ANTICANCER RESEARCH *39*: 5933-5942 (2019)
doi:10.21873/anticanres.13798

Modulation of BDNF–TRKB Interactions on Schwann Cell-induced Oral Squamous Cell Carcinoma Dispersion *In Vitro*

LILIANA EIN[1*], CHRISTINE MEI[1*], OLENA BRACHO[1], ESPERANZA BAS[1], PAULA MONJE[2], DONALD WEED[1], ZOUKAA SARGI[1], GIOVANA THOMAS[1] and CHRISTINE DINH[1]

[1]*Department of Otolaryngology, University of Miami Miller School of Medicine, Miami, FL, U.S.A.;*
[2]*Department of Neurological Surgery, University of Indianapolis School of Medicine, Indianapolis, IA, U.S.A.*

Abstract. *Background/Aim: Perineural invasion (PNI) is a significant pathological feature in head and neck cancer. The molecular mechanisms of PNI are poorly understood. Contrary to the previous belief that cancer cells invade nerves, recent studies have shown that Schwann cells (SC) can dedifferentiate, intercalate between cancer cells, and promote cancer dispersion. Communication between cells through brain-derived neurotrophic factor (BDNF) activation of its receptor tropomyosin receptor kinase B (TRKB) may contribute to these cellular events. We aimed to determine the effect of TRKB inhibitor ANA-12 on the direction of cell migration and degree of SC-induced oral cancer cell dispersion. Materials and Methods: Cell migration and dispersion assays were performed in vitro using murine SC and oral carcinoma cell lines. Assays were performed with and without ANA-12. Results: Although SCs preferentially migrated towards cancer cells in control medium, there was minimal SC-associated cancer cell dispersion. In contrast, treatment with ANA-12 reduced migration of SCs and cancer cells towards each other and initiated more SC-associated cancer cell dispersion. Conclusion: This pilot study shows that BDNF–TRKB signaling may have a role in regulating interactions between SC and oral cancer cells that affect cell migration, intercalation, and cancer cell dispersion. Further research into these interactions may provide important clues about the molecular and cellular mechanisms of PNI.*

*These Authors contributed equally to this work and should be considered co-first authors.

Correspondence to: Christine Dinh, MD, Assistant Professor of Otolaryngology, University of Miami Miller School of Medicine, 1120 NW 14th Street, Suite 579, Miami, FL 33136, U.S.A. Tel: +1 3052431484, Fax: +1 3052432009, e-mail: ctdinh@med.miami.edu

Key Words: Perineural invasion, Schwann cells, cancer cell dispersion, migration, head and neck cancer.

Head and neck cancer (HNC) is the sixth most common type of cancer in the world, with approximately 630,000 new cases diagnosed each year (1). Poor prognostic factors associated with HNC include increased age and advanced tumor staging (2, 3). Studies have shown that perineural invasion (PNI) is also a poor prognostic factor in HNC, particularly in oral cavity cancer (4-10).

PNI is the tracking of cancer cells along a neuron or within the neuronal sheath (7). Reports describing neoplastic invasion of peripheral nerves date as early as 1842 (11-13). In squamous cell HNC, PNI is associated with increased risk of local recurrence and reduced survival (14, 15), and therefore, presence of PNI in HNC is an indication for adjuvant radiotherapy (15). The exact mechanisms of PNI in HNC are not well understood and therefore effective therapies targeting neuronal involvement in cancer do not exist.

Although PNI was traditionally thought to be a cancer-driven process, the nerve microenvironment is now thought to be an important regulator. Schwann cells are glial cells that wrap around axons of peripheral nerves and aid in neuronal survival and repair after injury (16, 17). Schwann cells are able to extend cellular processes and dedifferentiate into migratory subtypes that act to repair neurons and guide neuronal growth (17-20). Using similar actions seen in nerve repair, Schwann cells can potentially promote or regulate PNI in cancer (21-27). Deborde and colleagues demonstrated how Schwann cells can dedifferentiate, become motile, and travel toward pancreatic cancer cells (22). They also showed how Schwann cells can intercalate and disperse cancer cells and aid in cancer cell migration towards nerves.

The mechanisms behind PNI may involve reciprocal signaling interactions between tumor cells and nerve components, in which nerves release trophic factors that stimulate cancer cells, and cancer cells release neurotrophic factors that stimulate nerve infiltration (9). In support of this theory, studies involving prostate and gastric cancer have shown that denervation can suppress tumorigenesis, tumor progression, and metastasis (28, 29). As it pertains to

squamous cell HNC, several neurotrophic factors have been proposed to contribute to neurite outgrowth and PNI (9), including nerve growth factor, brain-derived growth factor (BDNF), and neurotrophin-3 (Figure 1A), which interact with their high affinity receptors, tropomyosin receptor kinase A, B, and C (TRKA, TRKB, and TRKC), respectively. In particular, BDNF and its high affinity receptor TRKB are highly expressed in squamous cell HNC and have been shown to be involved in cell proliferation in other cancer types (9, 30-34).

In this pilot study, we created an *in vitro* migration and dispersion assay by co-culturing murine Schwann cells and oral cavity squamous cell carcinoma. We used the compound ANA-12 (Selleckchem, Houston, TX, USA), a non-competitive inhibitor of TRKB (35), to study the effect of BDNF–TRKB signaling on cell-to-cell interactions between cell types.

Materials and Methods

Cell lines and culture. The B4B8 oral squamous cell carcinoma cell line was characterized by Thomas *et al*. (36) and obtained for our study as a generous gift. In brief, mucosal keratinocytes of BALB/c mice were extracted and transformed *in vitro* with 4-nitroquinolone-1-oxide before inoculation into severe combined immunodeficient (SCID) mice. B4B8 cells were re-isolated from tumors in the SCID mice and confirmed to express markers of epithelial origin. B4B8 cells were cultured in keratinocyte-SFM medium with epidermal growth factor-1, bovine pituitary extract, and glutamine factors (Thermo Fisher, Waltham, MA, USA). Adult rat Schwann cells were isolated from normal peripheral nerve and cultured according to methods reported by Andersen and Monje (37) and were obtained from the Miami Project to Cure Paralysis (Monje Laboratory, Miami, FL, USA) as a generous gift. In short, the methods of Schwann cell isolation involved removal of epineurium, extensive teasing of nerve fibers, removal of myelin debris, dissociation of cells, expansion of initial cell population in medium with chemical mitogens, and preparation of Schwann cells as cryogenic stocks for experiments. The Schwann cells were transfected with lentiviral fluorescent protein vectors and expanded at 37˚C and 5% CO_2 on 0.01% poly-L-ornithine (Sigma–Aldrich, St. Louis, MO, USA) and laminin (5 μg/ml; Thermo Fisher) coated plates. Mitogenic media consisted of Dulbecco's modified Eagle's medium (DMEM), 10% heat-inactivated fetal bovine serum (FBS; Seradigm, VWR, Radnor, PA, USA), and heregulin (10 nM; Sigma–Aldrich).

