

Peer Review File

Manuscript Title: Neuronal substance-P drives metastasis via an extracellular RNA/TLR7 axis

Redactions – unpublished data

Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

Padmanaban et al. present a manuscript in which they describe how sensory neurons drive tumor invasion, proliferation and metastasis. They describe an interesting, putative mechanism via the neuronal secretion of substance-P that subsequently leads to the release of ssRNAs from tumor cells and an autocrine activation of TLR7.

Overall, I enjoyed reading the manuscript which is well-written. The mechanism is thought-provoking and the conceptual advance of this work would be certainly of high interest for the Cancer Neuroscience community. Furthermore, I commend the authors for their creative experimental ideas including the co-transplantation of DRG neurons and their work on figuring out the putative mechanisms of ssRNAs mediating tumor biological effects on tumor cells themselves.

However, several methodological aspects make me significantly less enthusiastic about the work and I believe that significantly more experiments and additional experimental readouts in vitro and in vivo are needed to support the conclusions made in this manuscript. Unfortunately, a lot of figure descriptions and details in the methods section left me wondering what exactly was done in the respective experiments and how it was analyzed.

Major Points:

1. I find the hypothesis of secreted ssRNA that mediate an autocrine loop via substance-P intriguing. However, I am wondering whether the authors can indeed demonstrate that ssRNA is secreted from tumor cells and not e.g. released via cell death of tumor cells. Could the authors make use of a e.g. a molecular beacon for RNA imaging and demonstrate that RNA is indeed released from the tumor cells upon substance-P signalling? Is this release of ssRNA unspecific or are only certain ssRNAs secreted? What role does the immune system play in conferring anti- or pro-tumorigenic effects?
2. The experimental readouts for invasion is not convincing and does not reflect what the authors want to demonstrate. The authors report that 'Invasiveness of organoids/spheroids was calculated as the inverse of the circularity of the structure.' $1/\text{circularity}$ does not reveal a good quantification of tumor invasion. The authors used live-cell imaging to observe their spheroid models. It looks to me that potentially neurites from the DRG simply sprout out of the spheroid rather than invasive cancer cells e.g. in Fig. 1f-g. It also seems to be the case that 48 h might be too little time to really comment on the invasiveness of tumor cells. Single tumor cells need to be imaged with a tumor-specific marker (with a stably transduced fluorophore e.g.) on a single cell level and invasion speed needs to be measured as the displacement of the centre of mass of the respective cells. Ideally, these

readouts should be done in vivo using intravital imaging although I understand that these experiments are probably beyond the scope of this study. Another measure ex vivo could be the determination of a dissemination coefficient (number of tumor cells in a certain distance from the main tumor mass that would need to be defined according to the model). Or the authors could use a three-dimensional quantification of their invasive strands e.g. together with their optical clearing approach. As this assay is used throughout the manuscript (e.g. Fig. 1f-g, 2b, 2d-e, 3a,c, d, 4c,g, Extended Data Fig. 3b,e, Extended Data Fig. 5b, Extended Data Fig. 6a,g,i, Extended Data Fig. 7a,b) they will need to be complemented with more appropriate readouts. For this purpose, at least a 2D co-culture assay needs to be repeated for all these experiments and I would highly recommend at least ex vivo readouts for key experimental results.

3. The authors report in a lot of experiments the percentage of Ki67 positive cells/spheroid (e.g. Fig. 1h, Fig. 2c, Fig. 3e). I am wondering how similar the spheroids look like with respect to cell numbers in them and I cannot find exact details in their methods section. Can the authors use the DAPI signal in 3D to quantify tumor cell numbers and define the proliferation index with respect to tumor cells?

4. The number of nerve bundles is quantified in Fig. 1b-c. How exactly was this quantified and normalized to tumor volume? The authors write in their methods section that 'Extent of innervation in tissue sections was estimated by manually counting the number of nerve bundles, positively identified by β III-tubulin or neurofilament-L.' Was a specific tumor marker stained for? Where does the tumor tissue come from? How does nerve density vary across tumor tissues? How specific is β III-tubulin for nerve bundles? An analysis of a single cell gene expression data showed me that β III-tubulin is expressed in human breast cancer cells and to a lesser extent neurofilament-L (dataset from Wu et al., Nature Genetics 2021). I understand that the authors show that in monoculture the tumor cells do not express β III-tubulin but this might be different if the tumors grow in vivo. This approach to quantifying nerve bundles is overly simplistic and needs to account at least for tumor regions and tumor cell area.

5. It is unclear to me from the figure panel 1d) what exactly is depicted. Is this a 3D rendering of the tumor? Is the black region in the middle still tumor bulk? I would like to also see representative sections of the raw data here and would ask the authors to provide access to the raw data. The exact image processing needs to be described both in summary in the figure description and in detail in the experimental methods. Furthermore, I am wondering if and how much more there might be additional sensory innervation that is not marked by the CGRP antibody labelling. Lastly, can the authors also quantify the density of sensory innervation? It would be good to use this approach to quantify the tumor innervation across their mouse models.

6. How do the authors define their highly and poorly metastatic tumor models? Can this be also quantified and can a correlation to nerve density be analyzed?

The authors correlate sensory innervation and SP expression with lymph node metastases. However, the two cohorts from Fig. 1 consisting of 30 patients and the cohort of 19 patients is not described in more detail in the methods (In their editorial checklist, there are also no human research participants listed and clinical data is also not checked? In the reporting summary human research participants are noted but it needs to be very clear where which data is coming from.). Why are the same cohorts not used in both Figures? How are lymph node metastases quantified through the

whole body ? Can other metastatic site also be used for this purpose and thoroughly quantified such as lung, liver or brain ?

7. Throughout the manuscript tumor burden is quantified. Unfortunately, I was not able to find in the Methods section how this was analyzed. Especially when co-injecting DRGs I would like to see that a specific (fluorescent) tumor cell signal was quantified in three dimensions. Was the whole volume of the breast cut and tumor cell quantity determined ? Or was simply the apparent tumor cell mass measured ? If so the authors need to also quantify the percentage of stromal cells within the tumor mass.

8. I really like the experimental approach of co-injecting DRG neurons with tumor cells to understand the functional consequence of neuron-tumor interactions. However, looking at the data from 1k) I am wondering what happens to the DRG neurons after injections ? Are they even viable ? How do they look like ? Are they functional as measured by electrophysiology ? When do they play a role for tumor behavior ? Do they play a role in the main tumor mass and/or at the infiltrative edge ?

9. 1p) How was the number of macrometastases quantified ? This is unclear from the Methods section to me. What about at least liver and brain macrometastases ?

10. 2f) Overall, comparing the mean fluorescence intensity to compare the abundance of substance P in different tumor models needs to be carefully calibrated. At first glance, the background level in the 4T1 looks significantly higher than in the 67NR model. But this could also be due to the visualization. Where in the tumor was this signal measured ? Can the authors report their positive and negative controls and whether the antibody works properly in their system ? What is the number in their statistics referring to ? Does this represent different sections ?

Minor points

Some of the following experimental additions and further analyses would make this study stronger:

1. I appreciate that the authors wanted to identify the source of peripheral innervation of the murine mammary gland by using a retrograde tracing system. I would be highly interested in understand whether and how this changes in breast tumors ? In other words: which DRGs are labeled when the GFP-tagged cholera toxin b is directly injected into the breast tumor ?
2. Can the authors comment on how their cell lines were repetitively authenticated and can they include these details in their manuscript ?
3. I am wondering how substance P is secreted and why this is increased when tumor are co-cultured. It would be highly interesting to understand whether this is an neuronal activity-dependent process and whether there might be a vicious cycle of neuronal hyperexcitability/hyperactivity and tumor progression as described for brain tumors although this might be beyond the scope of this work.
4. 1k) Please add to the figure description what mCh stands for (I assume these are the tumor cells transduced with a mCherry fluorophore). Please add a quantification of the collective invasion strands to the manuscript.

5. A scale bar is missing for 1p.
6. How do the authors define macro- and micrometastases ?
7. The authors use tail vein injections of cells as an experimental model of metastasis and subsequently analyze lung metastases. It would be interesting in addition to use heart injections that would allow to also quantify liver and/or brain metastases to see whether the effects potentially are specific to lung metastases.

Referee #2 (Remarks to the Author):

Tumors are complex environments that are innervated by peripheral neurons. It is well-established the density of nerve innervation is correlated with cancer metastasis. In the current study, Padmanaban and colleagues investigate if a causal relationship exists by asking if dorsal root ganglia neurons directly stimulate breast cancer growth. They use in vitro and in vivo assays to provide evidence that the presence of DRG neurons enhances the growth, invasion, and spread of cancer cells. They next seek to identify factors that could be released from neurons to cause these effects. Previous work has shown there is rich bi-directional communication take place where the cancerous cells release neurotrophic factors that promote infiltration and afferent branching and neurons release transmitters and other signaling molecules that can stimulate tumor growth. The authors assume the candidate molecules are likely neuropeptides and indeed show that substance P has pro-metastatic activity that involves the release of single stranded RNA and Tlr7 signaling.

