

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

Eun Jin Lim

Kosin University College of Medicine

Yu Jeong Yoon

Kosin University College of Medicine

Jeonghoon Heo

Kosin University College of Medicine

Seungwon Kim

University of Pittsburgh Medical Center

Yung-Hyun Choi

Dong-eui University

Young-Ho Kim

kimyh@kosin.ac.kr

Kosin University College of Medicine

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Abstract

Purpose

To investigate the role of neuregulin (NRG) signaling in promoting head and neck squamous cell carcinoma (HNSCC) migration through HER3-dependent pathways and to assess the therapeutic potential of targeting the NRG/HER3 axis in mitigating perineural invasion.

Methods

NRG-driven migration was studied using DRG co-culture, wound healing assays, and HER3 inhibition (shRNA, AV-203). The biological function and biochemical effects of the HER3/HER2/FAK axis in response to NRG were analyzed via phosphorylation assays, knockdown, western blotting, and cell staining for protein expression.

Results

NRG promoted directional migration of FaDu and TU138 HNSCC cells through HER3/HER2 and HER3/PI3K interactions. HER3 inhibition (shRNA or AV-203) abolished HER3 phosphorylation, disrupted HER3-HER2 interactions, and suppressed AKT and ERK signaling. Wound healing assays confirmed that NRG enhances migration via HER3 activation. NRG also induced HER3-dependent FAK phosphorylation, and FAK knockdown or inhibition with PF228 significantly reduced NRG-driven migration, highlighting the critical role of HER3-FAK signaling.

Conclusion

NRG promotes HNSCC cell migration by activating HER3, forming HER3-HER2 and HER3-FAK complexes, and driving downstream AKT, ERK, and FAK signaling. Targeting the NRG/HER3 axis holds potential as a therapeutic strategy to address perineural invasion and associated clinical challenges in HNC.

1 Introduction

Head and neck squamous cell carcinoma (HNSCC) 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 HNSCC 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 HNSCC.

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 HNSCC 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. Recent studies have shown a major role for HER3 signalling-mediated FAK activity in breast cancer [42], NRG-mediated *ErbB2/3*-dependent FAK signalling in Schwann cell migration [43], and Src-mediated FAK signalling in cell migration and growth [44]. HER3 signalling to Neuregulin was found to be involved in FAK phosphorylation. 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 Materials and methods

2.1. 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, USA). Recombinant human neuregulin (NRG1-beta, #396-HB-50) was prepared by R&D Systems (MN, USA). Antibodies against HER2 (#284), HER3 (#285), ERK (#153), FAK (#1688) and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-phospho-HER3 (#4791), anti-phospho-HER2 (#2247), anti-phospho-FAK (#8556 and #3284), anti-phospho-AKT (#9271), and anti-phospho-ERK (#9101) were purchased from Cell Signaling (Beverly, MA, USA). All other chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. 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) in PBS diluent for 24 h and controls were stimulated with PBS alone. Pre-treatment with lapatinib (a HER2 Tyrosine kinase inhibitor), LY294002 (LY, a PI3K inhibitor), AV-203 (a HER3 monoclonal antibody) and cetuximab (an EGFR monoclonal antibody) were performed for 24 h unless otherwise indicated.

2.3. 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).

2.4. 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-1 β 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.

2.5. 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 [45]. 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.

2.6. 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 μ g), 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 μ l of TBS and 2 x Laemmli buffer. Samples were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and visualized by immunostaining.

2.7. 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.

2.8. Transfection with small interfering RNA (siRNA) and Lentiviral shRNA

Cells (1×10^6) were transfected with 450 nM ONTARGET plus SMART pool Human *ERBB3* 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, 4 μ l 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 μ l 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 *ERBB3* 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.

2.9. Data analysis

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

3 Results

3.1. 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 HNSCC cells towards surrounding nerves, we employed a Matrigel dorsal root ganglion (DRG) co-culture assay, as described previously [46]. DRG activity was confirmed through the expression of neuronal and Schwann cell markers, with distinct expression patterns observed (sFig.1). During the culture of isolated tissue DRGs, inactivated DRGs, which do not undergo neurogenesis after 96 hours, were isolated and used in the experiments. Activated DRGs can be observed to develop neurons (sFig.1). The FaDu head and neck cancer cell line was co-cultured with DRGs to assess directional migration towards neurites extended by the DRG. Neuronal activity occurred in the DRG placed on matrigel, and after 24 hours, FaDu cells observed a significant increase in cell migration towards the DRG (Fig. 1a). In contrast, co-culture with inactive DRGs significantly reduced the migration of FaDu cells to DRG neurites (Fig. 1b). To generalize this observation, two HNSCC 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). The DRGs stimulated more cells to migrate in the developed environment of the neurons, while DRGs that lost their neuronal activity inhibited cell migration. These results suggest that DRG activation enhances the ability of HNSCC cells to migrate.

3.2. 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 HNSCC 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 HNSCC cell migration through activation of the HER3/AKT/ERK signaling axis. TU138 cells exhibit lower HER3 expression, whereas FaDu cells display relatively higher expression in HNSCC (sFig.2). Additional analysis confirmed that, regardless of the differences in origin and p53 mutations between the two cell lines, our study focused on the differences in HER3 expression in HNSCC. These results highlight the critical role of NRG-induced HER3/AKT/ERK signaling in enhancing the migratory ability of HNSCC cells.

