

Research Paper

Proliferation Enhanced By NGF-NTRK1 Signaling Makes Pancreatic Cancer Cells More Sensitive To 2DG-Induced Apoptosis

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Abstract

Rapidly proliferating cancer cells rely on increased glucose consumption for survival. The glucose analog 2-deoxy-D-glucose (2DG) cannot complete glycolysis and inhibits the growth of many types of cancers. It is unknown whether reduced glycolysis inhibits the growth of pancreatic cancer. Activation of nerve growth factor (NGF)-neurotrophic tyrosine kinase receptor type I (NTRK1) signaling leads to enhanced proliferation of these cells. We investigated the effect of 2DG treatment on the viability of NTRK1-transfected pancreatic cancer cells. After treatment with 2DG, the viability of pancreatic cancer cells was evaluated by MTT assay. SB203580 (a specific inhibitor of the p38-MAPK pathway) and PD98059 (an MAP2K1 [mitogen-activated protein kinase kinase 1, previously, MEK1] inhibitor) were used to inhibit p38-MAPK and ERKs, respectively. The percentage of apoptotic cells was determined by flow cytometry. Overexpression of NTRK1 in pancreatic cancer cells resulted in increased cell proliferation, which was reduced by PD98059-mediated inhibition of ERKs but not by suppression of p38-MAPK with SB203580. After treatment with 2DG, the percentage of apoptotic cells was greater in those with high expression of NTRK1 than in cells with low NTRK1 expression. Blocking the p38-MAPK pathway with SB203580 effectively abolished the apoptosis induced by 2DG. We conclude that pancreatic cancer cells with a high expression of NTRK1 are more sensitive to 2DG-induced apoptosis, through the p38-MAPK pathway.

Key words: NGF, NTRK1, pancreatic cancer, glycolysis, 2DG

Introduction

Nerve growth factor (NGF) is a member of the well-studied neurotrophin family, proteins with important roles in the regulation of cell growth, proliferation, survival, and death [1, 2]. NGF and its high affinity receptor, neurotrophic tyrosine kinase receptor type 1 (NTRK1), promote cell survival, proliferation, and resistance to cancer chemotherapy by activating the extracellular-signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/ AKT1,

and p38 mitogen-activated protein kinase (MAPK) signaling pathways [3-7]. However, in some cell types NGF induces cell differentiation or apoptosis, via p53[8] or c-Jun N-terminal kinases (JNKs) [9], respectively.

High levels of NGF and NTRK1 in pancreatic cancers and the perineurium of pancreatic nerves are closely correlated with perineural invasion [10], tumor aggressiveness, and unfavorable prognosis, and

are accompanied by activation of both ERKs and p38-MAPK signaling. The function of the activation of p38-MAPK signaling by NGF is not well understood. It has been shown that activation of p38-MAPK is associated with proliferation, migration, and resistance to apoptosis [11, 12]. Yet p38-MAPK also activates p53, induces cell-cycle arrest, apoptosis, and differentiation, and makes cells sensitive to chemotherapy [9, 13-17].

In previous studies [18] we demonstrated increased cell proliferation in NTRK1-transfected human pancreatic cancer cells, while knockdown of NTRK1 by small interfering RNA (siRNA) inhibited cell proliferation and sensitized pancreatic cancer to the chemotherapy drug gemcitabine. These results support targeting the NGF-NTRK1 pathway in pancreatic cancer therapy. However, the NTRK inhibitor CEP-701 (lestaurtinib) demonstrated significant damages to other tissues with high expression of NTRK1. Thus, it is important to develop an alternative strategy for the treatment of pancreatic cancers with increased levels of NGF and NTRK1 [19].

Unlike normal cells that gain energy through oxidative phosphorylation in mitochondria, the increased proliferation of cells in most types of cancers depends upon accelerated anaerobic glycolysis in the cytosol [20-22], a phenomenon known as the Warburg effect [23].

Therefore, targeting the anaerobic glycolytic pathway may preferentially kill malignant cells while sparing the normal [24, 25]. Since pancreatic cancer cells have been shown to have an increased utilization of glucose [26, 27], it is intriguing to target the glycolytic metabolic pathway for the treatment of pancreatic cancers [28].

