

# Effect of NFATc2- and Sp1-mediated TNFalpha Regulation on the Proliferation and Migration Behavior of Pancreatic Cancer Cells

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**Abstract.** *Background/Aim:* One in two people will develop a tumor during their lifetime. Adenocarcinoma of the pancreas is one of the most aggressive types of cancer in humans with very poor long-term survival. A central role in the carcinogenesis of pancreatic cancer has been attributed to NFAT transcription factors. Previous studies have identified the transcription factor Sp1 as a binding partner of NFATc2 in pancreatic cancer. Using expression profile analysis, our group was able to identify the tumor necrosis factor TNFalpha as a target gene of the interaction between NFATc2 and Sp1. The present study investigated the effect of TNFalpha over-expression via the transcription factors NFATc2 and Sp1 on the pancreatic cancer cell lines PaTu 8988t and PANC-1. *Materials and Methods:* Transient transfection of NFATc2, Sp1, and TNFalpha siRNAs and their effects on the expression were investigated with immunoblot. Cell proliferation was measured with the ELISA BrdU assay. Cell migration was assayed with a Cell Migration Assay Kit using a Boyden chamber. *Results:* Inhibition of the transfection factors NFATc2, Sp1, or TNFalpha by siRNA significantly inhibited proliferation, which was exacerbated when using the combination of NFATc2 and Sp1. TNFalpha was able to counterbalance this

effect. In contrast to proliferation, migration of pancreatic cancer cells was increased by inhibiting these transfection factors. *Conclusion:* Tumor progression is strongly influenced by transcriptional changes in signaling cascades and oncogene mutations as well as by changes in tumor suppressor genes. Further studies are needed to understand the underlying mechanisms of these processes.

Approximately 50 percent of men and 44 percent of women will develop a tumor during their lifetime. Thus, cancer is the second leading cause of death worldwide after cardiovascular disease (1). Adenocarcinoma of the pancreas is one of the most aggressive types of cancer in humans (2). Due to the absence of characteristic early symptoms and appropriate screening tests, most pancreatic tumors are classified as non-curable at the time of diagnosis and have a poor prognosis (3). Although some progress has been made in the molecular and biological understanding of pancreatic cancer, survival rates have not significantly improved, and long-term survival is still almost impossible (4).

A central role in the carcinogenesis of pancreatic cancer has been attributed to nuclear factor of activated T-cells (NFAT) transcription factors (5). These factors have been observed both inside and outside the immune system, in which, as transcription factors, they are involved in the induction of gene transcription (6). In this process, NFAT transcription factors, together with other binding partners, regulate many genes responsible for tumor dissemination (7). Yet, little is known about these binding partners so far. Previous studies have identified the transcription factor Sp1 as a binding partner of NFATc2 in pancreatic cancer, in which it is important for the transcriptional and functional activity of NFATc2 (8). In preliminary work by our group, the effects of analgesics and local anesthetics on gene transcription mediated by NFATc2 and Sp1 in pancreatic cancer were examined. It could be shown that 48 h

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stimulation with ketamine and s-ketamine significantly inhibited proliferation and contemporaneously decreased endogenous expression of NFATc2 in the nucleus (9). Furthermore, the tumor necrosis factor alpha (TNFalpha) was identified as the target gene of the interaction between NFATc2 and Sp1 using expression profile analysis (10).

TNFalpha is a pleiotropic cytokine with a broad spectrum of predominantly proinflammatory activity (11). In patients with cancer, TNFalpha has shown to have antitumor effects from the onset of infectious disease to remission (12). Moreover, deregulated TNFalpha expression in the tumor microenvironment appears to promote invasion, migration, and ultimately metastasis of malignant cells (13). NFAT-dependent TNFalpha expression has already been described in the literature in lymphocytes (14) and by our group also in pancreatic cancer cells (10).

The aim of the present study was to analyze the effect of TNFalpha over-expression *via* the transcription factors NFATc2 and Sp1 on the pancreatic cancer cell lines PaTu 8988t and PANC-1.

## Materials and Methods

**Cell lines.** The human pancreatic cancer cell lines PaTu 8988t and PANC-1 were received from Professor Ellenrieder (Philipps University of Marburg, Germany). The cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Gallen, Switzerland) supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich) and 5% Myco Zap (Lonza Verviers SPRL, Verviers, Belgium). PaTu 8988t and PANC-1 were cultured in a humidified CO<sub>2</sub> atmosphere (5%) at 37°C and maintained in monolayer culture. Experiments were performed with cells at approximately 70-80% confluence.