3.3. 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. These findings indicate that the formation of HER3-HER2 and HER3-PI3K complexes upon NRG stimulation is a key mechanism underlying HER3 activation.

3.4. AV-203 inhibits activation of HER3 and AKT/ERK in FaDu and TU138 cells

To determine whether NRG is activated by HER3 and EGFR signalling, we used a HER3 antibody, AV-203, and an EGFR antibody, Cetuximab (Cetu). Phosphorylation of HER3, HER2, AKT, and ERK proteins was compared in FaDu and TU138 cells pretreated with AV-203, Cetu, and then NRG. Reduced HER3 and HER2 phosphorylation was observed in FaDu cells after AV-203 and cetuximab treatment, and of course, the reduced HER3 phosphorylation was fully effective in TU138 cells. Notably, even without NRG treatment, TU138 cells consistently exhibited the characteristics of cells with increased HER3 phosphorylation, but AV-203 treatment reduced HER3 phosphorylation (Fig. 4a). We observed AKT and ERK phosphorylation in FaDu and TU138 cells after pretreatment with AV-203 and cetuximab antibodies prior to NRG treatment (Fig. 4b). After AV-203 treatment, AKT and ERK phosphorylation was reduced in both FaDu and TU138 cells. In TU138 cells, we also observed a decrease in both AKT/ERK phosphorylation overexpressed in control conditions. However, no significant changes in AKT/ERK phosphorylation were observed in Cetuximab pretreated cells. This suggests that NRG signalling is HER3-dependent and that EGFR activity can be ruled out. In addition, TU138 cells consistently exhibited

the characteristics of cells with increased HER3/AKT/ERK phosphorylation, but AV-203 treatment reduced phosphorylation. To investigate the role of the HER3/HER2 signaling pathway in HNSCC 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). TU138 cells are a cell line in which HER3, AKT/ERK phosphorylation is always active under control conditions. Therefore, we performed our experiments using FaDu cells, realising that the NRG effect on HER3 signalling in TU138 cells was weak, whereas the NRG effect in FaDu cells was dependent on HER3 signalling. However, it can also be suggested that the activated AKT/ERK signalling in TU138 cells would benefit from HER2 TKIs and AKT inhibitors (sFig.3). 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 results showed that AV-203 has a strong inhibitory activity on phosphorylation of HER3, AKT and ERK in HNSCC cells.

3.5. 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 Fig. 5a, in the parental cells, 100% of the cells migrated 24 hours after NRG treatment compared to the control, while in the *shHER3* cells, the migration of the control cells was about 18% and the NRG-treated cells migrated about 15%. In the wound healing assay, parental FaDu cells displayed robust migratory activity, with cell migration increasing by 3.6-fold and 6.6-fold compared to HER3 knockdown (*shHER3*) (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.

3.6. 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. We confirmed an effective siRNA targeting FAK by western blotting (sFig. 4). In Fig. 6b, siCon cells showed 100% migration after 24 hours of NRG treatment, whereas *siFAK* cells showed approximately 44% migration after NRG treatment. These results suggest that knockdown of FAK resulted in an approximately 2.2-fold decrease in basal migration compared to control cells. 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.

4 Discussion

This study investigated the mechanisms underlying NRG-induced cell migration in head and neck cancer, with a particular focus on the HER3 signaling pathway and its interactions with downstream effectors. Our findings provide strong evidence that NRG promotes HNSCC 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 HNSCC 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 HNSCC [7, 47–49]. 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. However, our results showed that TU138 cells

have weak HER3 signalling in response to NRG stimulation and overexpressed HER3, AKT and ERK phosphorylation even in the absence of NRG stimulation (Fig. 4a, 4b). These results suggest that TU138 cells are significantly desensitised to HER3 signalling, presumably by the constant secretion of NRG (sFig.2), and that the consistently active HER3 signalling, AKT/ERK pathway is involved in cell migration and growth of TU138 cells. Currently, we are conducting further studies on NRG-induced cell migration in other cell lines. In this study, we focused on FaDu cells to investigate the intracellular signalling in response to NRG stimulation. This differential activation reflects the heterogeneity of signaling dependencies in HNSCC 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]. In addition to targeting HER3, we propose that strategies that inhibit intracellular oncogenes to inhibit cell growth and migration may be a way to overcome HER3-resistant cancer cells. Using different antibody models of HER3, recent papers have documented the inhibitory ability of serivantumab, blocking HER2 and HER3 dimerization, in a colorectal [50], pancreatic [51], breast [52] and bladder [53] cancer cells.

Our results demonstrate that NRG-induced HER3/HER2 dimerization is pivotal for downstream signaling. Fluorescence microscopy confirmed HER3/HER2 co-localization upon NRG stimulation (data not shown), 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. Furthermore, in experiments to determine whether EGFR signalling is involved in NRG-mediated HER3 signalling (Fig. 4a, 4b), we found that NRG-mediated HER3 activation is dependent on AKT/ERK pathway but not dependent on EGFR signalling. 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. Overexpression of HER2 and HER3 in HNSCC patients is often associated with treatment resistance. Increased expression of HER3 may induce resistance to other anticancer therapies, highlighting the need for new targeted therapies. For example, a higher HER2/HER3 ratio may be associated with a lower response to anticancer drugs [54]. Recent studies have shown that high expression of HER3 and HER2 is associated with decreased overall survival (OS) and increased distant metastases. Some studies have also shown that cell membrane expression of HER3 is strongly associated with poor prognosis. These findings highlight the clinical utility of the HER2/HER3 axis in HNSCC patients [55]. One study showed that higher HER2 expression in HNSCC patients was associated with poorer survival and higher metastasis rates. These clinical connections may provide important guidance for developing targeted therapeutic approaches to HER3/HER2 [56, 57]. 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 Fig. 7, our findings demonstrate the potential of targeting the NRG-HER3 axis in HNSCC, 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 HNSCC 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 HNSCC progression.

