The Nerve Growth Factor controls EMT and Invasion of Castrate-Resistant Prostate Cancer

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Prostate cancer (PC) still remains the most common malignancy in men. Despite the significant efforts in early diagnosis and treatments, PC often progresses and escapes the therapies. As a result, many patients develop castration-resistant PC (CRPC), which is almost incurable (Parker et al 1).

While the role of steroid hormones and their receptors is well established in PC progression, other key events are involved. The nerve growth factor (NGF) turned out to be a multifaceted molecule in many solid neoplasias, including PC (Graham et al 2), which often exhibits derangements of NGF and its receptors, including the tropomyosin receptor kinase A (TrkA). However, the role of NGF signaling in EMT and aggressiveness of PC still remains pending.

By biochemical and functional assays, we found a link between the activation status of NGF signaling and aggressiveness in various PC cell lines (Di Donato et al., 2018 3, 2019 4). NGF sustains cell proliferation, drives the cell re-programming towards EMT and stimulates their invasion. Such features lead to the increase in the size of CRPC-derived spheroids. Secretoma analysis revealed a release of NGF by PC cells (Di Donato et al., 20215). As these cells express TrkA, an autocrine loop might sustain the survival and aggressive behavior of PC cells. The TrkA specific inhibitor, GW441756 reverses all the observed effects.

In addition to suggesting new therapeutic opportunities in PC, our findings might impact PC plasticity and its neuroendocrine differentiation.
Figure 1. TrkA activation mediates the NGF mitogenic effect in castration-resistant prostate cancer (CRPC) cells. C4-2B, DU145 and PC3 cells were used. In A, lysates from the indicated cell lines were prepared and proteins were analyzed by Western blot, using the antibodies against the indicated proteins. Quiescent C4-2B (B), DU145 (C) and PC3 (D) cells were left untreated (untr) or treated for 18h with the indicated compounds. After in vivo pulse with 100 µM BrdU, BrdU incorporation was analyzed by IF and expressed as % of total cells. Quiescent C4-2B, DU145 and PC3 cells were left untreated (untr) or treated for 18h with 20% (v/v) serum, in the absence or presence of GW441756. After in vivo pulse with 100 µM BrdU, BrdU incorporation was analyzed by IF and expressed as % of total cells. Under basal conditions, 9%, 12% and 14% of C4-2B, DU145 and PC3 cells, respectively, incorporated BrdU. Serum stimulation increased by 45%, 67% and 59% the number of BrdU incorporating C4-2B, DU145 and PC3 cells, respectively. Addition of GW441756 did not significantly inhibit this number (43%, 63% and 57% for C4-2B, DU145 and PC3 cells, respectively). Quiescent C4-2B (E), DU145 (F) and PC3 (G) were left untreated or treated for 24, 48 and 72 h with the indicated compounds. Cell proliferation was assayed using the WST-1 (water soluble tetrazolium-1) reagent. Graphs in E–G represent the ratio of proliferation, which was expressed as fold increase over the basal absorbance. NGF stimulation induced a significant variation of the proliferation as compared with untreated cells (p < 0.05). In H, LNCaP, DU-145 and PC3 cells were made quiescent. Conditioned media were collected after 48h and analysed by ELISA kits, as reported in Methods. Albeit at different extent, conditioned media from LNCaP, PC3 and DU-145 cells contain NGF. In B–G, NGF was used at 100 ng/mL; GW441756 (GW) was used at 1µM. When indicated, serum was used at 20% (v/v). Three independent experiments were done. Means and standard error of the means (SEMs) are shown. n represents the number of experiments. * p < 0.05 for the indicated experimental points vs. the corresponding untreated control.
Figure 2. NGF triggers migration and invasiveness in C4-2B cells. In A, quiescent C4-2B cells were wounded and left untreated or treated with NGF for the indicated times. GW441756 (GW) was added at 1 µM. Phase-contrast images are representative of three different experiments, each in duplicate. In (B), the wound area was measured using Leica Suite Software and data are presented as % in wound width over the control cells, analyzed at time 0. Means and standard error of the means (SEMs) are shown. n represents the number of experiments. Quiescent C4-2B cells were used for migration (C) and invasion (D) assays in Boyden’s chambers pre-coated with collagen or Matrigel, respectively. The indicated compounds were added to the upper and the lower chambers. NGF was used at 100 ng/mL and GW441756 (GW) at 1 µM. After 7 h (in C) or 24 h (in D), migrating or invading cells were counted as reported in Methods. Results from three different experiments were collected and expressed as fold increase. Means and SEMs are shown. n represents the number of experiments. * p < 0.05 for the indicated experimental points vs. the corresponding untreated control.
Figure 3. **NGF triggers migration and invasiveness in DU145 cells.** In A, quiescent DU145 cells were wounded and then left untreated or treated with NGF for the indicated time. When indicated, GW441756 (GW) was added at 1 µM. Phase-contrast images are representative of three different experiments, each in duplicate. In B, the wound area was measured as in Figure 2 B and data are presented as % in wound width over the control cells, analyzed at time 0. Means and SEMs are shown. n represents the number of experiments. Quiescent C4-2B cells were used for migration (C) and invasion (D) assays in Boyden’s chambers pre-coated with collagen or Matrigel, respectively. The indicated compounds were added to the upper and the lower chambers. NGF was used at 100 ng/mL and GW441756 (GW) was used at 1 µM. After 7 h (C) or 24 h (D), cells were counted as reported in Methods. Results from three different experiments were collected and expressed as fold increase. Means and standard error of the means (SEMs) are shown. n represents the number of experiments. * p < 0.05 for the indicated experimental points vs. the corresponding untreated control.

