HHS Public Access

Author manuscript

J Thorac Cardiovasc Surg. Author manuscript; available in PMC 2022 September 08.

Published in final edited form as:

J Thorac Cardiovasc Surg. 2021 August ; 162(2): 463–473. doi:10.1016/j.jtcvs.2020.03.166.

Neuropilin-2b facilitates resistance to tyrosine kinase inhibitors in non–small cell lung cancer

Anastasios Dimou, MD[a], **Cecile Nasarre, PhD**[b], **Yuri K. Peterson, PhD**[c], **Rose Pagano, BS**[a], **Monika Gooz, MD, PhD**[c], **Patrick Nasarre, PhD**[d], **Harry A. Drabkin, MD**[a], **Kent E. Armeson, MS**[e], **Barry C. Gibney, DO**[b], **Robert M. Gemmill, PhD**[a], **Chadrick E. Denlinger, MD**[b]

[a]Division of Medical Oncology, Department of Medicine, Medical University of South Carolina, Charleston, SC

[b]Division of Cardiothoracic Surgery, Department of Surgery, Medical University of South Carolina, Charleston, SC

[c]Department of Drug Discovery and Biomedical Sciences, Medical University of South Carolina, Charleston, SC

[d]Department of Surgery, Medical University of South Carolina, Charleston, SC

[e]Department of Public Health Sciences, Medical University of South Carolina, Charleston, SC

Abstract

Objective: Innate and acquired resistance is the principle factor limiting the efficacy of tyrosine kinase inhibitors in lung cancer. We have observed a dramatic upregulation of the cell surface co-receptor neuropilin-2b in lung cancers clinically treated with tyrosine kinase inhibitors correlating with acquired resistance. We hypothesize that neuropilin-2b plays a functional role in acquired tyrosine kinase inhibitor resistance.

Methods: Non–small cell lung cancer proliferation and survival were determined during chronic tyrosine kinase inhibitor exposure in the presence or absence of neuropilin-2b knock-down. Interactions of neuropilin-2a and neuropilin-2b isoforms with PTEN and GSK3β were assessed by immunoprecipitation. Neuropilin-2a and neuropilin-2b mutants deleted for their cytoplasmic domains were used to identify regions responsible for neuropilin-2b-GSK3β interaction. Because

Address for reprints: Chadrick E. Denlinger, MD, Division of Cardiothoracic Surgery, Department of Surgery, Medical University of South Carolina, 114 Doughty St, Charleston, SC 29425 (denlinge@musc.edu).
Drs Dimou and Nasarre contributed equally to this work.
Dr Dimou's current address is Division of Medical Oncology, Mayo Clinic College of Medicine, Rochester, Minn.
Dr Pagano's current address is Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, Pa.
Dr Drabkin's current address is University of Denver, Denver, Colo.

Read at the 99th Annual Meeting of The American Association for Thoracic Surgery, Toronto, Ontario, Canada, May 4-7, 2019.

Webcast
You can watch a Webcast of this AATS meeting presentation by going to: https://aats.blob.core.windows.net/media/19%20AM/Saturday_May4/203AC/203AC/S06%20-%20Lung%20cancer%20immunotherapy/S6_2_webcast_082135731.mp4.

Conflict of Interest Statement
Dr Dimou: personal fees from Roche/Genentech. Dr Nasarre: patent pending: mimetic peptides for the treatment of cancer. Dr Peterson: patent pending: US 62/835,200. Dr Gemmill: patent pending: mimetic peptides for the treatment of cancer. All other authors reported no conflicts of interest.
The *Journal* policy requires editors and reviewers to disclose conflicts of interest and to decline handling or reviewing manuscripts for which they may have a conflict of interest. The editors and reviewers of this article have no conflicts of interest.

GSK3β is known to phosphorylate and degrade PTEN, phospho-PTEN and total PTEN levels were assessed after transfection of neuropilin-2a and neuropilin-2b wild-type and mutant constructs.

Results: Non–small cell lung cancer cells chronically treated with gefitinib or osimertinib developed drug resistance and exhibited logarithmic growth in the presence of endothelial growth factor receptor tyrosine kinase inhibitors. However, neuropilin-2b knockdown cells remained sensitive to gefitinib. Likewise, neuropilin-2b knockdown suppressed and neuropilin-2a knockdown enhanced cellular migration. Acquired drug resistance and cell migration correlated with neuropilin-2b-dependent AKT activation with the intermediate step of GSK3β-dependent PTEN degradation. A specific binding site for GSK3β on the cytoplasmic domain of neuropilin-2b was identified with truncated protein constructs and computer modeling.

Conclusions: Neuropilin-2b facilitates non-small cell lung cancer resistance to tyrosine kinase inhibitors, and this biological effect relates to AKT activation. Neuropilin-2b GSK3β interactions appear to be essential for PTEN degradation and AKT activation in lung cancer cells. Disruption of the neuropilin-2b GSK3β interaction may represent a novel treatment strategy to preserve sensitivity to tyrosine kinase inhibitors in non-small cell lung cancer.

Keywords

lung cancer; tyrosine kinase inhibitor; resistance; migration; survival

---

Lung cancer remains the most frequent cause of cancer-related death in America and throughout the world. Lung adenocarcinoma is currently the dominant histologic subtype, and the incidence of adenocarcinoma continues to rise. The long-term survival for all patients with lung cancer, including patients who undergo surgery with curative intent, is limited by the development of distant metastatic disease, which is most effectively treated with chemotherapy, and by the emergence of therapeutic resistance.

Significant progress has been made regarding the treatment options for select populations of patients found to have specific actionable gene mutations. Molecularly targeted agents have improved the oncological outcomes of patients with lung adenocarcinoma while minimizing side effects. The greatest impact of this class of agents is seen in progression-free survival, whereas they have had a lesser impact on overall survival. Furthermore, the improved progression-free survival afforded by tyrosine kinase inhibitor (TKI) therapies is measured in months rather than years.[1] More robust improvements in patient outcomes are limited by the emergence of resistance to targeted agents, typically by the acquisition of secondary mutations and the development of alternative bypass signaling pathways.[2]

We recently linked drug resistance in lung adenocarcinomas to the expression of an alternatively spliced isoform of the cell surface co-receptor neuropilin-2 (NRP2).[3] NRP2 was originally identified as the receptor for the class 3 semaphorin, SEMA3F, an extracellular signaling molecule important for neuronal path finding and axonal development.[4] Subsequently, NRP2 was observed to interact with numerous cell surface receptor tyrosine kinases and their cognate ligands.[5] Alternative splicing generates 2 isoforms that are identical within the extracellular N-terminus but unique at the intracellular

C-terminus.[6] We reported that the isoforms NRP2a and NRP2b have distinct interactions with intracellular signaling pathways and distinct biological effects.[3]

NPR2a and NRP2b show differential expression throughout the body. In humans, NRP2a is highly expressed in the placenta, liver, and lung.[6] On the other hand, NRP2b is essentially absent from these tissues, but is abundant in skeletal muscle, where NRP2a expression is low. Although NPR2b is not normally abundant in lung or bronchial tissue, its presence is notable in lung cancer specimens, and its level of expression correlates with poor clinical outcomes in lung cancer.[3] Expression of NRP2, without data on specific isoforms, has also been correlated with poor clinical outcomes in other malignancies.[4,7–10]

We observed a dramatic upregulation of NRP2b in clinical specimens correlating with the development of resistance to TKIs.[3] In addition, NRP2b upregulation was found in lung cancer cell lines exposed to low-dose epidermal growth factor receptor (EGFR) TKI (gefitinib) and was important for the emergence of resistance in vitro. Furthermore, we reported that biological effects associated with NRP2a expression frequently opposed the effects seen with expression of NRP2b.[3] Clinically relevant cell behaviors, including migration, metastatic spread, stem-like properties, and the emergence of drug resistance, were linked specifically to NRP2b. Because clinical specimens with acquired resistance to the EGFR TKI gefitinib were most strongly upregulated for NRP2b, we hypothesized that this isoform was related to the development of drug resistance, although the mechanistic link was unclear. Therefore, we set out to clarify the mechanisms engaged by NRP2b to understand how it might contribute to acquired drug resistance. Our findings support this link and suggest that inhibition of NRP2b may prolong the sensitivity of non–small cell lung cancer (NSCLC) to treatment with TKIs by impeding the emergence of resistance (Figure 1).

