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Further Evidence for Expression and Function of the VEGF-C/VEGFR-3 Axis in Cancer Cells

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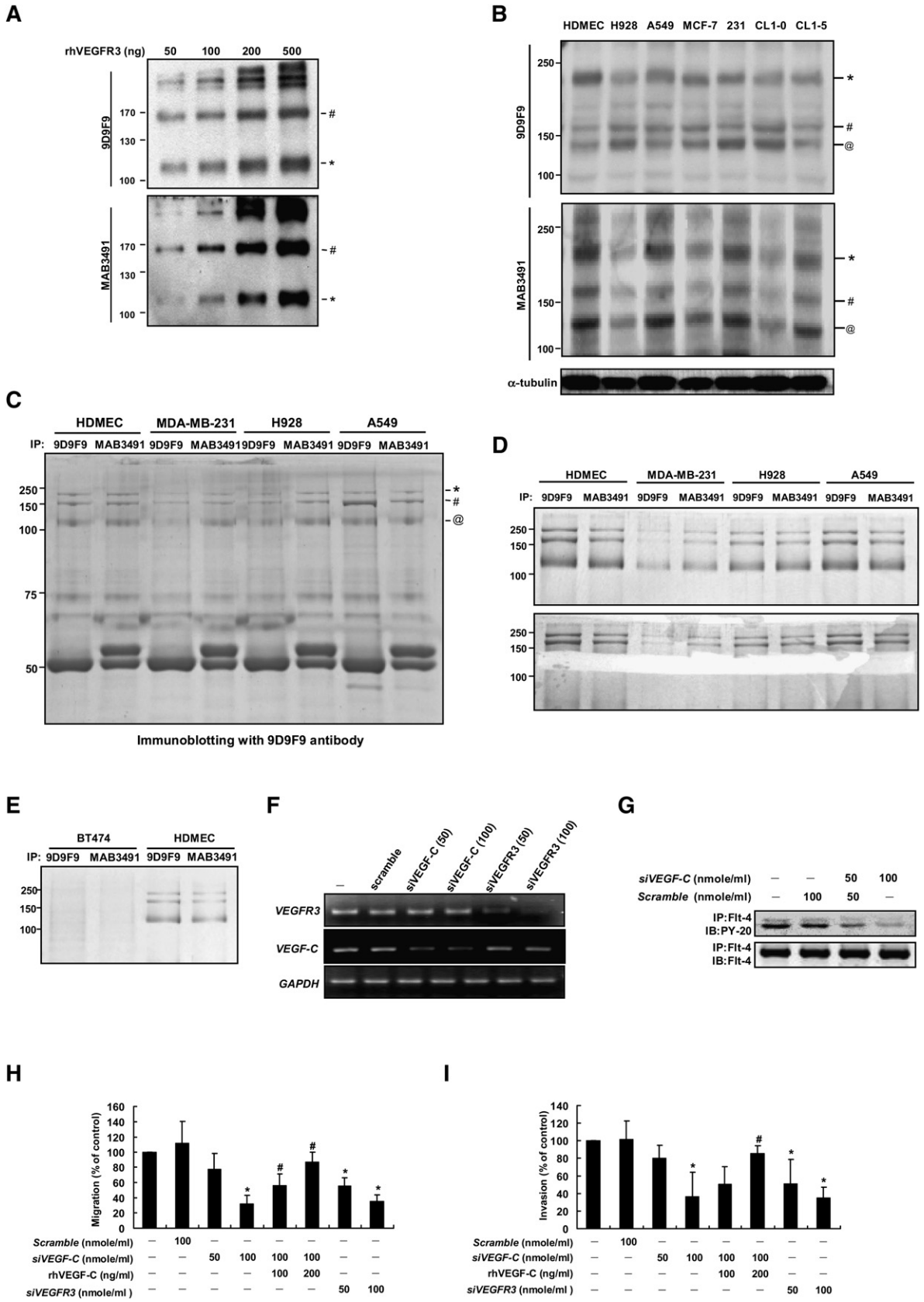
VEGFRs are primarily expressed by endothelial cells but are also expressed by nonendothelial cell types, including cancer cells (Witte et al., 2002; Li et al., 2003; Van Trappen et al., 2003; Wu et al., 2003; Su et al., 2007). VEGFR-3 influences the potential for a lymphangiogenic response (Alitalo and Carmeliet, 2002; Joukov et al., 1996; Skobe et al., 2001). Recent studies have indicated that VEGFR-3 is also expressed in a variety of human malignancies and that the VEGF-C/VEGFR-3 axis contributes to tumor progression (Dias et al., 2002; Marchio et al., 1999; Masood et al., 2003; Mylona et al., 2007; Van Trappen et al., 2003; Witte et al., 2002). We provided evidence in a recent study that the VEGF-C/VEGFR-3 axis enhances cancer cell mobility and invasion capabilities and promotes cancer cell metastasis (Su et al., 2006). Petrova et al. questioned the specificity of the VEGFR-3 antibody used in our previous study and the validity of our conclusion (Petrova et al., 2008), and we have taken their concerns to heart. Here we provide additional data to support our previous conclusions.