To test if inhibition of glycolysis of pancreatic cancer cells *in vitro* results in reduced cell proliferation, we evaluated cell viability after glucose deprivation induced by the nonmetabolizable glucose analog 2-deoxy-D-glucose (2DG) in NTRK1-transfected ASPC-1, BXPC-3, and Panc-1 pancreatic cancer cells.

Materials and Methods

Reagents

Dimethyl sulfoxide (DMSO), 2DG, G418 sulfate, β -NGF, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (USA). Radioimmunoprecipitation (RIPA) buffer and phenylmethylsulfonyl fluoride (PMSF) were obtained from Beyotime (China). Anti-NTRK1, Anti-p-p38, ERK inhibitor PD98059, and p38 inhibitor SB203580 were from Abcam (USA). An-

ti- β -actin antibody and secondary antibody were from Abnova.

Cell culture

Cells of the human pancreatic cancer cell lines ASPC-1, BXPC-3, and Panc-1 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). ASPC-1 cells were grown in RPMI-1640 and BXPC-3 or Panc-1 cells in Dulbecco's modified Eagle's medium (DMEM; both from Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone), penicillin 100 U/mL, and streptomycin 100 μ g/mL (Gibco). The cultures were maintained at 37 °C in a 5% CO₂ incubator.

Establishment of stable transfected cell lines

The plasmid pcDNA3.1-NTRK1 was provided by Wei Liu (Fourth Military Medical University, Xi'an, China). All transfections were performed using X-tremeGENE HP in accordance with the manufacturer's instructions, at the ratio of 1:1 (Roche). For generation of NTRK1-transfected or non-NTRK1-transfected cell lines, ASPC-1 cells were transfected with pcDNA3.1-NTRK1 vector or pcDNA3.1 blank vector, respectively. Cells were maintained in 200 ng/mL G418 for 21 days. Individual clones were screened for expression of NTRK1 by Western blot analysis.

Transfection of siRNA

Human *NTRK1*-specific and control siRNAs were purchased from Biocompare (SMARTpool, Charlottesville, VA). BXPC-3 or Panc-1 cells were plated in 6-well plates and incubated for 24 h prior to transfection. Eight microliters of siPORT Amine transfection reagent (Ambion) were added to 200 μ L serum-free medium and incubated at room temperature for 15 min. The transfection reagent/siRNA complexes were added to each well containing 800 μ L medium with 10% FBS. Six hours later, 1 mL DMEM containing 10% FBS was added into each well.

MTT assay of cell growth inhibition

Cells were seeded (3000/well) in 96-well plates for 24 hours. 2DG, PD98059, or SB203580 was added and incubated for the indicated time at 37 °C. MTT (0.5 mg/mL in PBS) was added to each well and incubated for 4 h at 37 °C. The media was then discarded and 100 μ L DMSO was added. After agitation for 10 min on a shaker, absorbance was read at 550 nm on a spectrophotometer. Data were normalized relative to the untreated group.

Western blot

Cells were lysed on ice with modified RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing 25 µg/mL leupeptin, 1 mM sodium orthovanadate, 2 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM PMSF. The concentration of protein was determined using the bicinchoninic acid assay (Beyotime). Twenty micrograms of total protein were loaded onto an 8% SDS-polyacrylamide gel, electrophoresed, and blotted onto a polyvinylidene difluoride (PVDF) membrane. Proteins were probed with the primary antibody overnight at 4 °C and the secondary antibody at room temperature for 1 hr. Immunoreactivity was detected by the electrochemiluminescence (ECL) system and normalized to β -actin.

Detection of apoptosis

Twenty-four hours after treatment, 10^6 of cells were harvested by centrifuging at $200 \times g$ for 5 min. Cells were resuspended in 100 µL of Annexin-V-FLUOS (Roche), incubated at room temperature for 15 min, and subjected to flow cytometry.

Statistical analysis

All experiments were repeated in triplicate. Data were analyzed by SPSS13.0 software using Student's *t*-test. Probability values (*P*) < 0.05 were considered statistically significant.