Cell viability assay. Cancer and Schwann cell viability were tested with different levels of ANA-12 (Selleckchem), a selective, non-competitive antagonist of TRKB. Cells were cultured on a 96-well plate that was precoated with laminin (5 mg/ml) and 0.01% poly-L-lysine. Approximately 20,000 cells were seeded in each well and incubated for 24 h at 37˚C and 5% CO_2. After 24 h, cells were cultured with DMEM with 10% FBS or different concentrations of ANA-12 up to 5 μM for 4 days. Subsequently, crystal violet colorimetric assay was performed and absorbances were measured for each well to determine cancer and Schwann cell viability in the presence of ANA-12. There was no significant difference in viability between control condition and within cancer and Schwann groups treated with different concentrations of ANA-12 (p=0.06 for cancer cells; p=0.58 for Schwann cells, data not shown). Therefore, it was determined that ANA-12 was not cytotoxic to the cancer or Schwann cells at the concentrations tested and ANA-12 at 5 μM was used for further experimentation.

Western blot. Western blots were performed to confirm expression of TRKB and BDNF in both cancer and Schwann cell lines. Protein was isolated using RIPA buffer (Thermo Fisher). Protein concentration was measured using the BCA Protein Assay Kit (Thermo Fisher), per manufacturer's instruction. For western blotting, 50 μg of proteins were loaded on a precast gel (Bio-Rad, Hercules, CA, USA), separated by gel electrophoresis, and transferred to a nitrocellulose membrane. Membranes were blocked in 2.5% bovine serum albumin and incubated overnight in primary antibody solution at 4˚C and Alexa Fluor 488 and 647-conjugated secondary antibodies (1:1000; Life Technologies, Carlsbad, CA, USA) for 2 h at room temperature. The following primary antibodies from Abcam (Cambridge, MA, USA) were used: BDNF (1:500, Ab108319), TRKB (1:250, Ab18987), beta-actin (1:1000, Ab8227), and beta-tubulin (1:1000, Ab78078). Fluorescent images were obtained using the ImageQuant LAS 4000 Gel Biomolecular Imager (GE Healthcare Life Sciences, Marlborough, MA, USA). Images of the western blot confirmed expression of TRKB and BDNF in both Schwann and cancer cells (Figure 2).

Migration and growth assay. Spheroids consisting of cancer cells and Schwann cells were cultivated in three-dimensional (3-D) hanging drop plates (Perfecta3D®, Sigma–Aldrich) (38). Spheroids were then plated in two-dimensional (2-D) culture on 6-well plates precoated with laminin (5 mg/ml) and 0.01% poly-L-lysine (Figure 3A). Four internal controls included cancer cells alone, Schwann cells alone, cancer cells exposed to ANA-12 (5 μM), and Schwann cells exposed to ANA-12 (5 μM). Once in 2-D culture, differential interference contrast (DIC) light and fluorescent microscopy images were taken daily for 4 days. Each image was divided into 12 equivalent pie-shaped sections with the center originating at the spheroid (Figure 3B). The cells were counted in each section daily for 4 days. The percentages relative to the total number of cells were calculated and graphed on radar cell plots.

Cell dispersion assay. For the co-culture cell dispersion assay, cancer and Schwann cell spheroids were plated simultaneously on 6-well plates and cultured in control media or with ANA-12 (5 μM). Once in 2-D culture, differential interference contrast light and fluorescent images were taken daily for 4 days. Once the cancer and Schwann cells came into contact, cancer cells became dispersed by Schwann cells in various conditions. Dispersed cells were defined as any cancer cell separated from the cancer cluster and completely surrounded by Schwann cells (Figure 4). The number of dispersed cancer cells were counted during the first 4 days.

Statistical analysis. Statistical analysis was performed with analysis of variance test with Tukey *post-hoc* testing or Kruskal–Wallis test with multiple pairwise comparison tests. Significance was set at $p<0.05$.

Results

Spheroid migration and growth assay. For the internal control groups in control medium, cancer cells and Schwann cells grew and migrated outward from the center of the

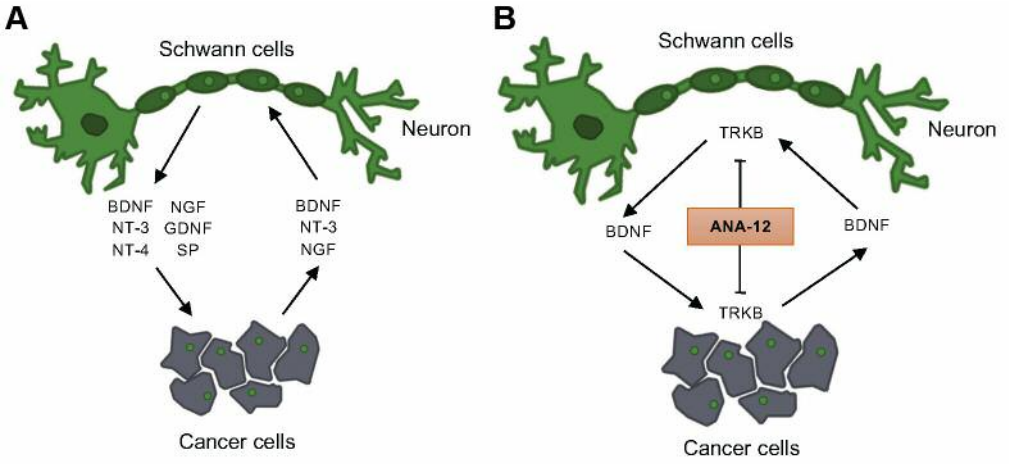

Figure 1. *Neurotrophins in perineural invasion in head and neck cancer. A: Neurotrophins secreted from both Schwann cells and head and neck squamous cell carcinoma in a communicative loop. B: An illustrative diagram demonstrating that Schwann cells and cancer cells express brain-derived neurotrophic factor (BDNF) and its high-affinity receptor, tropomyosin receptor kinase B (TRKB). ANA-12 is a novel TRKB non-competitive inhibitor that can modulate BDNF–TRKB signaling. NGF: Nerve growth factor; NT-3: neurotrophin-3; GDNF: glial cell-derived neurotrophic factor; NT-4: neurotrophin-4; SP: substance P.*

clusters over 4 days. Representative images are shown in Figure 4. The pattern of growth of both cancer cells and Schwann cells appeared to be random in all directions. When graphed on radar plots, the cancer cells in control medium showed a degree of nonrandom migration on days 1 and 2, however, the percentage of cells became consistent in each section on days 3 and 4. The Schwann cell plots remained circular, which demonstrated uniform migration in all directions in days 1-4 (Figure 5A, lower panel). We also noted that Schwann cells demonstrated a characteristic whorling pattern in culture (Figure 5A, upper panel).

Similarly to cancer cells and Schwann cells in control medium, both cancer cells and Schwann cells in the presence of ANA-12 demonstrated a random pattern of migration and growth (Figure 4B, lower panel). In addition, there was no effect of ANA-12 on the characteristic whorling pattern of Schwann cells. When exposed to ANA-12, the nonrandom migration by cancer cells seen on day 1 and 2 was less pronounced when compared to the control group (Figure 5B, upper panel). There was no difference in the random Schwann cell migration and growth in the ANA-12-treated group compared to control.

Co-culture migration and cell dispersion assay. Subsequently, Schwann cells and cancer cells were co-cultured in control medium. Time-lapse images initially showed intercalation of Schwann cells into cancer cell clusters; however, by day 4, the cancer appeared to be growing as a large mass, pushing Schwann cells to the periphery (Figure 6A). Co-cultures in control medium showed cancer and Schwann cells maintaining a well-defined border (Figure 6B and C) with minimal cancer cell dispersion.