Overall, the net result of this study is a somewhat complex model where neuronal signaling to cancer cells initiates an autocrine signaling cascade to promote growth and metastasis. While an intriguing concept, major questions remain about what is causing the peptide release in the first place. Technically, DRG neurons are quite diverse in function and neurochemistry, with only a subset expressing the Tac1 gene. Thus, it is not clear what is really going on in the in vitro co-culture and DRG transplantation assays. In terms of novelty, as the authors state, there already exists a literature on sensory innervation strongly correlating to metastasis. However, although not cited, the anti-tumor effects of Aprepitant, a TACR1 antagonist that is routinely prescribed for nausea associated with chemotherapy, seems to have already been established.

Major concerns.

1. Can the authors comment on how co-transplanting 200 DRG cells with the cancer cells would enhance the metastases (Fig 1)? Probably only half or less of these DRGs are peptidergic nociceptors that can release SP. And unlike the cancer cells, these neurons are postmitotic and can't divide. The authors use 100 DRGs per well of a 24 well plate in the in vitro experiments to enhance invasiveness, and the effect is dose-dependent with the number of DRGs. How can a similar order of magnitude of cells exert such strong effects via a chemical mediator when delivered in the vastly larger compartment of the mouse body? Wouldn't SP released from so few DRGs simply be diluted?
2. The authors perform a denervation experiment using capsaicin (Fig 1). However, from what I can see the authors have not shown evidence the denervation worked. Presumably they can

immunostain the tumors and show the axonogenesis into the tumors is decreased.

3. As it stands, the in vitro experiments do not fully support the model presented in Figure 4. I think the key contradictory results comes on page 7. The authors demonstrate that conditioned medium (CM) from DRG neurons cultured alone contains substantially less SP than CM from DRG neurons cultured with 67NR spheroids (Extended Data Fig 6b). Notably, DRG-only CM is not sufficient to enhance invasiveness (Extended Data Fig 6h-j). They explain this by saying there must be other tumoural factors present in CM from DRG/67NR co-cultures produced upon interaction of sensory neurons with cancer cells and that is sufficient to promote breast cancer invasiveness. They identify ssRNAs as the crucial factors. They then find exogenous SP increases invasiveness in a manner dependent on ssRNA. Thus, according to the authors, sensory neurons secrete SP, which in turn promotes release of ssRNA from breast cancer cells that drive invasiveness and proliferation.

But here the logic falls apart. If exogenous SP can promote invasiveness, why can't the lower levels of SP present in CM from DRG-only cultures? This SP should also be able to promote ssRNA release to enhance invasiveness. Given it can't, then the most parsimonious explanation is that CM from DRG-only cultures simply just doesn't contain enough SP. But the authors have no explanation for why DRG-only cultures produces less SP than DRGs cultured with the 67NR spheroid. For their model to make sense, we need to account for why the 67NR spheroids appear to increase SP release from the co-cultured DRG neurons.

Minimal SP release from DRG neurons cultured alone is unsurprising because SP release depends on depolarization, calcium influx and dense core vesicle release. But cultured DRG neurons do not typically show spontaneous activity and calcium influx unless they are stimulated with a pro-excitatory mediator such as ATP or capsaicin. Given SP release is far higher in the presence of the spheroids, it seems likely the spheroid is releasing a chemical mediator that is exciting the co-cultured DRG neurons to evoke additional SP release. Bidirectional interactions between nerve and tumor seem likely because tumors must secrete instructive signals that trigger both nerve innervation and the action potential-dependent efferent release of SP in vivo.

The authors should perform two simple experiments to test this. First, DRG neurons cultured-alone can be treated with 67NR CM and the amount of SP release measured – if there is a bidirectional interaction, we would expect 67NR CM to increase the amount of SP released to the same levels as DRGs cultured with the spheroids. Second, the authors should load cultured DRGs with a Fura calcium dye and perform live imaging. We would predict that DRGs cultured with 67NR spheroids, or treated with 67NR CM, would exhibit more spontaneous calcium activity than DRGs cultured alone. I think performing these experiments is critical to understanding why CM from DRGs cultured alone does not enhance invasiveness in vitro and would clarify why a normally silent neuron type is releasing SP into tumors in vivo.

4. The authors perform some in vitro experiments using DRGs from Tac1 KO mice. These animals are completely deficient in SP and are the strongest test of whether SP release from DRGs is necessary for tumor invasiveness. The authors should comment on the fact that even though galanin is sufficient to enhance invasiveness, galanin release apparently cannot compensate for the absence of SP. The manuscript would be improved significantly if the authors performed in vivo experiments of

metastasis using the Tac1 KO mice, as they clearly have access to them. For example, they could test tumor growth and metastasis in Tac1 KO bearing 4T1 tumors. Failure to metastasize in these KO animals would really strengthen the conclusions of this paper.

5. The authors provide convincing in vivo data that the NK1 antagonist aprepitant, an anti-emetic, can inhibit tumor growth and metastasis in multiple models. They conclude by saying this “warrants investigations into the clinical efficacy of this agent upon its prolonged use in breast cancer in combination with standard of care regimens for the prevention of metastatic relapse.” With these striking in vivo data, the paper does appear to be quite a substantial advance in breast cancer research.

Of course, aprepitant is commonly given to many patients with breast cancer to treat chemotherapy-induced nausea. And indeed, a quick google reveals there appears to be a rich literature of clinical observations showing additional anti-tumor effects of aprepitant.
<https://clinmedjournals.org/articles/ijccr/international-journal-of-cancer-and-clinical-research-ijccr-3-071.pdf> <https://www.spandidos-publications.com/10.3892/ijo.2014.2565>
<https://link.springer.com/article/10.1007/s10637-009-9218-8>

Indeed, there are also numerous in vitro and in vivo studies of various cancers showing anti-tumor effects of aprepitant. Could the authors explain their decision not to cite any of this literature?

6. Although I am not an expert in these types of experiments, the same measures between experiments seems to vary significantly with no explanation. For example, the reduction of the number of macromets with anti-Sp antibody is similar to the baseline with vehicle in the Aprepitant experiment. The tumor burden is very consistent throughout experiments - so this seems odd?

Other comments:

1. Primary DRG cultures will have many non-neuronal cells, even after gradient purification, that divide and take over the cultures. This is usually mitigated by supplementing media with anti-mitotics. Since this can't be done here, how do the authors avoid this contamination?

2. Although perhaps too much to ask, for the authors to convincingly prove a sensory requirement, there are genetic strategies to very specifically ablate distinct types of sensory neurons using Cre-dependent DTA and/or block transmitter and peptide release using Cre-dependent TetTOX.

3. The authors suggest the transplanted DRG are functional. I find this hard to believe. Normally these cells have their soma restricted to ganglia and make synaptic connections with the CNS. Functional proof of a higher burden than presented here is required.

4. The authors claim to have a function blocking Substance P antibody that works in vivo but provide no evidence of the efficacy. Validation (and a dose response) is required.

5. The images of the DRGs in the culture experiment suggest they may not be healthy. For example the image in Fig1F the soma is irregular with dense inclusions and the axon filaments look to be

fragmented. Data showing what type of DRG neurons these are and that they are alive and functional is needed.

Referee #3 (Remarks to the Author):

In their study entitled “Neuronal substance P drives breast cancer growth and metastasis via an extracellular RNA/TLR7 axis”, Padmanaban et al analyze mechanisms that underly the correlation of innervation of tumors and tumor growth as well as metastasis. The authors show that poorly metastatic tumors are less innervated. Using in vitro systems, in vivo models and patient derived material, the authors demonstrate that modulation of innervation or neuronal derived factors impacts tumor growth and metastasis and identify an SP/TACR1/ssRNA/TLR7 axis driving the observed effects. Based on these findings, the authors use an anti-nausea drug that targets TACR1 in two different in vivo models as well as in vitro 2D and 3D cultures.

The role of neurons in cancer progression and metastasis is increasingly recognized. However, mechanistic insight is still sparse. This study identified a potential mechanism how innervation or neuron derived factors affect cancer cells and increase their metastatic potential. The study provides interesting insight into a SP/TACR1/ssRNA/TLR7 axis driving tumor growth and metastasis together with a potential therapeutic intervention using Aprepitant to target TACR1. However, several questions and concerns remain to be addressed. The study is timely and the described results are novel and provide an interesting new perspective on the potential role of neurons in tumor growth and metastasis. The manuscript is well structured and the rationale for the performed experiments is clear. The used methodology is appropriate. Yet, there is much emphasis on in vitro systems especially for mechanistic insight that is not thoroughly validated in vivo. Numbers of animals for in vivo validation is low and there is no detailed evaluation of the in vivo data that would allow a more critical view on the role of the SP/TACR1/ssRNA/TLR7 axis in vivo. Therefore, the conclusions drawn in this manuscript should be better supported by thorough in vivo insight. References to previous literature is balanced and comprehensive.