Results

NGF-NTRK1 pathway enhances pancreatic cancer cell proliferation depends on ERKs

NGF-NTRK1 signaling enhances cell proliferation independently on the p38-MAPK pathway. NGF promotes proliferation, invasion and survival of many types of cells with high expression of NTRK1 including pancreatic cancer cells. In NTRK1-transfected ASPC-1 cells, 200 ng/mL NGF for 72 h increased the cell number by 1.5-fold in comparison to untreated group (*P* < 0.05). In contrast, NGF showed no obvious influence on the growth of ASPC-1 cells transfected with the empty vector (Fig. 1A). Thus the growth-stimulatory effects of NGF depend on the availability of its receptor NTRK1. Similar results were observed in BXPC-3 and Panc-1 cells (Fig. 1B and C).

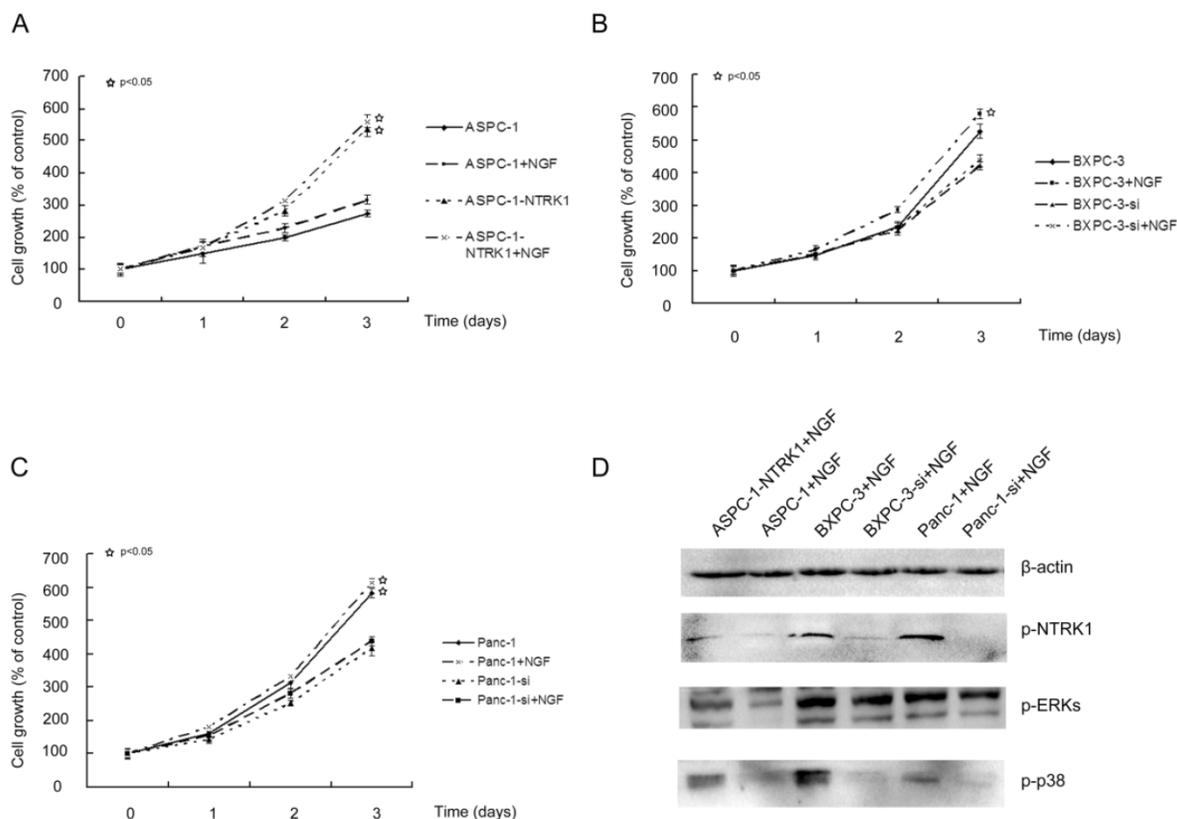


Figure 1. NTRK1 signaling enhances cell proliferation of pancreatic cancer cells. ASPC-1 (A), Panc-1 (B) and BXPC-3 (C) cells were treated as indicated. MTT was applied to determine cell viability spectrophotometrically at 24 h, 48 h, and 72 h following treatment. (D). Western blot analysis p-NTRK1, p-ERKs, and p-p38, with β -actin as loading control.