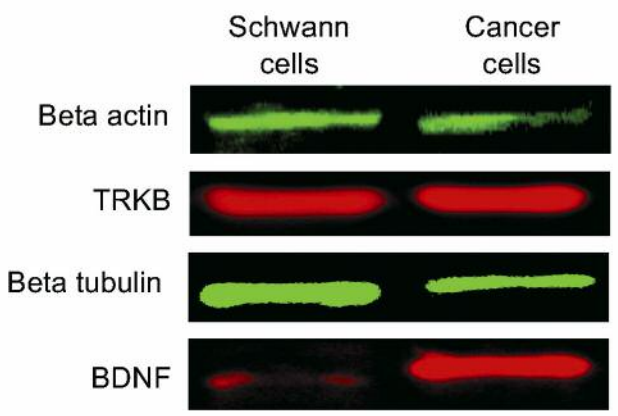

Figure 2. *Western blot demonstrating that Schwann and B4B8 cancer cells express tropomyosin receptor kinase B (TRKB) and brain-derived neurotrophic factor (BDNF). Expression levels of housekeeping proteins (beta actin and beta tubulin) are also shown.*

In contrast, when Schwann and cancer cells were co-cultured in ANA-12, a different pattern of growth and migration was appreciated (Figure 7A). The well-defined border between cancer cells and Schwann cells was no longer apparent. Schwann cells appeared more active, intercalating between cancer cell clusters, directing cancer cell migration between their whorls, leading to cancer cell dispersion (Figure 7B-D). In these co-cultures exposed to

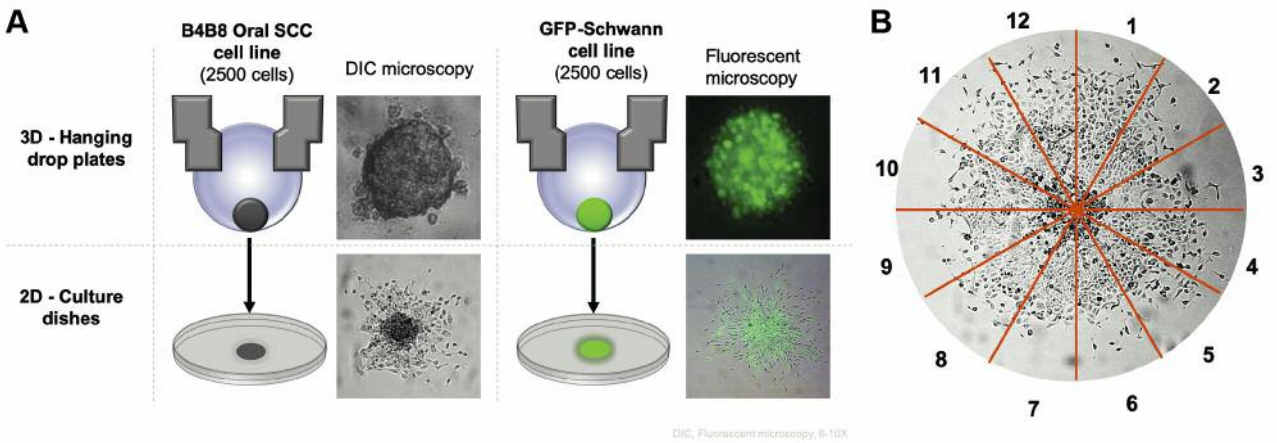

Figure 3. *Spheroid assays. An illustration and representative microscopy images from a three-dimensional (3-D) hanging drop plate to two-dimensional (2-D) culture dish method (A), and the subsequent division of images of cell clusters into 12 sections from the center of the cluster to determine cell migration and growth (B). SCC: Squamous cell carcinoma: GFP: green fluorescent protein: DIC: differential interference contrast.*

ANA-12, there was a significant increase in what appeared to be Schwann cell-associated cancer cell dispersion in the ANA-12-treated group at all time points when compared to the control (Figure 8A).

With the cancer and Schwann cell co-cultures, we also measured the direction of migration and growth using radar plots of the co-cultures on the first 2 days. In control medium, cancer cells demonstrated migration and growth towards Schwann cells, and Schwann cells demonstrated migration and growth toward cancer cells. These results demonstrate there is preferential migration and growth of cancer cells and Schwann cells towards each other. However, this preferential migration was not seen in the presence of ANA-12 (Figure 8B).

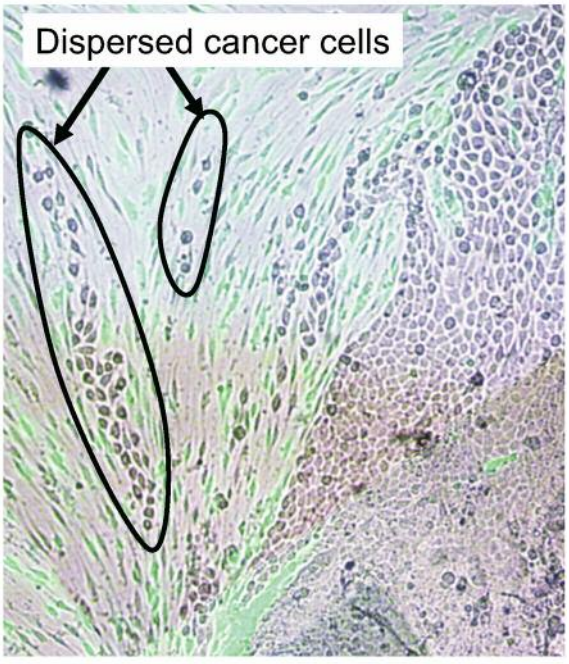

Figure 4. *Cancer cell dispersion as shown by fluorescent and differential interference contrast light microscopy (×10). Circled regions demonstrate dispersed cancer cells, which we defined as cancer cells completely surrounded by Schwann cells and separated from other cancer cell clusters.*

Discussion

Schwann cells have recently come into focus as having an important role in regulating the tumor microenvironment and PNI. In a 3-D *in vitro* experiment, Demir *et al*. demonstrated that Schwann cells exhibited early and targeted migration towards human pancreatic cancer cells prior to cancer cells migrating toward peripheral neurons (39). Deborde *et al*. demonstrated that dedifferentiated Schwann cells directed pancreatic cancer cell migration towards nerves in a neural cell adhesion molecule 1 (NCAM-1)-dependent mechanism (22, 40). These studies provide some clues that cancer cells have the potential to initiate Schwann cell migration and activity that can promote PNI in HNC.

