

Article

Not peer-reviewed version

Neuregulin-Induced HER3 Activation Drives Migration in Head and Neck Cancer via HER2 and FAK Signaling Pathways

Eun Jin Lim , Yu Jeong Yoon , Jeonghoon Heo , Seongwon Kim , [Yung-Hyun Choi](#) ^{*} , [Young-Ho Kim](#) ^{*}

Posted Date: 18 January 2025

doi: 10.20944/preprints202501.1319.v1

Keywords: neuregulin; HER3; focal adhesion kinase; head and neck cancer



Preprints.org is a free multidisciplinary platform providing preprint service that is dedicated to making early versions of research outputs permanently available and citable. Preprints posted at Preprints.org appear in Web of Science, Crossref, Google Scholar, Scilit, Europe PMC.

Copyright: This open access article is published under a Creative Commons CC BY 4.0 license, which permit the free download, distribution, and reuse, provided that the author and preprint are cited in any reuse.

Article

Neuregulin-Induced HER3 Activation Drives Migration in Head and Neck Cancer via HER2 and FAK Signaling Pathways

Eun Jin Lim ¹, Yu Jeong Yoon ¹, Jeonghoon Heo ¹, Seungwon Kim ², Yung-Hyun Choi ^{3,*} and Young-Ho Kim ^{1,*}

¹ Department of Molecular Biology and Immunology, Kosin University College of Medicine, Busan 49267, Republic of Korea

² Department of Otolaryngology, University of Pittsburgh Medical Center, Pittsburgh, PA, USA

³ Department of Biochemistry, College of Korean Medicine, Dong-eui University, Busan, 47227, Republic of Korea

* Correspondence: choiyh@deu.ac.kr (Y.-H.C.); kimyh@kosin.ac.kr (Y.-H.K.)

Abstract: Perineural invasion is a hallmark of aggressive head and neck cancer (HNC), often driven by interactions between tumor cells and surrounding nerves. Here, we investigate the role of neuregulin (NRG) signaling in promoting HNC cell migration through the activation of HER3-dependent pathways. Using Matrigel dorsal root ganglion (DRG) co-culture assays, we demonstrated that NRG secretion from active DRG promotes directional migration of FaDu and TU138 HNC cells, mediated by HER3/HER2 and HER3/PI3K interactions. Inhibition of HER3 using shRNA or the HER3-specific antibody AV-203 abolished HER3 phosphorylation, disrupted HER3-HER2 interactions, and suppressed downstream signaling through Akt and ERK. Further, wound healing assays revealed that NRG enhances cell migration via HER3 activation, with functional HER3 being critical for this effect. NRG stimulation also induced HER3-dependent phosphorylation of focal adhesion kinase (FAK), a key regulator of cell migration. Knockdown of FAK or pharmacological inhibition using PF228 significantly impaired NRG-driven migration, underscoring the importance of HER3-FAK signaling. These findings establish that NRG promotes HNC cell migration by activating HER3, forming HER3-HER2 and HER3-FAK complexes, and driving downstream signaling through Akt, ERK, and FAK pathways. Targeting the NRG/HER3 axis represents a promising therapeutic strategy to mitigate perineural invasion and its associated clinical challenges in head and neck cancer.

Keywords: neuregulin; HER3; focal adhesion kinase; head and neck cancer

1. Introduction

Head and neck cancers (HNC) are a heterogeneous group of malignancies that arise from the epithelial cells lining the oral cavity, pharynx, larynx, and other parts of the upper respiratory tract. They are associated with significant morbidity and mortality, with approximately 650,000 new cases diagnosed globally each year [1–5]. Despite advances in surgical techniques, radiotherapy, and chemotherapy, the prognosis for patients with advanced HNC remains poor, largely due to local recurrence, distant metastasis, and resistance to conventional therapies. A particularly aggressive feature of these cancers is their tendency to invade nearby nerves, a phenomenon known as perineural invasion (PNI) [6–8]. PNI is linked to poor clinical outcomes, including increased pain, difficulty in treatment management, and a higher likelihood of cancer recurrence [9–11].

Understanding the mechanisms that drive PNI is crucial to developing strategies to prevent or treat this aspect of HNC.

Neuregulins (NRGs) are a family of growth factors that play essential roles in cell signaling, particularly in the development of the nervous system and the regulation of various cellular functions, including proliferation, migration, and survival [12–15]. NRGs exert their effects through binding to ErbB receptors, a family of receptor tyrosine kinases that includes EGFR (HER1), HER2, HER3, and HER4 [16,17]. Among these, HER3 has emerged as a critical receptor in cancer progression. NRGs bind to HER3, leading to receptor dimerization and activation of downstream signaling pathways, such as the PI3K/Akt and MAPK/ERK pathways, which are known to regulate key cellular processes including cell migration, survival, and proliferation [18–21]. In particular, NRG-induced HER3 activation has been shown to contribute to cancer cell migration and metastasis in various cancer types, including breast, gastric, and ovarian cancers [22–27]. However, the role of NRG signaling in head and neck cancer, specifically in relation to perineural invasion, has not been fully explored.

Previous studies have suggested that NRG signaling may play a key role in facilitating PNI by promoting cancer cell migration along nerve fibers [28–30]. The interaction between cancer cells and nerves is believed to be driven by paracrine signaling, where NRG is secreted by the surrounding neural tissue and signals through HER3 on adjacent cancer cells. This interaction likely enhances the migratory and invasive capabilities of cancer cells, enabling them to infiltrate surrounding nerves and tissues [31,32]. Despite growing evidence that NRG signaling contributes to cancer progression, little is known about its specific role in head and neck cancers, particularly in the context of PNI. Thus, there is a critical need to better understand how NRG signaling regulates HNC cell migration and invasion, particularly toward nerves.