MATERIALS AND METHODS

Cell Lines, Tissue Culture, and Special Reagents

Validated cell lines were obtained from the University of Colorado Specialized Program of Research Excellence (Aurora, Colo; H358 and A549) and the Moffitt Cancer Center (Tampa, Fla; PC-9). Lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 under standard conditions and periodically verified by allele analysis. Selections used puromycin (2 μg/mL; lentiviral transfections) or hygromycin (100 μg/mL) and blasticidin (5 μg/mL; for Flp-In T-REx lines). Gefitinib was obtained from AstraZeneca Pharmaceuticals (Gaithersburg, Md). Other specialty chemicals (crystal violet, GSK3β inhibitors) were obtained from commercial sources. EGFR TKI-resistant cells were generated from the gefitinib-sensitive line, PC-9, by continuous culture at the IC_{50} for gefitinib (50 nM). Resistance was monitored by MTS viability assays according to the supplier's instruction (Promega Inc, Madison, Wis). Antibodies included phospho-AKT-S473-p, pan-AKT, phospho-mTOR-S2448-p, mTOR, GSK3β and PTEN (Cell Signaling Inc, Danvers, Mass), phospho-PTEN-T366-p (EP229, Abcam, Inc, Cambridge, Mass), total NRP2 (AF2215, R&D Systems, Minneapolis, Minn), and β-actin (Sigma, St Louis, Mo). The NRP2b-specific antibody 9080 was previously described.[3]

Short Hairpin RNA, Expression Constructs, and Protein Analyses

Isoform-specific short hairpin RNA (shRNA) has been described.[3] Western blots were performed as previously described.[3] Signal detection used a ChemiDoc image capture station (BioRad, Inc, Hercules, Calif) with chemiluminescence reagent from Advansta, Inc (San Jose, Calif). Captured digital images were analyzed using ImageJ. Expression constructs included GSK3β and pEGFP-tagged PTEN, which were gifts from S. Friedman and A. Ross, respectively (AddGene plasmids 49491 and 13039).

Migration and Invasion Assays

Transwell migration assays were performed using a Neuro Probe Reusable Multiwell Chemotaxis Chamber. Each well contained 10,000 cells in RPMI with 2% fetal bovine serum and with 20% fetal bovine serum in the lower chamber. Cells were allowed to migrate for 24 hours, fixed with 4% paraformaldehyde, stained with crystal violet, photographed, and counted. Each condition was performed in duplicate with 6 counted fields total and 3 biological replicates. Where indicated, GSK3β inhibitors were added to 10 μM.

Drug-Tolerant Persister Cells

PC-9 or HCC827 cell suspensions, with control or NRP2 isoform-specific knockdowns, were plated at 100,000 cells/100 mm dish, in triplicate. After attachment, gefitinib or osimertinib was added to 2.5 μM. Plates were incubated for 4 weeks (gefitinib) or 8 days (osimertinib) with medium (RPMI1640, 10% fetal bovine serum, fresh agent) replaced every 3 days. After selection, surviving cells were cultured with nonselective RPMI1640 for 3 to 4 weeks to permit growth of survivors into small clusters. Colonies were fixed with 4% paraformaldehyde, stained with crystal violet (0.1%), and counted.

Gefitinib-Sensitization Assay

NCI-H322 and NCI-H1703 cells, bearing control or NRP2b-specific knockdowns, were plated at 250,000 cells/60 mm dish, in triplicate. After attachment, gefitinib was added to 125 nM (1/2 of IC_{50} level). Vehicle (dimethyl sulfoxide) was added to a parallel set of control plates. Cells were harvested and counted weekly, and then replated with fresh medium ± gefitinib.

Immunoprecipitations

Flag-HA tagged NRP2a or NRP2b was transiently or stably transfected into HEK293 FlpIn TRex cells along with constructs expressing GSK3β or GFP-PTEN. Protein lysates in radio immunoprecipitation assay buffer (200-400 μg of protein) were tumbled overnight at 4°C with primary anti-NRP2 antibodies or control immunoglobulins along with 10 μL of pre-washed Protein G Dynabeads. Beads were washed 3 times with fresh radio immunoprecipitation assay buffer and co-precipitating proteins analyzed by Western blot.

Bioinformatics

A 3-dimensional model of the GSK3β-binding motif within the C-terminus of NRP2b (15AA peptide) was created initially. BLAST of the C-terminal domain against the pdb database showed modest similarity to pdb:3WOI (S520-L550)[11] and pdb:4U9O (A148-

L181)[12] and indicated a helical structure. 4U9O was selected as a template to build a 17mer homology model. Bimolecular protein docking was then performed between the NRP2b peptide homology model and the x-ray structure of GSK3β pdb:1PYX[13] using protein-protein rigid body docking methodology. Docking was performed using 10,000 preplacements, 1000 placements, and 100 refinements, and the final top 25 were analyzed for any consistency. This analysis indicated 28% of good poses interacted with Arg220. The best pose is presented. All operations and visualization were performed using MOE2018 (CCG).

RESULTS

NRP2b knockdown in NSCLC cell lines delays emergence of drug-tolerant persister cells. We previously found that inhibition of NRP2b modulated the sensitivity of several NSCLC cell lines to gefitinib using proliferation (MTS) assays.[3] The development of colonies in suspension culture (ie, "tumorspheres," a stem cell indicator) was observed to be NRP2b dependent.[3] Recent work has shown that bona fide resistance to targeted agents evolves from a residue of drug-tolerant persister cells with stem-like properties.[14–16] To determine whether NRP2b influenced the establishment of persister cells, the PC-9 NSCLC cell line, which possesses an activating EGFR mutation (delE746-A750 in exon 19), was knocked down with validated shRNAs targeting the individual NRP2 isoforms.[3] After puromycin selection, modified PC-9 cells (1×10^5) were plated in triplicate and treated with high-dose gefitinib (2.5 μM) for 4 weeks. Surviving drug-tolerant persister cells were allowed to recover in nonselective medium for 3 weeks, and the resulting colonies were quantified.

Approximately 100 surviving colonies were obtained from 1×10^5 initial control cells/plate (Figure 2, A). Selective knockdown of NRP2b suppressed the number of persister colonies approximately 100-fold. In contrast, knockdown of NRP2a led to a greater than 2- to 2.5-fold increase in the number of persister colonies. Equivalent experiments were conducted with the EGFR-mutant line HCC827 (exon 19 del) using osimertinib, the current standard of care for EGFR mutant lung tumors (Figure 2, B). Again, knockdown of NRP2b nearly eliminated persister colonies ($P < .05$), whereas knockdown of NRP2a yielded approximately 6-fold more persister colonies than controls ($P < .05$). Thus, the emergence of drug-tolerant persister cells appears to be dependent on expression of the NRP2b isoform.

The greatest clinical limitation of TKIs for the treatment of NSCLC is the fact that only small segments of the population harbor activating mutations required for susceptibility to these agents. Therefore, we were interested to determine whether NRP2b knockdown could sensitize NSCLC cells expressing wild-type EGFR. To assess this, NCI-H322 and NCI-H1703 cells were analyzed, both of which are wild-type for EGFR and KRAS. Control and NRP2b knockdown derivatives were continuously treated with gefitinib (125 nM) for 4 weeks, and cell numbers were counted at weekly intervals. As shown in Figure 3, A, gefitinib-treated control cells continued to proliferate despite the presence of drug. In contrast, knockdown of NRP2b sensitized H322 cells to gefitinib, reducing numbers approximately 25-fold, and impaired proliferation for the duration of the experiment. When the same cell lines were cultured in drug-free media, knockdown of NRP2b did not have a significant impact on proliferation (Figure 3, B). Both control and NRP2b knockdown cells proliferated exponentially in the absence of gefitinib. In contrast, knockdown of NRP2b had little effect on the growth of NCI-H1703 cells, whether or not gefitinib was present (Figure 3, C). These results suggest that specific knockdown of NRP2b can sensitize some (but not all) EGFR wild-type lung cancers to EGFR TKI.

NRP2b and AKT Activation

Recent reports suggested that neuropilins interact with the tumor suppressor and PI3K/AKT inhibitor PTEN.[17,18] We demonstrated that PTEN bound robustly to NRP2a but only weakly to NRP2b.[3] Such differential interaction could explain our previous findings that AKT activation downstream of hepatocyte growth factor (HGF)/MET was dependent on NRP2b expression.[3] To gain further insight, HEK293 cells bearing doxycycline-inducible NRP2a or NRP2b expression constructs were analyzed for the activation state of AKT and for PTEN levels (Figure 4, A). Cells expressing NRP2b consistently possessed higher levels of activated phospho-AKT (Ser473-P) than vector controls or NRP2a (Figure 4, A). When quantified, Ser473-specific phosphorylation (phos-AKT:pan-AKT) was observed to be approximately 2-fold higher in NRP2b-expressing cells compared with vector control or NRP2a-expressing cells (Figure 4, B). PTEN levels were substantially decreased in NRP2b cells (Figure 4, C). Real-time reverse transcription polymerase chain reaction showed that PTEN message was higher in NRP2b-expressing cells, indicating that PTEN loss occurred at the post-translational level (Figure 4, D). Reduced levels of PTEN protein are consistent with increased activation of AKT (ie, phospho-Ser473-AKT) and may underlie the biological effects of NRP2b including emergence of persister cells and drug resistance.

