**Molecular and Cellular Pathobiology**

**Autophagy Control by the VEGF-C/NRP-2 Axis in Cancer and Its Implication for Treatment Resistance**

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Abstract

A major contributor to cancer mortality is recurrence and subsequent metastatic transformation following therapeutic intervention. Therefore, in order to develop new treatment modalities and improve the efficacy of current ones, it is important to understand the molecular mechanisms that promote resistance to therapy in cancer cells. One pathway contributing to therapy resistance is autophagy, a self-digestive process that can eliminate unnecessary or damaged organelles to protect cancer cells from death. We have found that the VEGF-C/NRP-2 axis is involved in the activation of autophagy, which helps cancer cell survival following treatment. Inhibition of mTOR complex 1 activity by this axis is the underlying mechanism for the activation of autophagy. Furthermore, we identified two VEGF-C/NRP-2-regulated genes, LAMP-2 and WDFY-1, that have previously been suggested to participate in autophagy and vesicular trafficking. Upregulation of WDFY-1 following VEGF-C or NRP-2 depletion contributes to cytotoxic drug-mediated cell death. Together, these data suggest a link between the VEGF-C/NRP-2 axis and cancer cell survival despite the presence of chemotherapy-induced stress. Effective targeting of this pathway may lead to the development of new cancer therapies. *Cancer Res; 73(1); 160–71. ©2012 AACR.*

Introduction

A delicate balance between cell survival and cell death is crucial for maintaining normal physiologic homeostasis. This balance is dysregulated in cancer cells via alterations in pathways regulating apoptosis, necrosis, and autophagy, which constitutes an important mechanism of therapeutic resistance. The recurrence of cancer after therapy arises from a subset of cells that acquire the ability to survive during therapeutic stress. These cells also show enhanced metastatic properties and lead to cancer mortality (1). A key mechanism that confers stress tolerance and enables cancer cells to survive under stress is macroautophagy, most commonly known as autophagy (2, 3).

Autophagy is a regulated catabolic pathway that promotes lysosomal degradation of damaged proteins, cellular organelles, and other macromolecules (4–9). This self-digestion process, which facilitates the recycling of bioenergetic components, is activated by a number of stimuli, including the presence of reactive oxygen species, deprivation of growth factors, DNA damage, and cytotoxic drugs (10–12). Autophagy dysregulation is associated with a number of disease states, including cancer (6, 12, 13). Autophagy plays different roles during the initiation and progression of cancer (14, 15). While autophagy acts as a tumor suppressor during the initiation phase of cancer, it promotes tumor progression and metastasis in established cancers (2, 16). Metastatic cancer cells that usually grow in a nutrient-poor microenvironment use autophagy to fulfill their high metabolic demand. Autophagy can facilitate survival during anchorage-independent growth or anoikis, and promotes therapeutic resistance (17, 18). Furthermore, a recent study indicated that genetic or pharmacologic inhibition of autophagy sensitized tumor cells to anticancer treatment (19). During therapy resistance, autophagy protects cancer cells from necrotic death by removing organelles damaged by treatment with chemotherapeutic drugs (2). Autophagy has been shown to be a survival mechanism in castration-resistant prostate cancer cells (20–22). Additionally, pancreatic ductal adenocarcinoma cells display high basal levels of autophagy, which contributes to their intrinsic treatment resistance (23). VEGF-C, a member of the VEGF family of proteins, induces the formation of new lymphatic vessels, a process known as lymphangiogenesis (24). VEGF-C binds to a heterodimer, consisting of 1 of 2 tyrosine kinase receptors (VEGFR3 or VEGFR2) and a nontyrosine kinase receptor, neuropilin-2.
(NRP-2), on lymphatic endothelial cells (25–27). Notably, VEGF-C has lymphangiogenesis-independent functions. For example, VEGF-C is often overexpressed in glioblastoma patients, although brain tissue is void of lymphatics. VEGF-C is also a trophic factor for neural progenitors in vertebrate embryonic brain (28); and can stimulate the proliferation and survival of leukemic cells (29, 30), proliferation and migration of Kaposi sarcoma cells (31), and the invasion and metastasis of gastric, breast, and lung cancer cells (31–33).

Previously, we observed the expression of NRP-2 in cancer cells, suggesting an autocrine function of the VEGF-C/NRP-2 axis (34). Interestingly, we found that this axis can protect prostate and pancreatic cancer cells during chemotherapeutic stress by activating autophagy. Additionally, we have found evidence that inhibition of mTOR Complex 1 (mTORC1) activity by the VEGF-C/NRP-2 axis is a potential mechanism through which autophagy is induced in cancer cells for therapy resistance. These findings, therefore, provide a novel mechanism through which the VEGF-C/NRP-2 axis protects cancer cells from chemotherapy-induced stress.