HER3, in particular, plays a central role in NRG-induced signaling [33]. Unlike other members of the ErbB family, HER3 lacks intrinsic kinase activity and relies on heterodimerization with other ErbB receptors, such as HER2, to initiate signaling [24,34,35]. Upon binding NRG, HER3 forms heterodimers with HER2, leading to activation of downstream signaling pathways, including PI3K/Akt and MAPK/ERK. These pathways are crucial for the regulation of cell survival, migration, and invasion. HER3's role in regulating migration and invasion has been highlighted in various cancers, but its specific contribution to perineural invasion in head and neck cancer remains unclear. Furthermore, the downstream signaling events triggered by HER3 activation, such as phosphorylation of focal adhesion kinase (FAK) and activation of integrins, may play a key role in mediating cancer cell migration along nerve fibers.

Focal adhesion kinase (FAK) is a critical regulator of cell migration [36,37]. FAK is activated in response to integrin signaling and plays a key role in the formation and maturation of focal adhesions, which are critical for cell attachment and migration. FAK activation has been shown to be involved in cancer cell migration and invasion, including in the context of perineural invasion [38–41]. Phosphorylation of FAK at specific tyrosine residues, such as Tyr-397 and Tyr-925, is essential for its activation and subsequent signaling. HER3 signaling can regulate FAK phosphorylation, suggesting that HER3-dependent activation of FAK may be a key mechanism in cancer cell migration and perineural invasion. However, the specific relationship between NRG, HER3, FAK, and perineural invasion in head and neck cancer remains largely unexplored.

In this study, we explore the role of FAK phosphorylation in NRG-induced migration and investigate the interaction between HER3 and FAK in regulating cancer cell movement. Our findings provide new insights into the mechanisms by which NRG induces migration of head and neck cancer cells, particularly through HER3-dependent activation of HER2 and FAK. By understanding the molecular events that drive perineural invasion, we hope to identify potential therapeutic targets for inhibiting this process and improving the prognosis for patients with head and neck cancer. Moreover, targeting the NRG/HER3 signaling axis may offer a promising strategy for overcoming resistance to current therapies and preventing the spread of cancer through perineural invasion.

2. Results

2.1. NRG Signaling Pathway Induces the Ability of Head and Neck Cancer Cells to migrate Towards Neurites Extended From Dorsal Root Ganglia in a Matrigel Co-Culture Assay

To investigate the role of NRG signaling in mediating the migration of head and neck cancer (HNC) cells towards surrounding nerves, we employed a Matrigel dorsal root ganglion (DRG) co-culture assay, as described previously [42]. DRG activity was confirmed through the expression of neuronal and Schwann cell markers, with distinct expression patterns observed (data not shown). The FaDu head and neck cancer cell line was co-cultured with DRGs to assess directional migration towards neurites extended by the DRG. Cancer cells demonstrated a pronounced ability to migrate towards active DRG neurites, as quantified by migration distances (Fig. 1A). In contrast, co-culture with inactive DRG, or DRG with reduced secretion of NRG, resulted in a marked reduction in the migration of FaDu cells toward DRG neurites (Fig. 1B). To generalize this observation, two HNC cell lines, FaDu and TU138, were co-cultured with DRGs. Both cell lines displayed increased migration in the direction of DRG neurites, confirming the neurotropic behavior of these cancer cells (Fig. 1C). These results suggest that the paracrine signaling mediated by NRG, secreted by DRG, activates EGFR family receptors on HNC cells, enhancing their ability to migrate toward neural elements. This paracrine interaction likely contributes to the mechanisms underlying perineural invasion in head and neck cancers.

2.2. Effect of NRG-Stimulated Cells to Increase Migration and the Phosphorylation of HER3 and Akt

To further explore the effects of NRG on the migration of head and neck cancer (HNC) cells, wound healing assays were performed. Stimulation with NRG significantly enhanced the migratory abilities of FaDu and TU138 cells. After 24 hours of NRG stimulation, the number of migrating FaDu and TU138 cells increased by approximately 2-fold and 1.5-fold, respectively, compared to unstimulated controls (Fig. 2A). To elucidate the signaling mechanisms underlying NRG-driven migration, we assessed phosphorylation of HER family receptors and downstream signaling molecules. NRG stimulation markedly increased phosphorylation of HER3 and HER2 in FaDu cells and was weakly involved in HER3 phosphorylation in TU138 cells (Fig. 2B). Notably, HER3 phosphorylation was significantly higher in FaDu cells compared to TU138 cells, suggesting cell-line-specific activation of this receptor. Analysis of downstream signaling pathways revealed that Akt phosphorylation was NRG-dependent in FaDu cells, whereas constitutive Akt phosphorylation was observed in TU138 cells even in the absence of NRG stimulation (Fig. 2C). ERK phosphorylation, however, was strongly dependent on NRG stimulation in FaDu cells. These findings suggest that NRG promotes HNC cell migration through activation of the HER3/Akt/ERK signaling axis. The differential activation of HER3 and Akt in FaDu and TU138 cells may explain the varying dependence on specific signaling pathways in each cancer cell line. These results highlight the critical role of NRG-induced HER3/Akt/ERK signaling in enhancing the migratory ability of HNC cells.