BDNF and TRKB are highly expressed in HNC and their expression have been associated with tumor growth, invasion, and metastasis (32). In squamous cell HNC, BDNF–TRKB signaling was linked to aggressive tumor behavior, poor prognosis, and resistance to chemotherapy (30, 32, 41-43). Furthermore, overexpression of BDNF and TRKB in salivary adenoid cystic carcinoma was correlated with invasion, metastasis, and poor prognosis (44). It has been suggested

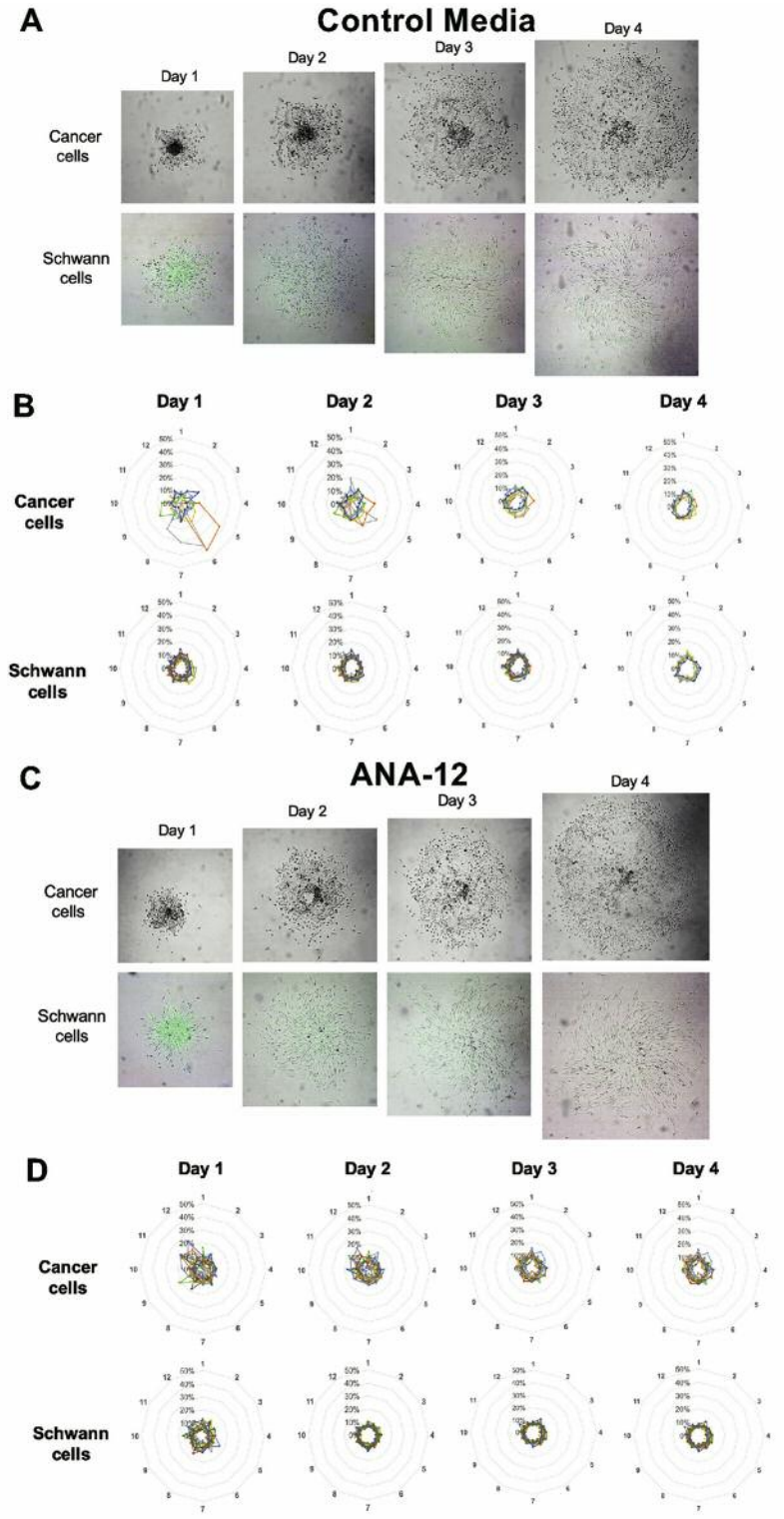

Figure 5. *Migration and growth assay. Migration and growth of cells were measured in control medium (A) and in culture with tropomyosin receptor kinase B (TRKB) inhibitor ANA-12 (B). Upper panel: Representative images of growing Schwann cells and cancer cells (fluorescent and differential interference contrast light microscopy, ×6). Lower panel: Radar plots of the number of cells in each of the 12 sections as a percentage of the total number of cells. Nonrandom migration was seen on days 1 and 2 of cancer cell migration and growth assays, but this evened out by day 4. Schwann cell migration and growth were random in all directions in control medium. Cancer cell and Schwann cell migration and growth were random in all directions in the presence of ANA-12.*

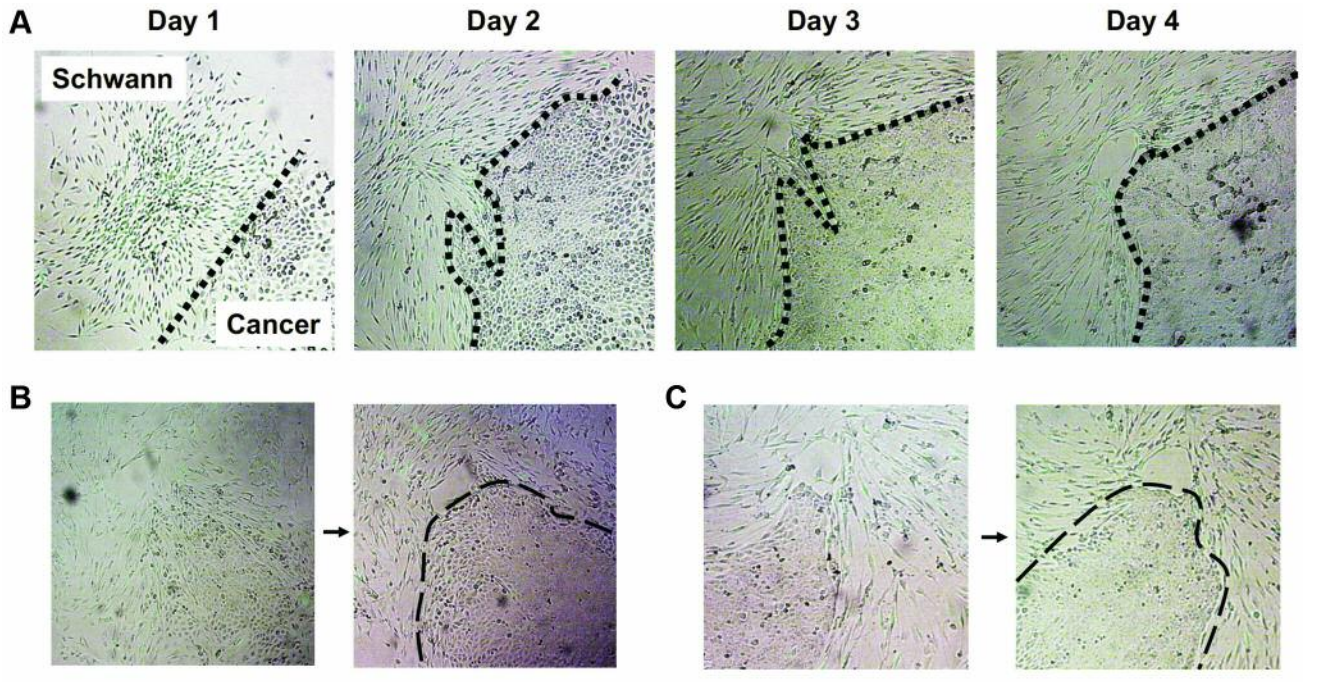

Figure 6. *Co-culture of Schwann cells and cancer cells in control medium. Schwann cells are visualized in green while cancer cells are seen in gray. A: Representative fluorescent and differential interference contrast light images of co-cultures in control medium on day 1 (×6) and days 2-4 (×10). The dotted lines demonstrate the border developing between cancer cells and Schwann cells over 4 days, as the cancer cluster grows, migrates, and invades into the Schwann cell clusters. This pattern of cancer cell invasion into Schwann cell clusters may represent a mechanism of direct perineural invasion. B, C: two separate examples showing the well-defined border that is formed between cancer and Schwann cells from days 3 to 4 (arrow; ×10).*

that BDNF and TRKB signaling may also be involved in the pathogenesis of PNI in HNC (9).