The authors show that the highly metastatic 4T1 line shows higher innervation than the low metastatic variant 67NR and also demonstrate in other models and patient samples that higher innervation correlates with increased metastasis. It remains unclear, which properties of cancer cells induce tumor innervation and thereby trigger interactions between neurons and cancer cells that promote tumor growth and metastasis.

In patient cohorts, the authors describe higher rates of lymph node metastasis. In the mouse model, the authors quantify lung metastasis. Throughout the manuscript, the general term of metastasis is used. However, based on the provided data it is not clear whether the SP/TACR1/ssRNA/TLR7 axis leads to a general increase in metastasis or whether preferential organ-tropism can be observed. What about other metastatic sites breast cancer is prone to metastasize to including bone, liver or brain? Can the authors provide insight from the patient cohorts regarding the occurrence of organ-

specific metastasis? Did the authors check for tumor cell spread to lymph nodes? The experiment shown in Figure 3 i-k could be extended by other injections routes than intravenous injection to analyze effects on bone, liver and brain metastasis.

When co-implanting DRGs together with 67NR cells, the authors observe increased tumor growth, higher numbers of CTCs as well as increased number of micrometastases. The authors state that 67NR tumor cells can intravasate but then a high proportion of cancer cells fails to extravasate. Does that mean, that experimentally induced innervation by co-implantation of DRGs locally affects 67NR tumor cells and increases their capacity in intravasate but the effect on invasiveness is lost within the circulation and cells fail to extravasate and colonize the target organ? Alternatively, one could assume that in naturally occurring innervation as observed in the 4T1 model, innervation is also observed at the metastatic site? That might mean that cancer cells that are able to induce innervation at the primary site can also induce innervation at the metastatic site. Whereas 67NR even with co-implantation of DRGs fail to accomplish this at the metastatic site. Analysis of innervation at the metastatic site is critical to further address this point.

In vivo trials in this study are designed to analyze only short-term effects. Growth kinetic is only evaluated up to 4 weeks after injection. To monitor the growth kinetic, tumor growth should be followed longer and survival experiments should be performed.

This is particularly important for experiments with the TACR1 antagonist. Here it looks like that Aprepitant treatment slows tumor growth for 1 week followed by tumor growth at similar rates compared to the control group. Furthermore, representation of growth curves of individual mice instead of mean values would better reflect the actual effects. The in vivo trials are performed with low replicate numbers. Metastatic burden is only represented as number of micro- or macrometastases. Looking at the provided representative gross overviews of the lungs it appears that the size of the individual metastasis is almost similar while numbers of lesions changes. Can the authors add information of the metastasis burden based on cumulative metastatic load and size of individual tumors.

The authors use ex vivo spheroid assays for critical mechanistic insight. While the use of such systems is valuable to gain first mechanistic insight, more thorough in vivo validation should be done. This is particularly important for effects where biodistribution or availability of factors is limited in a in vivo setting. In this study, there is lack of detailed in vivo validation of the observed effects.

Referee #4 (Remarks to the Author):

In this manuscript, Padmanaban et al. discovered that sensory innervation in tumor lesions promotes cancer growth and metastasis via substance P and single-stranded RNA (ssRNA). First, the authors showed that aggressive tumors tend to be innervated more in mouse and human cancers. Then they found that the sensory neuron innervation promotes tumor growth and invasive capacity by developing a 3D co-culture assay system. Substance P produced by neurons is likely to serve as a mediator between neuron-tumor interactions. Moreover, the authors identified that ssRNA is secreted when the tumor cells are co-cultured with neuronal cells, and the ssRNA in turn activates the transcriptional program to induce invasion and proliferation in a TLR7-, but not TLR3-, dependent manner. Interestingly, the authors further showed the efficacy of a clinically available inhibitor of substance P receptor. Taken together, the authors identified the novel link between neuron-tumor interactions to facilitate tumor progression.

Although this study is potentially interesting, there are various weaknesses that need to be addressed. In particular, the molecular mechanisms of how substance P promotes ssRNA secretion are not fully addressed, and the contribution of TLR7 needs to be more profoundly analyzed. Specific comments are following:

Major comments:

- 1) In several figures, the authors utilized the tumor cell injection into the tail vein as a model to evaluate metastasis. However, this is not a suitable model for metastasis since the tumor cells do not need to leave the primary tumor lesion, which is a key event in metastasis. Rather, it would be better to examine macro-metastasis using the method that generates primary lesions such as intraductal injections.
- 2) In Figure 1n, the authors showed that the denervating agent capsaicin inhibits breast cancer metastasis. However, a previous study has shown that this compound rather promotes metastasis using a similar animal model, which contradicts the authors' findings (PMID: 15161056). Therefore, the authors need to reconcile this discrepancy.
- 3) In Extended Figure 3d, the authors should test if the human neurons similarly promote invasion or proliferation capacity in human tumor cells, instead of using mouse DRG neurons. This is important for extrapolating their findings into clinics.
- 4) Figure 3A lacks proper negative control experiments (e.g. without any treatment). The authors should repeat this experiment in a more appropriate manner. Importantly, several lines of evidence suggested that some RNases can reduce tumor cell viability and/or metastatic capacity (PMID: 21194552, 23832341). Therefore, it would be meaningful to confirm that the RNase they used does not affect tumor invasiveness.
- 5) Although the authors' model suggests that substance P promotes the ssRNA secretion from tumor cells, this notion is not fully supported by experimental evidence. The authors should evaluate the amount of secreted ssRNA in spheroid culture conditions with and without substance P by using 4T1 and 67NR cell lines.
- 6) The authors used ssRNA40 and poly I:C to stimulate TLR7 and TLR3, respectively. However, methods for the treatment were not written. Given TLR7 is expressed in the endosome, ssRNA should be complexed with cationic lipids such as DOTAP to activate the cells. It is not clear if the authors used cationic lipids for the treatment. The authors should use other synthetic TLR7 ligands

such as imidazoquinolines, too. Similarly, the concentrations of poly I:C used in the study may not be sufficient to activate TLR3, if they simply added it to the media.

7) The molecular mechanisms of how substance P promotes the secretion of ssRNA is unclear and should be clarified. Is the apoptotic process activated to secrete as DAMPs? Alternatively, are there any secretory mechanisms that transport ssRNA specifically?

8) In Figure 4k, the authors did not treat the cells with substance P or ssRNA, which should be necessary for the activation of invasive capacity in their model. To further evaluate the role of substance P in TLR7 signaling, the authors should repeat this experiment by culturing control and Tlr7-deficient cells with or without substance P. By doing so, the authors can check if invasive programs are activated upon the treatment with substance P, and this effect is abrogated under Tlr7 deficiency.

9) The TLR7 signaling pathway is known to activate type I interferon signatures as well as inflammation. In this regard, the pathways differentially regulated by the depletion of TLR7 (Figure 4l) are surprising. Does this mean that a non-canonical signaling pathway is activated in this setting? The authors can analyze the question by knocking out MyD88, an adaptor protein critical for the canonical TLR7 signaling.

Minor comment:

1) The authors need to describe what CTC stands for.

Author Rebuttals to Initial Comments:

We thank the reviewers for their insightful comments on our manuscript. We have addressed all of the referee concerns experimentally—this has significantly strengthened our conclusions and expanded the scope of our manuscript. Some key findings from the revisions are summarized below:

1. Neuronal SP induces release of ssRNA from cancer cells by causing apoptotic cell death in a fraction of cancer cells. Inhibition of apoptosis prevents the ability of DRG neurons or SP to promote cancer cell invasion.
2. There is a significant increase in the amount of RNA released by cancer cells upon DRG co-culture or SP treatment. This ssRNA release is blocked in the presence of an apoptosis inhibitor.
3. Orthotopic transplantations of two independent cancer cell lines into *Tac1-null* mice (whose neurons do not produce SP) have conclusively demonstrated that SP release can drive breast cancer tumor growth and metastasis.
4. Calcium imaging studies have demonstrated that cancer cells enhance neuronal activity, which is consistent with increased SP release in the presence of cancer cells.
5. Neuronal activation of TLR7 signaling in cancer cells promotes cancer cell growth, invasion, and metastasis in a non-canonical, MyD88-independent, and immune independent manner.
6. Imaging-based studies have definitively demonstrated the viability and functionality of DRG neurons when co-transplanted with cancer cells into mice. Furthermore, we have shown that co-transplantation with DRG neurons promotes tumor growth *in vivo* in a cell-dose dependent manner.
7. Aprepitant significantly inhibited tumor growth in two independent patient-derived xenograft (PDX) models.
8. Intra-cardiac injections of cancer cells pre-treated with DRG conditioned medium showed a significant increase in metastases to the lung, liver, and brain of mice.
9. Retrograde tracing of DRG neurons innervating breast tumors has revealed that highly metastatic breast tumors are innervated by a significantly higher number of ganglia than poorly metastatic tumors.
10. Endothelial-derived Slit2, an axon guidance molecule, is a regulator of innervation in breast tumors.