Materials and Methods

Cell culture

Human prostate cancer cell lines PC3 (American Type Culture Collection, ATCC), LNCaP C4-2, LNCaP C4-2B, and Du145 as well as the pancreatic cancer cell line, CaPan-1 (ATCC), were cultured at 37°C either in RPMI-1640 with 1-glutamine (Invitrogen) or in Dulbecco’s Modified Eagle’s Medium (CellGro) media supplemented with 10% FBS (Invitrogen) and penicillin/streptomycin (Invitrogen). Stably transfected PC3 cell lines were grown on poly-L-lysine-coated coverslips (BD Biosciences). VEGF-C and NRP-2 were depleted 72 hours after transfection. Cells were harvested 48 hours after transfection. Cells were harvested 24 hours after rapamycin addition.

mRNA isolation and quantitative real-time PCR

Total RNA was isolated using an RNeasy mini kit (Qiagen) according to the manufacturer’s protocol. cDNA was prepared from approximately 1 μg mRNA by Transcriptor First Strand Synthesis Kit (Roche) with random hexamer primer. Quantitative real-time PCR (qRT-PCR) analyses were carried out in an Applied Biosystems machine using Power SYBR Green master mix following our previously published protocol (35; for details, please see supplementary material).

Detailed description of the microarray analysis has been provided in the supplementary material.

Western blot and ELISA

Western blots were conducted using antibodies against LAMP-2 (The Developmental Hybridoma Bank, University of Iowa, Iowa City, Iowa), Akt, pAkt (Ser473), LC3, pS6K (Ser389), S6K (Cell Signaling), NRP-2, and rho-GDI (Santa Cruz Biotechnology, ATG13, WDFY-1 (GeneTex), β-actin (Sigma), and pATG13 (Ser318) (Rockland Immunochemicals). Secreted VEGF-C in conditioned media was analyzed using an ELISA kit (R&D Systems, Inc.) according to manufacturer’s protocol.

Apoptosis assay and confocal microscopy

Vybrant Apoptosis Assay Kit #7, purchased from Molecular Probes (Invitrogen), was used according to the manufacturer’s protocol. Briefly, PC3, Du145, and CaPan-1 cells were seeded at a density of 1.5 × 10^5 cells per well of a 2-well chamber slide (LabTek). VEGF-C, NRP-2, WDFY-1, and LAMP-2 were depleted and, 24 hours later, the cells were treated with docetaxel (5, 10, or 20 nmol/L, doses) for prostate cancer cell lines and gemcitabine (20 nmol/L) for pancreatic cancer cell lines; 48 hours later, bafilomycin A1 (BAFM, 10 nmol/L; Sigma) was added. After 24 hours of BAFM treatment, adherent cells were incubated with 1 μL of Hoechst 33342, YO-PRO-1, and propidium iodide (PI) at room temperature for 15 minutes. The cells were then viewed under a confocal microscope using appropriate filters. The apoptotic cells were stained with green fluorescent YO-PRO-1 dye and the dead cells were stained with red fluorescent PI dye. The blue fluorescent dye, Hoechst 33342, stains the chromatin of the cells. Experiments were repeated at least 3 times. A t test was conducted with the data sets obtained to determine whether the differences between experimental groups were significant (P ≤ 0.05).

To determine the autophagic activity, stably transfected PC3 cells grown on poly-L-lysine-coated coverslips (BD Biosciences). VEGF-C and NRP-2 were depleted 72 hours before cell fixation. BAFM and docetaxel at the concentrations listed above were added. At the end of the experiment, the cells were fixed with 4% paraformaldehyde and counter-stained with 4', 6-diamidino-2-phenylindole (DAPI). Respective filters were used to image red and green LC-3II puncta. All confocal images were captured using a Zeiss 710 Confocal Laser Scanning Microscope equipped with 4 lasers, and images analyzed using Zeiss Zen 2010 software. For quantification, the
arithmetic mean intensities of red and green fluorescence images in each field were measured. The ratio of intensities of red fluorescence over green were then calculated and represented graphically. An increased ratio of red to green corresponds to increased autolysosome formation.