In our study, we chose to investigate how BDNF–TRKB signaling affects cell migration and cancer dispersion in co-cultures of murine Schwann cells and oral squamous cell carcinoma cells. To do this, we first performed a western blot to confirm that both Schwann cells and cancer cells express BDNF and TRKB (Figure 2). Then, we utilized a 3-D spheroid to 2-D plate assay (Figure 3) for our investigation and performed cell migration and growth assays individually for Schwann and cancer cells in the presence and absence of a TRKB inhibitor (ANA-12). Our study found that Schwann cells and cancer cells exhibited random growth and migration overall and that ANA-12 did not affect this finding (Figure 5). Lastly, we performed co-cultures of Schwann and cancer cell spheroids and performed migration and cell dispersion assays with and without ANA-12. Our findings demonstrated that Schwann cells and cancer cells preferentially migrate towards each other and that TRKB inhibition with ANA-12 blocked the preferential migration that was seen (Figure 8). However, although TRKB inhibitor ANA-12 reduced the preferential migration of Schwann and cancer cells towards each other, noticeable differences in the cell-to-cell interactions were observed under both treatment conditions when the two cell types came into direct contact with each other.

Upon cell contact, it appeared that the cancer cells were growing as a single mass invading into the Schwann cell cluster and herding Schwann cells to the periphery. Under the control conditions, it appears that the cancer cells disregard the boundaries set up by the Schwann cells (Figure 6). This pattern of cancer cell invasion may mimic what potentially occurs in direct PNI and perhaps the reciprocal signaling interactions between cell types is responsible for what was observed. However, in the presence of ANA-12, Schwann cells became more active against intrusion by the cancer cell cluster. Schwann cells intercalated between cancer cells, ultimately dispersing small collections of cancer cells from the much larger cancer cell cluster (Figures 4, 7 and 8). The Schwann cell activity seen is similar to that seen with dedifferentiated Schwann cells in nerve injury (24-27), which suggests that TRKB inhibition may activate intrinsic properties of Schwann cells that might regulate the cancer-and-nerve interactions associated with PNI. In addition, the cancer did not penetrate toward the center of the Schwann cell cluster

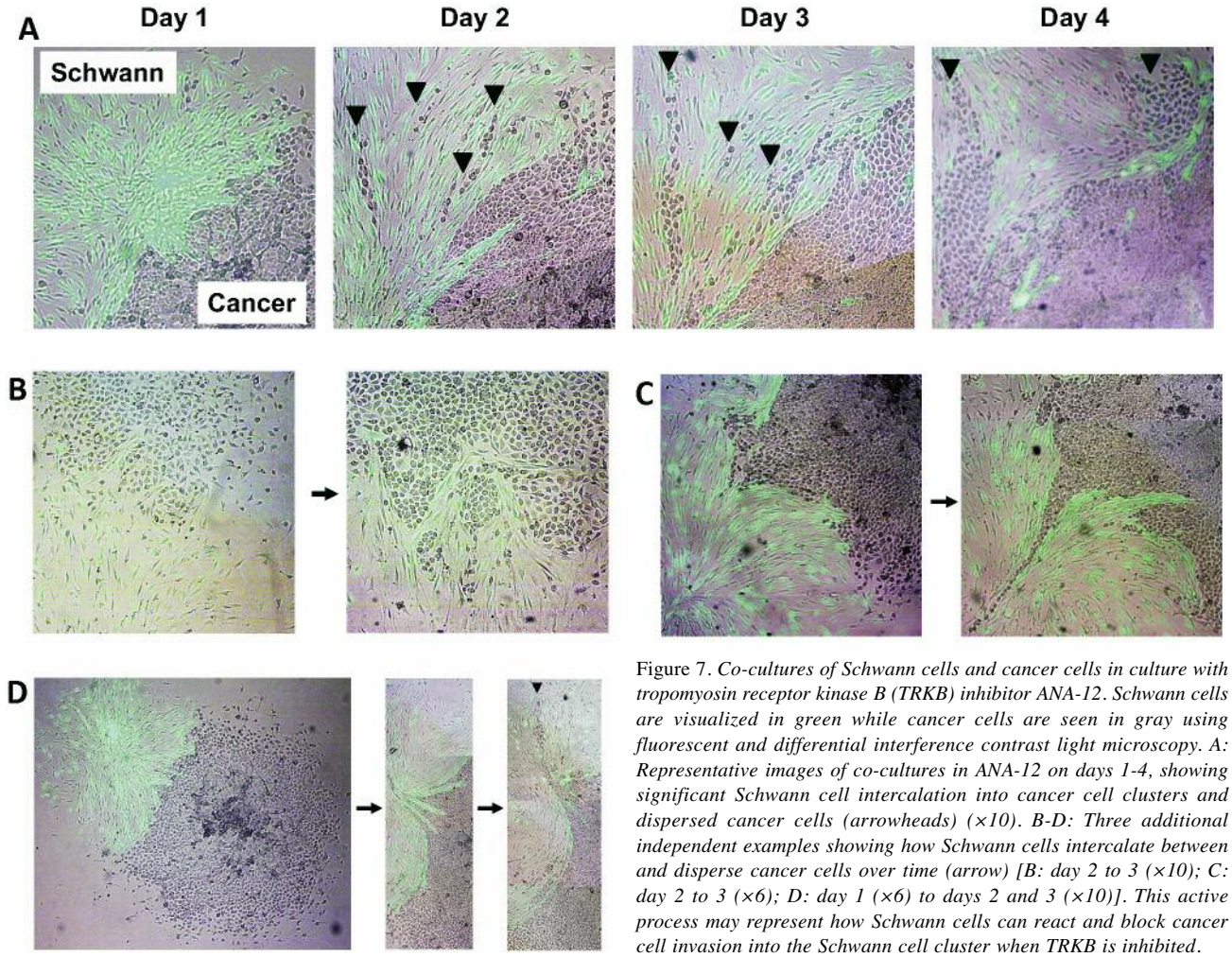

Figure 7. *Co-cultures of Schwann cells and cancer cells in culture with tropomyosin receptor kinase B (TRKB) inhibitor ANA-12. Schwann cells are visualized in green while cancer cells are seen in gray using fluorescent and differential interference contrast light microscopy. A: Representative images of co-cultures in ANA-12 on days 1-4, showing significant Schwann cell intercalation into cancer cell clusters and dispersed cancer cells (arrowheads) (×10). B-D: Three additional independent examples showing how Schwann cells intercalate between and disperse cancer cells over time (arrow) [B: day 2 to 3 (×10); C: day 2 to 3 (×6); D: day 1 (×6) to days 2 and 3 (×10)]. This active process may represent how Schwann cells can react and block cancer cell invasion into the Schwann cell cluster when TRKB is inhibited.*

as it did under the control conditions, suggesting that TRKB inhibition with ANA-12 may have some utility against perineural spread and PNI.