2.3. NRG Stimulation Leads to Activation of HER3, Resulting in Receptor Clustering and Activation of the PI3K-Akt Pathway

To assess whether regulating HER3 hetero-oligomerization influences HER3 phosphorylation, we analyzed receptor interactions in response to NRG stimulation. In FaDu cells, NRG significantly enhanced the interaction between HER3 and HER2, as well as HER3 and PI3K, while no notable increase in HER3 and EGFR interaction was observed (Fig. 3A). In contrast, NRG stimulation did not significantly increase HER3 interactions in TU138 cells. However, constant interaction between HER2 and EGFR, independent of HER3, was observed in TU138 cells (Fig. 3B). To further validate the role of HER3 in these interactions, a HER3 antibody was utilized. The HER3-specific antibody AV-203, which blocks NRG binding to HER3, inhibited NRG-induced interactions between HER3 and HER2 or PI3K (Fig. 3C). This blockade further confirmed the critical role of NRG in driving HER3/HER2

and HER3/PI3K interactions. To observe HER3/HER2 interactions, fluorescent antibodies were used to visualize the cells. After NRG treatment, HER3 and HER2 antibodies were observed to overlap in the cells, confirming HER3/HER2 dimerization induced by NRG (Fig. 3D). These findings indicate that the formation of HER3-HER2 and HER3-PI3K complexes upon NRG stimulation is a key mechanism underlying HER3 activation.

2.4. AV-203 Inhibits Activation of HER3 and AKT/ERK in FaDu and TU138 Cells

To evaluate the inhibitory effect of AV-203 on HER3 activation and downstream signaling, we tested its activity in FaDu and TU138 cells. Treatment with AV-203 at a dose of 1 µg/mL effectively inhibited HER3 phosphorylation in both FaDu and TU138 cells (Fig. 4A). Furthermore, 24-hour treatment with AV-203 completely blocked the activation of downstream signaling pathways, including Akt and ERK, in both cell lines (Fig. 4B). In comparison, cetuximab, an EGFR-targeting monoclonal antibody, failed to interfere with NRG-induced HER3 phosphorylation. This indicates that HER3 activation via NRG operates independently of EGFR signaling. To investigate the role of the HER3/HER2 signaling pathway in HNC cells, we blocked HER2/Akt signaling using specific inhibitors. FaDu and TU138 cells were treated with NRG in the presence of the HER2 tyrosine kinase inhibitor (TKI) Lapatinib (Lapa) and the PI3K inhibitor LY294002 (LY) to assess their effects on HER3, Akt, and ERK activation. In FaDu cells, treatment with Lapatinib slightly inhibited HER3 phosphorylation, demonstrating its potent ability to block HER3 activation in response to NRG. LY294002 treatment inhibited HER2 activation, while only weakly reducing HER3 activation, suggesting a less direct role in regulating HER3 phosphorylation (Fig. 4C). These findings suggest that HER3 plays a critical role in facilitating HER2 activation, while PI3K contributes to a feedback mechanism that partially supports HER2 signaling. The combined inhibition of HER2 and PI3K may represent a strategic approach to disrupt the HER3/AKT/ERK pathway in HNC cells. The results showed that AV-203 has a strong inhibitory activity on phosphorylation of HER3, AKT and ERK in HNC cells.

2.5. NRG Induces HER3-Dependent Cell Migration and HER2 Interaction

To evaluate the role of NRG-induced HER3 signaling in cell migration and HER2 interaction, we conducted a series of experiments using FaDu cells, including western blotting, wound healing assays, and HER3 knockdown (shHER3). NRG stimulation significantly enhanced HER3 and HER2 phosphorylation in FaDu cells, as confirmed by western blot analysis (Fig 2B). In the wound healing assay, parental FaDu cells displayed robust migratory activity, with cell migration increasing by 2-fold compared to HER3 knockdown (shHER3) cells (Fig. 5A). In shHER3 cells, HER3 phosphorylation was completely abolished, further confirming the dependence on HER3 for NRG-induced migration. Time-course studies demonstrated that NRG stimulation induced a time-dependent increase in HER3, Akt, and ERK phosphorylation in parental cells (Fig. 5B). However, this activation was significantly suppressed in shHER3 cells, indicating the critical role of HER3 in mediating downstream signaling. Furthermore, interaction between HER3 and HER2, as detected by co-immunoprecipitation, was markedly reduced in shHER3 cells (Fig. 5C, 5D). These findings suggest that HER3 is essential for the formation of HER3-HER2 complexes in response to NRG and for activation of the downstream Akt and ERK pathways. These results demonstrate that NRG induces HER3-dependent cell migration and signaling through the interaction of HER3 with HER2 and the activation of downstream pathways, such as Akt and ERK.

2.6. NRG Induces FAK-Dependent Cell Migration and HER3 Interactions

To dissect the role of HER3-dependent signaling in NRG-induced migration, we investigated its impact on focal adhesion kinase (FAK) phosphorylation and interaction in HER3-deficient cells. FAK phosphorylation, occurring at key tyrosine sites (Tyr-397 and Tyr-925), was assessed in response to NRG stimulation. In parental cells, NRG stimulation significantly increased FAK phosphorylation in

a time-dependent manner. However, this effect was completely abolished in HER3 knockdown (shHER3) cells, indicating that HER3 is essential for NRG-induced FAK activation (Fig. 6A). To evaluate the role of FAK in cell migration, we compared the migratory capacity of parental cells (siCon) with FAK knockdown (siFAK) cells using scratch wound assays. Knockdown of FAK resulted in an approximately 2.2-fold decrease in basal migration compared to control cells (Fig. 6B). Similarly, treatment with the FAK inhibitor PF228 significantly reduced the migratory capacity of parental cells in scratch wound assays (Fig. 6C). These findings underscore the critical role of FAK in NRG-induced migration. We further investigated the interaction between HER3 and FAK upon NRG stimulation. Co-immunoprecipitation studies demonstrated that HER3 interacted with FAK in response to NRG, whereas this interaction was absent in shHER3 cells (Fig. 6D). These results demonstrate that NRG induces HER3-dependent activation and interaction of FAK, which plays a pivotal role in driving cell migration. The absence of HER3 disrupts FAK activation and interaction, further emphasizing the importance of HER3-FAK signaling in NRG-stimulated migratory processes.