NRP2b Recruits GSK3β to Phosphorylate and Destabilize PTEN

NRP2 has been reported to work in concert with GSK3β during the migration of newly born neurons in the adult dentate gyrus.[19] GSK3β is known to phosphorylate PTEN on Thr366, leading to its destabilization by ubiquitylation and proteasome-mediated degradation.[20,21] We used co-immunoprecipitation to explore whether NRP2 isoforms might bind and recruit GSK3β as a mechanism to reduce PTEN levels. NRP2a or NPR2b isoforms were separately expressed in HEK293 cells and lysates immunoprecipitated with anti–pan-NRP2 antibodies. Western blot analysis of the precipitates confirmed that GSK3β co-precipitated with NRP2 (Figure 5, A). The interaction was strongly biased to the NRP2b isoform and was lost by deletion of the cytoplasmic domain (2bΔC). This set of experiments established the preferential association of GSK3β with NRP2b, in contrast with the previously described preferential association of PTEN with NRP2a.[3]

NRP2b-Related PTEN Loss Depends on GSK3β and the Proteasome

Given the preferential interaction of GSK3β with NRP2b (Figure 5, A), we wanted to determine whether this kinase was important for the loss of PTEN in NRP2b-expressing cells. HEK293 cells were induced to express NRP2a or NRP2b by doxycycline treatment and lysates compared with vector controls by Western blot. Expression of NRP2b correlated with reduced PTEN and with increased phosphorylation on Thr366 (Figure 5, B). The addition of TDZD-8, a specific GSK3β inhibitor,[22,23] prevented PTEN phosphorylation and preserved PTEN levels in NRP2b-expressing cells. Likewise, addition of the proteasome inhibitor MG-132 also preserved PTEN levels (Figure 5, C and D). Together, these results support our hypothesis that expression of NRP2b results in a GSK3β-dependent phosphorylation of PTEN leading to its proteosomal degradation, providing a mechanistic link between NRP2b expression and activation of AKT-related survival pathways.

Direct Interaction of NRP2b and GSK3β

Further definition of the NRP2b-GSK3β interaction was obtained using a series of nested C-terminal deletions of NRP2b (Figure 6, A). Co-immunoprecipitation showed that both full-length NRP2b and a deletion of 6 C-terminal amino acids (2b-Δ6) retained binding to GSK3β. However, the interaction was abrogated by deletion of the entire cytoplasmic domain (2b-ΔC) or deletion of the last 21 amino acids (Figure 6, B; 2b-Δ21). These results localized the putative GSK3β binding site within a 15 amino acid segment spanning amino acids A881 to Q895 (−21 to −6), within the NRP2b C-terminus. In silico modeling of this peptide using MOE2018 (CCG) software suggested it formed an amphipathic α-helical structure, segregating hydrophobic and hydrophilic side chains on opposing faces (Figure 6, C). Docking of this α-helix onto the x-ray structure of GSK3β revealed a potential high-affinity binding site with a calculated $\Delta G^{binding}$ of −45 Kcal (Figure 6, D). These results support the co-IP analysis and suggest that NRP2b specifically recruits GSK3β, potentially as part of its mechanism of action.

NRP2b-Mediated Migration and Persister-Cell Formation Require GSK3β Activity

To determine whether GSK3β recruitment by NRP2b had functional significance in NSCLC, migration assays were performed with A549 cells manipulated to express one or the other NRP2 isoform. Serum-starved cells bearing total NRP2 or isoform-specific knockdowns were treated with vehicle or HGF and then allowed to migrate across a porous polycarbonate membrane. HGF was chosen because it induces migration downstream of MET activation, and we had previously demonstrated that MET signaling was differentially enhanced by NRP2b while being suppressed by NRP2a.[3] Both control cells and cells knocked down for NRP2a (leaving NRP2b as the principle isoform) readily migrated in response to HGF (Figure 7, A). However, HGF-induced migration of shNRP2a cells was completely inhibited when treated with the GSK3β inhibitor, TDZD-8. TDZD-8 alone had no effect; it only blocked migration of shNRP2a cells exposed to HGF. Conversely, when NRP2b (or total NRP2) was knocked down, HGF inhibited migration, an effect that was relieved by TDZD-8. These results suggest that migration of A549 cells in the presence of HGF is dependent on NRP2b and GSK3β. Moreover, HGF/MET signaling appears to direct cellular resources away from endogenous migration to a form that is dependent on MET, NRP2b, and GSK3β activity. PC-9 cells behaved in a similar manner (Figure 7, B), supporting the general validity of these findings.

To determine whether GSK3β was important for NRP2b-promoted drug resistance, persister cell assays were performed in the presence or absence of TDZD-8 (Figure 8). PC-9 (Figure 8, A and B) or HCC827 cells (Figure 8, C), bearing control or isoform-specific knockdowns, were cultured in the presence of gefitinib (Figure 8, A) or osimertinib (Figure 8, B, C; all at 2.5 μM) for 4 weeks or 9 days, respectively. Surviving drug-tolerant persister cells were then grown without selection into small colonies for 3 to 4 weeks and counted. As noted previously (Figure 2), knockdown of NRP2b strongly suppressed the development of persister cells. Inhibition of GSK3β (GSK3βi) in both PC-9 and HCC827 cells reduced the excess persister colonies observed with NRP2a knockdown (Figure 8, A–C). Equivalent results were obtained using the clinically relevant EGFR inhibitor, osimertinib (Figure 8, B and C). Collectively, these experiments demonstrate that interaction of NRP2b with GSK3β in NSCLC cells facilitates AKT activation, leading to cell migration and persister cell emergence when stressed with clinically relevant EGFR inhibitors.

DISCUSSION

The greatest limitation to the long-term outcomes of patients with NSCLC is the lack of a sustained response to chemotherapeutic agents ranging from traditional cytotoxic agents, molecularly targeted agents, to immunotherapy. We have discovered that differential functions of the NRP2a and NRP2b isoforms significantly influence the responsiveness of NSCLC cells to 2 representative TKIs, gefitinib and osimertinib. Knockdown of NRP2b drastically reduced the number of persister NSCLC cells that survived treatment with gefitinib or osimertinib. In contrast, knockdown of NRP2a, which enhances NRP2b activity, facilitated persister cell development after treatment with either EGFR inhibitor as demonstrated in Figure 1.

The major mechanistic discovery reported in this article is that NRP2b interacts with GSK3β, whereas NRP2a does not. Previous studies by Ng and colleagues[19] showed that NRP2 and GSK3β functioned together in controlling migration of newly born neurons in the dentate gyrus of the hippocampus. We have shown that GSK3β binds to NRP2b, but not NRP2a, and that GSK3β activity is important for NRP2b functionality. In support of this relationship, we found that NRP2b-dependent drug-tolerant persister cells were suppressed by GSK3β inhibitors. Likewise, cell migration also depended on combined signaling through NRP2b and GSK3β. One consequence of GSK3β recruitment was the reduction of PTEN levels in NRP2b-expressing cells, a putative explanation for the enhanced AKT activity reported previously[3] and shown in Figure 4. The importance of the NRP2b- GSK3β recruitment suggests it represents a novel therapeutic target. A schematic model depicting interactions among PTEN, NRP2a, NRP2b, and GSK3β in cell survival, and resistance to TKIs targeting the EGF receptor is illustrated in the Figure 1.

NRP2b knockdown alone had essentially no impact on the growth of NSCLC cells. In addition, no phenotypic changes were noted. This suggests that cells tolerate the lack of NRP2b function well, and it is only with the additional stress of a TKI that cytotoxic effects were observed. These observations enhance the potential for clinical application of our in vitro observations.

Previous studies have reported disparate results after NPR2 manipulation, with some showing promotion of growth and tumor-associated lymphangiogenesis, whereas others showed tumor suppressive effects. These differences have been attributed to interactions with different intracellular cofactors, but our results suggest they may be related to contradictory effects of the NRP2a and NPR2b isoforms.[9,24–26]

NPR2 expression has also been identified in tumor infiltrating macrophages where its function has been implicated in efferocytosis, the process of phagocytosis of apoptotic tumor cells.[27] Efferocytosis suppresses an immunological response to dying tumor cells by preventing the release of tumor-specific neoantigens.[28] Therefore, NPR2 inhibition may hold some potential for enhancing antigen exposure and responsiveness of tumors to immunotherapy.

Study Limitations

The primary limitation of this study is the fact it was performed in vitro. Although the data show an encouraging response of NSCLC cells, more work must be done to determine if treatments exploiting the importance of the NRP2b–GSK3β interaction can be effectively delivered in vivo and whether these treatments are tolerable. It is currently unclear what other important cellular processes may be disrupted when targeting this pathway and how such targeting might affect cell function. It is our hope that the selective disruption of the NRP2b-GSK3β axis without disrupting other activities of either protein will minimize impacts on vital cell functions and minimize toxic effects.

CONCLUSIONS

We have demonstrated the importance of NRP2b and its interaction with GSK3β in the resistance of NSCLC cells to TKIs. The NRP2b-GSK3β signaling pathway deserves continued investigation in other cancer types and animal models to further explore the potential for a clinically relevant treatment option.

Acknowledgments

This work was supported by grants from the Veterans Administration (VA Merit award BX00333), the South Carolina Research Institute (SCTR #1803), and pilot funding from the Hollings Cancer Center Support Grant P30 CA138313 at the Medical University of South Carolina. In addition, this work was supported by the Biostatistics and Cell & Molecular Imaging shared resources of the Hollings Cancer Center (P30 CA138313).