**Transient transfection of siRNA.** For siRNA transfection, NFATc2, Sp1, and TNFalpha siRNAs were acquired from Santa Cruz (Dallas, TX, USA). Cells were transfected for 24 h with siRNA transfection reagent (Santa Cruz) according to the manufacturer's protocol.

**Subcellular fractionation and immunoblotting.** Cells were washed twice with cold Dulbecco's phosphate-buffered saline (DPBS) and collected by centrifugation for 10 min at 4,000 rpm at 4°C. Afterwards, the lysates were resuspended in RIPAE buffer (5 ml Triton X100, 0.5 g SDS, 190 mg EDTA, 2.5 g deoxycolic acid, 500 ml DPBS, proteinase inhibitors) for 15 min and then centrifuged at 13,000 rpm for 30 min. The supernatants were transferred to new cups and incubated on ice. For western blotting, 30 µg protein extracts were analyzed using SDS-PAGE and blotted onto nitrocellulose. After protein separation and gel transfer, the membranes were probed with antibodies against, NFATc2 (Cell signaling, Leiden, Netherlands), Sp1 (Cell signaling), TNFalpha (Santa Cruz), and β-actin (Sigma-Aldrich). Afterwards, the membranes were washed in TBS wash buffer and incubated with peroxidase-conjugated secondary antibodies. By using an enhanced chemiluminescence detection system (Western Blotting Detection Reagent, GE Healthcare, Chicago, IL, USA) the immunoreactive proteins were visualized.

**Cell proliferation.** Cell proliferation was quantified by measuring BrdU incorporation during DNA synthesis. The test was conducted according to the manufacturer's records (cell proliferation ELISA-BrdU, Roche applied science, Basel, Switzerland). In short, cells were seeded onto 48 well plates and cultured in medium containing 10% FCS. 24 h after the siRNA transfection, the cells were additionally treated with BrdU labeling solution for almost 24 h. The culture medium was removed, and the cells were fixed, and finally the DNA was denatured. Afterwards, the cells were incubated with Anti-BrdU-POD solution for 90 min and washed three times to remove unbound or non-specifically bound antibody conjugates. Subsequently, the immune complexes were detected with TMB substrate for 15 min and quantified by measuring the absorbance at 405 nm and 490 nm. Eight wells per treatment group were used and the tests were repeated at least three times.

**Cell migration assay.** Cell migration was evaluated using a cell migration assay kit (Abcam, Cambridge, UK). The assay uses a Boyden chamber, in which cells migrate through a semi-permeable membrane under different stimuli. A total of 200,000 cells of the human pancreatic cancer cell lines PaTu 8988t or PANC-1 were transfected with siRNA and placed in the upper chamber. A stimulant was placed into the lower chamber, incubated at 37°C for 24 h. The migrating cells were passed through the semi-permeable membrane and migrated into the lower chamber or adhered to the bottom of the upper chamber. After 48 h, cell migration was analyzed by reading fluorescence (Ex/Em=490/405 nm) in a plate reader. All experiments were repeated twice and were done with four wells per treatment group.

**Statistical analysis.** The data are presented as mean±SD. The non-parametric Mann-Whitney *U*-test was used for statistical evaluation of the data. *p*-Values of <0.05 were considered significant. For statistical analysis the IBM SPSS Statistics (Vs. 27; IBM, Armonk, NY, USA) and Excel Vs. 2019 (Microsoft, Redmond, WA, USA) packages were used.

## Results

**Knockdown of the interaction partners NFATc2 and Sp1 or TNFalpha with RNAi technology significantly reduced cell proliferation.** The impact of the interaction between NFATc2, Sp1, and TNFalpha on cell proliferation was investigated by means of an ELISA BrdU assay (Figure 1). For this purpose, pancreatic cancer cells were transiently transfected with a silencer-RNA sequence for NFATc2, Sp1, TNFalpha, both (siNFATc2 + siSp1, siNFATc2 + siTNFalpha, siSp1 + siTNFalpha) or all transcription factors (siNFATc2 + siSp1 + siTNFalpha).