3. Discussion

This study investigated the mechanisms underlying neuregulin (NRG)-induced cell migration in head and neck cancer (HNC), with a particular focus on the HER3 signaling pathway and its interactions with downstream effectors. Our findings provide strong evidence that NRG promotes HNC cell migration through the activation of HER3-dependent pathways, including HER2, PI3K/Akt, ERK, and FAK signaling.

The Matrigel DRG co-culture assay revealed that NRG secretion from active DRGs stimulates HNC cells, enhancing their ability to migrate toward neural elements. This neurotropic behavior, mediated by paracrine signaling, highlights a potential mechanism for perineural invasion, a hallmark of aggressive HNC [7,43–45]. Specifically, FaDu and TU138 cells displayed significant migration toward active DRG neurites, whereas migration was markedly reduced when DRGs were inactive or secreted lower levels of NRG. These results emphasize the importance of the tumor-nerve microenvironment and its contribution to cancer progression. NRG stimulation triggered the phosphorylation of HER3 and HER2, which was essential for initiating downstream signaling pathways [24,33]. Our findings indicate cell-line-specific variations in HER3 activation. In FaDu cells, HER3 phosphorylation was robust and NRG-dependent, whereas in TU138 cells, HER3 phosphorylation was weak. This differential activation reflects the heterogeneity of signaling dependencies in HNC and suggests that FaDu cells rely more heavily on the HER3/Akt/ERK axis for migration, while TU138 cells utilize alternative pathways. Further analysis revealed that NRG stimulation enhanced HER3/HER2 and HER3/PI3K interactions in FaDu cells, leading to strong activation of the PI3K-Akt pathway. This pathway is a well-established regulator of cancer cell survival and motility, supporting the notion that HER3/PI3K interactions are critical for NRG-induced migratory responses. The use of the HER3-specific antibody AV-203 effectively blocked HER3 phosphorylation and disrupted these interactions, confirming the central role of HER3 in NRG signaling [38].

Our results demonstrate that NRG-induced HER3/HER2 dimerization is pivotal for downstream signaling. Fluorescence microscopy confirmed HER3/HER2 co-localization upon NRG stimulation, and co-immunoprecipitation studies further validated their interaction. Interestingly, HER3 knockdown (shHER3) cells exhibited significant reductions in HER3/HER2 interactions, downstream Akt/ERK activation, and migration, highlighting the indispensable role of HER3 in this signaling cascade. HER2 also plays a complementary role, as Lapatinib, a HER2-specific inhibitor, slightly reduced HER3 phosphorylation. This suggests that HER2 supports HER3 activation, albeit to a lesser extent than NRG. The partial involvement of HER2 underscores the complexity of HER receptor signaling and suggests that targeting both HER3 and HER2 could provide a synergistic therapeutic approach.

NRG stimulation also induced HER3-dependent FAK phosphorylation, a critical event for cell migration [36,37]. FAK phosphorylation at Tyr-397 and Tyr-925 was abolished in shHER3 cells,

indicating that HER3 is essential for NRG-induced FAK activation. Functional studies revealed that both genetic (siFAK) and pharmacological (PF228) inhibition of FAK significantly impaired HNC cell migration. These findings establish that HER3 regulates FAK activation, further linking HER3 to focal adhesion dynamics and migratory processes. Notably, co-immunoprecipitation studies demonstrated that HER3 interacts directly with FAK in an NRG-dependent manner, further supporting the idea that HER3 serves as a central hub for coordinating migratory signaling. The absence of HER3 disrupted this interaction, emphasizing the importance of HER3-FAK signaling in driving cell migration.

In Figure 7, our findings demonstrate the potential of targeting the NRG-HER3 axis in HNC, as the HER3-specific antibody AV-203 robustly inhibits HER3 phosphorylation and downstream Akt and ERK signaling, while the FAK inhibitor PF228 suppresses NRG-induced cell migration by disrupting intracellular signaling, suggesting that AV-203 effectively disrupts pathways promoting HNC cell migration and perineural invasion, with further evidence that partial HER3 inhibition by Lapatinib and the complementary effects of PI3K inhibitors like LY294002 highlight the promise of combination therapies targeting HER3, HER2, and PI3K to enhance therapeutic efficacy and suppress downstream pathways involved in HNC progression.

While this study provides valuable insights, several considerations and questions remain. The differential activation of HER3 and its downstream pathways in FaDu and TU138 cells underscores the importance of understanding the molecular heterogeneity of HNC. Future studies should explore the signaling dependencies of additional HNC cell lines and primary tumors to identify subsets of patients who may benefit most from HER3-targeted therapies. The partial effects of Lapatinib and LY294002 suggest that combination therapies targeting multiple nodes in the HER3 signaling network may be more effective. The design and testing of such combinations should consider the potential for additive or synergistic effects in reducing migration and invasion. The critical role of FAK in NRG-induced migration highlights its potential as a therapeutic target. Combining HER3 inhibitors with FAK inhibitors, such as PF228, may further reduce HNC cell motility and invasion.

In summary, our study establishes the pivotal role of NRG-HER3 signaling in promoting HNC cell migration through HER3/HER2 and HER3/FAK interactions. These findings not only provide mechanistic insights into perineural invasion but also identify the NRG-HER3 axis as a promising therapeutic target. By combining HER3-targeted therapies with inhibitors of HER2, PI3K, or FAK, it may be possible to effectively suppress the migratory and invasive behavior of HNC cells, improving patient outcomes in this aggressive cancer.