Discussion Presenter: Dr Chadrick E. Denlinger

Dr Jessica S. Donington *(Chicago, Ill)*. Chad, congratulations; this is a tremendous amount of work and very sophisticated work. Looking at the subtle mechanistic differences between these isoforms, I think you guys are the first group to recognize the different impact of the neuropilin isoforms in this setting. This is groundbreaking work. That being said, I have a couple of questions. Isoform work is always a little challenging; I think it's hard to convince the world that they really exist and are different. Has any of this moved out of cell lines and into any preclinical models?

Author Manuscript
Author Manuscript
Author Manuscript

Dr Chadrick E. Denlinger *(Charlestown, SC)*. That's an important question, and this is a direction we want to go. At this point, we have not moved into animal models. We have just sorted out the difference between A and B isoforms, which are significant. We're seeing significant differences in tissue culture. The important questions that we are needing to address are how can we get this into at least a mouse model? The second and bigger question is, once we get there, how toxic will it be? The neuropilins are expressed throughout the body; in the skeletal muscle, they are important for angiogenesis, and they have a lot of other effects beyond our current understanding. We are heading there and will get there in the coming years.

Dr Donington. That takes me directly to my next question. My understanding of the different isoforms is that the extracellular component between A and B are the same, what would be the ways to target it? A small molecule or antibody isn't going to work in this setting, is it?

Dr Denlinger. That's an important question. Antibodies targeting the extracellular domain are in existence and in clinical trials for sarcoidosis. As you are saying, that won't tease out the A versus B.

Dr Donington. That seems to be important here.

Dr Denlinger. We believe that's true. What we are looking at is the interaction between the GS3Kβ and neuropilin 2, and finding ways to disrupt that particular interaction. Our belief at this point is that disrupting this interaction, rather than knocking out each individual protein, it might be less toxic and still achieve the intended effects.

Dr Donington. My last question is to better understand from a clinical standpoint. You don't think that this interaction really pushes the cells toward any specific pattern of resistance—this isn't pushing cells toward T790M, or it's not pushing them toward MET amplification, application, or a small-cell phenotype—this is just allowing them to live longer. Is that the concept?

Dr Denlinger. We initially believed that it would drive other site mutations such as the common T790M point mutation, but we have drifted away from this belief. At this point, we believe that neuropilin 2b signaling leads to AKT activation leading to cell survival. Therefore, neropilin 2b activation allows cells to survive while they evolve other mechanisms of resistance.

Dr Donington. This is a big breakthrough in this area of therapy, because one of the hang-ups that we've seen with the targeted agents is that they can be cytostatic and not cytotoxic.

Dr Denlinger. We see that they are efficacious for 6 to 9 months, or perhaps a year before the cancers develop resistance and resume growth. The hope is that we can at least prolong that sensitive period.

Dr Donington. Very nice work. Congratulations.

Abbreviations and Acronyms

EGFR	epidermal growth factor receptor
HGF	hepatocyte growth factor
NRP2	neuropilin-2
NSCLC	non–small cell lung cancer
RPMI	Roswell Park Memorial Institute
shRNA	short hairpin RNA
TKI	tyrosine kinase inhibitor

References

1. Lee SH. Chemotherapy for lung cancer in the era of personalized medicine. Tuberc Respir Dis (Seoul). 2019;82:179–89. [PubMed: 30841023]
2. Bordi P, Del Re M, Minari R, Rofi E, Buti S, Restante G, et al. From the beginning to resistance: study of plasma monitoring and resistance mechanisms in a cohort of patients treated with osimertinib for advanced T790M-positive NSCLC. Lung Cancer. 2019;131:78–85. [PubMed: 31027702]
3. Gemmill RM, Nasarre P, Nair-Menon J, Cappuzzo F, Landi F, D'Incecco A, et al. The neuropilin 2 isoform NRP2b uniquely supports TGFbeta-mediated progression in lung cancer. Sci Signaling. 2017;10:eaag0528.
4. Nasarre P, Gemmill RM, Drabkin HA. The emerging role of class-3 semaphorins and their neuropilin receptors in oncology. Oncotargets Ther. 2014;7:1663–87.

5. West DC, Rees CG, Duchesne L, Patey SJ, Terry CJ, Turnbull JE, et al. Interactions of multiple heparin binding growth factors with neuropilin-1 and potentiation of the activity of fibroblast growth factor-2. J Biol Chem. 2005;280:13457–64. [PubMed: 15695515]
6. Rossignol M, Gagnon ML, Klagsbrun M. Genomic organization of human neuropilin-1 and neuropilin-2 genes: identification and distribution of splice variants and soluble isoforms. Genomics. 2000;70:211–22. [PubMed: 11112349]
7. Ong HS, Gokavarapu S, Xu Q, Tian Z, Li J, Ji T, et al. Cytoplasmic neuropilin 2 is associated with metastasis and a poor prognosis in early tongue cancer patients. Int J Oral Maxillofac Surg. 2017;46:1205–19. [PubMed: 28602571]
8. Burton JB, Priceman SJ, Sung JL, Brakenhielm E, An DS, Pytowski B, et al. Suppression of prostate cancer nodal and systemic metastasis by blockade of the lymphangiogenic axis. Cancer Res. 2008;68:7828–37. [PubMed: 18829538]
9. Caunt M, Mak J, Liang W-C, Stawicki S, Pan Q, Tong RK, et al. Blocking neuropilin-2 function inhibits tumor cell metastasis. Cancer Cell. 2008;13:331–42. [PubMed: 18394556]
10. Rossi M, Tuck J, Kim OJ, Panova I, Symanowski JT, Mahalingam M, et al. Neuropilin-2 gene expression correlates with malignant progression in cutaneous melanoma. Br J Dermatol. 2014;171:403–8. [PubMed: 24359286]
11. Sakamoto Y, Suzuki Y, Iizuka I, Tateoka C, Roppongi S, Fujimoto M, et al. S46 peptidases are the first exopeptidases to be members of clan PA. Sci Rep. 2014;4:4977. [PubMed: 24827749]
12. Steuber J, Vohl G, Casutt MS, Vorburger T, Diederichs K, Fritz G. Structure of the V. cholerae Na+-pumping NADH:quinone oxidoreductase. Nature. 2014;516:62–7. [PubMed: 25471880]
13. Bertrand JA, Thieffine S, Vulpetti A, Cristiani C, Valsasina B, Knapp S, et al. Structural characterization of the GSK-3beta active site using selective and non-selective ATP-mimetic inhibitors. J Mol Biol. 2003;333:393–407. [PubMed: 14529625]
14. Hangauer MJ, Viswanathan VS, Ryan MJ, Bole D, Eaton JK, Matov A, et al. Drug-tolerant persister cancer cells are vulnerable to GPX4 inhibition. Nature. 2017;551:247–50. [PubMed: 29088702]
15. Ramirez M, Rajaram S, teininger RJ, Osipchuk D, Roth MA, Morinishi LS, et al. Diverse drug-resistance mechanisms can emerge from drug-tolerant cancer persister cells. Nat Commun. 2016;7:10690. [PubMed: 26891683]
16. Hata AN, Niederst MJ, Archibald HL, Gomez-Caraballo M, Siddiqui FM, Mulvey HE, et al. Tumor cells can follow distinct evolutionary paths to become resistant to epidermal growth factor receptor inhibition. Nat Med. 2016;22:262–9. [PubMed: 26828195]
17. Delgoffe GM, Woo S-R, Turnis ME, Gravano DM, Guy C, Overacre AE, et al. Stability and function of regulatory T cells is maintained by a neuropilin-1-semaphorin-4a axis. Nature. 2013;501:252–6. [PubMed: 23913274]
18. Nakayama H, Bruneau S, Kochupurakkal N, Coma S, Briscoe DM, Klagsbrun M. Regulation of mTOR signaling by semaphorin 3f-neuropilin 2 interactions in vitro and in vivo. Sci Rep. 2015;5:11789. [PubMed: 26156437]
19. Ng T, Hor CHH, Chew B, Zhao J, Zhong Z, Ryu JR, et al. Neuropilin 2 signaling is involved in cell positioning of adult-born neurons through glycogen synthase kinase-3beta (GSK3beta). J Biol Chem. 2016;291:25088–95. [PubMed: 27687730]
20. Al-Khouri AM, Ma Y, Togo SH, Williams S, Mustelin T. Cooperative phosphorylation of the tumor suppressor phosphatase and tensin homologue (PTEN) by casein kinases and glycogen synthase kinase 3beta. J Biol Chem. 2005;280:35195–202. [PubMed: 16107342]
21. Martinez A, Castro A, Dorronsoro I, Alonso M. PTEN is destabilized by phosphorylation on Thr366. Biochem J. 2007;405:439–44. [PubMed: 17444818]
22. Martinez A, Alonso M, Castro A, Pérez C, Moreno FJ. First non-ATP competitive glycogen synthase kinase 3 beta (GSK-3beta) inhibitors: thiadiazolidinones (TDZD) as potential drugs for the treatment of Alzheimer's disease. J Med Chem. 2002;45:1292–9. [PubMed: 11881998]
23. Martinez A, Castro A, Dorronsoro I, Alonso M. Glycogen synthase kinase 3 (GSK-3) inhibitors as new promising drugs for diabetes, neurodegeneration, cancer, and inflammation. Med Res Rev. 2002;22:373–84. [PubMed: 12111750]