Petrova et al. claim that the antibody used in our previous study (Su et al., 2006) may detect nonspecific antigens, while their antibodies are more specific. To resolve these concerns, we used the antibody we used before (MAB3491 purchased from R&D) and one of the antibodies used by Petrova et al. (9D9F9 purchased from Chemicon as MAB 3757) to detect pure recombinant human VEGFR-3 protein (rhVEGFR-3) using western blot analysis. We found that both antibodies detected the unglycosylated (~112 kDa) and glycosylated (~160 kDa) forms of recombinant human VEGFR-3 protein in a dose-dependent manner (Figure 1A). Both antibodies also detected similar bands higher than 170 kDa. Furthermore, both antibodies detected endogenous VEGFR-3 in various cancer cell lines and, as expected, in human dermal microvascular endothelial cells (HDMEC) (Figure 1B), as well as VEGFR-3 ectopically expressed in cancer cell lines (Figure S1A available online). By immunoprecipitation-immunoblotting analysis, we found that VEGFR-3 protein can be immunoprecipitated by both antibodies (Figure 1C). Using immunofluorescence staining, VEGFR-3 protein in A549 lung cancer cells and in human lymphatic microvascular endothelial cells (HLMEC) was detected by both antibodies (Figure S1B). These data suggest that the sensitivity and specificity of 9D9F9 antibody and MAB3491 antibody are comparable.

A similar pattern of proteins were immunoprecipitated by both antibodies as detected by SDS-PAGE followed by staining with SimplyBlue SafeStain (purchased from Invitrogen) (Figure 1D). The 125 kDa bands were subjected to in-gel digestion and PMF using the nanoLC/MS/MS analysis for protein identification (Table S1). The representative peptide peaks from MS/MS analysis were detected as human VEGFR-3 protein precursor in the immunoprecipitant of either 9D9F9 antibody or MAB3491 antibody (Figure S2). The search results indicated that these proteins showed the best match, with a protein score of greater than or equal to 1008, considered to be significant using the MASCOT search algorithm (Table S1). There was no significant difference between the data from the MS/MS analysis of immunoprecipitants using the 9D9F9 antibody and the data from the analysis using the MAB3491 antibody. In agreement with the results from Petrova et al., some cancer cell lines do not express VEGFR-3; for example, we can not detect the expression of VEGFR-3 in BT474 cells using either antibody (Figure 1E). Together, these data indicated that VEGFR-3 is expressed in certain cancer cells, and that MAB3491 and 9D9F9 antibodies recognize human VEGFR-3.