**Results**

**Disruption of the VEGF-C/NRP-2 axis alters the expression of autophagy-related genes in prostate cancer cells**

We previously observed that the expression of VEGF-C and NRP-2 promoted the survival of prostate cancer cells following treatment with H2O2 (34). To identify potential pathways involved in this VEGF-C-mediated cell survival, we conducted a microarray study in PC3 prostate cancer cells depleted of either VEGF-C or NRP-2 using SmartPool siRNAs and with scrambled siRNA as a control (Detailed microarray data at GEArray, Geo accession number GSE36085; NCBI tracking system number 16273855; ref. 36). We found 34 gene-tags that were commonly up- or downregulated in both NRP-2- and VEGF-C-depleted cells. A subset of 10 genes was validated via quantitative PCR (qPCR; Fig. 1A). Two genes, LAMP-2 and WDFY-1, were studied in-depth, on the basis of previous reports of function and cellular localization of the proteins. LAMP-2 plays an important role in autophagy by facilitating the fusion of autophagosomes to lysosomes (37). WDFY-1 participates in vesicular trafficking (38) and shares homology with WDFY-3, which participates in aggrephagy, a selective process that degrades protein aggregates using the autophagy machinery (39). We found that both the LAMP-2 and WDFY-1 proteins were upregulated in PC3 cells following VEGF-C/NRP-2 depletion (Fig. 1, B, and C). Because both these proteins are involved in autophagy, we hypothesized that the VEGF-C/NRP-2 axis regulates autophagy. Depletion of VEGF-C was confirmed by both ELISA and qPCR (Supplementary Fig. S1A and B), while NRP-2 depletion was confirmed by both qPCR and Western blot analysis (Supplementary Fig. S1C and D).

**VEGF-C and NRP-2 promote the activation of autophagy following stress**

Starvation of cells initiates autophagy (12); therefore, we examined the effects of starvation on autophagy in VEGF-C–depleted PC3 cells. During autophagy induction, microtubule-associated protein 1A/1B-light chain 3 (LC3) becomes post-translationally modified by phosphatidyl ethanolamine and is incorporated into the membrane of the forming autophagosomes. Detection of this lipiddated version of LC3 (LC3-II) by Western blot is frequently used in the laboratory to monitor autophagy (12). We monitored the level of LC3-II protein via Western blotting in PC3 cells following starvation in PBS at various time points. As illustrated in Fig. 2A, following VEGF-C depletion, the basal LC3-II level increased compared with controls (scrambled siRNA-treated cells). LC3-II accumulation was maximal at the 1-hour time interval in VEGF-C–depleted samples. Increases in WDFY-1 and LAMP-2 levels were detected following VEGF-C depletion at every time point (data not shown). This increase in LC3-II level does not necessarily represent an enhanced autophagic activation, as LC3-II is eventually degraded by the lysosome during autophagy. Therefore, LC3-II accumulates in a time-dependent manner when autophagy is inhibited as a result of decreased lysosomal degradation. To determine whether VEGF-C depletion induces or inhibits autophagy, we conducted autophagic flux experiments. In this assay, the degradation of LC3-II is inhibited by treatment with BAFM, which prevents the fusion of the autophagosome with the lysosome. The fold-change in LC3-II

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**Figure 1. Differential gene expression analysis following depletion of the VEGF-C/NRP-2 Axis:** VEGF-C or NRP-2 was depleted from PC3 cells via cocktail siRNAs for 72 hours. A, total mRNA was isolated from cells, reverse transcribed into cDNA, and used in qPCR analysis using primers specific to genes discovered via microarray analysis. B, immunoblot analysis of WDFY-1 and LAMP-2 following the depletion of VEGF-C. C, immunoblot analysis of WDFY-1 and LAMP-2 following the NRP-2 depletion.
VEGF-C depletion blocked autophagic flux, suggesting an inhibition of autophagy (13, 40, 41). We found that VEGF-C depletion induced autophagy and a lower fold-change in LC3-II level in a particular condition compared with control cells. Inhibition of autophagy was calculated. A higher fold-change in autophagic flux was observed in BAFM-treated cells compared with the level of LC3-II in untreated cells. Immunoblot analysis is shown (right). The density of these bands was calculated and the ratio of LC3-II in BAFM-treated cells was divided by LC3-II in untreated cells. This value is illustrated graphically (left).

Inhibition of autophagic flux occurring in the cells, the level of LC3-II in cells treated with Bafilomycin A1 (BAFM) was compared with the level of LC3-II in untreated cells. Immunoblot analysis is shown (right). The density of these bands was calculated and the ratio of LC3-II in BAFM-treated cells was divided by LC3-II in untreated cells. This value is illustrated graphically (left).

To confirm that the depletion of VEGF-C or NRP-2 prevents autophagic trafficking, PC3 cells stably expressing a mCherry-GFP-LC3 construct were transfected with VEGF-C or NRP-2 siRNAs, incubated with docetaxel for 48 hours, and imaged via confocal microscopy. Following autophagic initiation red, yellow, or green LC3 punctae are visible (13). While yellow or green puncta correspond to autophagosomes, as the autophagosomes mature and fuse with the lysosomes (autolysosome), only red puncta should be visible as GFP is acid-labile, and the lysosomal lumen is highly acidified. As shown in Fig. 3F, we saw both small green/yellow puncta as well as large, red, perinuclear structures indicative of autophagic activity in control cells. In VEGF-C- and NRP-2–depleted cells, however, we observed only diffuse green staining or green puncta, indicating a decrease in autolysosomal turnover and, therefore, an inhibition of autophagy. When we calculated the ratio between levels in each experimental condition in the presence or absence of BAFM, we observed an induction of autophagy and a lower fold-change in LC3-II level in a particular condition compared with control cells. Inhibition of autophagy was calculated. A higher fold-change in autophagic flux was observed in BAFM-treated cells compared with the level of LC3-II in untreated cells. Immunoblot analysis is shown (right). The density of these bands was calculated and the ratio of LC3-II in BAFM-treated cells was divided by LC3-II in untreated cells. This value is illustrated graphically (left).