Our findings with oral squamous cell carcinoma are consistent with investigations in other cancer types that show Schwann cells can migrate toward cancer cells and promote cancer progression (39, 40). However, instead of Schwann cell migration as an initial event in migration and invasion assays, we show both Schwann and cancer cells migrate and grow towards each other in a similar manner. The addition of ANA-12 blocked this preferential migration of Schwann and cancer cells towards each other, possibly by preventing BDNF activation of the TRKB receptor. This is consistent with a study by Yamuachi *et al*., who showed how BDNF inhibits Schwann cell migration, while removal of BDNF using the BDNF scavenger TRKB-Fc enhanced Schwann cell migration and activity (45).

Upon contact though, we found cancer cells invaded Schwann cell clusters and pushed Schwann cells to the periphery, which may potentially represent a mechanism of perineural spread and PNI in oral squamous cell carcinoma. This pattern of cell-to-cell interaction seen with murine oral squamous cell carcinoma is similar to an investigation using pancreatic adenocarcinoma that showed Schwann cell contact-mediated cancer cell migration towards the nerve (22). Although we did not have neurons in our co-cultures, we did demonstrate how cancer cells grew and migrated toward the center of the Schwann cell cluster.

Unlike Deborde *et al*. (22), we did not show that Schwann cells induced cancer cell dispersion under the control conditions, but rather demonstrated an intriguing response from the Schwann cells when co-cultures were treated with ANA-12. With ANA-12, the passive Schwann cells appeared very active, intercalating between cancer cells and dispersing

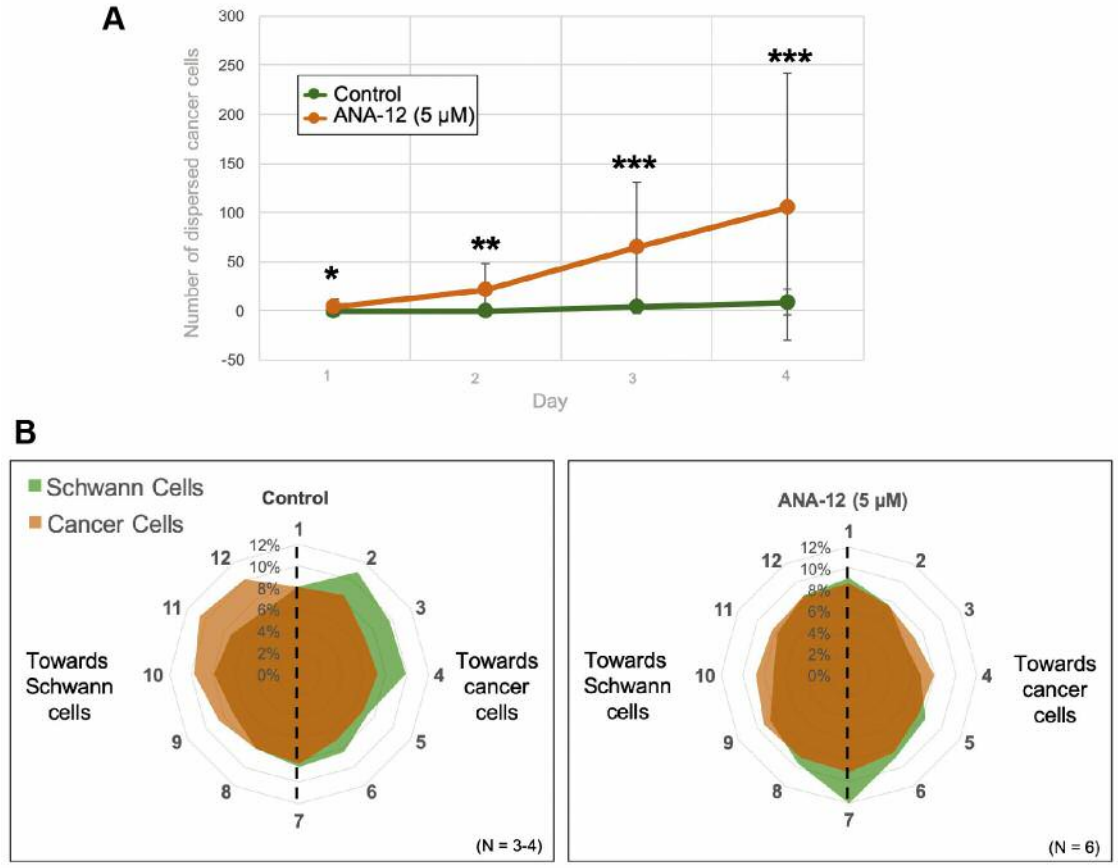

Figure 8. *Cancer cell dispersion and cell migration in co-culture. A: There was a significant increase in Schwann-induced cancer cell dispersion in cells treated with tropomyosin receptor kinase B (TRKB) inhibitor ANA-12 group on all days when compared to the control. Significantly different at: *p<0.05, **p<0.01, ***p<0.001 (n=6-9). B: Radar plots of the co-cultures on the first 2 days of co-culture assays. The image on the left demonstrates co-cultures in control medium. Cancer cells are shown in orange growing towards Schwann cells. Schwann cells are shown in green growing towards cancer cells. These results demonstrate there is a preferential migration of cancer cells and Schwann cells towards each other. The image on the right shows that this preferential migration was not seen in the presence of ANA-12.*

groups of cancer cells from the cancer cluster. This contradicts what is seen when pancreatic adenocarcinoma is co-cultured with dorsal root ganglia (22), which shows Schwann cell-induced cancer dispersion occurs through a NCAM1-dependent manner. Differences in these findings related to cancer cell dispersion *in vitro* may be related to differences in cell culture, assays, and cancer types. Further investigation into the role of Schwann cells in cancer cell dispersion and its relationship to PNI is warranted.

Although we demonstrate that BDNF and TRKB signaling regulates Schwann and cancer cell interactions that may contribute to PNI in HNC, there are several limitations to this study. Migration and dispersion assays were *in vitro* and performed on 2-D plates. In addition, human cell lines were not used in this pilot study. Furthermore, neurons were not included in co-cultures to avoid confounding elements in this preliminary investigation involving Schwann and cancer cells. In future studies, we hope to further uncover the mechanisms of PNI, using primary human squamous cell carcinoma and adenoid cystic cell lines, as well as 3-D *in vitro* and *in vivo* models of PNI.

Conclusion

BDNF–TRKB signaling can regulate Schwann cell activation and migration, and may contribute to cellular events involved with PNI in HNC. Our findings warrant further investigation to clearly delineate these relationships *in vitro* and *in vivo*. As we further understand the interactions between cancer cells and Schwann cells that promote PNI, we can begin to identify target-directed therapies for patients with HNC.

Conflicts of Interest

No conflicts of interest exist for any Author.