A significant correlation was observed between NRG1 expression levels and cancer growth inhibition by AV-203. Cancer growth inhibition was not correlated with *ERBB3* expression, and the correlation between NRG1 expression levels in tumors and response to *ERBB3* inhibition by AV-203 was further validated using a patient-derived tumor xenograft model [58]. Huilan et al [59] colleagues explain their findings as follows. Because HER3 has weak tyrosine kinase activity, upon the appearance of HER3 ligands, neuregulin 1 (NRG1) or neuregulin 2 (NRG2), activated HER3 forms heterodimers with other receptors, mainly epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2), contributing to cancer development and drug resistance. Inhibition of HER3 and its downstream signalling, PI3K/AKT, MEK/MAPK, JAK/STAT, and Src kinases, is believed to be necessary to overcome drug resistance and improve treatment efficiency. To date, several anti-HER3 antibodies are in preclinical and clinical studies, but no HER3-targeted therapies have been licensed for use in clinical cancer treatment due to safety and efficacy concerns.

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 HNSCC. Future studies should explore the signaling dependencies of additional HNSCC 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 HNSCC cell motility and invasion.

In summary, our study establishes the pivotal role of NRG-HER3 signaling in promoting HNSCC 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 HNSCC cells, improving patient outcomes in this aggressive cancer.

Abbreviations

DRG Dorsal root ganglion

EGFR Epidermal growth factor receptor

FAK Focal adhesion kinase

FBS Fetal bovine serum

HER3 Human epidermal growth factor receptor 3

HNSCC Head and neck squamous cell carcinoma

NRG Neuregulin

PNI Perineural invasion

Declarations

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.

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Figures

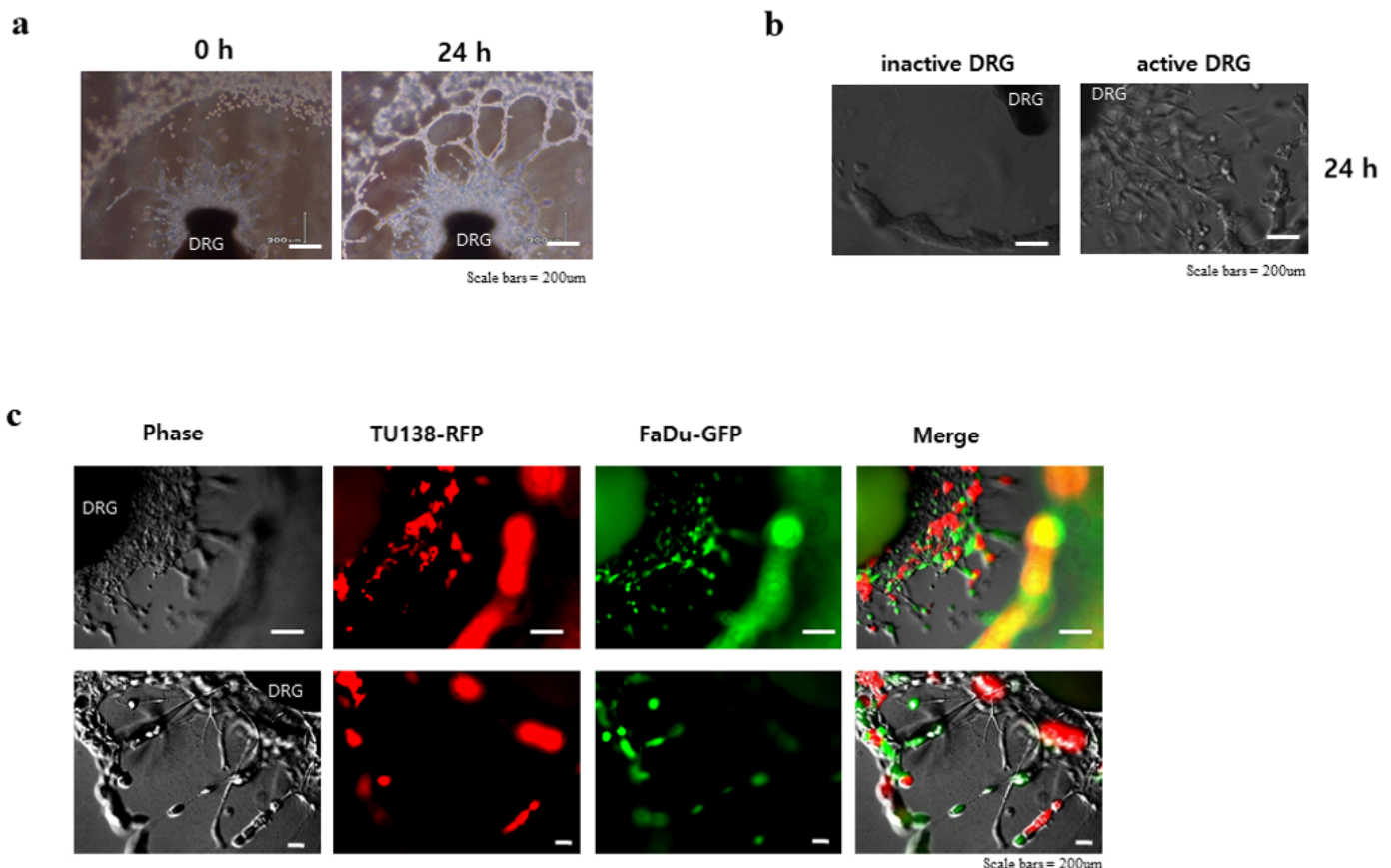


Figure 1

Effect of NRG on the ability of head and neck cells to migrate towards neurites extends from dorsal root ganglia (DRG).