VEGF-C depletion in the presence of therapeutic stress led to a dysregulation of autophagy similar to that observed during starvation. We used docetaxel, which is commonly used to treat patients with metastatic prostate cancer, at different doses (5, 10, and 20 nmol/L) to induce autophagy. Reduced autophagic flux was observed in docetaxel-treated PC3 cells following VEGF-C depletion with smart-pool siRNA cocktail (Fig. 3A) or with 2 individual VEGF-C specific siRNAs (Fig. 3B). Similar to cocktail siRNA, the individual siRNAs also significantly inhibited VEGF-C expression (Supplementary Fig. S2A and B) and were capable of increasing the expression of Lamp-2 and WDFY-1 (Supplementary Fig. S2C). NRP-2 depletion in docetaxel-treated PC3 cells also led to an inhibition of autophagy (Fig. 3C). We repeated the autophagic-flux experiment in PC-3 cells using function-blocking antibody against VEGF-C (anti-human VEGF-C; AF752; R&D Systems). Similar to VEGF-C siRNA, the blocking antibody inhibited the autophagic flux in the presence of docetaxel (Fig. 3D). NRP-2 function was specifically inhibited by using soluble NRP-2-Fc fused protein. This soluble chimeric protein competes with the membrane bound receptor for ligand binding and, therefore, inhibits its function. We again observed a decrease in autophagic flux in docetaxel-treated PC-3 cells following addition of soluble NRP-2-Fc protein (Fig. 3E). Combined, these results suggest the VEGF-C/NRP-2 axis is involved in autophagy maintenance in cancer cells during therapeutic stress.
the red and green fluorescence intensities (a measure of autophagy activity) in 20 randomly selected fields for control and VEGF-C/-NRP-2-depleted cells (Fig. 3G), we found a decrease in the ratio of red to green, which validated our previous results. Representative images for Fig. 3G are shown in Supplementary Fig. S3. Interestingly VEGF-A, which is functionally redundant with VEGF-C in the regulation of lymphangiogenesis and angiogenesis (42), does not regulate autophagy in PC3 during therapeutic stress. We did not observe any increase in WDFY-1 and LAMP-2 in PC3 cells (Supplementary Fig. S4A) and no change in autophagic flux in docetaxel-treated PC3 cells following VEGF-A-depletion (Supplementary Fig. S4B–D). These results emphasize the specificity of the VEGF-C axis in regulating autophagy during stress.

To confirm that the results we obtained in PC3 cells are neither cell-line nor tumor-type specific, we repeated the autophagic flux assays in Du145 prostate cancer cells treated with 5 and 10 nmol/L doses of docetaxel (Supplementary Fig. S5A), as well as CaPan-1 pancreatic cancer cells treated with a 20 nmol/L dose of gemcitabine (Supplementary Fig. S5B). Gemcitabine is frequently used to treat pancreatic cancer patients. We found a decrease in autophagic flux following VEGF-C-depletion compared with control cells, suggesting that VEGF-C depletion dysregulates autophagy initiated by cytotoxic drugs. NRP-2 depletion in gemcitabine-treated CaPan-1
cells dysregulated autophagy similarly (Supplementary Fig. S5B). Similar to PC-3 cells, the VEGF-C function-blocking antibody (Supplementary Fig. 5C, left) and soluble NRP-2-Fc chimera (Supplementary Fig. S5C, right) also inhibited autophagic flux in CaPan-1 cells and, therefore, confirmed the siRNA results. Overall, these data indicate that autophagic activation by the VEGF-C axis acts as a generalized stress response in cancer cells and may be responsible for their survival following chemotherapy treatment.

**VEGF-C and NRP-2 depletion in cancer cells promotes enhanced cell death following chemotherapy**

To assess whether VEGF-C depletion promotes cancer cell death, VEGF-C–depleted PC3 cells and control cells were treated with docetaxel for 48 hours before staining with Yo-Pro, Hoechst 33342, and PI and subsequent confocal imaging. Representative images of control and VEGF-C–depleted PC3 cells are shown in Fig. 4A, left. To quantify the amount of dead and dying cells, the numbers of green, red, and hybrid red-green cells were divided by the numbers of blue cells per field (Fig. 4A, right). We observed enhanced cell death in untreated VEGF-C–depleted cells compared with controls and the addition of docetaxel significantly increased the amount of cell death in VEGF-C–depleted cells compared with control cells (P = 0.0069). Similar results were observed in NRP-2–depleted PC3 cells (Figs. 4B and C and Supplementary Figs. S6 and S7). Interestingly, we observed increased cell death in untreated PC3 cells following NRP-2 depletion than those in which VEGF-C was depleted (Figs. 4A and B). Because NRP-2 is a receptor, we believe its depletion exerts greater downstream effect than VEGF-C and, thus, a greater consequence to the survival of the cells.