Authors' Contributions

Ein, L: Conceived the experimental design of the study, performed experiments/data collection, data analysis and interpretation, provided revisions to scientific content, provided revisions to the article. Mei, C: Performed experiments/data collection, data analysis and interpretation, primary author of the article, provided revisions to the article. Bracho, O: Performed experiments/data collection, data analysis and interpretation, provided revisions to the article. Bas, E: Performed experiments/data collection, data analysis and interpretation, provided revisions to the article. Monje, P: Provided access to crucial research components, data interpretation, provided revisions to the article. Weed, D: Contributions to experimental design of the study, data interpretation, provided revisions to scientific content, provided stylistic/grammatical revisions to the article. Thomas, G: Contributions to experimental design of the study, data interpretation, provided revisions to scientific content, provided stylistic/grammatical revisions to the article, provided access to crucial research components. Sargi, Z: Contributions to experimental design of the study, data interpretation, provided revisions to scientific content, provided stylistic/grammatical revisions to the article. Dinh, C: Principal investigator, conceived the experimental design of the study, performed experiments/data collection, data analysis and interpretation, provided revisions to scientific content, provided stylistic/grammatical revisions to the article, provided access to crucial research components.

References

1 Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: Globocan 2008. Int J Cancer *127(12)*: 2893-2917, 2010. PMID: 21351269. DOI: 10.1002/ijc.25516

2 Cadoni G, Giraldi L, Petrelli L, Pandolfini M, Giuliani M, Paludetti G, Pastorino R, Leoncini E, Arzani D, Almadori G and Boccia S: Prognostic factors in head and neck cancer: A 10-year retrospective analysis in a single-institution in Italy. Acta Otorhinolaryngol Ital *37(6)*: 458-466, 2017. PMID: 28663597. DOI: 10.14639/0392-100X-1246

3 Argiris A and Eng C: Epidemiology, staging, and screening of head and neck cancer. Cancer Treat Res *114*: 15-60, 2003. PMID: 12619537. DOI: 10.1007/0-306-48060-3_2

4 Hatten KM, Gupta N and Strome SE: What additional treatment is indicated for oral cavity cancer with isolated perineural invasion? Laryngoscope *127(9)*: 1965-1966, 2017. PMID: 28349551. DOI: 10.1002/lary.26577

5 Chatzistefanou I, Lubek J, Markou K and Ord RA: The role of perineural invasion in treatment decisions for oral cancer patients: A review of the literature. J Craniomaxillofac Surg *45(6)*: 821-825, 2017. PMID: 28359633. DOI: 10.1016/j.jcms.2017.02.022

6 Binmadi NO and Basile JR: Perineural invasion in oral squamous cell carcinoma: A discussion of significance and review of the literature. Oral Oncol 47(11): 1005-1010, 2011. PMID: 21865078. DOI: 10.1016/j.oraloncology.2011.08.002

7 Liebig C, Ayala G, Wilks JA, Berger DH and Albo D: Perineural invasion in cancer: A review of the literature. Cancer 115(15): 3379-3391, 2009. PMID: 19484787. DOI: 10.1002/cncr.24396

8 Johnston M, Yu E and Kim J: Perineural invasion and spread in head and neck cancer. Expert Rev Anticancer Ther 12(3): 359-371, 2012. PMID: 22369327. DOI: 10.1586/era.12.9

9 Roh J, Muelleman T, Tawfik O and Thomas SM: Perineural growth in head and neck squamous cell carcinoma: A review. Oral Oncol 51(1): 16-23, 2015. PMID: 25456006. DOI: 10.1016/j.oraloncology.2014.10.004

10 Adelstein D, Gillison ML, Pfister DG, Spencer S, Adkins D, Brizel DM, Burtness B, Busse PM, Caudell JJ, Cmelak AJ, Colevas AD, Eisele DW, Fenton M, Foote RL, Gilbert J, Haddad RI, Hicks WL, Jr., Hitchcock YJ, Jimeno A, Leizman D, Lydiatt WM, Maghami E, Mell LK, Mittal BB, Pinto HA, Ridge JA, Rocco J, Rodriguez CP, Shah JP, Weber RS, Witek M, Worden F, Yom SS, Zhen W, Burns JL and Darlow SD: NCCN guideline insights: Head and neck cancers, version 2.2017. J Natl Compr Canc Netw *15(6)*: 761-770, 2017. PMID: 28596256. DOI: 10.6004/jnccn.2017.0101

11 Ballantyne AJ, McCarten AB and Ibanez ML: The extension of cancer of the head and neck through peripheral nerves. Am J Surg *106*: 651-667, 1963. PMID: 14070757.

12 Cruveilheir J: Maladies des nerfs. Anatomie Pathologique du Dorps Humain 2nd ed. Pt 35 Paris: J.B. Bailliére pp. 1829-1842, 1835.

13 Neumann E: Secundäre cancroidinfiltration des nervus mentalis bei einem fall von lippencancroid. Virchows Arch *24(1-2)*: 201-202, 1862.

14 Frunza A, Slavescu D and Lascar I: Perineural invasion in head and neck cancers – a review. J Med Life *7(2)*: 121-123, 2014. PMID: 25408713.

15 Seiwert TY, Salama JK and Vokes EE: The chemoradiation paradigm in head and neck cancer. Nat Clin Pract Oncol *4(3)*: 156-171, 2007. PMID: 17327856. DOI: 10.1038/ncponc0750

16 Jessen KR: Glial cells. Int J Biochem Cell Biol 36(10): 1861-1867, 2004. PMID: 15203098. DOI: 10.1016/j.biocel.2004.02.023

17 Scheib J and Hoke A: Advances in peripheral nerve regeneration. Nat Rev Neurol *9(12)*: 668-676, 2013. PMID: 24217518. DOI: 10.1038/nrneurol.2013.227

18 Glenn TD and Talbot WS: Signals regulating myelination in peripheral nerves and the Schwann cell response to injury. Curr Opin Neurobiol *23(6)*: 1041-1048, 2013. PMID: 23896313. DOI: 10.1016/j.conb.2013.06.010

19 Kang H, Tian L and Thompson W: Terminal Schwann cells guide the reinnervation of muscle after nerve injury. J Neurocytol *32(5-8)*: 975-985, 2003. PMID: 15034280. DOI: 10.1023/B:NEUR.0000020636.27222.2d

20 Son YJ and Thompson WJ: Nerve sprouting in muscle is induced and guided by processes extended by Schwann cells. Neuron *14(1)*: 133-141, 1995. PMID: 7826631. DOI: 10.1016/0896-6273(95)90247-3

21 Bunimovich YL, Keskinov AA, Shurin GV and Shurin MR: Schwann cells: A new player in the tumor microenvironment. Cancer Immunol Immunother *66(8)*: 959-968, 2017. PMID: 27885383. DOI: 10.1007/s00262-016-1929-z

22 Deborde S, Omelchenko T, Lyubchik A, Zhou Y, He S, McNamara WF, Chernichenko N, Lee SY, Barajas F, Chen CH, Bakst RL, Vakiani E, He S, Hall A and Wong RJ: Schwann cells

induce cancer cell dispersion and invasion. J Clin Invest *126(4)*: 1538-1554, 2016. PMID: 26999607. DOI: 10.1172/JCI82658

23 Azam SH and Pecot CV: Cancer's got nerve: Schwann cells drive perineural invasion. J Clin Invest *126(4)*: 1242-1244, 2016. PMID: 26999601. DOI: 10.1172/JCI86801

24 Daniloff JK, Levi G, Grumet M, Rieger F and Edelman GM: Altered expression of neuronal cell adhesion molecules induced by nerve injury and repair. J Cell Biol *103(3)*: 929-945, 1986. PMID: 2427528. DOI: 10.1083/jcb.103.3.929