Referee #1 (Remarks to the Author):

Padmanaban et al. present a manuscript in which they describe how sensory neurons drive tumor invasion, proliferation and metastasis. They describe an interesting, putative mechanism via the neuronal secretion of substance-P that subsequently leads to the release of ssRNAs from tumor cells and an autocrine activation of TLR7. Overall, I enjoyed reading the manuscript which is well-written. The mechanism is thought-provoking and the conceptual advance of this work would be certainly of high interest for the Cancer Neuroscience community. Furthermore, I commend the authors for their creative experimental ideas including the co-transplantation of DRG neurons and their work on figuring out the putative mechanisms of ssRNAs mediating tumor biological effects on tumor cells themselves.

We sincerely thank the reviewer for sharing our excitement about the significance of our study and for making great suggestions as to how we can strengthen our conclusions. We have worked very hard on addressing all your concerns and believe that the manuscript has greatly benefited from your useful insights.

However, several methodological aspects make me significantly less enthusiastic about the work and I believe that significantly more experiments and additional experimental readouts *in vitro* and *in vivo* are needed to support the conclusions made in this manuscript. Unfortunately, a lot of figure descriptions and details in the methods section left me wondering what exactly was done in the respective experiments and how it was analyzed.

As you will see, we have conducted substantial additional *in vivo* and *in vitro* experimental studies, which have strengthened our conclusions and enhanced the scope of the manuscript. In addition to addressing the experimental concerns below, we have extensively revised our Methods and figure legends to add more detail.

Major Points:

1. I find the hypothesis of secreted ssRNA that mediate an autocrine loop via substance-P intriguing. However, I am wondering whether the authors can indeed demonstrate that ssRNA is secreted from tumor cells and not e.g. released via cell death of tumor cells.

We thank the reviewer for this comment since it directly led us to make some unexpected and exciting discoveries. We hypothesized that ssRNA release from cancer cells could result from either regulated secretory mechanisms or from cell death. In support of the latter, we found that a small number of cancer cells undergo apoptosis when cultured with DRG neurons or upon treatment with substance-P (Fig. 3m). Importantly, neither DRGs nor SP are able to drive

invasion of cancer cell spheroids in the presence of a pan-caspase inhibitor (Fig. 3n). In further confirmation that cancer cell death is contributing to ssRNA release, there is a significant decrease in the amount of RNA in the conditioned medium (DRG-CM) upon treatment with a caspase inhibitor (Fig. 3o). These interesting findings reveal that neuronal substance-P causes apoptosis in a subset of cancer cells, releasing ssRNA which binds TLR7 receptors on neighboring cancer cells to drive metastasis.

Could the authors make use of a e.g. a molecular beacon for RNA imaging and demonstrate that RNA is indeed released from the tumor cells upon substance-P signalling? Is this release of ssRNA unspecific or are only certain ssRNAs secreted? What role does the immune system play in conferring anti- or pro-tumorigenic effects?

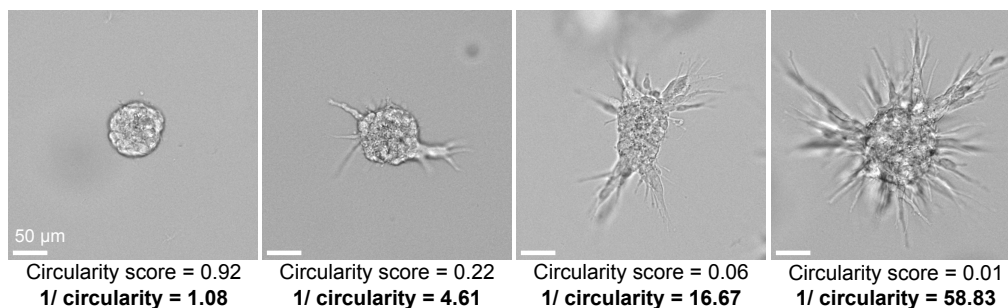
To address this point, we isolated RNA from conditioned medium and ran it through a Bioanalyzer to quantify the amount of RNA. We found that DRG-CM (from a 67NR-DRG co-culture) has significantly higher amounts of RNA compared to DRG only-CM or 67NR only-CM. There is also a significant decrease in the amount of RNA in DRG-CM when in the presence of an apoptosis inhibitor (Fig. 3o).

There is a significant increase in the amount of RNA released when 67NR cancer cell spheroids are treated with SP – confirming that RNA is released from the cancer cell compartment (Fig. 3o).

Given that cancer cell death is the source the extracellular RNA, multiple RNA species would be released. From the Bioanalyzer traces, we find that extracellular RNA species in CM are <200 nt (Fig. 3o). Our data shows that the extracellular ssRNA signals via tumoral TLR7 receptors (Fig. 4 a-e). TLR7 receptors largely sense ssRNAs based on structural elements, not their nucleotide sequence (PMIDs: 22617878, 18071655). Given these new data, the use of tools that recognize sequence specific RNAs such as molecular beacons were not appropriate.

2. The experimental readouts for invasion is not convincing and does not reflect what the authors want to demonstrate. The authors report that ‘Invasiveness of organoids/spheroids was calculated as the inverse of the circularity of the structure.’ $1/\text{circularity}$ does not reveal a good quantification of tumor invasion.

We thank you for this comment as it is important for us to be clear about the method we used to quantify invasion. To summarize, we use phase-contrast images of spheroids embedded in 3D collagen, manually trace the spheroid boundary, use ImageJ to measure circularity of the boundary, and we then plot the inverse of circularity as invasiveness. The circularity score tells us how close a shape is to a perfect circle (score=1); the more distorted (or invasive) the boundary, lower the score. For invasion analysis of 3D cultures, this circularity score is the standard in the field (five example PMIDs that use this method: 31485072, 28874803, 33578855, 34081437, 28186196). Other measures of invasion in 3D cultures include quantifying the number of invasion strands or length of the invasion strand (PMIDs: 32777015, 35292774). Both measurements are captured in the circularity score (Rebuttal Fig. #1).



Rebuttal Fig. #1: Range of circularity scores observed for 67NR spheroids in collagen I.

The authors used live-cell imaging to observe their spheroid models. It looks to me that potentially neurites from the DRG simply sprout out of the spheroid rather than invasive cancer cells e.g in Fig. 1f-g.

Neurites are small fibers (~1µm) that are clearly distinguishable from cancer cells (~10 µm) due to the difference in scale and morphology. Spheroid/ organoid invasion data reported in the manuscript are all from collective invasion of cancer cells (tens of µms). Additionally, most experiments in Fig 2 – 4 (for example, those with SP) involve cancer cells being cultured in the absence of neurons. The invasive morphology observed here is similar to that observed in the co-culture system and the same readout for invasion (circularity score) is used. Therefore, we are confident that we are definitively measuring the cancer spheroid boundary.

It also seems to be the case that 48 h might too little time to really comment on the invasiveness of tumor cells.

The endpoint for invasion assays is defined by the cancer model we use. Spheroids generated from cancer cell lines invade into collagen I quite readily, often within the first 24-48 hours. In contrast, primary organoids such as those from the MMTV-PyMT model or from patient samples do not invade for ~3-5 days. We use these corresponding endpoints for each of those model systems. If we culture cancer cells spheroids for longer than these times, we frequently see spheroids growing into each other, making any invasion quantifications very challenging. We have updated the Methods section to reflect these times.

Single tumor cells need to be imaged with a tumor-specific marker (with a stably transduced fluorophore e.g.) on a single cell level and invasion speed needs to be measured as the displacement of the centre of mass of the respective cells. Ideally, these readouts should be done *in vivo* using intravital imaging although I understand that these experiments are probably beyond the scope of this study.

An example of a fluorescently labeled (mCherry+) spheroid and a β III-tubulin+ DRG neuron co-cultured for 48 hours is now shown in Extended Data Fig. 2b. There is little physical contact between neurites and cancer cells. Additionally, cancer cells frequently invade by extending collective invasion strands into the collagen (or into the stroma *in vivo*, see Fig. 1m). Each strand contains multiple cells and one spheroid has multiple invasion strands. We do not believe that it would be possible to accurately measure cell displacement under these conditions. We agree with the referee's comment that intravital imaging experiments are beyond the scope of study.

Another measure *ex vivo* could be the determination of a dissemination coefficient (number of tumor cells in a certain distance from the main tumor mass that would need to be defined according to the model). Or the authors could use a three-dimensional quantification of their invasive strands e.g. together with their optical clearing approach. See above re: circularity score.

The amount of dissemination observed in 3D collagen varies across various models of breast cancer. While 4T1 cancer cell spheroids disseminate frequently, we observe little dissemination from 67NR, MMTV-PyMT, or EO771 cancer cells, or primary human samples. Invasion is a more robust phenotype in this assay— it is more uniformly comparable across these systems and models.

However, we did quantify the number of circulating tumor cells (a measure for systemic dissemination) when 67NR cancer cells were transplanted with or without DRG neurons. We observed that co-transplantation with DRG neurons significantly increased the number of circulating tumor cells in these animals (Extended Data Fig. 4f).