a) FaDu cells migration around the DRG was assessed on the matrix at 0 and 24 hours, analyzing movement to evaluate migratory capacity. b) Migration was compared between DRG-activated and inactivated conditions to determine DRG activity's impact on FaDu cells movement. c) FaDu-GFP (green) and TU138-RFP (red) cells were co-cultured with DRG, and migration was analyzed by overlapping their fluorescence images (Different scale bars in the top and bottom panels).

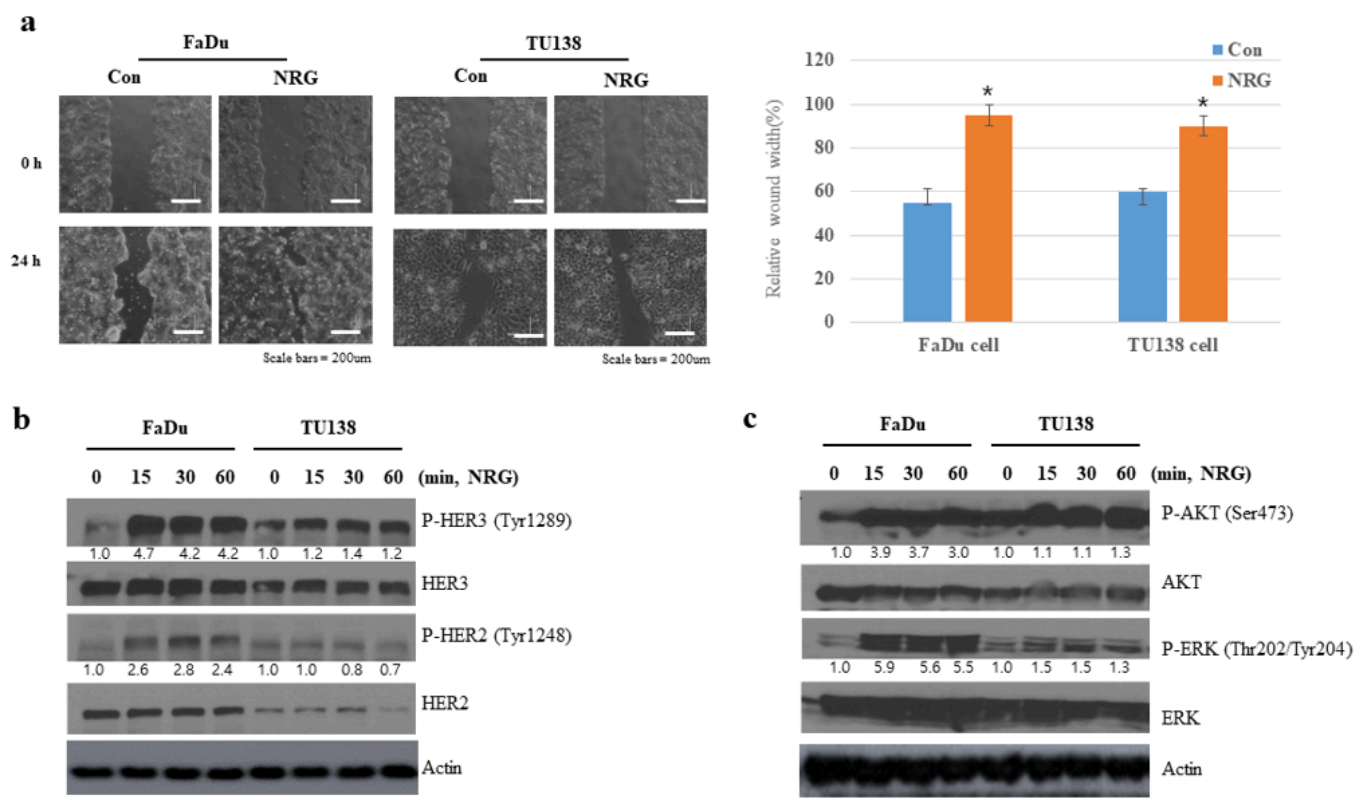


Figure 2

HER3/AKT signaling pathway on NRG treatment in HNSCC

a) FaDu and TU138 cell migration was measured after 24 hours of treatment with 10 ng/ml NRG, comparing treated and untreated groups. b) HER3/HER2 phosphorylation in FaDu and TU138 cells was analyzed over 0–60 minutes following 10 ng/ml NRG treatment. c) AKT and ERK phosphorylation were also assessed under the same conditions. Blots represent NRG-induced changes, with results shown as mean $\pm$ SD. Statistical significance: *p < 0.01 vs. control.