Transfection of siNFATc2 reduced proliferation to 85% (PaTu 8988t) compared to the control group treated with medium. Transfection of siSp1 decreased proliferation to 63% (PaTu 8988t) and 56% (PANC-1), and transfection of siTNFalpha decreased proliferation to 46% (PaTu 8988t) and 55% (PANC-1) (Figure 1A). Inhibition of both transcription factors NFATc2 and Sp1 by siRNA knockdown resulted in a further significant reduction of cell proliferation to 36% (PaTu 8988t) and 51% (PANC-1) (Figure 1B).

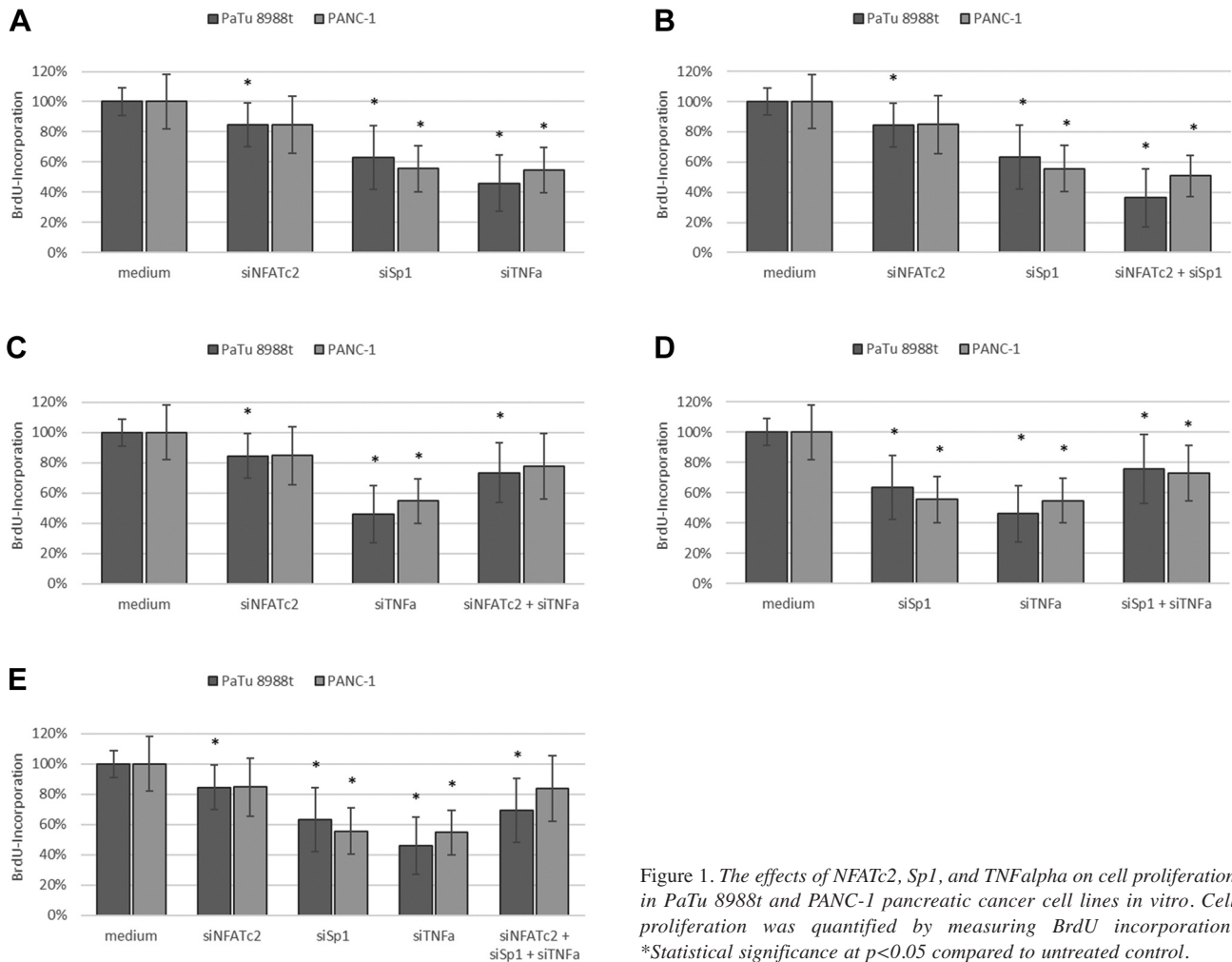


Figure 1. The effects of NFATc2, Sp1, and TNFalpha on cell proliferation in PaTu 8988t and PANC-1 pancreatic cancer cell lines in vitro. Cell proliferation was quantified by measuring BrdU incorporation. \*Statistical significance at  $p < 0.05$  compared to untreated control.