Figure 4. The consequence of VEGF-C/NRP-2 depletion or autophagic blockade and subsequent drug treatment on cell viability. VEGF-C or NRP-2 were depleted from PC3 for either 72 or 48 hours before treatment with a 10 nmol/L dose of docetaxel. The cells were then stained with Yo-Pro, propidium iodide (PI), and Hoechst 33342. A, cells were visualized at ×63 using an LSM 710 microscope with appropriate filters. Three representative images for each treatment are shown (left). In order to quantify the amount of cell death caused by each treatment, the total number of green, red, and hybrid cells were counted in 10 randomly selected fields and divided by the number of Hoechst-positive cells. The results of this analysis for PC3 cells treated with scrambled- or VEGF-C-siRNA in the presence and absence of docetaxel are shown (right). The effect of BAFM on the survival of LNCaPC4-2 cells expressing exogenous VEGF-C. NRP-2 siRNA was added to the cells 48 hours before analysis. Eighteen hours before staining and confocal microscopy, a 10 nmol/L dose of docetaxel was added to the cells. G, the effect of BAFM treatment on the survival of LNCaPC4-2B cells expressing exogenous VEGF-C-siRNA in the presence and absence of docetaxel are shown (right). B, cell death analysis in NRP-2–depleted PC3 cells and control cells were treated with 10 nmol/L doses of docetaxel for 72 hours. C, the resulting cell death in PC3 cells treated with scrambled- or NRP-2 siRNA following treatment with a 10 nmol/L dose of docetaxel for 48 hours. D and E, cell death analysis in PC3 cells treated with VEGF-C function-blocking antibody or soluble NRP-2-Fc chimera (Supplementary Fig. S5C, right) also inhibited autophagic flux in CaPan-1 cells and, therefore, confirmed the siRNA results. Overall, these data indicate that autophagic activation by the VEGF-C axis acts as a generalized stress response in cancer cells and may be responsible for their survival following chemotherapy treatment.

V C EGF and NRP-2 depletion in cancer cells promotes enhanced cell death following chemotherapy

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PC-3 cells. Therefore, to observe a further increase in cell death in NRP-2–depleted PC3 cells following docetaxel treatment, doses of docetaxel greater than 10 nmol/L (Fig. 4B) or the depletion of NRP-2 for shorter time period (48 hours instead of 72 hours; Fig. 4C) was required. Similar increases in cell death were observed when the function of VEGF-C or NRP-2 was inhibited in PC-3 cells by VEGF-C function-blocking antibody (Fig. 4D) or soluble NRP-2-Fc chimera (Fig. 4E). As such, these data indicate that signaling from the VEGF-C/NRP-2 axis protects cancer cells from therapy-induced stress. To determine whether autophagy is involved in the response of cancer cells to docetaxel treatment, PC3 cells were incubated with docetaxel, BAFM, or both agents. Autophagic inhibition by BAFM was confirmed by analyzing mCherry-GFP-LC3-expressing cells using confocal microscopy (Supplementary Fig. S8A). We observed a significant (P = 0.0345) increase in cell death following treatment with both docetaxel and BAFM (Fig. 4F and Supplementary Fig. S8B), although we did not observe a significant increase in cell death following docetaxel treatment alone. Overall, the extent of cell death was similar to that observed in VEGF-C- or NRP-2–depleted cells. To further show that the VEGF-C axis-regulated autophagy is important for docetaxel resistance, we conducted the following experiments. We over-expressed VEGF-C in LNCaP C4-2B and LNCaP C4-2 cells. LNCaP C4-2B is a variant of LNCaP C4-2 cells, which is capable of metastasizing to the bone. These cells express low levels of endogenous VEGF-C. Interestingly, LNCaP C4-2B expresses similar endogenous level of NRP-2 to PC3. The C4-2 expresses detectable level of NRP-2 although lower than both C4-2B and PC3. By overexpressing VEGF-C, we activated the VEGF-C/NRP-2 axis in both LNCaP C4-2B and LNCaP C4-2 cell lines. As expected, the overexpression of VEGF-C induced autophagy and protected the cells from docetaxel-induced death (Figs. 4G and H and Supplementary Figs. S9A and B and S10). NRP-2 depletion in either cell line prevented VEGF-C-dependent rescue of cell survival. The survival of C4-2B and C4-2 cells due to VEGF-C overexpression was also completely abolished when autophagy was inhibited via treatment with the autophagy inhibitor, BAFM. These results, therefore, suggest that VEGF-C/NRP-2 axis-dependent autophagy is required for its survival-promoting function during chemotherapeutic stress. This conclusion was again validated in PC3 cells (Supplementary Fig. S11). Increased cell survival was observed in VEGF-C siRNA-treated PC3 cells, when VEGF-C was exogenously overexpressed. This rescue of cell survival was not detected following autophagy inhibition by BAFM treatment. Thus, we conclude that the cell death we observed in VEGF-C- and NRP-2–depleted cells treated with docetaxel is due to an inhibition of autophagy.