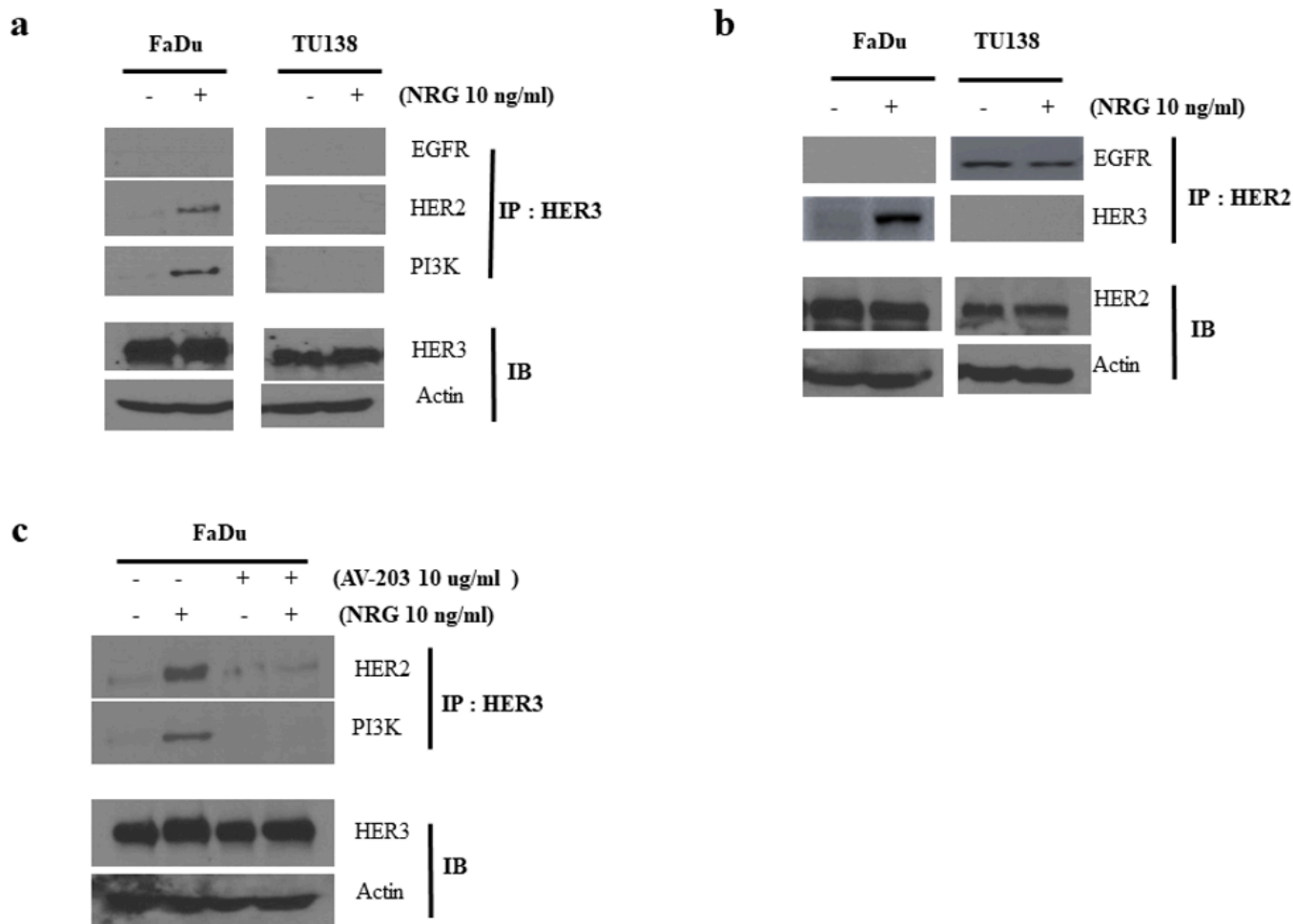


Figure 3

Interaction of HER3 and HER2 on NRG treatment in HNSCC

a, b) FaDu and TU138 cells were treated with 10 ng/ml NRG for 30 minutes. Immunoprecipitation with anti-HER3 or anti-HER2 antibodies, followed by immunoblotting, assessed HER3-HER2 interactions. c) Parent and shHER3 cells were treated with NRG (10 ng/ml) $\pm$ AV203 (1 μ g/ml) for 30 minutes. HER3 immunoprecipitation and immunoblotting analyzed receptor interactions. The blots shown are representative of multiple independent experiments.