NGF-treated NTRK1-transfected cells displayed a dramatic increase in the phosphorylation of ERK and p38 (Fig. 1D). This indicates that NGF-NTRK1 pathway upregulates the p38-MAPK pathway and ERKs [29, 30]. To test whether these signaling pathways are involved in the enhanced proliferation response to NGF-NTRK1 pathway activation, we inhibited p38-MAPK with SB203580 and ERKs with PD98059. We found that, in the presence of NGF, PD98059 but not SB203580 significantly decreased the cell viability of cells transfected with NTRK1 after 72 h of incubation (Fig. 2). These data showed that the enhanced cell proliferation in response to NGF-NTRK1 activation is correlated with ERKs, and is independent of the p38-MAPK pathway.

2DG-induced apoptosis depends on p38-MAPK signaling but not ERKs

The growth of NTRK1-transfected ASPC-1 cells treated with 15 mM 2DG was 40% that of non-treated NTRK1-transfected ASPC-1 cells, in a time-dependent

association. The cell growth of non-transfected ASPC-1 cells was inhibited 17% at 72 h. BXPc-3 and Panc-1 cells in which NTRK1 expression was down-regulated via siRNA were less sensitive to 2DG than cells with normal NTRK1 expression (Figure 3C-F). As increased p38-MAPK and ERK activity was observed in NTRK1-transfected cells, we next checked whether upregulation of p38-MAPK or ERKs via the NGF-NTRK1 pathway were involved in 2DG-induced cell growth. For this, we treated cells with SB203580 or PD98059, and found that SB203580 but not PD98059 significantly attenuated the number of apoptotic cells caused by 2DG in all cell types that overexpressed NTRK1 (Fig. 3A-F). Consistent with this result, blocking p38-MAPK with SB203580 greatly reduced apoptosis induced by 2DG, at 24 h (Fig. 3G). These results indicate that apoptosis induced by 2DG in pancreatic cancer cells can be reduced by inhibition of the p38-MAPK pathway.