To show that autophagic activation by the VEGF-C axis was a general mechanism of tumor cell survival, we repeated the cell-survival experiments in Du145 cells as well as in CaPan-1 cells. In both cell lines, we observed a statistically significant decrease in survival following VEGF-C depletion and subsequent treatment with chemotherapeutic agents compared with treated control cells (Fig. 5A and B and Supplementary Figs. S12 and S13). Similarly, NRP-2 depletion in CaPan-1 cells also leads to increased cell death following gemcitabine treatment (Fig. 5C and Supplementary Fig. S14). Again, increase in cell death was observed when CaPan-1 cells were treated with

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**Figure 5.** The effect of VEGF-C/NRP-2 depletion on the survival of other cell types. A, Du145 cells were treated with either scrambled or VEGF-C siRNA. Twenty-four hours later, the cells were treated with a 5 nmol/L dose of docetaxel. Cell death was then analyzed using the aforementioned stains and confocal microscopy. The quantification of 10 fields is illustrated. B, CaPan-1 cells were seeded onto coverslips and transfected with either scrambled or VEGF-C siRNAs. Twenty-four hours after siRNA treatment, 10 and 20 nmol/L doses of gemcitabine were added to the cells. Cell death was then visualized and the quantification of 10 fields is shown. C, NRP-2 was depleted from CaPan-1 cells and the cells were then treated with 0, 20, 30, and 50 nmol/L doses of gemcitabine for 48 hours. Cell death was then visualized via confocal microscopy, the quantification of which is shown here.
Figure 6. WDFY-1 promotes cell death on inhibition of the VEGF-C/NRP-2 axis. A, immunoblot analysis of WDFY-1 and LAMP-2 in PC3, Du145, and CaPan-1 cells treated with BAFM. B, immunoblot analysis of WDFY-1 expression in PC3 cells depleted of VEGF-C alone or both VEGF-C and WDFY-1 siRNAs. C, PC3 cells were plated and treated with scrambled, VEGF-C, or both VEGF-C and WDFY-1 siRNAs, and the amount of cell death was calculated as described previously. D, quantitation of cell death in PC3 cells depleted of NRP-2 and both NRP-2 and WDFY-1. Again, in all graphs, the standard deviation is depicted. Furthermore, the statistical difference \( P < 0.05 \) between samples was calculated using a t-test.

VEGF-C function-blocking antibody or NRP-2 Fc chimera along with gemcitabine (Supplementary Fig. S15A and B). These data indicate that the VEGF-C axis promotes autophagy, which contributes to survival in cancer cells exposed to cytotoxic stress.

**Upregulation of WDFY-1 following VEGF-C and NRP-2 depletion contributes to decreased cell viability in chemotherapy-treated cells**

We previously showed that LAMP-2 and WDFY-1 levels increased following VEGF-C and NRP-2 depletion (Fig. 1). We observed a similar increase in LAMP-2 and WDFY-1 following inhibition of autophagy by BAFM treatment in all cell lines (Fig. 6A). We, therefore, concluded that the upregulation of WDFY-1 and LAMP-2 occur when autophagy is inhibited through an inhibitory mechanism. Next, we tested whether increases in the levels of WDFY-1 and LAMP-2 influence the survival of cancer cells following therapeutic intervention. We found that VEGF-C depletion led to a significant \( P = 0.0119 \) increase in cell death following docetaxel treatment. Intriguingly, the codepletion of VEGF-C and WDFY-1, which we verified by Western blot (Fig. 6B), maintains cell viability following docetaxel treatment (Fig. 6C and Supplementary Fig. S16). Enhanced cell viability was also observed in PC3 cells codepleted of NRP-2 and WDFY-1 following docetaxel treatment compared to PC3 cells depleted of NRP-2 alone (Fig. 6D and Supplementary Fig. S16). Similarly, the depletion of both LAMP-2 and VEGF-C also led to a decrease in cell death compared with the depletion of VEGF-C alone (data not shown), although we did not find that this difference was statistically significant. Together, these results suggest that increased levels of WDFY-1 and LAMP-2 (to a lesser extent) promote cell death following the inhibition of autophagy.