As this assay is used throughout the manuscript (e.g. Fig. 1f-g, 2b, 2d-e, 3a,c, d, 4c,g, Extended Data Fig. 3b,e, Extended Data Fig. 5b, Extended Data Fig. 6a,g,i, Extended Data Fig. 7a,b) they will need to be complemented with more appropriate readouts. For this purpose, at least a 2D co-culture assay needs to be repeated for all these experiments and I would highly recommend at least *ex vivo* readouts for key experimental results.

We have conducted the requested experiments and performed more traditional trans-well invasion assays using 67NR cancer cells +/- DRG neurons or +/- SP. Consistent with our findings in 3D culture, we find that both DRG neurons and SP promote cancer cell invasion (Extended Data Fig. 2 c-d, 6a respectively).

3. The authors report in a lot of experiments the percentage of Ki67 positive cells/spheroid (e.g. Fig. 1h, Fig. 2c, Fig. 3e). I am wondering how similar the spheroids look like with respect to cell numbers in them and I cannot find exact details in their methods section. Can the authors use the DAPI signal in 3D to quantify tumor cell numbers and define the proliferation index with respect to tumor cells?

We apologize for not clarifying this in the legends/ Methods section. All Ki67 measurements reported in the manuscript are %Ki67+ nuclei (of the total number of nuclei marked by DAPI). This method normalizes for any difference in spheroid size. We have updated our Methods section to state this more explicitly.

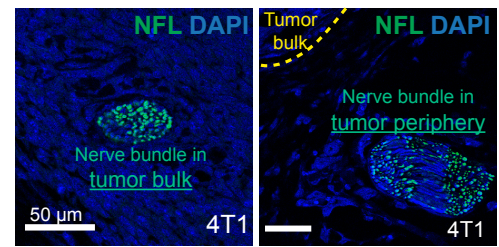
4. The number of nerve bundles is quantified in Fig. 1b-c. How exactly was this quantified and normalized to tumor volume? The authors write in their methods section that 'Extent of innervation in tissue sections was estimated by manually counting the number of nerve bundles, positively identified by β III-tubulin or neurofilament-L.' Was a specific tumor marker stained for?

Innervation was quantified in size-matched tumors (at the time of harvest). Immunofluorescence for CGRP (Fig. 1d) or NFL/ β III-tubulin (Extended Data Fig. 1b) was used to positively identify the nerve bundles. Two sections per tumor were averaged and plotted as a single data point. The tumor mass can easily be distinguished based on DAPI staining

within the stroma to visualize nuclei density and nuclei size (Rebuttal Fig. #2). Any innervation in the surrounding tissue was not included in the analysis. We have clarified these points in the Methods section.

Where does the tumor tissue come from? How does nerve density vary across tumor tissues?

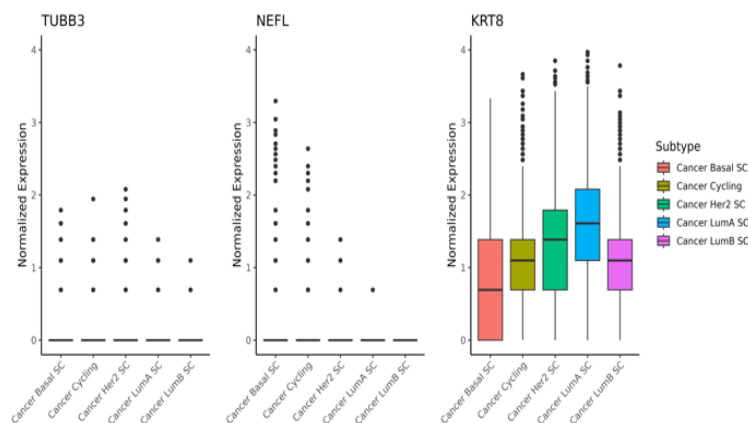
For syngeneic models (67NR/ 4T1/ EO771/ HCC1806), cancer cells were orthotopically transplanted into the mammary gland, and tumors were allowed to grow until they reached ~1500 mm³ in volume prior to harvest. For genetic models (MMTV-PyMT/ C31(T), mice spontaneously form mammary tumors in multiple mammary glands. We harvested late-stage (~1500 mm³) tumors.



Rebuttal Fig. #2: Representative nerve bundles within the tumor bulk or tumor periphery.

How specific is β III-tubulin for nerve bundles? An analysis of at single cell gene expression data showed me that β III-tubulin is expressed in human breast cancer cells and to a lesser extent neurofilament-L (dataset from Wu et al., Nature Genetics 2021). I understand that the authors show that in monoculture the tumor cells do not express β III-tubulin but this might be different if the tumors grow *in vivo*.

Immunofluorescence for both β III-tubulin and NFL can clearly identify neuronal structures (morphology) and there is no staining of cancer cells observed *in vivo*. The same is true for CGRP staining *in vivo* (representative nerve bundle in Fig 1d). Additionally, in our WB experiments, we include tumor organoids that were freshly isolated from tumors grown in mice (these organoids were not cultured or passaged). There is no observed expression for β III-tubulin in these organoids (Extended Data Fig. 1d). Finally, we investigated the data from Wu *et al.* and found that *Tubb3* (gene encoding β III-tubulin) or *Nefl* (gene encoding NFL) transcripts are not detected in breast tumors. In contrast, expression of a luminal epithelial marker, Keratin-8 (KRT8) was significantly upregulated in human breast tumors (Rebuttal Fig. #3).



Rebuttal Fig. #3: Relative expression of neuronal markers (*Tubb3* or *Nefl*). Data plotted includes 24489 single cells from 20 patients (3 HER2+, 8 triple-negative, 9 ER+ breast cancers). To generate the figures above, raw counts were imported, metadata provided by the authors was appended, normalization and variance-stabilization was performed with SCTransform v2.

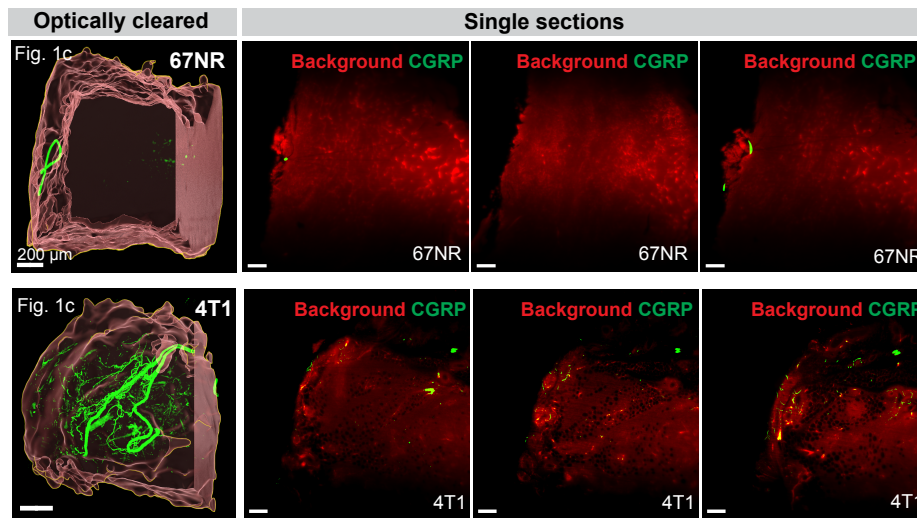
This approach to quantifying nerve bundles is overly simplistic and needs to account at least for tumor regions and tumor cell area. As described above, both are accounted for.

5. It is unclear to me from the figure panel 1d) what exactly is depicted. Is this a 3D rendering of the tumor? Is the black region in the middle still tumor bulk? I would like to also see representative sections of the raw data here and would ask the authors to provide access to the raw data. The exact image processing needs to be described both in summary in the figure description and in detail in the experimental methods.

We apologize for not describing the methods for the rendering of optically cleared tumors.

It is a maximum intensity projection of CGRP staining within a 3D tumor mass (~1 cm in diameter). Autofluorescence in the 488 channel is typically used to mark the tumor boundary. This mode of representation is standard for cleared images (for another example, see Fig. 1b from the original iDISCO clearing paper (PMID: 25417164) that was inspired by the CLARITY method of tissue clearing (PMID: 24945384)).

For cleared samples, there are ~2000-5000 individual sections and total data is over 100 GB/ sample. We are happy to submit the raw files to Nature if the reviewer would like to examine them. We have presented 3 representative sections each from 67NR and 4T1 tumors (Rebuttal Fig. #4).



Rebuttal Fig. #4: Representative single slices from optically cleared 67NR or 4T1 primary tumors.

Furthermore, I am wondering if and how much more there might be additional sensory innervation that is not marked by the CGRP antibody labelling.

CGRP is the most expressed peptide in sensory neurons. Importantly, all SP expressing neurons are CGRP+ (PMID: 2434236). Since our data support a model in which neuronal SP drives metastasis, we believe that we are capturing the most relevant sensory neuronal population in our analyses.