4. Materials and Methods

4.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and all other tissue culture reagents were obtained from GIBCO/BRL Life technologies (Grand Island, NY). The neuregulin (NRG) was acquired from Aldrich (Milwaukee, WI, USA). Antibodies against HER2, HER3, ERK, FAK and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-phospho-HER3, anti-phospho-HER2, anti-phospho-FAK, anti-phospho-Akt, and anti-phospho-ERK were purchased from Cell Signaling (Beverly, MA, USA). All other chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.2. Cell Culture and Treatments

Human head and neck squamous cell carcinoma (HNSCC), FaDu and TU138 cell lines were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM with 10 % (vol/vol) FBS, 50 U/ml penicillin and streptomycin at 37 °C in a humidified 95 % air and 5 % CO₂ atmosphere. After incubation, the cells were harvested and extracted for further analysis. Cells were stimulated with 10 ng/ml neuregulin-1 β (NRG-1 β) for 24 h unless otherwise indicated. Pre-

treatment with lapatinib, LY, AV-203 and cetuximab were performed for 24 h unless otherwise indicated.

4.3. Wound Healing/Scratch Assay

At 90% confluence, cell layers were wounded by scratching using 1-mm pipette tips. Cells were stimulated with NRG as indicated, and different scratch regions ($n=30$) were photographed at the indicated times. Scratch area was determined using Image-Pro Plus (Media Cybernetics Inc., Rockville, MD).

4.4. Cell Migration Assay

Cultured cells re-suspending in serum-free DMEM were added to the top well of each migration (Boyden) chamber with the pore membrane size of 8 mm (Transwell, Corning Life Sciences, Acton, MA, USA). Cell migration was induced by serum-free DMEM with or without NRG b1 treatment (10 ng/ml) in a CO₂ incubator at 37 °C for 24 hours. After that, the membrane was removed and the cells on the top side of the membrane were wiped off. The remaining migrating cells on the membrane were then fixed with 100 % methanol and subsequently stained with crystal violet for 3 min. Photomicrographs were taken under light microscopy (Olympus CX31RTSF, Japan) and the number of migrating cells from six random fields per chamber was counted.

4.5. Dorsal Root Ganglion (DRG) Co-Culture Assay

DRGs were isolated from mice provided by Taconic Bioscience (Hudson, NY) following a protocol adapted from Malin et al [46]. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). Mice were euthanized, and their dorsal surfaces were prepared by clipping the fur and sterilizing the area with 70% ethanol. Using a dorsal approach, the vertebral column was exposed, and the spinal cord was carefully removed. DRGs were dissected from the lumbar, thoracic, and cervical regions, then placed in tissue culture media containing antibiotics to maintain sterility. FaDu and TU138 cells, stably labeled with GFP and RFP, respectively, were cultured under standard conditions. Cells were trypsinized, counted, and resuspended in chilled Matrigel (BD Biosciences) at a final concentration of 5×10^6 cells/mL. The cell suspension was maintained on ice to prevent premature solidification. Individual DRGs were transferred to a 12-well tissue culture plate. A 20 μ L drop of Matrigel was carefully applied over each DRG and allowed to solidify at 37°C for 15 minutes. Adjacent to the DRG, a 25 μ L drop of the Matrigel-embedded cancer cell suspension was placed, ensuring contact between the two drops. The plates were incubated at 37°C for an additional 15 minutes to complete Matrigel solidification. After solidification, each well was flooded with 2 mL of warm culture media containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). The co-culture plates were maintained at 37°C in a humidified incubator with 5% CO₂. The migration of GFP-labeled FaDu cells and RFP-labeled TU138 cells towards the DRG neurites was observed on Days 2 and 3 using fluorescence microscopy. The movement and directionality of the cells were quantified by measuring the distance traveled towards the neurites. Each experiment was repeated independently at least five times. Results were averaged and expressed as mean $\pm$ standard error (SE). Statistical analyses were performed to assess the significance of migration differences under experimental conditions.

4.6. Immunoblotting, Immunoprecipitation, and SDS-PAGE

For immunoblotting analyses, cells were lysed with 1 x Laemmli lysis buffer (2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M SDS, 0.3 mM bromophenol blue) and boiled for 10 minutes. Protein content was measured with the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). The samples were diluted with 1 x Laemmli lysis buffer containing 1.28 M β -mercaptoethanol, and equal amounts of protein were loaded on 8-12% SDS-polyacrylamide gels. Proteins were separated by SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. The nitrocellulose membrane was

blocked with 5% nonfat dry milk in PBS-Tween-20 (0.1 %, v/v) for one hour. The membrane was incubated with primary antibody (diluted according to the manufacturer's instructions) at room temperature for 1.5 hours. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (ECL, Amersham, Arlington Heights, IL, USA). To ensure equal protein loading, each nitrocellulose membrane was stripped and reprobed with an anti-actin antibody after the experiment was complete. The proteins of interest were immunoprecipitated by a 1-h incubation with a specific antibody (2 µg), followed by a 30-min incubation with protein G-Sepharose (Amersham Biosciences). Immune complexes were washed three times with TBS (50 mM Tris-HCl, pH 7.4, and 150 mM NaCl) and resolved in 20 µl of TBS and 2 x Laemmli buffer. Samples were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and visualized by immunostaining.