Compared to singular inhibition, proliferation increased again when cells were transiently inhibited in combination with siNFATc2 and siTNFalpha (Figure 1C), siSp1 and siTNFalpha (Figure 1D), or with all three transcription factors simultaneously (Figure 1E). However, overall proliferation was still significantly reduced in PaTu 8988t compared to the untreated control group.

Figure 2 shows the knockdown efficiency of the siRNA used. The pancreatic cancer cells PaTu 8988t were transiently transfected with a silencer-RNA sequence for NFATc2, Sp1, or TNFalpha. The expression of the transfection factors NFATc2 (column 1), Sp1 (column 3), and TNFalpha (column 5) was decreased compared to the untreated control group. The endogenous expression of  $\beta$ -actin serving as a loading control is shown in the lower blot (column 2 + 4 + 6).

*Analysis of migration of pancreatic cancer cells after transient inhibition of NFATc2, Sp1, or TNFalpha.* PaTu 8988t and PANC-1 pancreatic cancer cells were transfected with silencer-

RNA sequence for NFATc2, Sp1, TNFalpha, both (siNFATc2 + siSp1, siNFATc2 + siTNFalpha, siSp1 + siTNFalpha), or all three (siNFATc2 + siSp1 + siTNFalpha) (Figure 3).

In the PANC-1 cell line, incubation with silencer-RNA for NFATc2, Sp1, and TNFalpha resulted in a statistically significant increase in migration compared to the control group treated with medium (Figure 3A). The combination of siNFATc2 and siSp1 (Figure 3B) as well as that of siNFATc2 and siTNFalpha (Figure 3C) also significantly increased the migration of PANC-1 pancreatic cancer cells after 24 h of transfection.

In contrast, the pancreatic cancer cell line PaTu 8988t showed a decrease in migration after transfection with siNFATc2 and siSp1 (Figure 3B) and an increase after transfection with siSp1 and siTNFalpha (Figure 3D).

## Discussion

In recent years, some progress has been made in the molecular and biological understanding of pancreatic cancer.

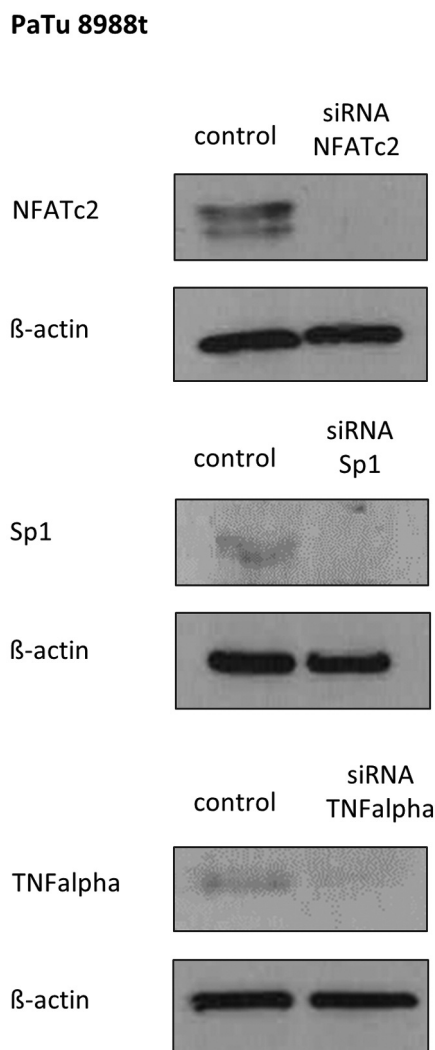


Figure 2. Western Blot analysis of the expression of NFATc2, Sp1 and TNFalpha after treatment with siRNA.

However, the exact mechanism by which this type of cancer develops remains unclear (15). In terms of cell morphology, the carcinogenesis of pancreatic cancer is characterized by the following stages: PanIN stage 1A refers to the development of a flat, cylindrical epithelial hyperplasia from healthy pancreatic epithelial tissue, which changes to papillary hyperplasia with unfolding lobules in PanIN stage 1B. PanIN stage 2 includes structural histologic abnormalities such as enlargement of the nucleus and loss of cell polarity, and PanIN stage 3 represents the progression to carcinoma in situ with highly altered epithelia and severe cellular atypia. From PanIN stage 3 onwards, progression to invasive pancreatic cancer is possible (16).