Lastly, can the authors also quantify the density of sensory innervation? It would be good to use this approach to quantify the tumor innervation across their mouse models.

We thank the reviewer for bringing up this important point. Indeed, we quantified the number of CGRP+ nerve bundles in poorly metastatic (67NR, EO771, HCC1806) and highly metastatic (4T1, EO771 LM2, HCC1806 LM2) tumors. We found that the extent of CGRP+ sensory innervation correlates with its metastatic potential (Fig. 1d).

6. How do the authors define their highly and poorly metastatic tumor models? Can this be also quantified and can a correlation to nerve density be analyzed?

Isogenic cell lines of differential metastatic potential (Eg: 67NR vs 4T1) were originally described by Miller in 1992 (PMID: 1540948) and are widely used in breast cancer research (a few example PMIDs: 20064377, 32999457). These cell lines were generated by performing *in vivo* selection (repeated injections of cancer cells *in vivo* to select for highly metastatic cancer cells). The 67NR cell line was selected for its inability to metastasize (cells appear to be trapped in the lung endothelium when transplanted orthotopically, Extended Data Fig. 4e) while the 4T1 model results in large macro-metastases.

In the original manuscript, we had included quantification for the total number of nerve bundles in poorly vs highly metastatic tumors (now Extended Data Fig. 1b). We have now updated this to include sensory-specific nerves. Highly metastatic tumors have significantly higher innervation from sensory neurons (Fig. 1d).

The authors correlate sensory innervation and SP expression with lymph node metastases. However, the two cohorts from Fig. 1 consisting of 30 patients and the cohort of 19 patients is not described in more detail in the methods (In their editorial checklist, there are also no human research participants listed and clinical data is also not checked? In the reporting summary human research participants are noted but it needs to be very clear where which data is coming from.). Why are the same cohorts not used in both Figures?

We apologize for not including a more detailed description of these methods in the original manuscript. The two patient cohorts correspond to different mediums in which the tumor sample was received. For Fig. 1a (11 patients), fresh tumor samples were received which were snap-frozen and processed for WB. For Fig. 1b (19 patients), we received FFPE tumor blocks that were compatible only with IF analyses. We have now explicitly stated this in the Methods section.

“No human participants” was selected since this study is IRB-exempt. Samples were received from the Cooperative Human Tissue Network (CHTN) and were de-identified. We receive pathology reports from these samples that contained clinically relevant information including the patient’s lymph node status.

How are lymph node metastases quantified through the whole body? Can other metastatic site also be used for this purpose and thoroughly quantified such as lung, liver or brain?

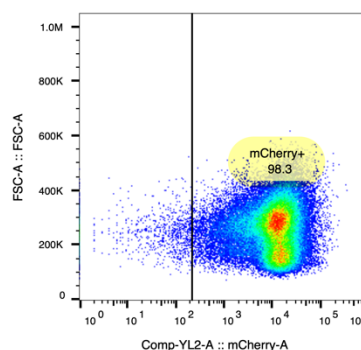
Sentinel and auxiliary lymph nodes are frequently excised from patients at the time of surgery for diagnostic and staging purposes. Lymph node metastases are indicative of additional distant metastases in the patients and overall prognosis (PMIDs: 32466193, 17342568). The lymph node status of each patient was provided to us in the pathology report that accompanied each tumor sample.

Most mouse breast cancer models, including all the models we use in the study, metastasize predominantly to the lung when injected orthotopically into the mammary gland. We thank the reviewer for encouraging us to look at other metastatic sites since it led to some striking data. We pre-treated mCherry+ 67NR cancer cells with either tumor-CM or DRG-CM prior to intra-cardiac injections into mice. We observed that pre-treatment with DRG-CM significantly increases metastasis to the lungs, liver, and brain (Extended Data Fig. 7 i-k).

7. Throughout the manuscript tumor burden is quantified. Unfortunately, I was not able to find in the Methods section how this was analyzed. Especially when co-injecting DRGs I would like to see that a specific (fluorescent) tumor cell signal was quantified in three dimensions. Was the whole volume of the breast cut and tumor cell quantity determined? Or was simply the apparent tumor cell mass measured? If so the authors need to also quantify the percentage of stromal cells within the tumor mass.

Tumor size is measured 1-2x each week using digital calipers. Tumor volume is then calculated according to $r \times h^2/2$, as is commonly used in the breast cancer field (PMID: 23689461). Since neurons are not proliferative, and only a small number of neurons were injected (~250), they do not contribute meaningfully to the tumor volume. As the tumor grows in mice, it almost completely replaces the normal mammary gland – only a distinct tumor mass remains, which was measured.

When a mCherry+ 67NR tumor is run through the flow cytometer, over 98% of the cells are cancer cells (Rebuttal Fig. #5). Therefore, stromal cells do not contribute significantly to the tumor mass.



Rebuttal Fig. #5: Flow cytometry plot of a mCherry+ tumor demonstrating that most of the cells in the tumor are mCherry+ cancer cells.

8. I really like the experimental approach of co-injecting DRG neurons with tumor cells to understand the functional consequence of neuron-tumor interactions. However, looking at the data from 1k) I am wondering what happens to the DRG neurons after injections? Are they even viable? How do they look like? Are they functional as measured by electrophysiology? When do they play a role for tumor behavior? Do they play a role in the main tumor mass and/or at the infiltrative edge?

We thank the reviewer for recognizing the creativity of the co-transplantation assays – it certainly has greatly improved our basic understanding of the role neurons play in the breast tumor microenvironment.

In the original manuscript, we had included a tile scan (2D sections) of 67NR and 67NR+DRG tumors. The latter had increased nerve bundles. Our co-transplantation experiments are performed with only ~200-250 neurons in a tumor that grows to contain millions of cancer cells making electrophysiology studies not technically feasible. Given the referee's suggestions along with some comments from Reviewer #2, we have now significantly strengthened our conclusion that neurons are both viable and functional when co-transplanted with cancer cells *via* the following:

(1) We performed optical tissue clearing of 67NR and 67NR+DRG tumors and immuno-stained for the sensory nerve marker, CGRP. While control 67NR tumors have little sensory innervation, sensory nerve fibers robustly innervate co-transplanted tumors (Extended Data Fig. 4a).

(2) There is a significant increase in the amount of SP in 67NR+DRG tumors, relative to control 67NR tumors (Extended Data Fig. 4b).

(3) We performed co-transplantations of 67NR cancer cells with increasing numbers of DRG neurons (25, 250, or 1000 DRGs injected per mouse). We found a cell-dose dependent increase in tumor growth (Extended Data Fig. 4 c-d).

Additionally, in our tail-vein experiments in which we co-injected DRG neurons with 67NR cancer cells, we observed CGRP+ neuronal cell bodies within the lungs of these mice (Extended Data Fig. 4h). Since there are no resident neurons in the lung, the neuronal cell bodies observed are coming from the ones injected into the mice. These neurons are branched, express neuropeptides, and therefore are viable.

We do not observe any preferential localization of nerves within the tumor bulk vs invasive edge.

9. How was the number of macrometastases quantified? This is unclear from the Methods section to me. What about at least liver and brain macrometastases?

Thank you for raising this concern. Macro-metastases were quantified using H&E staining of lungs harvested from size-matched tumors. In the original manuscript, we merely counted the number of macro-metastases (typically >100 cells). However, in response to Reviewer #3's comments, we now revised the quantification to reflect percentage metastatic area. For this, we first measured the total lung area, then manually traced the metastatic area within each lung. This was repeated for 3-4 lung sections per mouse, the average of which is a single data-point in our graphs. This new quantification method accounts for differences in both the number and size of metastases. Importantly, we continue to find that sensory neurons, SP, ssRNA, and TLR7 all promote breast cancer metastasis. We have now included a description of these in our Methods section.

As mentioned above in response to point 6, our revised manuscript includes data from intra-cardiac injections that demonstrate that pre-treatment of 67NR cancer cells with DRG-CM significantly promotes metastatic colonization of the lung, liver, and brain (Extended Data Fig. 7 i-k).

10. 2f) Overall, comparing the mean fluorescence intensity to compare the abundance of substance P in different tumor models needs to be carefully calibrated. At first glance, the background level in the 4T1 looks significantly higher than in the 67NR model. But this could also be due to the visualization. Where in the tumor was this signal measured? Can the authors report their positive and negative controls and whether the antibody works properly in their system? What is the n-number in their statistics referring to? Does this represent different sections?

We thank you for pointing out that we were less clear than we needed to be on this key issue. All quantifications were performed using images collected at the same laser power and detector gain.

For mouse tumors: Each dot in the plot corresponds to a distinct ROI within the tumor. This was repeated across 3 independent tumors per condition (Fig. 2f and Extended Data Fig. 6e, g, k).

For human tumors: We manually traced ROIs around cancer cells within tumors. We measured the SP MFI within this region for four independent ROIs within each tumor. The average MFI value per tumor is plotted for 19 independent tumors (Fig. 2g).