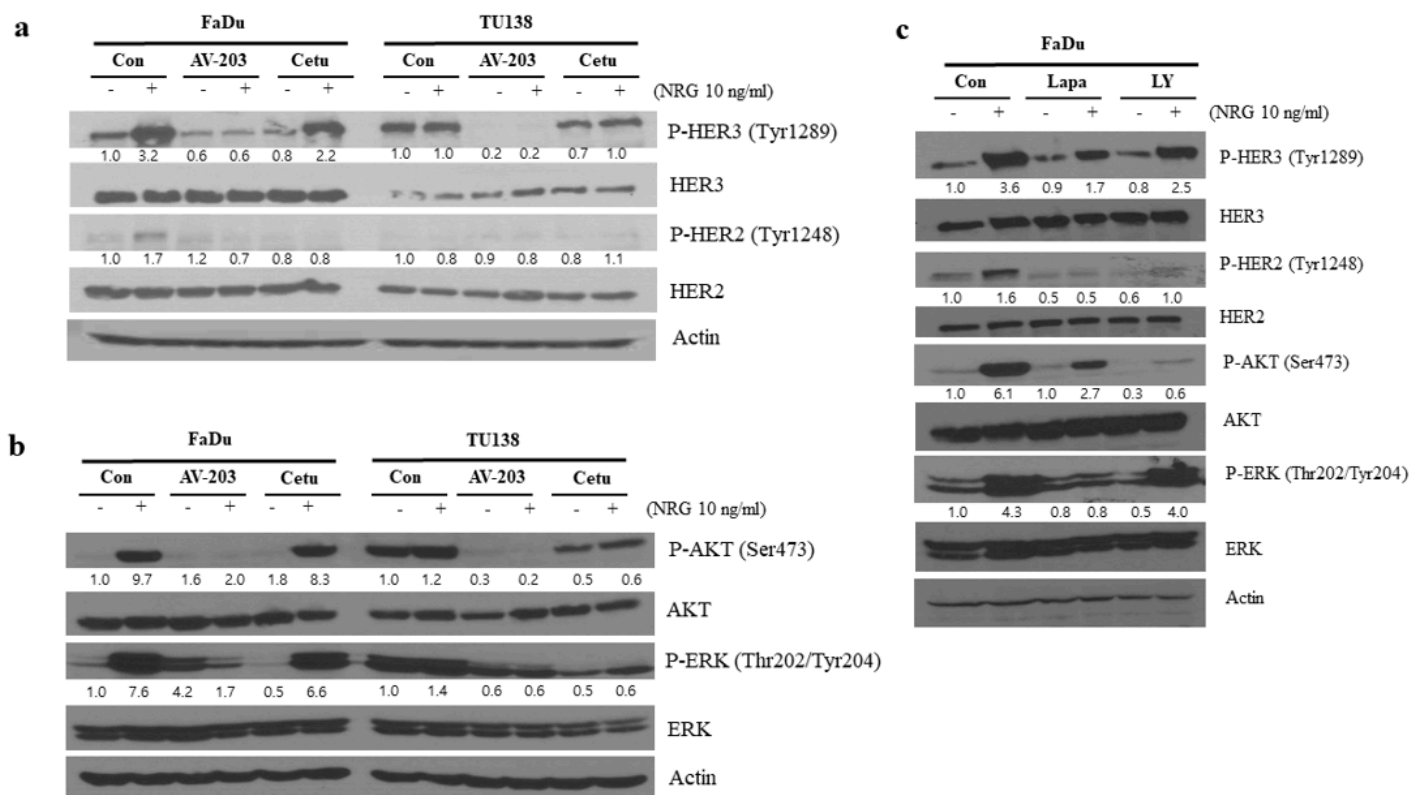


Figure 4

Effects of NRG on HER3-AKT pathway in HNSCC

a) FaDu and TU138 cells were treated with 10 ng/ml NRG for 30 minutes, with or without pre-treatment with 1 μ g/ml AV-203 or 1 μ g/ml Cetuximab (Cetu) for 30 minutes. Western blotting analyzed phosphorylated HER3/HER2 (P-HER3, P-HER2), total HER3/HER2 to evaluate receptor inhibition. b) Phosphorylation of AKT (P-AKT) and ERK (P-ERK) was examined in FaDu and TU138 cells following NRG treatment for 30 minutes with or without pre-treatment using AV-203 or Cetuximab. Western blotting was used to analyze the levels of phosphorylated AKT (P-AKT) and ERK (P-ERK). c) FaDu cells were treated with 10 ng/ml NRG for 30 minutes and pre-treated with either 1 μ M Lapatinib (Lapa) or 1 μ M LY294002 (LY) for 30 minutes. Cell lysates were analyzed by western blotting for phosphorylation of HER3 (P-HER3), HER2 (P-HER2), AKT (P-AKT), and ERK (P-ERK). The blots shown are representative of multiple independent experiments.