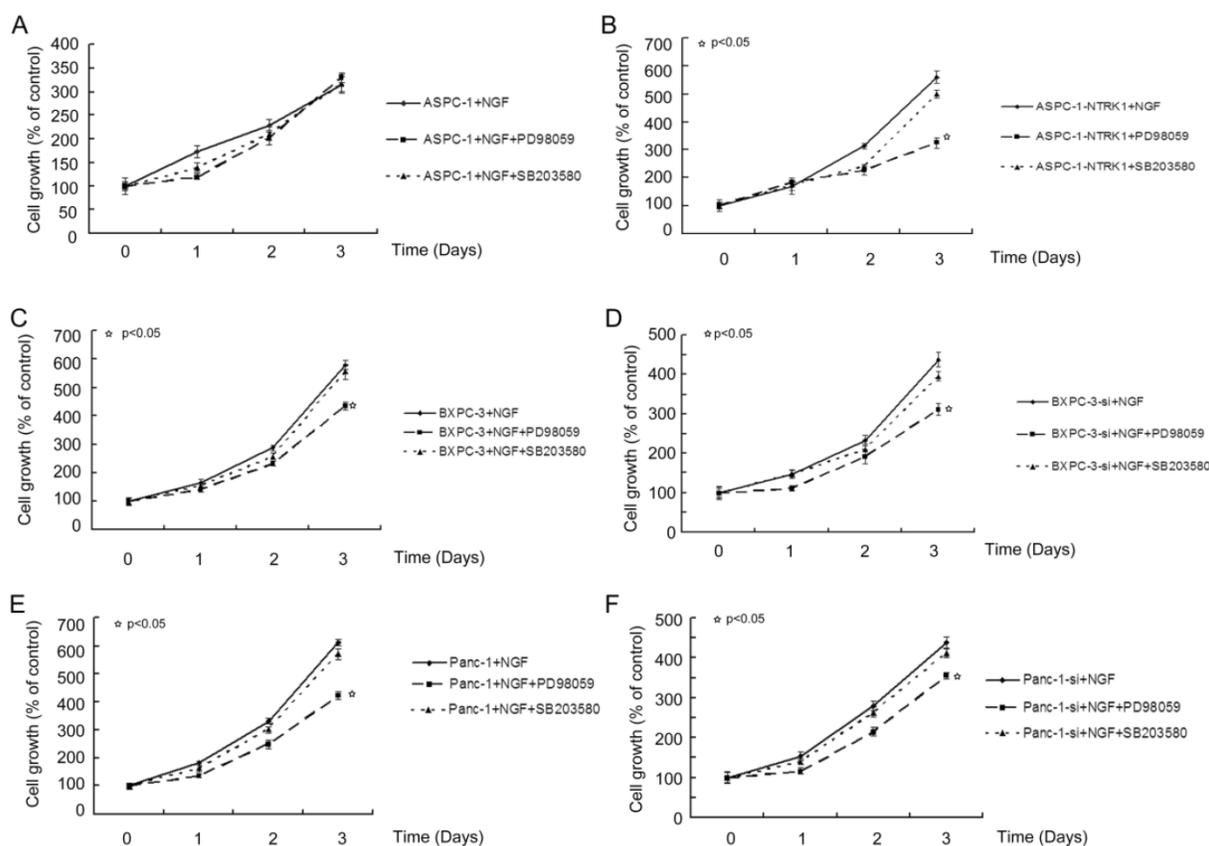


Figure 2. Blocking ERKs by PD98059 greatly suppressed the proliferation of cells with overexpression of NTRK1. Results are expressed as a percentage of control cells at 8 h after plating.