Furthermore, tumors that were grown in *Tac1-null* mice had little to no signal for SP (Extended Data Fig. 6k) – demonstrating the specificity of the antibody used.

Minor points

Some of the following experimental additions and further analyses would make this study stronger:

1. I appreciate that the authors wanted to identify the source of peripheral innervation of the murine mammary gland by using a retrograde tracing system. I would be highly interested in understand whether and how this changes in breast tumors? In other words: which DRGs are labeled when the GFP-tagged cholera toxin b is directly injected into the breast tumor?

We thank the reviewer for this great suggestion. We injected GFP-tagged cholera toxin B (CTB-GFP) into 67NR or 4T1 tumors and found that 4T1 tumors were innervated by several neurons within the T12-L3 ganglia (Fig. 1 e-f). In contrast, 67NR tumors were innervated by very few neurons within the T12-T13 ganglia (Fig. 1 e-f). These new data further support our conclusions that the poorly metastatic 67NR tumors are poorly innervated, while highly metastatic 4T1 tumors have abundant sensory innervation.

2. Can the authors comment on how their cell lines were repetitively authenticated and can they include these details in their manuscript?

As noted in the Methods section, all cell lines used in the manuscript were purchased from ATCC that provides authentication. According to their website: "As a biological resource center, ATCC comprehensively performs authentication and quality-control tests on all distribution lots of cell lines. Therefore, obtaining and using low-passage cell lines from ATCC is a sure way to work and publish with confidence."

All cell lines used in the study were tested for mycoplasma contamination every ~3 months.

3. I am wondering how substance P is secreted and why this is increased when tumor are co-cultured. It would be highly interesting to understand whether this is an neuronal activity-dependent process and whether there might be a vicious cycle of neuronal hyperexcitability/hyperactivity and tumor progression as described for brain tumors although this might be beyond the scope of this work.

We thank the reviewer for stimulating us to think more deeply about the mechanisms of SP release. This along with some comments from Reviewer #2 led us to some very exciting discoveries that expand the scope of the manuscript and are summarized below:

(1) We found that DRG neurons cultured in the presence of cancer cells frequently exhibit spontaneous calcium activity (seen by using the calcium dye, Fluo-4; Fig. 3a, b, Extended Data Fig. 7d). Such alteration in neural activity has previously been shown for tumors in the brain such as gliomas (PMID: 31534222) but has not been studied in the context of epithelial tumors in the periphery.

(2) It is known that SP release from sensory neurons is initiated by these calcium transients. Consistently, we observe that DRG-CM (from a 67NR-DRG co-culture) has more SP than DRG only-CM. Culturing DRG neurons in the presence of tumor-CM drives SP release from the neurons (Extended Data Fig. 6d).

Taken together, as you alluded to in this comment, these data support a model in which neuronal activity within breast tumors promotes metastasis.

4. 1k) Please add to the figure description what mCh stands for (I assume these are the tumor cells transduced with a mCherry fluorophore).

You are correct that mCherry is transduced into cancer cells so they can be reliably identified in a non-fluorescent host. We have added this description to the figure legends.

Please add a quantification of the collective invasion strands to the manuscript.

Thank you for this suggestion. We imaged ~20 ROIs within 67NR or 67NR+DRG tumors across 3 independent tumors per condition. We called the ROI to be invasive even if there was 1 invasion strand in that field of view. Using this method, we found that control 67NR tumors have invasive borders only in ~10% of the cases, while co-transplanted tumors extend invasion strands into the stroma ~95% of the time (Fig. 1n).

5. A scale bar is missing for 1p.

Scale bars have been added, thank you.

6. How do the authors define macro- and micrometastases?

Macro-metastases are typically ~100 cells or more and are readily identified by H&E staining with low magnification. Micro-metastases were counted manually in lung sections and typically consist of <50 cells. We have included this description to the Methods section.

7. The authors use tail vein injections of cells as an experimental model of metastasis and subsequently analyze lung metastases. It would be interesting in addition to use heart injections that would allow to also quantify liver and/or brain metastases to see whether the effects potentially are specific to lung metastases.

As mentioned above in response to major points 6 and 9, intracardiac injections revealed an increase in the number of lung, liver, and brain metastases arising from 67NR cancer cells pre-treated with DRG-CM (Extended Data Fig. 7 i-k).

We thank the reviewer for these carefully considered comments and suggestions. Collectively they were very effective in stimulating us to do important new experiments and analyses. Our manuscript has benefitted significantly from your time and expertise.

Referee #2 (Remarks to the Author):

Tumors are complex environments that are innervated by peripheral neurons. It is well-established the density of nerve innervation is correlated with cancer metastasis. In the current study, Padmanaban and colleagues investigate if a causal relationship exists by asking if dorsal root ganglia neurons directly stimulate breast cancer growth. They use *in vitro* and *in vivo* assays to provide evidence that the presence of DRG neurons enhances the growth, invasion, and spread of cancer cells. They next seek to identify factors that could be released from neurons to cause these effects. Previous work has shown there is rich bi-directional communication take place where the cancerous cells release neurotrophic factors that promote infiltration and afferent branching and neurons release transmitters and other signaling molecules that can stimulate tumor growth. The authors assume the candidate molecules are likely neuropeptides and indeed show that substance P has pro-metastatic activity that involves the release of single stranded RNA and Tlr7 signaling.

Overall, the net result of this study is a somewhat complex model where neuronal signaling to cancer cells initiates an autocrine signaling cascade to promote growth and metastasis. While an intriguing concept, major questions remain about what is causing the peptide release in the first place. Technically, DRG neurons are quite diverse in function and neurochemistry, with only a subset expressing the Tac1 gene. Thus, it is not clear what is really going on the *in vitro* co-culture and DRG transplantation assays. In terms of novelty, as the authors state, there already exists a literature on sensory innervation strongly correlating to metastasis. However, although not cited, the anti-tumor effects of Aprepitant, a TACR1 antagonist that is routinely prescribed for nausea associated with chemotherapy, seems to have already been established.

We thank the reviewer for their valuable suggestions on our manuscript. We have worked very diligently to address all of the reviewer's comments. As detailed below, we present new data that (i) support viability and functionality of neurons when co-transplanted *in vivo*, (ii) demonstrate a change in neuronal activity in response to epithelial cancer cells, (iii) identify apoptosis in a small fraction of cancer cells to be the source of extracellular ssRNA that promotes metastasis, and (iv) establish a requirement for neuronal SP in metastasis (using genetic KO mice).

We emphasize that while innervation has been associated with adverse outcomes, our report describes a previously unreported and remarkable finding whereby sensory DRG neurons promote multiple metastatic phenotypes through a neuropeptide-driven extracellular RNA-mediated signaling axis that nominates multiple new targets (SP, TACR1, ssRNA, TLR7) and therapeutic vulnerabilities. Given our new findings as well as the positive comments from the other referees regarding the novelty of our findings, we hope to have satisfied the referee.

Major concerns.

1. Can the authors comment on how co-transplanting 200 DRG cells with the cancer cells would enhance the metastases (Fig 1)? Probably only half or less of these DRGs are peptidergic nociceptors that can release SP. And unlike the cancer cells, these neurons are postmitotic and can't divide. The authors use 100 DRGs per well of a 24 well plate in the *in vitro* experiments to enhance invasiveness, and the effect is dose-dependent with the number of DRGs. How can a similar order of magnitude of cells exert such strong effects via a chemical mediator when delivered in the vastly larger compartment of the mouse body? Wouldn't SP released from so few DRGs simply be diluted?

We thank the reviewer for challenging us to think more deeply about our experiments. Quite honestly, we were equally surprised that we had such a strong phenotype with our co-transplantation experiments. The reviewer is correct in that a relatively small number of neurons were co-injected with cancer cells. Release of SP, however, is not limited to the neuronal cell bodies – nerve terminals release SP too. As a part of the revised manuscript, we have included optically cleared 67NR and 67NR+DRG tumors which demonstrate that upon co-transplantation, DRG neurons extend a robust network of axons through the bulk of the tumor (Extended Data Fig. 4a). In further support of the functionality of these co-transplanted neurons, we see that 67NR+DRG tumors have significantly more SP than control 67NR tumors (Extended Data Fig. 4b). Therefore, a relatively small number of neuronal cell bodies are still able to secrete biologically meaningful amounts of SP into the tumor compartment. Finally, during the revisions we performed an *in vivo* co-transplantation with 50, 250, or 1000 neurons/ mouse (Extended Data Fig. 4c). We found that co-transplantation of DRG neurons increased tumor growth in a cell-dose dependent manner (Extended Data Fig. 4d).

Survival of DRGs when co-injected with cancer cells is supported by two additional experiments – (i) We see that co-cultured DRG neurons have improved survival compared to DRGs cultured alone (Extended Data Fig. 2a). (ii) When co-injected with cancer cells *via* the tail vein of mice, we detect neuronal cell bodies with robust axonal projections within the lungs of these mice – the only source of which could be the injected neurons (Extended Data Fig. 4h).