25 Jessen KR and Mirsky R: The origin and development of glial cells in peripheral nerves. Nat Rev Neurosci *6(9)*: 671-682, 2005. PMID: 16136171. DOI: 10.1038/nrn1746

26 Martini R: Expression and functional roles of neural cell surface molecules and extracellular matrix components during development and regeneration of peripheral nerves. J Neurocytol 23(1): 1-28, 1994. PMID: 8176415. DOI: 10.10007/bf01189813

27 Neuberger TJ and Cornbrooks CJ: Transient modulation of Schwann cell antigens after peripheral nerve transection and subsequent regeneration. J Neurocytol 18(5): 695-710, 1989. PMID: 2515258. DOI: 10.1007/bf01187088

28 Magnon C, Hall SJ, Lin J, Xue X, Gerber L, Freedland SJ and Frenette PS: Autonomic nerve development contributes to prostate cancer progression. Science *341(6142)*: 1236361, 2013. PMID: 23846904. DOI: 10.1126/science.1236361

29 Zhao CM, Hayakawa Y, Kodama Y, Muthupalani S, Westphalen CB, Andersen GT, Flatberg A, Johannessen H, Friedman RA, Renz BW, Sandvik AK, Beisvag V, Tomita H, Hara A, Quante M, Li Z, Gershon MD, Kaneko K, Fox JG, Wang TC and Chen D: Denervation suppresses gastric tumorigenesis. Sci Transl Med *6(250)*: 250ra115, 2014. PMID: 25143365. DOI: 10.1126/scitranslmed.3009569

30 Yilmaz T, Jiffar T, de la Garza G, Lin H, Milas Z, Takahashi Y, Hanna E, MacIntyre T, Brown JL, Myers JN and Kupferman ME: Theraputic targeting of Trk supresses tumor proliferation and enhances cisplatin activity in HNSCC. Cancer Biol Ther *10(6)*: 644-653, 2010. PMID: 20703101. DOI: 10.4161/cbt.10.6.12782

31 Ketterer K, Rao S, Friess H, Weiss J, Buchler MW and Korc M: Reverse transcription-PCR analysis of laser-captured cells points to potential paracrine and autocrine actions of neurotrophins in pancreatic cancer. Clin Cancer Res *9(14)*: 5127-5136, 2003. PMID: 14613990.

32 Kowalski PJ and Paulino AF: Perineural invasion in adenoid cystic carcinoma: Its causation/promotion by brain-derived neurotrophic factor. Hum Pathol *33(9)*: 933-936, 2002. PMID: 12378520. DOI: 10.1053/hupa.2002.128249

33 Miknyoczki SJ, Lang D, Huang L, Klein-Szanto AJ, Dionne CA and Ruggeri BA: Neurotrophins and TRK receptors in human pancreatic ductal adenocarcinoma: Expression patterns and effects on *in vitro* invasive behavior. Int J Cancer *81(3)*: 417-427, 1999. PMID: 10209957. DOI: 10.1002/(sici)1097-0215(19990505)81:3<417::aid-ijc16>3.0.co;2-6

34 Okugawa Y, Tanaka K, Inoue Y, Kawamura M, Kawamoto A, Hiro J, Saigusa S, Toiyama Y, Ohi M, Uchida K, Mohri Y and Kusunoki M: Brain-derived neurotrophic factor/tropomyosin-related kinase B pathway in gastric cancer. Br J Cancer *108(1)*: 121-130, 2013. PMID: 23175149. DOI: 10.1038/bjc.2012.499

35 Cazorla M, Premont J, Mann A, Girard N, Kellendonk C and Rognan D: Identification of a low-molecular weight TRKB antagonist with anxiolytic and antidepressant activity in mice. J Clin Invest *121(5)*: 1846-1857, 2011. PMID: 21505263. DOI: 10.1172/JCI43992

36 Thomas GR, Chen Z, Oechsli MN, Hendler FJ and Van Waes C: Decreased expression of CD80 is a marker for increased tumorigenicity in a new murine model of oral squamous-cell carcinoma. Int J Cancer *82(3)*: 377-384, 1999. PMID: 10399955. DOI: 10.1002/(sici)1097-0215(19990730)82:3<377::aid-ijc11>3.0.co;2-9

37 Andersen ND and Monje PV: Isolation, culture, and cryopreservation of adult rodent schwann cells derived from immediately dissociated teased fibers. Methods Mol Biol *1739*: 49-66, 2018. PMID: 29546700. DOI: 10.1007/978-1-4939-7649-2_4

38 Laib AM, Bartol A, Alajati A, Korff T, Weber H and Augustin HG: Spheroid-based human endothelial cell microvessel formation *in vivo*. Nat Protoc *4(8)*: 1202-1215, 2009. PMID: 19644460. DOI: 10.1038/nprot.2009.96

39 Demir IE, Boldis A, Pfitzinger PL, Teller S, Brunner E, Klose N, Kehl T, Maak M, Lesina M, Laschinger M, Janssen KP, Algul H, Friess H and Ceyhan GO: Investigation of Schwann cells at neoplastic cell sites before the onset of cancer invasion. J Natl Cancer Inst 106(8), 2014. PMID: 25106646. DOI: 10.1093/jnci/dju184

40 Deborde S and Wong RJ: How Schwann cells facilitate cancer progression in nerves. Cell Mol Life Sci *74(24)*: 4405-4420, 2017. PMID: 28631007. DOI: 10.1007/s00018-017-2578-x

41 de Moraes JK, Wagner VP, Fonseca FP, Vargas PA, de Farias CB, Roesler R and Martins MD: Uncovering the role of brain-derived neurotrophic factor/tyrosine kinase receptor B signaling in head and neck malignancies. J Oral Pathol Med *47(3)*: 221-227, 2018. PMID: 28650560. DOI: 10.1111/jop.12611

42 Kupferman ME, Jiffar T, El-Naggar A, Yilmaz T, Zhou G, Xie T, Feng L, Wang J, Holsinger FC, Yu D and Myers JN: TRKB induces EMT and has a key role in invasion of head and neck squamous cell carcinoma. Oncogene *29(14)*: 2047-2059, 2010. PMID: 20101235. DOI: 10.1038/onc.2009.486

43 Lee J, Jiffar T and Kupferman ME: A novel role for BDNF-TRKB in the regulation of chemotherapy resistance in head and neck squamous cell carcinoma. PLoS One *7(1)*: e30246, 2012. PMID: 22276165. DOI: 10.1371/journal.pone.0030246

44 Jia S, Wang W, Hu Z, Shan C, Wang L, Wu B, Yang Z, Yang X and Lei D: Bdnf mediated TRKB activation contributes to the EMT progression and the poor prognosis in human salivary adenoid cystic carcinoma. Oral Oncol *51(1)*: 64-70, 2015. PMID: 25456007. DOI: 10.1016/j.oraloncology.2014.10.008

45 Yamauchi J, Chan JR and Shooter EM: Neurotrophins regulate Schwann cell migration by activating divergent signaling pathways dependent on RHO GTPases. Proc Natl Acad Sci USA *101(23)*: 8774-8779, 2004. PMID: 15161978. DOI: 10.1073/pnas.0402795101

Received August 19, 2019
Revised September 23, 2019
Accepted September 30, 2019