Figure 4. **NGF triggers migration and invasiveness in PC3 cells.** In A, quiescent PC3 cells were wounded and then left untreated or treated with NGF for the indicated times. GW441756 (GW) was added at 1µM. Phase-
contrast images are representative of three different experiments, each in duplicate. In (B), the wound area was measured as in Figure 2B and data are presented as % in wound width over the control cells analyzed at time 0. Means and SEMs are shown. n represents the number of experiments. Quiescent C4-2B cells were used for migration (C) and invasion (D) assays in Boyden’s chambers pre-coated with collagen or Matrigel, respectively. The indicated compounds were added to the upper and the lower chambers. NGF was used at 100 ng/mL and GW441756 (GW) was used at 1 µM. After 7 h (C) or 24 h (D), migrating or invading cells were counted as reported in Methods. Results from three different experiments were collected and expressed as fold increase. Means and standard error of the means (SEMs) are shown. n represents the number of experiments. *p < 0.05 for the indicated experimental points vs. the corresponding untreated control.

Figure 5. NGF promotes epithelial-mesenchymal transition (EMT) in DU145 and PC3 cells and silencing of Androgen Receptor (AR) does not affect EMT, migration, invasiveness and DNA synthesis of NGF-treated C4-2B cells. Quiescent C4-2B (A), DU145 (B) and PC3 (C) cells were left untreated or treated for 72h with the indicated compounds. NGF was used at 100 ng/mL and GW441756 (GW) at 1 µM. In A-C (left panels), protein lysates were analyzed by Western blot, using the antibodies against the indicated proteins. The blots are representative of three different experiments. In A-C (right panels), Phase-contrast images are representative of 3 different experiments, each in duplicate. Scale bar, 10 µm in A and 90 µm in B and C. In D-G, C4-2B cells were transfected with AR siRNA or control siRNA, as reported in Methods. After transfection, the cells were made quiescent and then left un-stimulated or stimulated as reported. In D, cells were stimulated as in A and protein lysates were analyzed by western blot using the antibodies against the indicated proteins. Cells were used for migration (E) and invasion (F) assays in Boyden’s chambers pre-coated with collagen or Matrigel, respectively. The indicated compounds were added to the upper and the lower chambers. After 7 h (in E) or 24 h (in F), migrating or invading cells were counted as reported in Methods. In G, transfected C4-2B cells on coverslips were left untreated (untr) or treated for 18h with the indicated compounds. After in vivo pulse with 100 µM BrdU, BrdU incorporation was analyzed by immunofluorescence (IF) and expressed as % of total cells. In E–G, the cells were co-transfected with siRNA Alexa Fluor 488 to help identification of transfected cells. Results from three different experiments were collected and expressed as fold increase. Means and standard error of the means (SEMs) are shown. n represents the number of experiments. *p < 0.05 for the indicated experimental points vs. the corresponding untreated control.
Figure 6. Expression of p75NTR and signaling effectors involved in locomotion of CRPC cells. C4-2B, DU145 and PC3 cells were used. In A, lysates from the indicated cell lines were prepared and proteins were analyzed by western blot, using the antibodies against p75NTR. In B, lysates from the indicated cell lines were prepared and proteins were analyzed by Western blot, using the antibodies against the indicated proteins. Filters were re-probed using anti tubulin antibody, as a loading control. Western blots in A and B are representative of three different experiments. Expression levels of proteins were analyzed by densitometry analysis, using NIH Image J Software. The ratio between Filamin A/tubulin (C), FAK/tubulin (D) and Paxillin/tubulin (E) was evaluated. Results were expressed as relative ratio. Means and SEMs are shown, n represents the number of the experiments.

