoo CY, Ko MS, Park CS, Yang SH : NRP-1 expression is strongly associated with the progression of pituitary adenomas. *Oncol Rep* 32 : 1537-1542, 2014
- Zhu H, Cai H, Tang M, Tang J : Neuropilin-1 is overexpressed in osteosarcoma and contributes to tumor progression and poor prognosis. *Clin Transl Oncol* 16 : 732-738, 2014