In their article "The hallmarks of cancer" published in 2000, Hanahan and Weinberg defined six characteristic properties of

tumor cell physiology. For example, transformed cells show the properties of independence from growth signals, insensitivity to growth-inhibitory signals, avoidance of programmed cell death (apoptosis), unlimited division potential, the ability to undergo neoangiogenesis, and the capacity for tissue invasion and metastasis (17). In their subsequent publication, the authors extended these properties by including the adaptation of tumor cell energy metabolism and the avoidance of destruction by the immune system (18).

Following the publication by Hanahan and Weinberg, Cavallo *et al.* finally defined the "immune hallmarks of cancer". According to Cavallo *et al.*, tumor cells are able to grow in a chronically inflammatory environment by evading recognition by the immune system through various mechanisms, thereby suppressing antitumor immune reactivity (19).

The main messengers between the individual components of the immune system are the cytokines, which consist of a heterogeneous group of peptides and proteins that serve to transmit signals from cells and to control their proliferation and differentiation. Cytokines are produced and released, among others, by macrophages, lymphocytes, natural killer cells, and fibroblasts (20). TNFalpha plays a central role among the cytokines (21). It is produced in various cells and proteolytically split by the "tumor necrosis factor-alpha converting enzyme". From there, it finally enters tissues and blood as a soluble protein and binds to specific receptors (TNFR1 or TNFR2) (22). This interaction subsequently triggers several intracellular transcriptional cascades and signaling pathways (23). Using expression profile analysis, our group identified TNFalpha as a target gene of the interaction between NFATc2 and Sp1 (10). In the present study, we therefore inhibited NFATc2, Sp1, and TNFalpha with siRNA in the pancreatic cancer cells PaTu 8988t and PANC-1 and analyzed the effect on cell proliferation and migration. This assay showed that transient inhibition of the transcription factors NFATc2, Sp1, and TNFalpha significantly inhibited cell proliferation in the pancreatic cancer cells PaTu 8988t and PANC-1.

The effect of cytokines in the carcinogenesis of tumors has been discussed controversially in the literature. TNFalpha can act as a tumor suppressor through vascular destruction, tumor necrosis, and immune stimulation but also as a tumor promoter by inducing cellular transformation, survival, proliferation, and angiogenesis (24, 25). Mocellin *et al.* also described that deregulated TNFalpha expression in the tumor microenvironment promotes invasion, migration, and ultimately metastasis in malignant cells (13). Thus, treatment of ductal adenocarcinoma with TNFalpha has significantly increased tumor growth and metastasis in animal studies (24). Similarly, treatment with infliximab and etanercept has reduced tumor growth in metastases, for instance, in the liver (25). Transient inhibition of the transcription factors NFATc2, Sp1, and TNFalpha significantly inhibited cell proliferation in the pancreatic cancer cells PaTu 8988t and PANC-1. Inhibition of