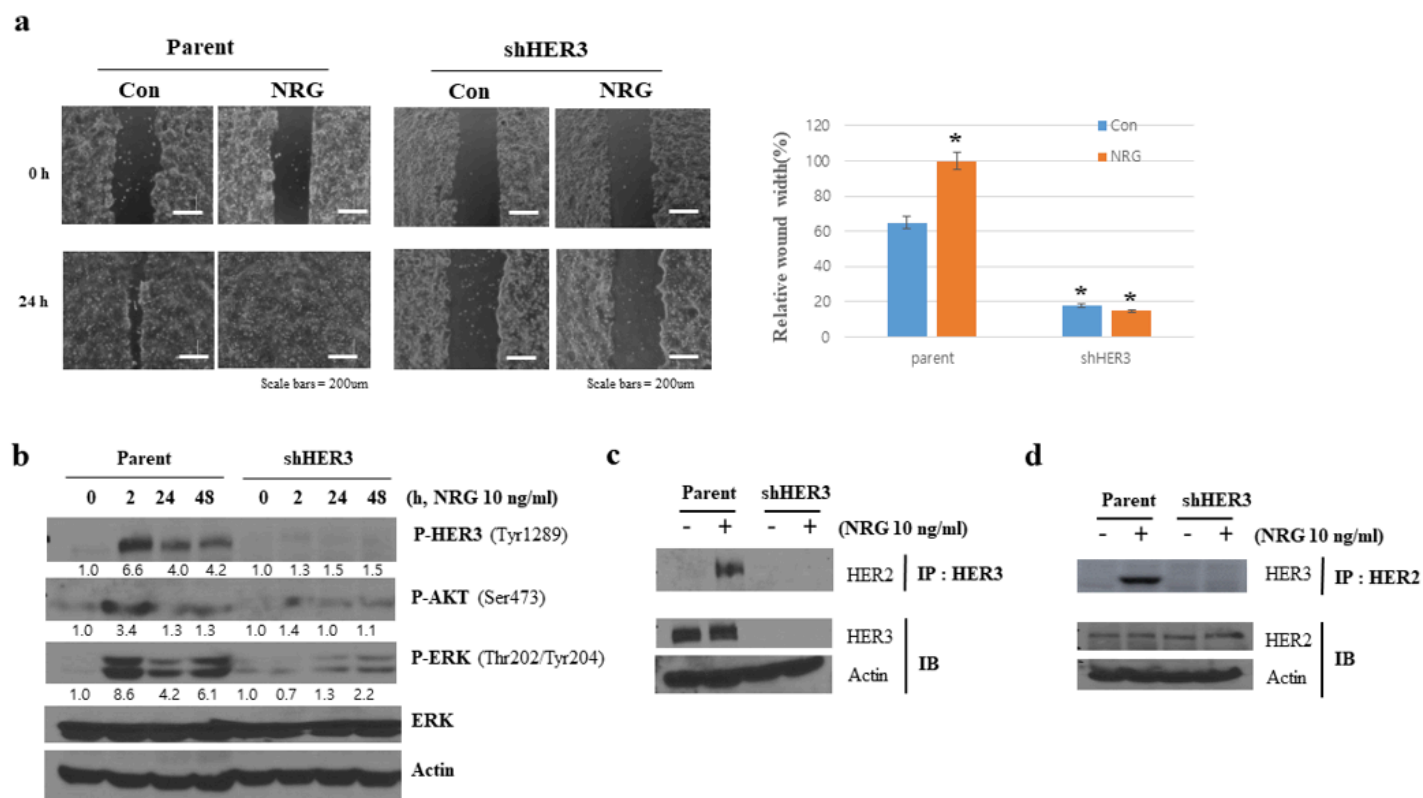


Figure 5

Migration of NRG on HER3 signaling pathway in HNSCC

a) Migration of parent and shHER3 cells was assessed after 24 hours of 10 ng/ml NRG treatment, comparing migration distances between treated and untreated groups to evaluate HER3's role in migration. b) Parent and shHER3 cells were treated with 10 ng/ml NRG for 2–48 hours, and western blotting was performed on cell lysates to analyze time-dependent phosphorylation of HER3, HER2, and downstream effectors. c) After 30 minutes of 10 ng/ml NRG treatment, cell extracts were immunoprecipitated with anti-HER3 antibodies to assess HER3-HER2 interaction. d) HER2-HER3 interaction was examined using anti-HER2 antibodies under the same conditions. Blots represent multiple experiments, with results shown as mean $\pm$ SD. Statistical significance: * $p < 0.01$ vs. parent control cells.