To investigate the expression of *VEGFR-3* mRNA in cancer cells, we performed RT-PCR using three different sets of primers and clearly detected the presence of *VEGFR-3* mRNA (Figure S3). Furthermore, we used absolute quantitative real-time RT-PCR to determine levels of *VEGFR-3* mRNA expressed in HDMEC and cancer cell lines (Table S2). The expression of *VEGFR-3* mRNA in BT474, MDA-MB-453, and HBL100 cell lines were undetectable under the same condition (Table S2). To investigate whether our A549 cells were contaminated with lymphatic endothelial cells, we analyzed the expression of a lymphatic endothelial marker, LYVE-1, in A549 cells, HDMEC, and purified HLMEC (at least 95% double-positive for CD31 and podoplanin by flow cytometry analysis). As shown in Figures S1C and S1D, *VEGFR-3* is expressed in all three, whereas *LYVE-1* is expressed only in HDMEC and HLMEC but not in A549 cells. These data indicated that *VEGFR-3* transcripts are indeed expressed in some cancer cells.


A
rhVEGFR3 (ng) 50 100 200 500
9D9F9
MAB3491
170
130
100
#
*
B
HDMEC H928 A549 MCF-7 231 CL1-0 CL1-5
9D9F9
MAB3491
250
150
100
*
#
@
α-tubulin
C
HDMEC MDA-MB-231 H928 A549
IP: 9D9F9 MAB3491 9D9F9 MAB3491 9D9F9 MAB3491 9D9F9 MAB3491
250
150
100
75
50
*
#
@
Immunoblotting with 9D9F9 antibody
D
HDMEC MDA-MB-231 H928 A549
IP: 9D9F9 MAB3491 9D9F9 MAB3491 9D9F9 MAB3491 9D9F9 MAB3491
250
150
100
E
BT474 HDMEC
IP: 9D9F9 MAB3491 9D9F9 MAB3491
250
150
100
F
–
scramble
siVEGF-C (50)
siVEGF-C (100)
siVEGFR3 (50)
siVEGFR3 (100)
VEGFR3
VEGF-C
GAPDH
G
siVEGF-C (nmole/ml) – – 50 100
Scramble (nmole/ml) – 100 50 –
IP:Flt-4
IB:PY-20
IP:Flt-4
IB:Flt-4
H
Migration (% of control)
160
140
120
100
80
60
40
20
0
#
*
Scramble (nmole/ml) – 100 – – – – – –
siVEGF-C (nmole/ml) – – 50 100 100 100 – –
rhVEGF-C (ng/ml) – – – – 100 200 – –
siVEGFR3 (nmole/ml) – – – – – – 50 100
I
Invasion (% of control)
140
120
100
80
60
40
20
0
#
*
Scramble (nmole/ml) – 100 – – – – – –
siVEGF-C (nmole/ml) – – 50 100 100 100 – –
rhVEGF-C (ng/ml) – – – – 100 200 – –
siVEGFR3 (nmole/ml) – – – – – – 50 100

The expression of both VEGF-C and VEGFR-3 at high levels in A549 cells is consistent with the presence of a VEGF-C/VEGFR-3 autocrine loop (Su et al., 2006). To study the role of VEGF-C/VEGFR-3 axis in the invasiveness of cancer cells, expression of VEGF-C in A549 cells was knocked down using VEGF-C-specific siRNA. Transfection with VEGF-C-specific siRNA decreased the expression of VEGF-C in A549 cells (Figure 1F) and reduced the phosphorylation of VEGFR-3 (Figure 1G). Knockdown of VEGF-C also reduced the migratory and invasive activities of A549 cells in a dose-dependent manner (Figures 1H and 1I, lane 1 versus lanes 3 and 4), which were restored by treatment with recombinant human VEGF-C protein (Figures 1H and 1I, lane 4 versus lanes 5 and 6). Likewise, transfection of A549 cells with VEGFR-3-specific siRNA to decrease the expression of VEGFR-3 (Figure 1F) reduced their migratory and invasive activities (Figures 1H and 1I, lanes 7 and 8). Similar results were obtained using the MDA-MB-231 cell line (Figure S4). In addition to using pooled siRNAs, we also found similar results in transfection of A549 cells with two VEGF-C- or VEGFR-3-specific siRNAs individually (Figure S5). These findings strongly support our previous conclusion that the VEGF-C/VEGFR-3 axis is actively involved in regulating the migratory and invasive activities of cancer cells.