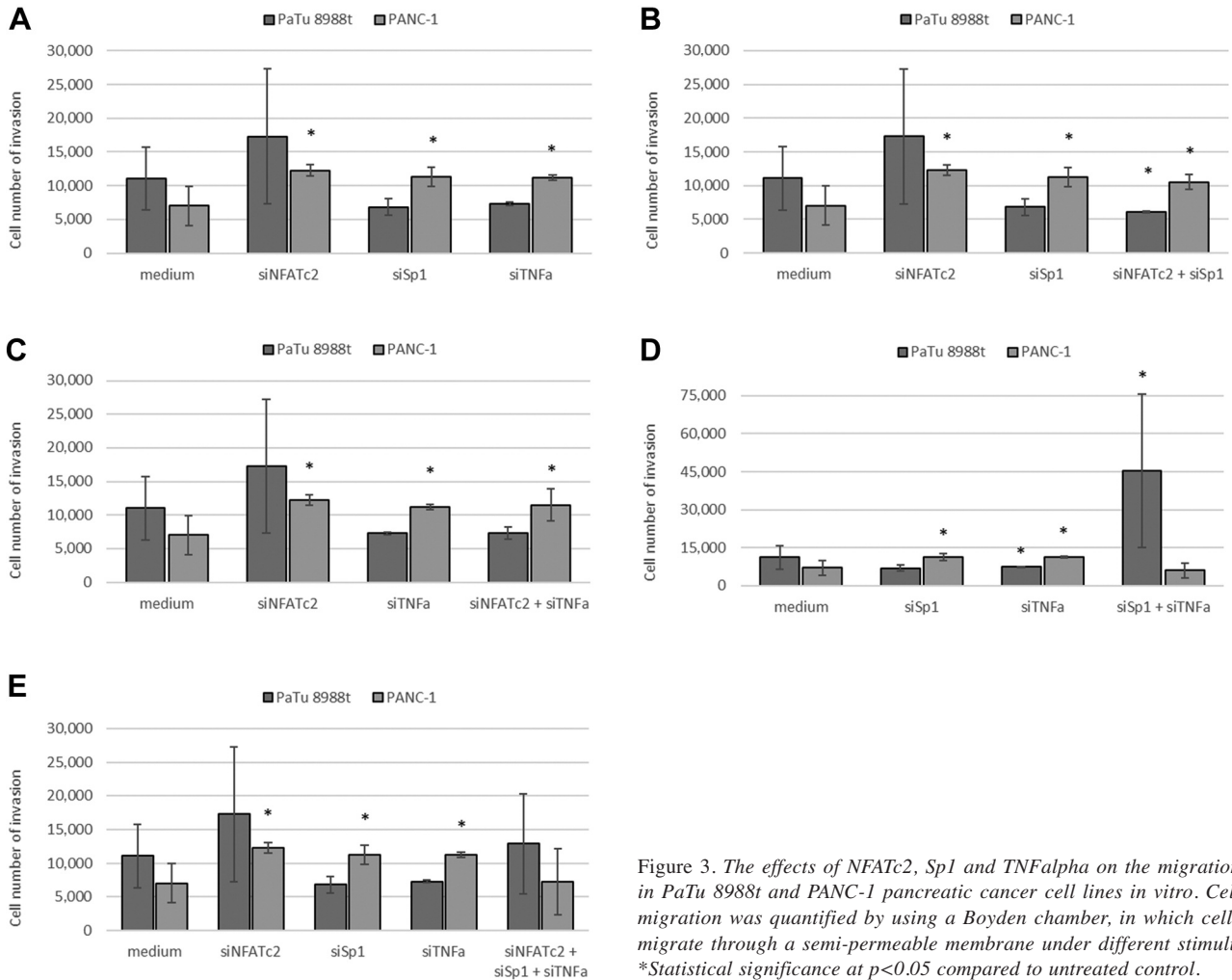


Figure 3. The effects of NFATc2, Sp1 and TNFalpha on the migration in PaTu 8988t and PANC-1 pancreatic cancer cell lines in vitro. Cell migration was quantified by using a Boyden chamber, in which cells migrate through a semi-permeable membrane under different stimuli. \*Statistical significance at  $p < 0.05$  compared to untreated control.

both NFATc2 and Sp1 enhanced this effect, and cell proliferation continued to decrease. Interestingly, this effect was only seen when NFATc2 and Sp1 were combined. When cells were transiently transfected with the combination of siNFATc2 and siTNFalpha, siSp1 and siTNFalpha, or with all three transcription factors simultaneously, proliferation increased again compared to singular inhibition. However, overall proliferation was still significantly reduced in PaTu 8988t compared to untreated control. Therefore, one possible interpretation of the results may be that TNFalpha acts as a tumor suppressor in combination with other transcription factors and reduces cell proliferation, but alone it acts as a tumor promoter.

In contrast, the migration behavior of the pancreatic cancer cell PANC-1 shows the opposite effect. Transient inhibition of the transfection factors NFATc2, Sp1, or TNFalpha by siRNA increased migration, suggesting that the transcription factors may act as tumor suppressors in this case. No exacerbation of the effect was observed when the transcription factors were combined.

## Conclusion

Although some progress has been made in the molecular and biological understanding of pancreatic cancer in recent years, many questions remain unanswered, not only about the mechanisms of development of this type of cancer with its oncogene mutations and tumor suppressor gene alterations but also about the impact of transcriptional changes in signaling cascades on early diagnosis, risk assessment, and especially cancer therapy. Further studies are needed to understand the underlying mechanisms of these processes. The identification and characterization of the transcription process will help establish new therapeutic options in the sense of a "targeted therapy" and ultimately improve the long-term survival of patients with pancreatic cancer.

## Conflicts of Interest

The Authors declare that they have no competing interests.

## Authors' Contributions

All Authors have made substantial contributions to the conception, design, analysis and the interpretation of the data in this research article. They have been involved in the critical revision of the manuscript with regard to important intellectual content. All Authors have given their final approval for the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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