24. Bielenberg DR, Pettaway CA, Takashima S, Klagsbrun M. Neuropilins in neoplasms: expression, regulation, and function. Exp Cell Res. 2006;312:584–93. [PubMed: 16445911]
25. Coma S, Shimizu A, Klagsbrun M. Hypoxia induces tumor and endothelial cell migration in a semaphorin 3F- and VEGF-dependent manner via transcriptional repression of their common receptor neuropilin 2. Cell Adh Migr. 2011;5:266–75. [PubMed: 21610314]
26. Achen MG, Mann GB, Stacker SA. Targeting lymphangiogenesis to prevent tumour metastasis. Br J Cancer. 2006;94:1355–60. [PubMed: 16641900]
27. Roy S, Bag AK, Dutta S, Polavaram NS, Islam R, Schellenburg S, et al. Macrophage-derived neuropilin-2 exhibits novel tumor-promoting functions. Cancer Res. 2018;78:5600–17. [PubMed: 30111533]
28. Stanford JC, Young C, Hicks D, Owens P, Williams A, Vaught DB, et al. Efferocytosis produces a prometastatic landscape during postpartum mammary gland involution. J Clin Invest. 2014;124:4737–52. [PubMed: 25250573]

CENTRAL MESSAGE

Inhibition of the cell surface receptor NRP2b and associated GSK3b prevents the development of resistance to TKIs and migration in NSCLC.

PERSPECTIVE

Innate and acquired resistance to TKIs is the greatest limitation to the use of these agents in NSCLC. In this preclinical study, inhibition of the cell surface receptor NRP2b prevented the emergence of resistance in NSCLC cells. This signaling pathway should be further explored for potential clinical translation.

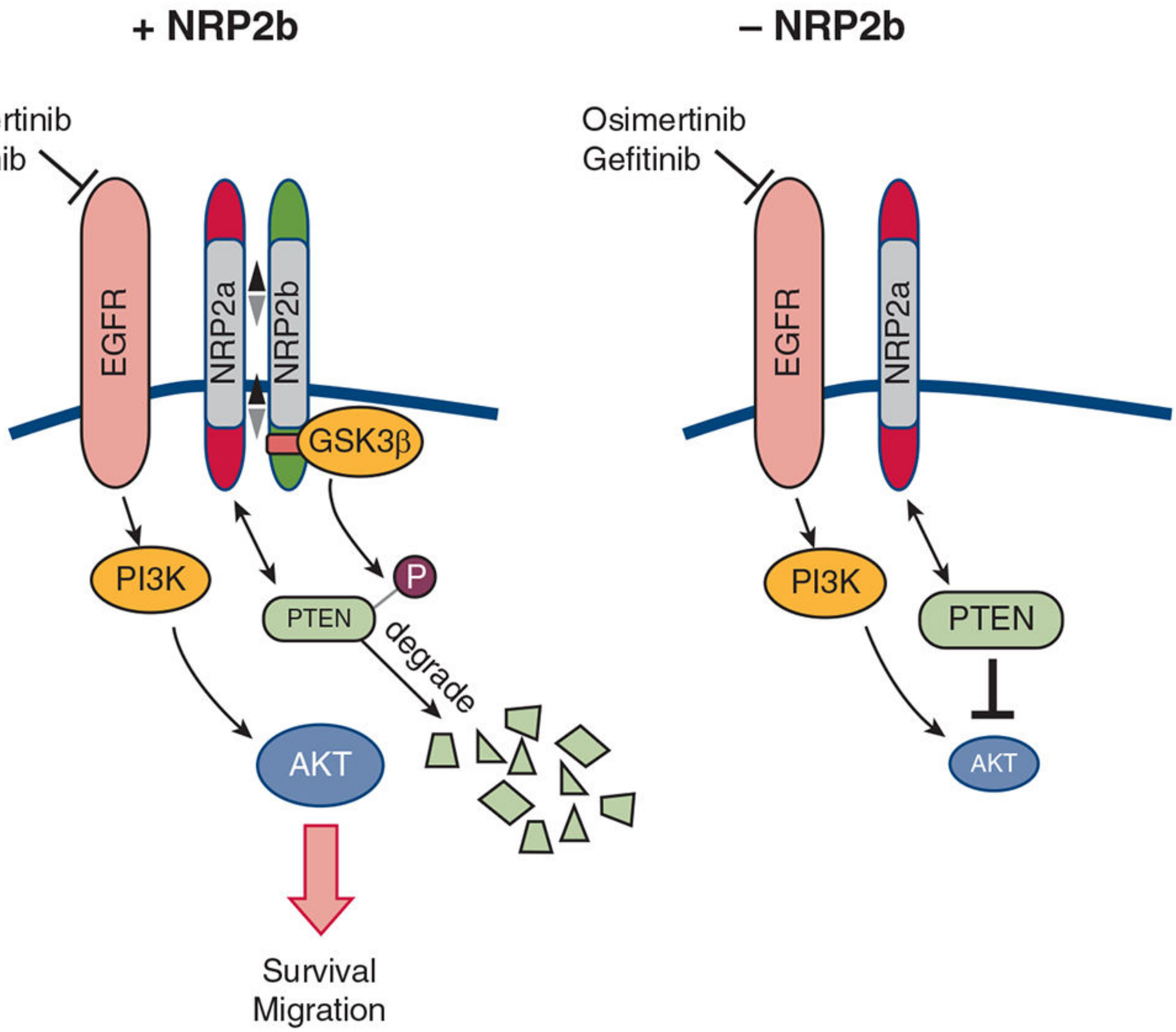

FIGURE 1.

Schematic molecular model of the relationship between cellular growth of EGFR-mutant lung tumors and the link to phosphatidylinositol-3 kinase (PI3K) and activation of AKT, a kinase known to promote cell survival and migration. TKIs, such as gefitinib or osimertinib, block this pathway at the level of the EGFR receptor, stagnating cell growth and inducing apoptosis. When NRP2b is present, GSK3β is recruited to phosphorylate and degrade NRP2a-recruited PTEN, leading to enhanced AKT activation with promotion of survival and metastasis. This effect is predicted to enhance the effect of residual EGFR activity remaining in the presence of EGFR inhibitors. When NRP2b is absent, NRP2a-recruited PTEN is stabilized, opposing PI3,4,5P3 production by PI3K and thereby inhibiting AKT, survival, and metastatic spread, thus enhancing the effect of EGFR TKI.

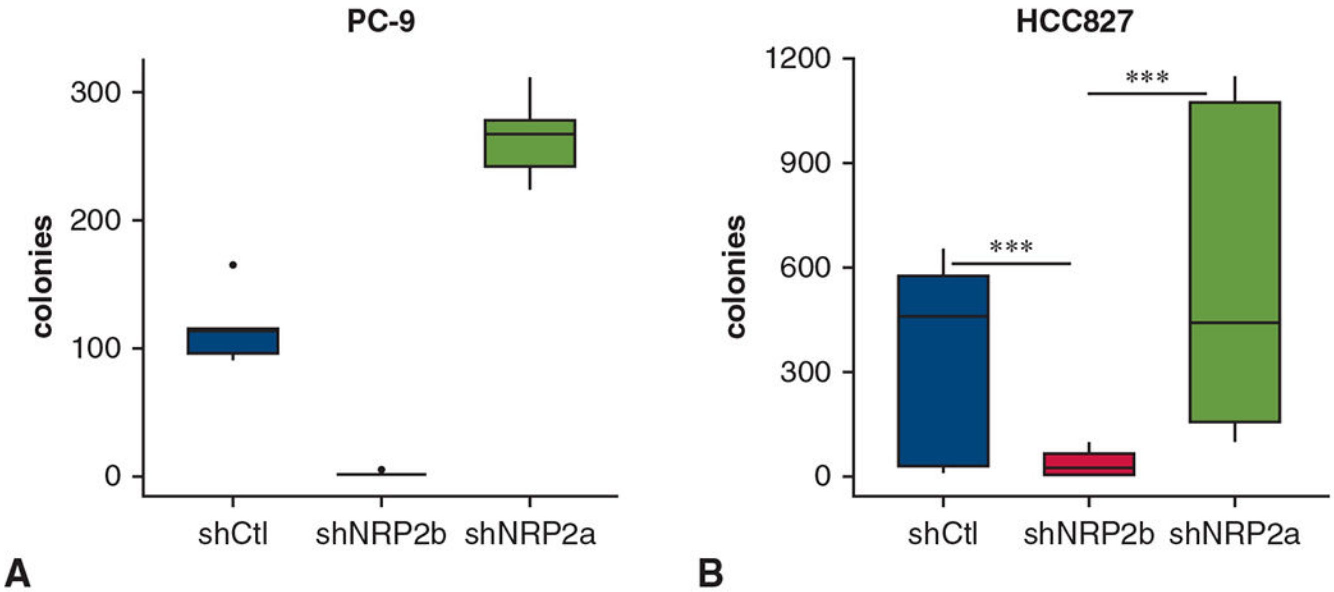

FIGURE 2.