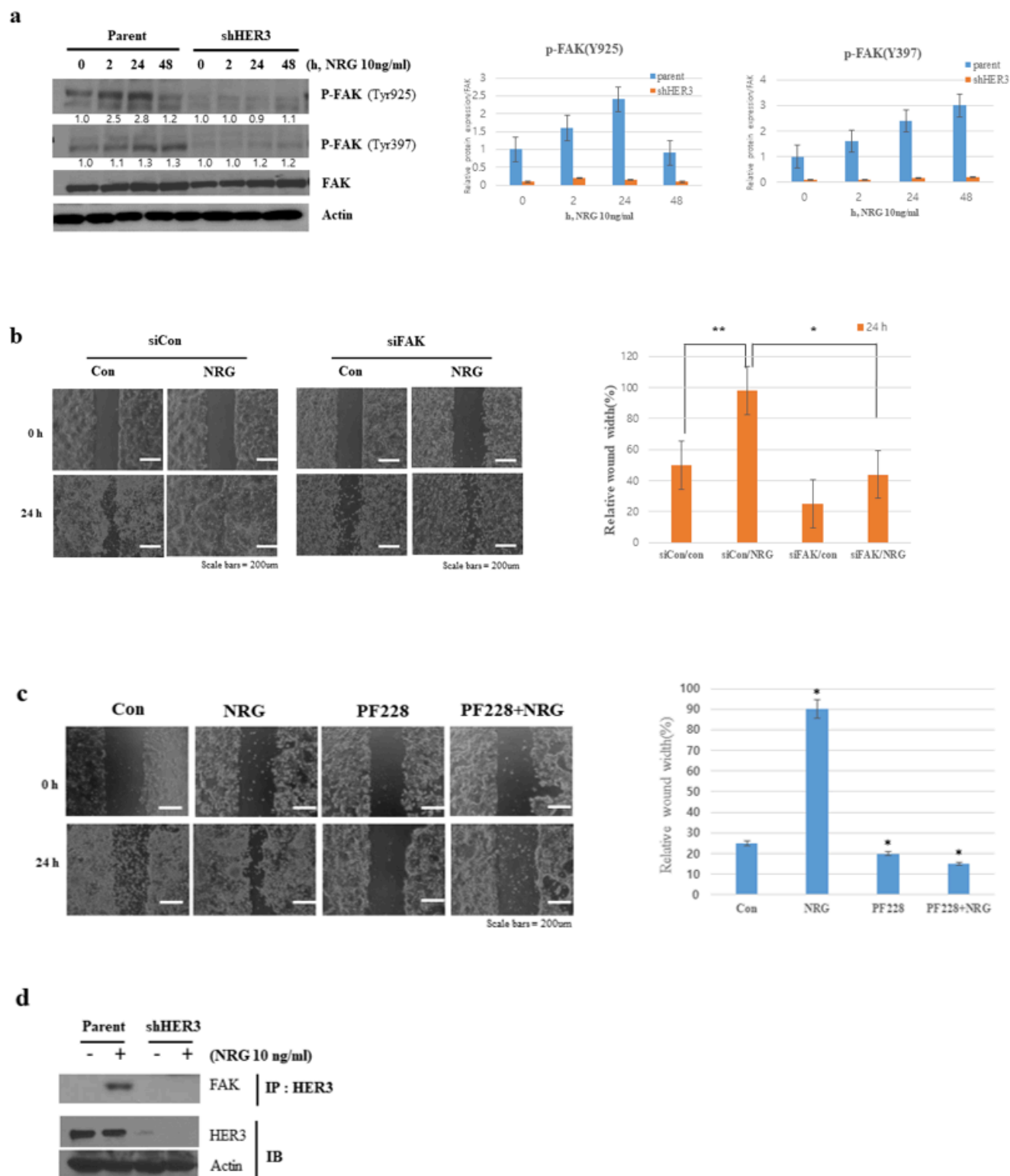


Figure 6

Effects of NRG on migration of HER3-FAK signaling dependent in HNSCC

a) Parent and shHER3 cells were treated with 10 ng/ml NRG for time periods ranging from 2 to 48 hours. Cell lysates were collected, and the expression levels of key signaling proteins were analyzed by western blotting to examine the time-dependent phosphorylation of FAK at tyrosine sites Tyr397(Y397) and

Tyr925(Y925), providing insight into FAK activation in response to NRG treatment. b, c) The migration of siCon and siFAK transfected cells, as well as cells treated with the FAK inhibitor PF228, was assessed after 24 hours of treatment with 10 ng/ml NRG. The migration distance of the cells was compared between NRG-treated and untreated groups to evaluate the role of FAK in NRG-induced cell migration. d) Parent (control) and shHER3 cells were treated with 10 ng/ml NRG for 30 minutes. Cell extracts were immunoprecipitated with anti-HER3 antibodies to investigate the interaction between HER3 and FAK. The blots show the changes in the HER3-FAK interaction in response to NRG treatment. The blots shown that are representative of multiple independent experiments. Results are presented as the mean $\pm$ standard deviation (SD) of the experiments. Statistical significance is indicated as $**p < 0.01$ compared to siCon cells, $*p < 0.05$ compared to siCon/NGR treated cells.

Figure 7

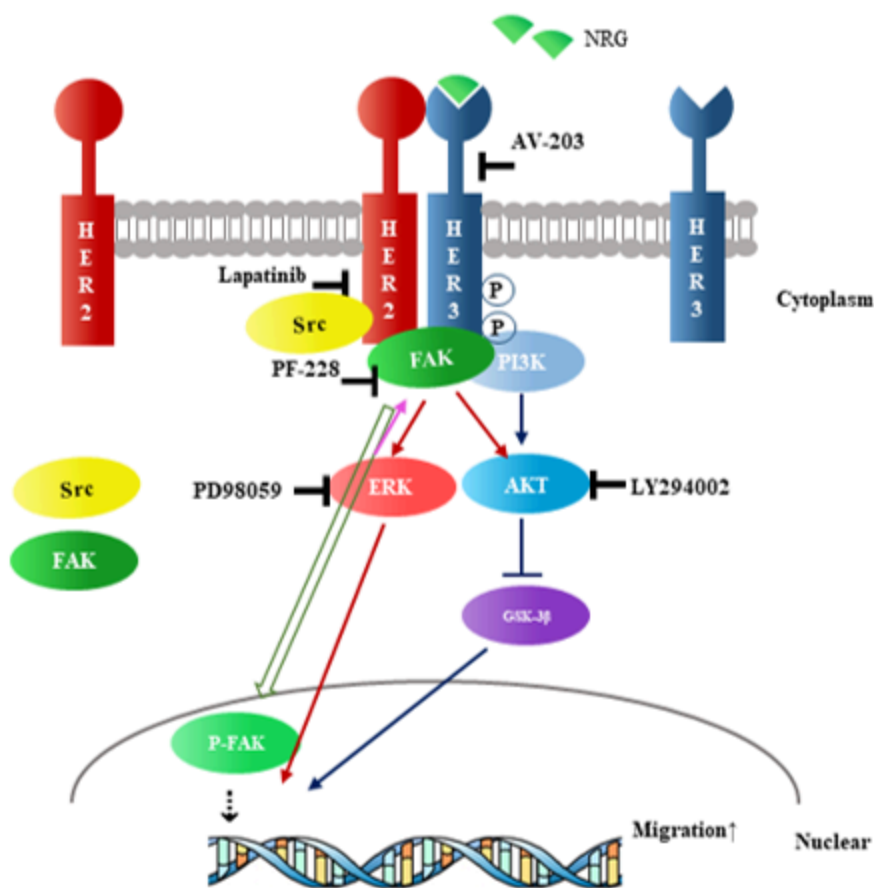


Figure 7

A schematic diagram indicates the proposed mechanism of the NRG-HER3/HER2-FAK axis regulation in HNSCC

Supplementary Files

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