**Inactivation of mTORC1 activity by the VEGF-C/NRP-2 axis is a potential mechanism for autophagy induction**

We have shown that the accumulation of WDFY-1 and LAMP-2 driven by the depletion of VEGF-C and NRP-2 in cancer cells (Figs. 6A) might be a consequence of autophagic inhibition. These results, therefore, indicated that these proteins might not regulate autophagy in cancer cells downstream of the VEGF-C axis. In search of the potential mechanisms through which the VEGF-C axis regulates stress-induced autophagy, we turned our attention to our previously reported findings. Earlier, we showed that during oxidative stress the VEGF-C axis maintains mTOR Complex 2 (mTORC2) activity, which is upstream of AKT (34). Interestingly, the downstream mediators of the AKT-mTOR Complex 1 (mTORC1) signaling axis, the S6 protein or 4EBP (43), were not activated under these conditions in prostate cancer cells. The reason for this unexpected result remained unanswered in our previous publication (34). As active mTORC1 blocks the induction of autophagy (9), we hypothesized that the VEGF-C/NRP-2 axis actually inhibits mTORC1 activity to promote autophagy during stress (44). Indeed, we observed a significant increase in phospho-S6K following the depletion of either VEGF-C or NRP-2 in PC-3 (Fig. 7A) or a decrease in phospho-S6K level in docetaxel-treated LNCaP C4-2 cells (34) following addition of
VEGF-C (Supplementary Fig. S1A and B), suggesting an inhibitory role of VEGF-C axis on mTORC1 activity. This increase in S6K phosphorylation on VEG-C depletion seemed to be independent of AKT as knocking down the AKT level did not significantly influence phospho-S6K levels (Supplementary Fig. S1C). A decrease in ATG13 phosphorylation following the depletion of VEG-C or NRP-2 in PC3 treated with docetaxel was observed (Fig. 7A). Downregulation of ATG13 was regulated by mTORC1 for the inhibition of autophagy. To further confirm that this increased mTORC1 activity is responsible for the observed dysregulation of autophagy, we used rapamycin to block mTORC1 activity in VEG-C- and NRP-2-depleted PC3 cells. Rapamycin treatment rescued the autophagic flux in PC3 cells despite decreased levels of VEG-C and NRP-2 (Fig. 7B). Similar results were obtained when we monitored autophagy by confocal microscopy in the docetaxel-treated stably expressing mCherry-GFP-LC3 PC3 cell line (Fig. 7C). An increased number of mCherry-positive red punctae of autolysosomes, indicative of increased autophagy, were observed in VEG-C or NRP-2-depleted PC3 cells following treatment with rapamycin. A similar decrease in ATG13 activity and an upregulation of pS6K activity following the inhibition of the VEG-C/NRP-2 axis was also observed in CaPan-1 cells (Fig. 7D). Rapamycin also restored the autophagic flux in CaPan-1 cells, despite the depletion of VEG-C or NRP-2 (Fig. 7E and F). We, therefore, conclude that the VEG-C/NRP-2 axis promotes autophagy via downregulation of mTORC1 function especially during therapeutic stress.

Discussion

The ability of tumor cells to survive under stress is a major obstacle to effective cancer therapy (1, 19). Autophagy has been shown to promote cancer cell survival in the presence of metabolic, hypoxic, or cytotoxic stress (6, 12) that can result

![Image of Figure 7](https://example.com/image7.png)
in tumor dormancy and subsequent progression and metastasis (2, 17). Previously, we reported a survival-promoting function of the VEGF-C axis during oxidative stress (34). Our subsequent studies suggested that the VEGF-C axis can regulate distinct cellular mechanisms to facilitate the survival of cancer cells under different stress conditions. A clue that the VEGF-C/NRP-2 axis regulates autophagy in cancer cells came from a microarray study to identify genes that are regulated by this axis. We knocked down endogenous VEGF-C and NRP-2 separately in prostate cancer cells and identified genes whose expression was influenced by reduced levels of endogenous VEGF-C and NRP-2. We identified 10 genes that were equally up- or downregulated following the depletion either VEGF-C or NRP-2 and considered them as the genes regulated by VEGF-C axis in prostate cancer. Interestingly, most of these genes are similarly regulated by VEGF-C or NRP-2 in other cancer cells, which suggests the importance of this axis in different cancers. Among these genes, we took particular interest in WDFY-1 and LAMP-2 (Fig. 1). LAMP-2 is a lysosomal membrane protein, which plays a role in autophagy (36). WDFY-1 (also known as FENS-1), which contains a single FYVE domain and multiple WD-40 repeats, plays a role in vesicular trafficking (37). Interestingly, a related protein WDFY-3 (ALFY) is involved in selective autophagy (aggrephagy; ref. 38), suggesting a potential connection between WDFY-1 and autophagy. We, therefore, tested the hypothesis that the VEGF-C/NRP-2 axis regulates autophagy and that WDFY-1 and LAMP-2 function as downstream effectors in this pathway.