Figure 7. NGF increases the growth of organoids from CRPC cells through TrkA activation. C4-2B (upper panel), DU145 (middle panel) and PC3 (lower panel) were used in miniaturized 3D cultures in extracellular matrix (ECM), as reported in Methods. Four days after cells embedding in VitroGel-3D-RGD,
representative images were acquired as described in Methods. 3D cultures were left untreated or treated with 100 ng/mL NGF, in the absence or presence of GW441756 (GW; 1 µM) for 14 days. Shown are phase-contrast images captured at 14th day. Scale bar, 100 µ.

Figure 8

Figure 8. TrkA activation by NGF triggers MAPKs and Akt activation in CRPC cells. Quiescent C4-2B (A, E and H), DU145 (B, F and I) and PC3 (C, G and L) cells were used. In A–C, cells were left un-stimulated or stimulated for the indicated times with 100 ng/mL NGF. In D, the indicated cycling cells were used. In E–L, cells were left un-stimulated or stimulated for the indicated times with 100 ng/mL NGF, in the absence or presence of GW441756 (GW; 1µM). In A–C, protein lysates were analyzed by western blot, using the indicated antibodies. Filters were re-probed using anti-ERK (Extracellular signal-regulated kinase) or anti-Akt or anti-tubulin (tub) antibodies, as a loading control. In (D), protein lysates were analyzed by Western blot, using the indicated antibodies. In E–F, protein lysates were analyzed by western blot, using the antibodies against the indicated proteins (p-TrkA stands for P-Tyr-490 TrkA). In H–L, cells were left untreated (untr) or treated for the indicated times with NGF (100 ng/mL), in the absence or presence of 1µM GW441756 as reported in E–G. Lysates proteins were analyzed by western blot, using the antibodies against the indicated proteins. In A, B, C, H, I, L, p-ERK stands for P-Tyr 204 ERK 1, and the corresponding phosphorylated ERK 2; p-Akt stands for P-Ser 473 Akt. The filters were re-probed using anti-ERK or anti-Akt or anti-tubulin (tub) antibodies, as loading controls.
Figure 9. Results from Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) network and effect of TrkA knockdown in CRPC cells. Combined screenshots from the STRING website, which has been queried with TrkA (NTRK1) and extracellular signal-regulated kinase (ERK; MAPK1; A) or TrkA (NTRK1; neurotrophic tyrosine kinase) and Akt (or PKB, protein kinase B, AKT1; B) are shown. Colored lines between the proteins indicate the various type of interaction, as described in the inset in A. Protein nodes also indicate the availability of 3D protein structure information. C4-2B (C), DU145 (D) and PC3 (E) cells were transfected with TrkA siRNA or control siRNA, as reported in Methods. After transfection, the cells were made quiescent and then left un-stimulated or stimulated with Nerve growth factor (NGF). In C–E, protein lysates were analyzed by western blot using the antibodies against the indicated proteins. Transfected C4-2B (F), DU145 (G) and PC3 (H) cells on coverslips were left untreated or treated for 18h with 100 ng/mL NGF. After in vivo pulse with 100 µM BrdU, BrdU incorporation was analyzed by IF and expressed as % of total cells. Transfected C4-2B (I), DU145 (L) and PC3 (M) cells were used for invasion assays in Boyden’s chambers pre-coated with Matrigel. NGF (100 ng/mL) was added to the upper and the lower chambers. After 24 h, invading cells were counted as reported in Methods. In F–M, the cells were co-transfected with siRNA Alexa Fluor 488 to help identification of transfected cells. Results from three different experiments were collected and expressed as fold increase. Means and standard error of the means (SEMs) are shown. n represents the number of experiments. * p < 0.05 for the indicated experimental points vs. the corresponding untreated control.
Figure 10. Inhibition of Akt or ERK impairs the NGF-challenged proliferation and migration in CRPC cells. C4-2B (A), DU145 (B) and PC3 (C) cells were left untreated (untr) or treated for 18h with the indicated compounds. After in vivo pulse with 100 µM BrdU, BrdU incorporation was analyzed by IF and expressed as % of total cells. C4-2B (D), DU145 (E) and PC3 (F) cells were used for migration assay in collagen pre-coated Boyden’s chambers. The indicated compounds were added to the upper and the lower chambers. After 7 h, migrated cells were counted as described in Methods and results expressed as fold increase. In A-F, NGF was used at 100 ng/mL; PD98059 (PD) and LY-294002 (LY) were used at 10 µM. Results from three different experiments have been collected. Means and standard error of the means (SEMs) are shown. n represents the number of experiments. * p < 0.05 for the indicated experimental points vs. the corresponding untreated control.

References

2. Graham et al., J.Urol, 1992 147, 1444-1447
3. Di Donato et al., Cell Death and Discovery, 2018 31, 4-5
4. Di Donato et al., Cancers, 2019 11, 784-807
5. Di Donato et al., Cell Death and Disease 2021 12, 127-144