4.7. Immunofluorescence Microscopy

Cultured cells grown on glass coverslips were first fixed for 30 min. with 4% paraformaldehyde. After blocking with 1% BSA, 0.3% (vol/vol) Triton X-100 and 1% normal goat serum for 30 min, the cells were incubated with blocking buffer containing primary antibodies against active form of HER2 and HER3 (dilution 1:100, Santa Cruz, CA, USA) at 4 °C overnight. After washing with PBS thoroughly, cells were incubated with cy3-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 1 hour at room temperature. Following that, cells were washed and mounted with ProLong Gold anti-fade reagent with DAPI nuclear (Invitrogen, Carlsbad, CA, USA). The immuno-expression of erbB2 and erbB3 were photomicrographed with the Leica TCS SP5 spectral confocal system (Leica, Wetzlar, Germany). Images shown depict single confocal sections. Images were processed using NIH ImageJ.

4.8. Transfection with Small Interfering RNA (siRNA) and Lentiviral shRNA

Cells (1×10⁶) were transfected with 450 nM ONTARGET plus SMART pool Human HER3 siRNA (Thermo-Fisher Scientific–Dharmacon, Epsom, UK) or control siRNA (AllStars Negative Control siRNA, Qiagen, Hilden, Germany) by Lipofectamin 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, 4µl of lipofectamin and 100nM siRNA were mixed together in equal volumes, in serum free medium (SFM) in poly-styrene tubes and allowed to incubate at room temperature for 30 minutes. All the media from the wells was aspirated off and 500 µl of the siRNA mix was added to each well along with 1.5ml of media. Downregulation of FAK expression was determined by western blot analysis. pLKO.1-puro vectors encoding shRNA directed against HER3 and the nontarget sequence control were obtained from Sigma-Aldrich. The ViraPower lentiviral expression system (Invitrogen) was used for an optimized mixture of packaging plasmids that supply the structural and replication proteins that are required to produce lentivirus in HEK293T cells.

4.9. Data Analysis

All experiments were repeated three or more times. The results are represented as means ± standard deviations (SDs). The difference between two mean values was analyzed using Student's *t*-test and was considered statistically significant when *p* < 0.05.

5. Conclusions

This study investigates how neuregulin (NRG) promotes migration in head and neck cancer (HNC) cells via HER3 signaling pathways, including HER2, PI3K/Akt, ERK, and FAK. NRG secretion from active dorsal root ganglia (DRGs) enhances cancer cell migration toward neural elements, contributing to perineural invasion. HER3/HER2 dimerization and HER3-FAK interactions are essential for downstream signaling, and HER3 knockdown or inhibition significantly reduces migration. The HER3-specific antibody AV-203 disrupts NRG-HER3 signaling, suggesting its

potential as a therapeutic target. The findings highlight the importance of targeting HER3, possibly in combination with HER2, PI3K, or FAK inhibitors, to suppress migration and improve HNC outcomes.

Author Contributions: Y.-H.K. and E.J.L. conceived and designed the experiments; E.J.L. and Y.J. Y. performed the experiments; E.J. L., Y.J. Y., J. H., S. K. and Y.-H. K. analyzed the data; E.J. L., Y.J. Y., Y.-H.C. and Y.-H.K. contributed reagents/materials/analysis tools; E.J.L., Y.-H.C. and Y.-H. K wrote the paper.

Funding: This work was supported by Basic Science Research Program through the NRF funded by the Ministry of Education, Science and Technology (2015R1D1A1A01058476) and the Korea government (MSIT) (RS2023-00270936).

Institutional Review Board Statement: The animal experiments were conducted under the approval and guidance of the Experimental Animal Management and Use Committee of Kosin University College of Medicine, Busan, Korea (approval number: KMAP -19-18).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We thank Dr. Seungwon Kim of the University of Pittsburgh School of Medicine for his kind assistance with the mouse DRG experiments and results.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Johnson, D.E.; Burtneess B.; Leemans C.R.; Lui V.W.Y.; Bauman J.E.; Grandis J.R. Head and neck squamous cell carcinoma. *Nat. Rev. Dis. Primers*. **2020**, *6*, 92.
2. Bhat, G.R.; Hyole, R.G.; Li, J. Head and neck cancer: Current challenges and future perspectives. *Adv. Cancer Res*. **2021**, *152*, 67-102.
3. Ghosh, S.; Shah, P.A.; Johnson, F.M. Novel Systemic Treatment Modalities Including Immunotherapy and Molecular Targeted Therapy for Recurrent and Metastatic Head and Neck Squamous Cell Carcinoma. *Int. J. Mol. Sci*. **2022**, *23*, 7889.
4. Samara, P.; Athanasopoulos, M.; Mastronikolis, S.; Kyrodimos, E.; Athanasopoulos, I.; Mastronikolis, N.S. The Role of Oncogenic Viruses in Head and Neck Cancers: Epidemiology, Pathogenesis, and Advancements in Detection Methods. *Microorganisms*. **2024**, *12*, 1482.
5. Prieto-Fernández, L.; Montoro-Jiménez, I.; de Luxan-Delgado, B.; Otero-Rosales, M.; Rodrigo, J.P.; Calvo, F.; García-Pedrero, J.M.; Álvarez-Teijeiro, S. Dissecting the functions of cancer-associated fibroblasts to therapeutically target head and neck cancer microenvironment. *Biomed. Pharmacother*. **2023**, *161*, 114502.
6. Wang, H.; Zheng, Q.; Lu, Z.; Wang, L.; Ding, L.; Xia, L.; Zhang, H.; Wang, M.; Chen, Y.; Li, G. Role of the nervous system in cancers: a review. *Cell Death Discov*. **2021**, *7*, 76.
7. Misztal, C.I.; Green, C.; Mei, C.; Bhatia, R.; Torres, J.M.V.; Kamrava, B.; Moon, S.; Nicolli, E.; Weed, D.; Sargi, Z.; Dinh, C.T. Molecular and Cellular Mechanisms of Perineural Invasion in Oral Squamous Cell Carcinoma: Potential Targets for Therapeutic Intervention. *Cancers (Basel)*. **2021**, *13*, 6011.
8. Liang, S.; Hess, J. Tumor Neurobiology in the Pathogenesis and Therapy of Head and Neck Cancer. *Cells*. **2024**, *13*, 256.
9. Fagan, J.J.; Collins B.; Barnes, L.; D'Amico, F.; Myers, E. N.; Johnson, J.T. Perineural invasion in squamous cell carcinoma of the head and neck. *Arch. Otolaryngol. Head Neck Surg*. **1998**, *124*, 637-640.