The experimental proof of this hypothesis came from studies where we tested autophagic activity in VEGF-C- or NRP-2-depleted PC3 cells during nutrient deprivation (Fig. 2) and observed an autophagy-inducing function by both VEGF-C and NRP-2. To our knowledge, this is a novel observation and underscores the importance of this axis in cancer stress-resistance. It has been postulated by researchers in this field that autophagy and apoptosis are mutually exclusive (10). Because autophagy is viewed as primarily cytoprotective, it has been hypothesized that its induction should be coupled with the activation of antiapoptotic pathways (10). Our previous study on the function of the VEGF-C axis in cancer validates this hypothesis (34). We previously showed that the VEGF-C axis activates mTORC2 and promotes cancer cell survival during stress through the activation of antiapoptotic pathways. Our current study suggests that this axis also controls autophagy. We, therefore, propose that due to its ability to couple autophagy and antiapoptotic pathways, the VEGF-C/NRP-2 axis is a significant pathway in promoting cancer cell survival during stress.

We then examined the interaction between the VEGF-C axis and autophagy in the presence of docetaxel-induced cytotoxic stress. Docetaxel has been used to treat patients with metastatic, castration-resistant prostate cancer (45) and independent clinical trials have shown that docetaxel-based treatment can increase median overall survival compared with mitoxantrone and prednisone (46, 47). Docetaxel functions by stabilizing microtubules, thereby inhibiting mitotic spindle depolymerization and cell proliferation. Docetaxel also induces cell death by inhibiting various cell-survival mechanisms. The cell death-inducing function of docetaxel may be therapeutically more significant in prostate cancer as this cancer usually proliferates at a slower rate, and lethality of metastatic castration-resistant prostate cancer is due to its ability to resist death (45). Resistance to docetaxel in men with metastatic castration-resistant disease has been observed in almost 50% of patients. Accordingly, we determined whether the autophagy-inducing activity of the VEGF-C axis in prostate cancer cells is responsible for its ability to resist docetaxel-induced cell death. In VEGF-C- and NRP-2-depleted cells treated with docetaxel, we observed decrease in autophagic flux compared with control cells (Fig. 3A–E). We have confirmed this observation morphologically using mCherry-GFP-LC3-expressing cells (Fig. 3F and G). As expected, the depletion of VEGF-C and NRP-2 in PC3 cells also made them more vulnerable to docetaxel-induced death (Fig. 4A–E; Supplementary Figs. S6 and S7). A similar increase in cell death was observed when PC3 cells were treated with docetaxel and, at the same time, autophagy was inhibited chemically (Fig. 4F; Supplementary Figs. S8A, B, and S11). Furthermore, the addition of VEGF-C to LNCaP C4-2B and C4-2 cells failed to rescue cell survival following autophagy inhibition (Fig. 4G and H; Supplementary Figs. S9 and S10). These results indicate a VEGF-C/NRP-2-dependent mechanism of docetaxel resistance in prostate cancer. Because both VEGF-C and NRP-2 are expressed in high levels in metastatic prostate cancer (48, 49), we assume that this axis could contribute to prostate cancer recurrence following docetaxel treatment.

To prove that our observation was not a cell-type specific phenomenon, we conducted similar experiments in another prostate cancer cell line, Du145, as well as in a pancreatic cancer cell line, CaPan-1. We selected these lines because of their aggressive nature, metastatic potential, and high endogenous expression of both VEGF-C and NRP-2 (data not shown). We treated CaPan-1 cells with gemcitabine as it is the standard chemotherapeutic agent used to treat metastatic pancreatic cancer (50). Like docetaxel, resistance to gemcitabine is the major reason for its limited therapeutic efficacy (50). We observed a similar decrease in autophagic flux and increase in cell death in both Du145 treated with docetaxel as well as CaPan1 cells treated with gemcitabine (Fig. 5; Supplementary Figs. S4, S9–12), which suggests that upregulation of the VEGF-C axis is a general adaptation that promotes tumor cell survival under adverse conditions. Overall, these data strongly suggest the importance of this axis in autophagic activation.

Although the increased levels of WDFY-1 and LAMP-2 (to a lesser extent) following VEGF-C and NRP-2 depletion can function in the induction of cell death (Fig. 6; Supplementary Fig. S13), they are not likely to be involved in regulating autophagy. In future studies, we will unravel their roles in promoting cell death following autophagy inhibition. Our finding that the VEGF-C axis inhibits mTORC1 to activate autophagy (Fig. 7) is novel and provides a mechanism through which this axis promotes resistance against treatment.

In conclusion, we have established that the VEGF-C/NRP-2 axis is important in the activation of autophagy following treatment with chemotherapy. Our data suggest that VEGF-C-induced autophagy protects cancer cells from the cytotoxic

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stress-induced death as well as the potential of the VEGF-C axis as a therapeutic target. Our future studies will focus on determining the mechanism(s) through which WDFY-1 activates cell death downstream of autophagy inhibition. Unraveling these signaling pathways may assist in the development of novel treatments to overcome chemotherapy resistance.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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