Drug-tolerant persister cells require NRP2b. A, EGFR mutant PC-9 NSCLC cells (1×10^5/dish), knocked down for NRP2b, NRP2a, or with scrambled control shRNA (as indicated), were treated with gefitinib (2.5 μM) for 4 weeks. Surviving drug-tolerant persister cells were then cultured in drug-free medium to permit colony formation and quantitation. B, HCC827 NSCLC cells were treated as in (A) except the EGFR TKI osimertinib was used (2.5 μM) for 9 days before expansion into colonies. Significance was determined by 2-tailed *t* tests. ***$P < .001$.

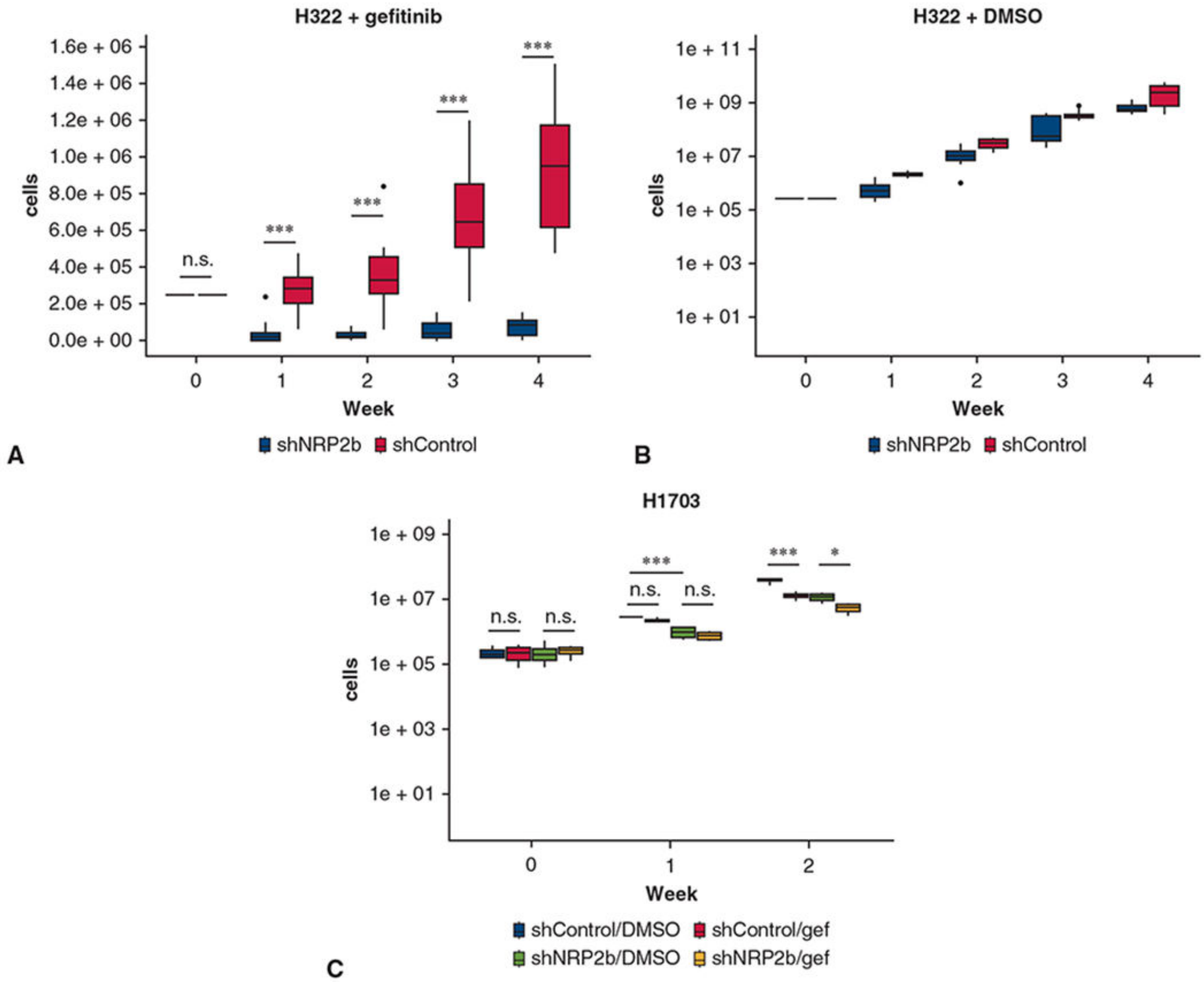

FIGURE 3.

Sensitization to EGFR-TKI by NRP2b knockdown. A, NCI-H322 NSCLC cells (EGFR-wild type), knocked down with shNRP2b or nontargeting shCtl, were cultured with 125 nM gefitinib for 4 weeks. Weekly cell counts for the 2 genotypes are plotted on a linear scale versus time. B, Weekly cell counts for dimethyl sulfoxide–treated NCI-H322 (shNRP2b or shCtl) are plotted on a $\log_{10}$ scale versus time. C, EGFR-wt NCI-H1703 cells, knocked down with shNRP2b or shCtl, were cultured in gefitinib or dimethyl sulfoxide, as indicated. Weekly cell counts for shNRP2b or shCtl cells are plotted on a $\log_{10}$ scale versus time. Significance determined by 2-tailed *t* tests; *$P < .05$. ***$P < .001$. *n.s.*, Not significant.

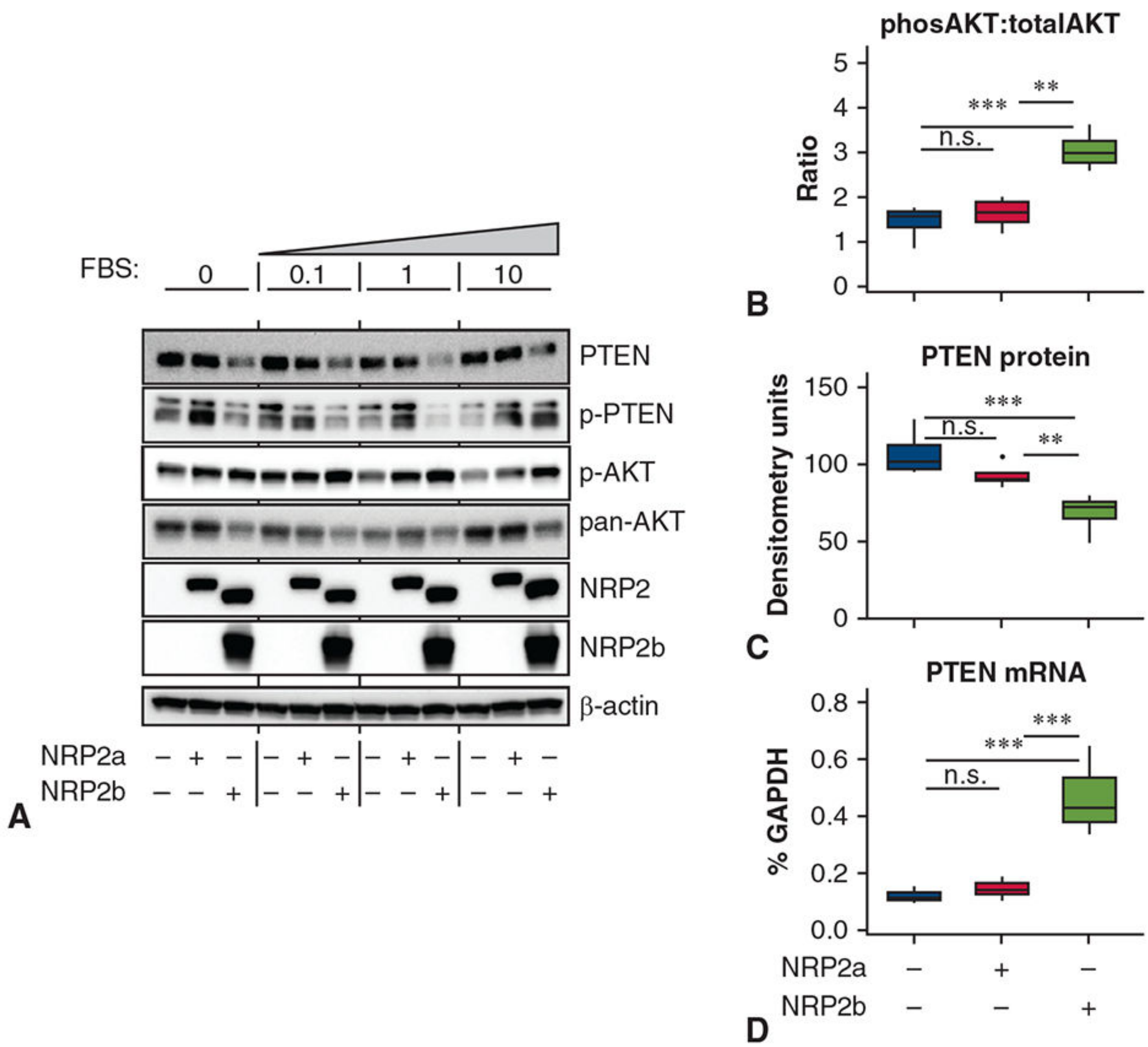

FIGURE 4.