We agree with the points raised by Petrova et al. that careful characterization of antibodies is very important, especially when they produce unexpected results. In our hands, both 9D9F9 antibody and MAB3491 antibody can detect VEGFR-3 expression in several cancer cell lines in addition to HDMEC. We have provided additional data to support our earlier conclusion that the VEGF-C/VEGFR-3 axis plays a role in cancer cell mobility. It is unclear why our results are different from those of Petrova et al., but different cell culture conditions might have contributed to this difference. Our findings and other studies support that VEGFR-3 is expressed not only in endothelial cells but also in some nonendothelial cells, including some cancer cells. The VEGF-C/VEGFR-3 axis may have many undefined functions and mediate tumor progression via many as yet unknown molecular mechanisms; thus, further study of the axis is needed.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, five supplemental figures, and three supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/13/6/557/DC1/.

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REFERENCES

Alitalo, K., and Carmeliet, P. (2002). Molecular mechanisms of lymphangiogenesis in health and disease. Cancer Cell *1*, 219–227.

Dias, S., Choy, M., Alitalo, K., and Rafii, S. (2002). Vascular endothelial growth factor (VEGF)-C signaling through FLT-4 (VEGFR-3) mediates leukemic cell proliferation, survival, and resistance to chemotherapy. Blood *99*, 2179–2184.

Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lahtinen, I., Kukk, E., Saksela, O., Kalkkinen, N., and Alitalo, K. (1996). A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J. *15*, 290–298.

Li, Q., Dong, X., Gu, W., Qiu, X., and Wang, E. (2003). Clinical significance of co-expression of VEGF-C and VEGFR-3 in non-small cell lung cancer. Chin. Med. J. (Engl.) *116*, 727–730.

Marchio, S., Primo, L., Pagano, M., Palestro, G., Albini, A., Veikkola, T., Cascone, I., Alitalo, K., and Bussolino, F. (1999). Vascular endothelial growth factor-C stimulates the migration and proliferation of Kaposi's sarcoma cells. J. Biol. Chem. *274*, 27617–27622.

Masood, R., Kundra, A., Zhu, S., Xia, G., Scalia, P., Smith, D.L., and Gill, P.S. (2003). Malignant mesothelioma growth inhibition by agents that target the VEGF and VEGF-C autocrine loops. Int. J. Cancer *104*, 603–610.

Mylona, E., Alexandrou, P., Mpakali, A., Giannopoulou, I., Liapis, G., Markaki, S., Keramopoulos, A., and Nakopoulou, L. (2007). Clinicopathological and prognostic significance of vascular endothelial growth factors (VEGF)-C

Figure 1. Analysis of VEGFR-3 Expression and Cellular Function

(A) Both 9D9F9 and MAB3491 antibodies recognize pure recombinant human VEGFR-3 protein. rhVEGFR-3 (50, 100, 200, or 500 ng) was resolved by SDS-PAGE and then immunoblotted using 9D9F9 (upper panel) or MAB3491 (lower panel) antibody. According to the instructions for rhVEGFR-3 from R&D Systems, the rhVEGFR-3 has a calculated molecular mass of approximately 112 kDa (*). As a result of glycosylation, the recombinant protein migrates as an approximately 160 kDa protein (#) in SDS-PAGE.