10. Balamucki, C.J.; Mancuso, A.A.; Amdur, R.J.; Kirwan, J.M.; Morris, C.G.; Flowers, F.P.; Stoer, C.B.; Coggnetta, A.B.; Mendenhall, W.M. Skin carcinoma of the head and neck with perineural invasion. *Am. J. Otolaryngol.* **2012**, *33*, 447-454.
11. Guo, J.A.; Hoffman, H.I.; Shroff, S.G.; Chen, P.; Hwang, P.G.; Kim, D.Y.; Kim, D.W.; Cheng, S.W.; Zhao, D.; Mahal, B.A.; Alshalalfa, M.; Niemierko, A.; Wo, J.Y.; Jay S Loeffler, J.S.; Castillo, C.F.; Jacks, T.; Aguirre, A.J.; Hong, T.S.; Mino-Kenudson, M.; Hwang, W.L. Pan-cancer Transcriptomic Predictors of Perineural Invasion Improve Occult Histopathologic Detection. *Clin. Cancer Res.* **2021**, *27*, 2807-2815.
12. Britsch, S. The neuregulin-I/ErbB signaling system in development and disease. *Adv. Anat. Embryol Cell Biol.* **2007**, *190*, 1-65.
13. Lin, M.; Klaus-Armin, N. Neuregulin-ERBB signaling in nervous system development and neuropsychiatric diseases. *Neuron.* **2014**, *83*, 27-49.
14. Brinkmann, B.G.; Agarwal, A.; Sereda, M.W.; Garratt, A.N.; Müller, T.; Wende, H.; Stassart, R.M.; Nawaz, S.; Humml, C.; Velanac, V.; Radyushkin, K.; Goebbels, S.; Fischer, T.M.; Franklin, R.J.; Lai, C.; Ehrenreich, H.; Birchmeier, C.; Schwab, M.H.; Nave, K.A. Neuregulin-1/ErbB signaling serves distinct functions in myelination of the peripheral and central nervous system. *Neuron.* **2008**, *59*, 581-595.
15. Hardeep, K.; Arsalan, A.; Soheila, K.A. Neuregulin-1/ErbB network: An emerging modulator of nervous system injury and repair. *Prog. Neurobiol.* **2019**, *180*, 101643.
16. Hsieh, A.C.; M M Moasser, M.M. Targeting HER proteins in cancer therapy and the role of the non-target HER3. *Br J Cancer.* **2007**, *97*(4), 453-457.
17. Sergina, N.V.; Rausch, M.; Wang, D.; Blair, J.; Hann, B.; Shokat, K.M.; Moasser, M.M. Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. *Nature.* **2007**, *445*, 437-441.
18. Gao, L.; Zhang, Y.; Feng, M.; Shen, M.; Yang, L.; Wei B.; 1, Zhou, Y.; Zhang, Z. HER3: Updates and current biology function, targeted therapy and pathologic detecting methods. *Life Sci.* **2024**, *357*, 123087.
19. Mishra, R.; Patel, H.; Alanazi, S.; Yuan, L.; Garrett, J.T. HER3 signaling and targeted therapy in cancer. *Oncol. Rev.* **2018**, *12*, 355.
20. Bahar, M.E.; Kim, H.J.; Kim, D.R. Targeting the RAS/RAF/MAPK pathway for cancer therapy: from mechanism to clinical studies. *Signal. Transduct. Target Ther.* **2023**, *8*, 455.
21. Albanell, J.; Codony, J.; Rovira, A.; Mellado, B.; Gascón, P. Mechanism of action of anti-HER2 monoclonal antibodies: scientific update on trastuzumab and 2C4. *Adv. Exp. Med. Biol.* **2003**, *532*, 253-268.
22. de Visser K.E.; Joyce J.A. The evolving tumor microenvironment: From cancer initiation to metastatic outgrowth. *Cancer Cell.* **2023**, *41*(3), 374-403.
23. Gandullo-Sánchez, L.; Ocaña, A.; Pandiella, A. HER3 in cancer: from the bench to the bedside. *J. Exp. Clin. Cancer Res.* **2022**, *41*, 310.
24. Gala, K.; Chandarlapaty, S. Molecular pathways: HER3 targeted therapy. *Clin Cancer Res.* **2014**, *20*, 1410-1416.
25. Choi, B.K.; Fan, X.; Deng, H.; Zhang, N.; An, Z. ERBB3 (HER3) is a key sensor in the regulation of ERBB-mediated signaling in both low and high ERBB2 (HER2) expressing cancer cells. *Cancer Med.* **2012**, *1*, 28-38.