NRP2b inhibits PTEN expression. A, HEK293 cells were induced for 48 hours with doxycycline to express NRP2a or NRP2b, as indicated, and serum starved for the final 24 hours. Cultures were then exposed briefly (10 minutes) to fetal bovine serum at concentrations ranging from 0% to 10%, harvested and analyzed by western blot for PTEN, phosphorylated PTEN (p-PTEN; on Thr366), phosphorylated-AKT (p-AKT; on Ser473), total AKT (pan-AKT), NRP2, and NRP2b. A, β-actin control verified equal loading. B, Densitometric quantitation of mean phospho-AKT and total-AKT signals from (A); control (v), NRP2a (a), and NRP2b (b) expressing samples are plotted as ratios (pAKT:AKT). C, Quantitation of mean PTEN levels from (A). D, Quantitative real-time reverse transcription polymerase chain reaction analysis of PTEN mRNA levels from (A). Significance determined by 2-tailed *t* tests; $^{**}P < .01$. $^{***}P < .001$. *n.s.*, Not significant.

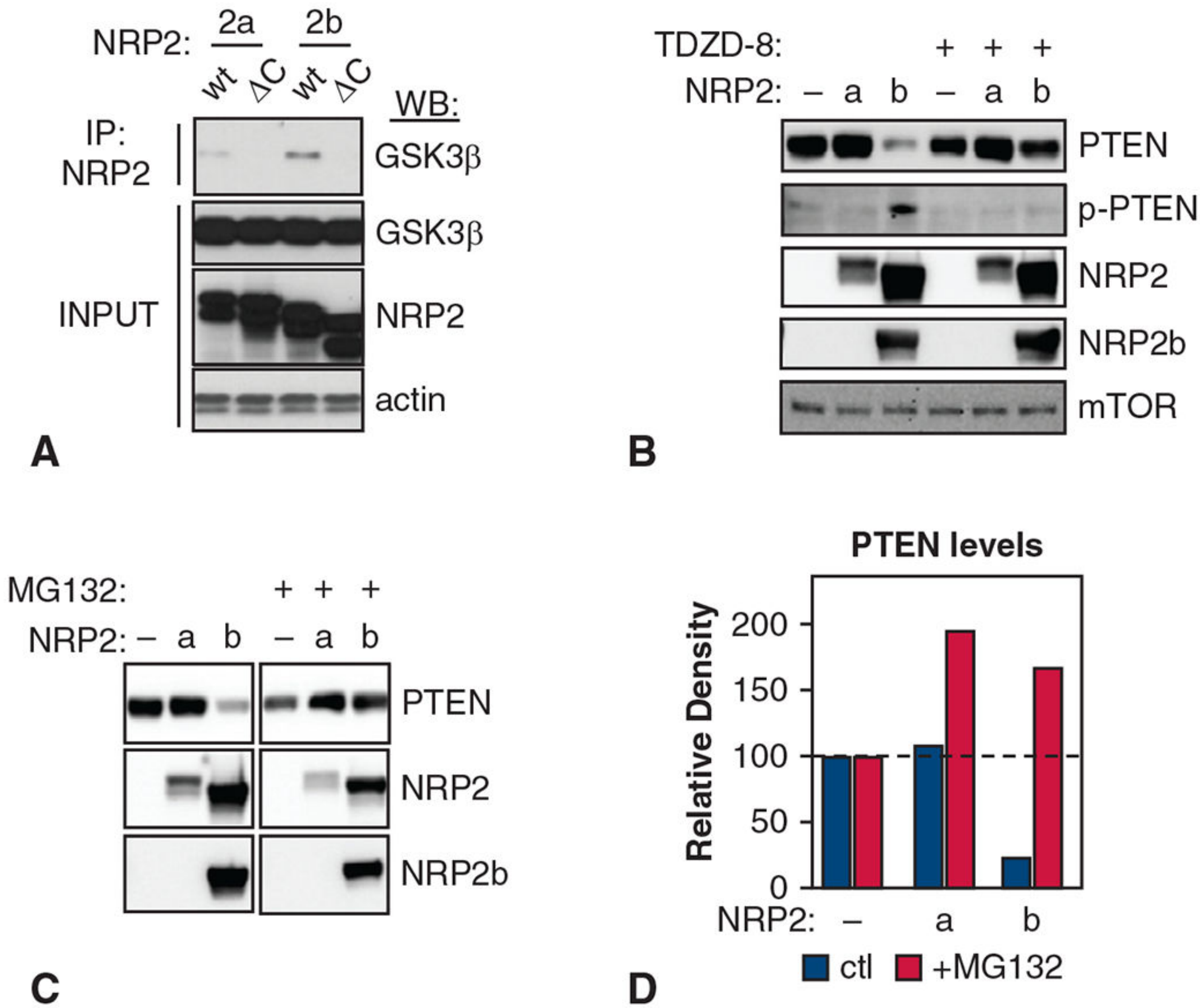

FIGURE 5.

GSK3β interacts with NRP2b to regulate PTEN. A, HEK293 cells expressing wild-type NRP2a or NRP2b, or with deletion of the cytoplasmic domains (ΔC), were immunoprecipitated with antibodies against NRP2. Precipitates were probed for co-precipitated GSK3β by Western blot. Lysates before immunoprecipitation were analyzed simultaneously for input levels. B, PTEN levels were assessed by Western blot in lysates from HEK293 cells expressing NRP2a, NPR2b, or vector control, +/− treatment with the GSK3β inhibitor, TZDZ-8. Blots were probed for total PTEN, phosphorylated PTEN [p-PTEN (T366-P)], total NRP2, NRP2b, and mTOR (control for equal loading). C, PTEN levels were assessed as in (B) in lysates of HEK293 cells expressing NRP2a, NPR2b, or vector control treated or not with the proteasome inhibitor MG132. D, Normalized PTEN levels from (C) determined by quantitation.