(B and C) VEGFR-3 expressed in HDMEC, H928, A549, MCF-7, MDA-MB-231, CL1-0, and CL1-5 cells is detected by immunoblotting (B) or by immunoprecipitation using 9D9F9 or MAB3491 as indicated followed by immunoblotting with 9D9F9 antibody (C). Cell lysates containing 1 mg of total proteins were subjected to immunoprecipitation by incubation with the primary antibody (2 μl 9D9F9 antibody or 2 μg MAB3491 antibody) at 4°C overnight and subjected to immunoblotting assay. The uncleaved, unglycosylated (precursor), and cleaved VEGFR-3 have calculated molecular masses of approximately 195 kDa (*), 175 kDa (#), and 125 kDa (@), respectively.

(D and E) Cell lysates (10 mg protein/condition) were subjected to immunoprecipitation by incubation with the primary antibody (7.5 μl of 9D9F9 antibody or 7.5 μg of MAB3491; 4°C overnight), and the SDS-PAGE gels were stained. The protein in the ≈125 kDa bands was identified by mass spectrometry.

(F) A549 cells were transfected with scramble control siRNA or indicated specific siRNA. RT-PCR was performed to confirm the inhibitory effects of siRNA on *VEGF-C* and *VEGFR-3* expression.

(G–H) Immunoprecipitation-immunoblot assay was performed to analyze the inhibitory effects of siVEGF-C on VEGFR-3 phosphorylation (G). Thirty-six hours after transfection with scramble control siRNA or indicated specific siRNA, cells were harvested and assayed using migration (H) or invasion (I) assay in the presence or absence of rhVEGF-C. Bars represent means ± SD. Asterisks denote a statistically significant induction (*$p < 0.05$) compared with values of lane 1. The # symbol indicates a significant induction compared with values of lane 4. Results represent the findings of three separate experiments, and all incubations were conducted in triplicate.

and -D and VEGF receptor 3 in invasive breast carcinoma. Eur. J. Surg. Oncol. *33*, 294–300.

Petrova, T.V., Bono, P., Holnthoner, W., Chesnes, J., Pytowski, B., Sihto, H., Laakkonen, P., Heikkilä, P., Joensuu, H., and Alitalo, K. (2008). VEGFR-3 expression is restricted to blood and lymphatic vessels in solid tumors. Cancer Cell *13*, this issue, 554–556.

Skobe, M., Hawighorst, T., Jackson, D.G., Prevo, R., Janes, L., Velasco, P., Riccardi, L., Alitalo, K., Claffey, K., and Detmar, M. (2001). Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat. Med. *7*, 192–198.

Su, J.L., Yang, P.C., Shih, J.Y., Yang, C.Y., Wei, L.H., Hsieh, C.Y., Chou, C.H., Jeng, Y.M., Wang, M.Y., Chang, K.J., et al. (2006). The VEGF-C/Flt-4 axis promotes invasion and metastasis of cancer cells. Cancer Cell *9*, 209–223.

Su, J.L., Yen, C.J., Chen, P.S., Chuang, S.E., Hong, C.C., Kuo, I.H., Chen, H.Y., Hung, M.C., and Kuo, M.L. (2007). The role of the VEGF-C/VEGFR-3 axis in cancer progression. Br. J. Cancer *96*, 541–545.

Van Trappen, P.O., Steele, D., Lowe, D.G., Baithun, S., Beasley, N., Thiele, W., Weich, H., Krishnan, J., Shepherd, J.H., Pepper, M.S., et al. (2003). Expression of vascular endothelial growth factor (VEGF)-C and VEGF-D, and their receptor VEGFR-3, during different stages of cervical carcinogenesis. J. Pathol. *201*, 544–554.

Witte, D., Thomas, A., Ali, N., Carlson, N., and Younes, M. (2002). Expression of the vascular endothelial growth factor receptor-3 (VEGFR-3) and its ligand VEGF-C in human colorectal adenocarcinoma. Anticancer Res. *22*, 1463–1466.

Wu, W., Shu, X., Hovsepyan, H., Mosteller, R.D., and Broek, D. (2003). VEGF receptor expression and signaling in human bladder tumors. Oncogene *22*, 3361–3370.