26. Liu, L.; Soler, J.; Reckamp, K.L.; Sankar, K. Emerging Targets in Non-Small Cell Lung Cancer. *Int. J. Mol. Sci.* **2024**, *25*, 10046.
27. Xiao, Z.; Carrasco, R.A.; Schifferli, K.; Kinneer, K.; Tammali, R.; Chen, H.; Rothstein, R.; Wetzel, L.; Yang, C.; Chowdhury, P.; Tsui, P.; Steiner, P.; Jallal, B.; Herbst, R.; Hollingsworth, R.E.; Tice, D.A. A Potent HER3 Monoclonal Antibody That Blocks Both Ligand-Dependent and -Independent Activities: Differential Impacts of PTEN Status on Tumor Response. *Mol. Cancer Ther.* **2016**, *15*, 689-701.
28. Wei, C.; Guo, Y.; Ci, Z.; Li, M.; Zhang, Y.; Zhou, Y. Advances of Schwann cells in peripheral nerve regeneration: From mechanism to cell therapy. *Biomed. Pharmacother.* **2024**, *175*, 116645.
29. Chen, Z.; Fang, Y.; Jiang, W. Important Cells and Factors from Tumor Microenvironment Participated in Perineural Invasion. *Cancers (Basel)*. **2023**, *15*, 1360.
30. Chen, S.H.; Zhang, B.Y.; Zhou, B.; Zhu, C.Z.; Sun, L.Q.; Feng, Y.J. Perineural invasion of cancer: a complex crosstalk between cells and molecules in the perineural niche. *Am. J. Cancer Res.* **2019**, *9*, 1-21.
31. de Visser, K.E.; Joyce, J.A. The evolving tumor microenvironment: From cancer initiation to metastatic outgrowth. *Cancer Cell*. **2023**, *41*, 374-403.
32. Cui, Q.; Jiang, D.; Zhang, Y.; Chen, C. The tumor-nerve circuit in breast cancer. *Cancer Metastasis Rev.* **2023**, *42*, 543-574.
33. Lengerich, B.V.; Agnew, C.; Puchner, E.M.; Huang, B.; Jura, N. EGF and NRG induce phosphorylation of HER3/ERBB3 by EGFR using distinct oligomeric mechanisms. *Proc. Natl. Acad. Sci. USA*. **2017**, *114*, E2836-E2845.
34. Wu, Y.; Zhang, Y.; Wang, M.; Li, Q.; Qu, Z.; Shi, V.; Kraft, P.; Kim, S.; Gao, Y.; Pak, J.; Youngster, S.; Horak, I.D.; Greenberger, L.M. Downregulation of HER3 by a novel antisense oligonucleotide, EZN-3920, improves the antitumor activity of EGFR and HER2 tyrosine kinase inhibitors in animal models. *Mol. Cancer Ther.* **2013**, *12*, 427-437.
35. Udagawa, H.; Nilsson, M.B.; Robichaux, J.P.; He, J.; Poteete, A.; Jiang, H.; Heeke, S.; Elamin, Y.Y.; Shibata, Y.; Matsumoto, S.; Yoh, K.; Okazaki, S.; Masuko, T.; Odintsov, I.; Somwar, R.; Ladanyi, M.; Goto, K.; Heymach, J.V. HER4 and EGFR Activate Cell Signaling in NRG1 Fusion-Driven Cancers: Implications for HER2-HER3-specific Versus Pan-HER Targeting Strategies. *J. Thorac. Oncol.* **2024**, *19*, 106-118.
36. Hauck, C.R.; Hsia, D.A.; Schlaepfer, D.D. The focal adhesion kinase-a regulator of cell migration and invasion. *IUBMB Life*. **2002**, *53*, 115-119.
37. Zhao, J.; Guan, J.L. Signal transduction by focal adhesion kinase in cancer. *Cancer Metastasis Rev.* **2009**, *28*, 35-49.
38. Zeng, H.; Wang, W.; Lin, Z. HER3-targeted therapy: the mechanism of drug resistance and the development of anticancer drugs. *Cancer Drug Resist.* **2024**, *7*, 14.
39. Chuang, H.H.; Zhen, Y.Y.; Tsai, Y.C.; Chuang, C.H.; Hsiao, M.; Huang, M.S.; Yang, C.J. FAK in Cancer: From Mechanisms to Therapeutic Strategies. *Int. J. Mol. Sci.* **2022**, *23*, 1726.
40. Lee, B.Y.; Timpson, P.; Horvath, L.G.; Daly, R.J. FAK signaling in human cancer as a target for therapeutics. *Pharmacol. Ther.* **2015**, *146*, 132-149.
41. Kwantwi, L.B.; Tandoh, T. Focal adhesion kinase-mediated interaction between tumor and immune cells in the tumor microenvironment: implications for cancer-associated therapies and tumor progression. *Clin. Transl. Oncol.* **2024**,

42. Dai, H.; Li, R.; Wheeler, T.; Ozen, M.; Ittmann, M.; Anderson, M.; Wang, Y.; Rowley, D.; Younes, M.; Ayala, G.E. Enhanced survival in perineural invasion of pancreatic cancer: an in vitro approach. *Hum. Pathol.* **2007**, *38*, 299-307.
43. Schmitd, L.B.; Scanlon, C.S.; D'Silva, N.J. Perineural Invasion in Head and Neck Cancer. *J. Dent. Res.* **2018**, *97*, 742–750.
44. Frunza, A.; Slavescu, D.; Lascar, I. Perineural invasion in head and neck cancers - a review- *J. Med. Life.* **2014**, *7*, 121–123.
45. Pei, M.; Wiefels, M.; Harris, D.; Velez Torres, J.M.; Gomez-Fernandez, C.; Tang J.C.; Hernandez Aya, L.; Samuels, S.E.; Sargi, Z.; Weed, D.; Dinh, C.; Kaye, E.R. Perineural Invasion in Head and Neck Cutaneous Squamous Cell Carcinoma. *Cancers (Basel)*. **2024**, *16*, 3695.
46. Malin, S.A.; Davis, B.M.; Molliver, D.C. Production of dissociated sensory neuron cultures and considerations for their use in studying neuronal function and plasticity. *Nat. Protoc.* **2007**, *2*, 152-160.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.