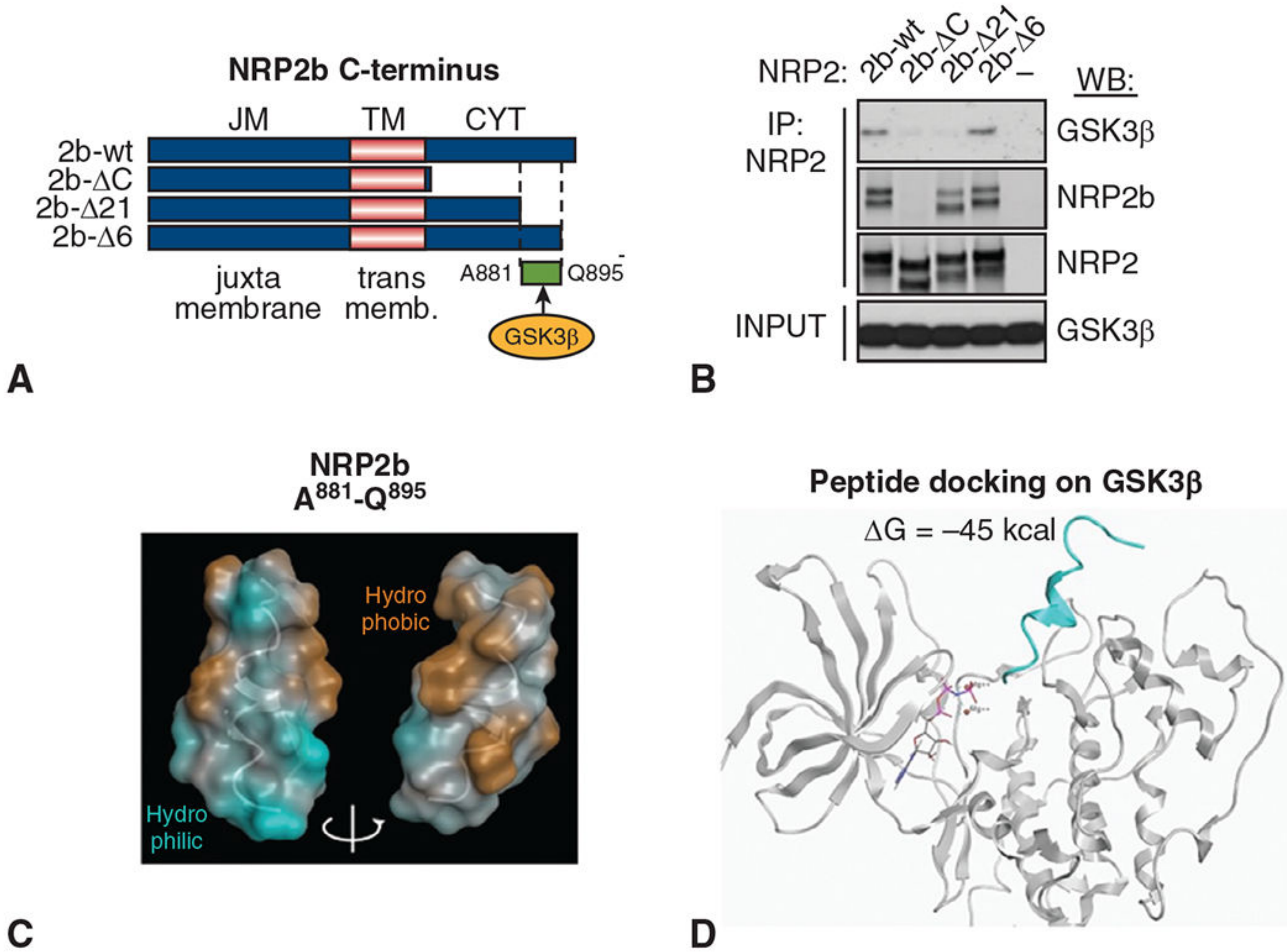

FIGURE 6.

GSK3β interacts with a 15 amino acid motif within NRP2b. A, Diagram of C-terminal deletion mutants of NRP2b used for GSK3β co-immunoprecipitation. *From top*; full-length wild-type (2b-wt), cytoplasmic-domain deletion (2b-ΔC), deletion of the last 21 amino acids (2b-Δ21), and deletion of the last 6 amino acids (2b-Δ6). B, Lysates of HEK293 cells transfected with GSK3β and the NRP2b constructs diagrammed in (A) were immunoprecipitated with antibodies against NRP2. Precipitates were analyzed for GSK3β by Western blot. C, In silico modeling of the GSK3β-interacting motif (A881-Q895) of NPR2b suggested an amphipathic α-helical structure with hydrophilic (teal) and hydrophobic (tan) side-chains clustered on opposite faces. D, In silico modeling identified a putative high-affinity docking site (−45 kcal) for the NRP2b aliphatic helix on the 3-dimensional x-ray structure of GSK3β (pdb:1PYX).[13]

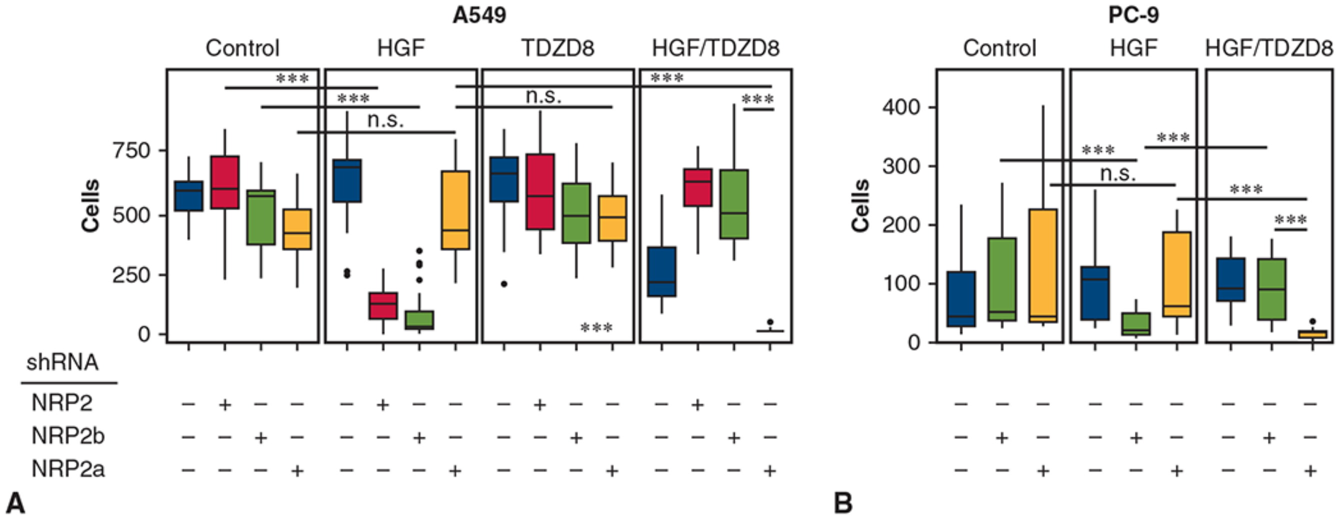

FIGURE 7.

NRP2b-dependent migration requires GSK3β activity. A, A549 lung cancer cells, modified by shRNAs targeting NRP2, NRP2b, or NRP2a, were assayed for migration towards 20% fetal bovine serum in the lower chamber (see "Materials and Methods"). Cells in the upper chamber were treated with HGF (50 ng/mL), TDZD-8 (10 μM GSK3β inhibitor), or both, as indicated. Migrated cells were stained, photographed, and counted; results are reported using *box* and *whisker plots*; *solid line* is the median. B, Equivalent migration assays of PC-9 cells. Significance determined by 2-tailed *t* tests. ***$P < .001$. *n.s.*, Not significant.

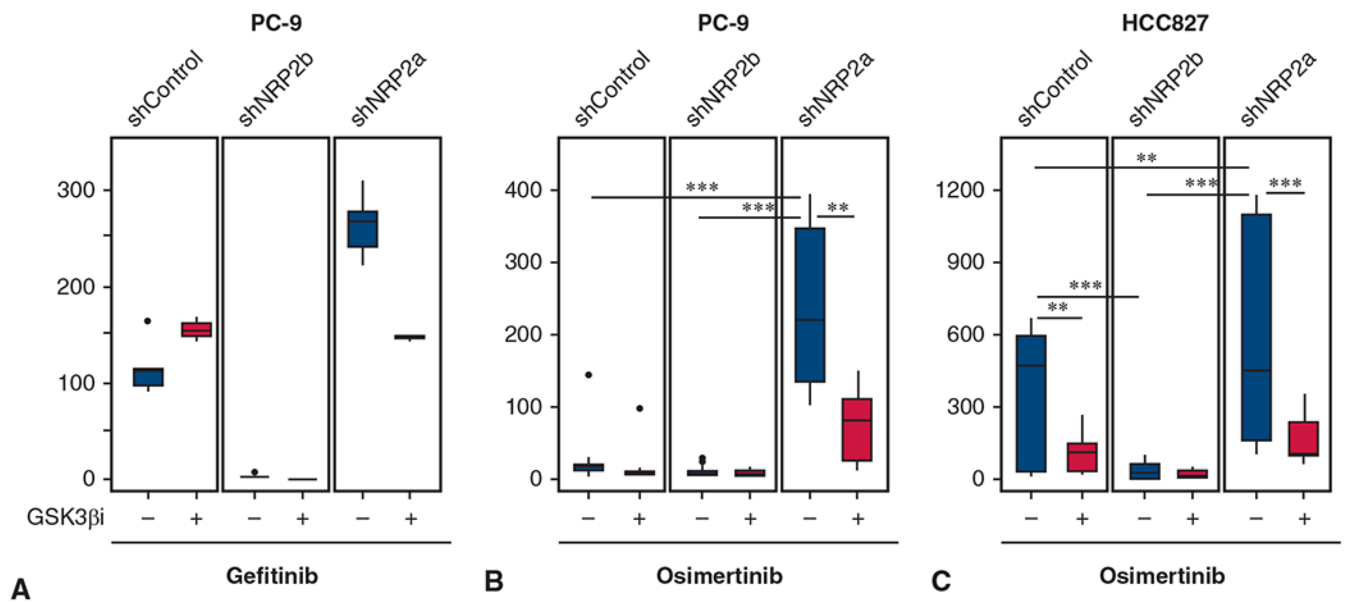

FIGURE 8.

Emergence of NRP2b-dependent, drug-tolerant persister cells requires GSK3*β* activity. A, PC-9 cells knocked down for NRP2a, NRP2b, or nontargeting controls were treated with gefitinib (2.5 *μ*M) for 4 weeks with or without the GSK3*β* inhibitor, TZDZ-8 (5 *μ*M). For quantitation, surviving persister cells were grown into colonies in nonselective medium for an additional 3 to 4 weeks. *Box* and *whisker plots* indicate colony counts. B, Same as (A) except PC-9 cells were treated with osimertinib (2.5 μM) with or without TZDZ-8 (5 *μ*M) for 9 days; surviving cells were grown into colonies for counting, as in (A). C, Same as (B) except HCC827 cells with knockdown of NPR2a, NRP2b, or nontargeted controls were treated with osimertinib (2.5 *μ*M) with or without TZDZ-8 (5 *μ*M) for 9 days. Significance determined by 2-tailed *t* tests. ** $P < .01$. *** $P < .001$.