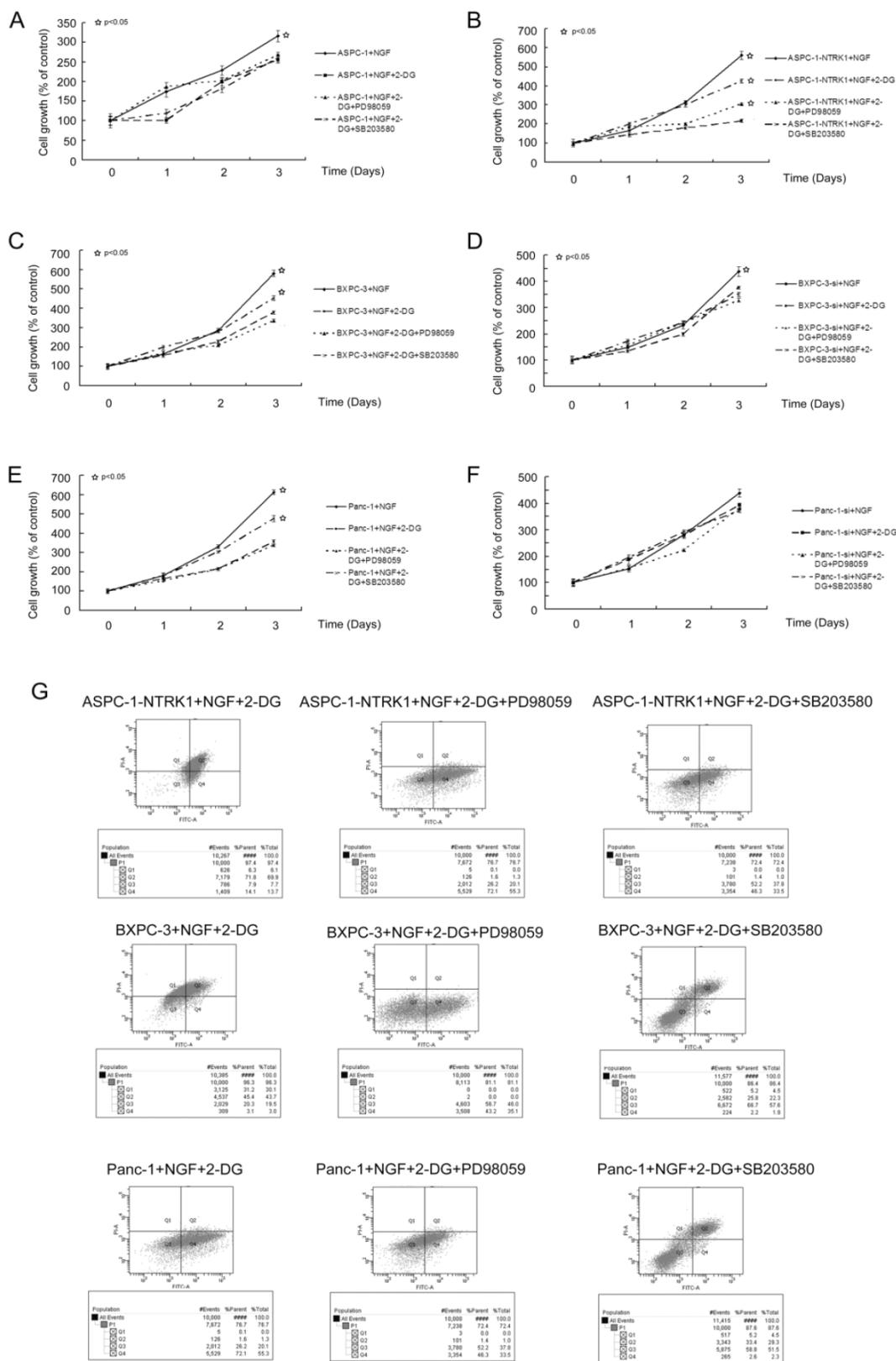


Figure 3. 2DG-induced apoptosis depends on p38-MAPK signaling but not ERKs. (A-F) 2DG significantly reduced the growth of six types of cells, while inhibition of ERKs did not show any obvious influence. However, by inhibiting p38-MAPK with SB203580, the growth inhibition was greatly alleviated. This phenomenon was not observed in untreated ASPC-I, BXPC-3-si and Panc-I-si cells, which had a low expression of NTRK1. (G) 2DG induced apoptosis in cells with higher expression of NTRK1 after 24 h of treatment. Treatment of PD98059 did not alleviate this phenomenon, while SB203580 did.

Discussion

NTRK1 is overexpressed in various types of tumors, suggesting that NTRK1 may be a valuable therapeutic target. Unfortunately, it was found that pan-Trk inhibitors could not discriminate among NTRK1, NTRK2, and NTRK3, and therefore have relatively poor specificity. They also inhibited other targets, such as fms-related tyrosine kinase 3 (FLT3) [31]. Thus, it remains imperative to develop specifically targeted cytotoxic drugs, especially when targeted toward the hallmarks of cancer cells. For example, it is widely accepted that malignant tumors are characterized by higher rates of glucose usage and glycolysis [32]. The higher cellular proliferation rate of these malignancies depends on elevated ATP consumption [33, 34]. Thus targeting the glycolytic metabolism of cancer cells is a promising strategy for cancer therapy.

The glucose analog 2DG acts as a competitive inhibitor of glucose metabolism, causing a depletion of cellular ATP and leading to cessation of the cell cycle and apoptosis [35]. In addition, 2DG can inhibit protein glycosylation and induce accumulation of misfolded proteins in the endoplasmic reticulum, leading to endoplasmic reticulum stress and constant apoptosis [36]. 2DG has been proposed to be an anti-cancer drug for a variety of cancer cells.

In the present study, we utilized the NGF-NTRK1 pathway to enhance proliferation of pancreatic cancer cells. We found that activation of NGF-NTRK1 signaling induced cell proliferation and progression, which is in agreement with previous studies [37-39]. Interestingly, the proliferation of cells with elevated NTRK1 expression was significantly inhibited by treatment with 2DG. Our data indicates that p38-MAKP is activated by NGF-NTRK1 signaling, but may not be required for NGF-NTRK1-enhanced cell proliferation. However p38-MAPK is required for 2DG-induced apoptosis. Similarly, enhanced proliferation due to loss of PTEN makes cells more sensitive to 2DG [33].

Our results showed that NGF-NTRK1 signaling induced pancreatic cancer cell proliferation and made cells more vulnerable to glycolysis inhibition by 2DG. As enhanced dependence on glucose glycolysis is a hallmark of cancer cells, our results suggest that targeting glucose glycolysis may be an avenue for the development of anti-cancer drugs.

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Competing Interests

The authors have declared that no competing interest exists.

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