2. The authors perform a denervation experiment using capsaicin (Fig 1). However, from what I can see the authors have not shown evidence the denervation worked. Presumably they can immunostain the tumors and show the axonogenesis into the tumors is decreased.

We apologize for not stating our observations more explicitly. In the original manuscript, we had included immunofluorescence images from mammary glands 7 days post-capsaicin administration (day of cancer cell inoculation; Fig. 1p). Sensory nerve fibers, but not sympathetic nerve fibers, were almost completely abolished in the capsaicin-treated condition (revised Extended Data Fig. 5a). In response to your comment, we also performed immunofluorescence for CGRP in vehicle or capsaicin-treated 4T1 tumors. We found a significant decrease in the number of sensory nerve fibers in capsaicin-treated tumors (Extended Data Fig. 5b). These data demonstrate the success of our experimental approach to denervate both tissue-resident nerves and tumor-induced axonogenesis.

3. As it stands, the in vitro experiments do not fully support the model presented in Figure 4. I think the key contradictory results comes on page 7. The authors demonstrate that conditioned medium (CM) from DRG neurons cultured alone contains substantially less SP than CM from DRG neurons cultured with 67NR spheroids (Extended Data Fig 6b). Notably, DRG-only CM is not sufficient to enhance invasiveness (Extended Data Fig 6h-j). They explain this by saying there must be other tumoural factors present in CM from DRG/67NR co-cultures produced upon interaction of sensory neurons with cancer cells and that is sufficient to promote breast cancer invasiveness. They identify ssRNAs as the crucial factors. They then find exogenous SP increases invasiveness in a manner dependent on ssRNA. Thus, according to the authors, sensory neurons secrete SP, which in turn promotes release of ssRNA from breast cancer cells that drive invasiveness and proliferation.

But here the logic falls apart. We are thankful for the reviewer's helpful suggestions and firmly believe that the manuscript has greatly benefited from it. It is important to clarify that our original and additional data remain consistent with the model presented in the original manuscript as described below.

If exogenous SP can promote invasiveness, why can't the lower levels of SP present in CM from DRG-only cultures? This SP should also be able to promote ssRNA release to enhance invasiveness. Given it can't, then the most parsimonious explanation is that CM from DRG-only cultures simply just doesn't contain enough SP. But the authors have no explanation for why DRG-only cultures produces less SP than DRGs cultured with the 67NR spheroid. For their model to make sense, we need to account for why the 67NR spheroids appear to increase SP release from the co-cultured DRG neurons. Minimal SP release from DRG neurons cultured alone is unsurprising because SP release depends on depolarization, calcium influx and dense core vesicle release. But cultured DRG neurons do not typically show spontaneous activity and calcium influx unless they are stimulated with a pro-excitatory mediator such as ATP or capsaicin. Given SP release is far higher in the presence of the spheroids, it seems likely the spheroid is releasing a chemical mediator that is exciting the co-cultured DRG neurons to evoke additional SP release. Bidirectional interactions between nerve and tumor seem likely because tumors must secrete instructive signals that trigger both nerve innervation and the action potential-dependent efferent release of SP in vivo. The hypothesis of the referee is spot on and in agreement with our model, whereby the magnitude of SP release is critical, with breast cancer cells inducing a greater degree of release that hits the threshold for mediating the phenotypic consequences. While we described this bi-directional reprogramming of neurons and cancer cells in the results and discussion sections, we agree that our original manuscript lacked direct evidence to show an alteration in neuronal function in response to cancer cells. We have now characterized the nature of these interactions in response to the useful suggestions by the reviewer. Our new findings support our model of cancer→neuron→cancer interactions driving this phenotype.

The authors should perform two simple experiments to test this. First, DRG neurons cultured-alone can be treated with 67NR CM and the amount of SP release measured – if there is a bidirectional interaction, we would expect 67NR CM to increase the amount of SP released to the same levels as DRGs cultured with the spheroids.

This was a great suggestion, thank you! We did indeed observe a significant increase in the amount of SP released when DRG neurons were cultured in the presence of tumor-CM (Extended Data Fig. 6d).

Second, the authors should load cultured DRGs with a Fura calcium dye and perform live imaging. We would predict that DRGs cultured with 67NR spheroids, or treated with 67NR CM, would exhibit more spontaneous calcium activity than DRGs cultured alone.

I think performing these experiments is critical to understanding why CM from DRGs cultured alone does not enhance invasiveness in vitro and would clarify why a normally silent neuron type is releasing SP into tumors in vivo.

We thank the reviewer once again for this great suggestion. We used the Fluo-4 calcium dye to monitor calcium transients in DRG neurons that were cultured by themselves or co-cultured with 67NR cancer cells. As the reviewer pointed out, we observed no evidence for spontaneous activity in DRG neurons cultured alone. However, in the presence of cancer cells, DRGs frequently exhibit spontaneous calcium transients (Fig. 3a, b, Extended Data Fig. 7d). It is known that SP release from these neurons is initiated by calcium spikes. Collectively, the two experiments described above present a model in which changes in neuronal activity upon exposure to cancer cells cause the release of SP, which, in turn, drives invasion and metastasis. To our knowledge, changes in neuronal activity in response to cancer cells have only been reported for brain tumors (PMID: 31534222). Neuronal activity-dependent modulation of metastasis in an epithelial cancer is a major finding of our revised manuscript, one that directly resulted from the reviewer's insightful comments.

4. The authors perform some *in vitro* experiments using DRGs from Tac1 KO mice. These animals are completely deficient in SP and are the strongest test of whether SP release from DRGs is necessary for tumor invasiveness. The authors should comment on the fact that even though galanin is sufficient to enhance invasiveness, galanin release apparently cannot compensate for the absence of SP.

Thank you for raising this important point. The reviewer is correct in that galanin promotes invasion and proliferation of cancer cell spheroids (Extended Data Fig. 6b, c). We chose to focus on substance-P since it had the strongest effect on cancer cell invasion (Fig. 2d). In the original manuscript, we had also shown that addition of RNase doesn't alter the ability of galanin to promote invasion (Extended Data Fig. 6b). As a part of the revisions, we discovered that neuronal SP is causing cell death in a subset of cancer cells, causing ssRNA release (Fig. 3 m-o). Addition of galanin, however, does not cause apoptosis in cancer cells (Extended Data Fig. 9a). Therefore, galanin regulates invasion of cancer cells *in vitro* via an independent mechanism. The significance of galanin in regulating metastasis should be further explored *in vivo* and is out of the scope of the current work.

The manuscript would be improved significantly if the authors performed *in vivo* experiments of metastasis using the Tac1 KO mice, as they clearly have access to them. For example, they could test tumor growth and metastasis in Tac1 KO bearing 4T1 tumors. Failure to metastasize in these KO animals would really strengthen the conclusions of this paper.

We thank the reviewer for this comment. We were indeed very interested in evaluating the role of SP *in vivo* using the *Tac1-null* mice which we used for *in vitro* experiments in the original manuscript. We received these mice from Jackson Laboratories in the C57BL/6J background. However, the 67NR/4T1 breast cancer cells predominantly used in the manuscript are in the BALB/c background. After ~2 years of backcrossing, we finally had the opportunity to perform this important experiment. We orthotopically transplanted 4T1 cancer cells into the mammary fat pad of *Tac1-WT* or *Tac1-null* mice (Fig. 2k). *Tac1-null* mice showed significant reduction in 4T1 tumor growth and metastatic burden (Fig. 2 k-m). We also repeated these experiments using a second breast cancer cell line, EO771 LM2 and observed a strong requirement for host-derived Tac1 in tumor growth and metastasis (Extended Data Fig. 6 h-j).

5. The authors provide convincing *in vivo* data that the NK1R antagonist aprepitant, an anti-emetic, can inhibit tumor growth and metastasis in multiple models. They conclude by saying this "warrants investigations into the clinical efficacy of this agent upon its prolonged use in breast cancer in combination with standard of care regimens for the prevention of metastatic relapse." With these striking *in vivo* data, the paper does appear to be quite a substantial advance in breast cancer research.

We thank the reviewer for recognizing the potential clinical significance of our findings. In the revised manuscript, we have further strengthened these conclusions in PDX models (see below).

Of course, aprepitant is commonly given to many patients with breast cancer to treat chemotherapy-induced nausea. And indeed, a quick google reveals there appears to be a rich literature of clinical observations.

To our knowledge, only one clinical case report (of one patient) exists describing an anecdotal reduction in blood tumor markers in this patient while on aprepitant (Lee *et al.*, Int J Can Clin Research, 2016). This has little weight even though it is consistent with the findings of our study. Moreover, this report does not provide imaging evidence of anti-metastatic effects. This patient already had metastatic disease and thus this clinical anecdote is not demonstrating metastasis prevention effects of aprepitant. However, per the reviewer's suggestion, we have included a discussion of this in the revised manuscript: "These striking anti-tumour effects of aprepitant are consistent with an anecdotal clinical observation of decreased tumour markers in a patient upon aprepitant administration."

Indeed, there are also numerous *in vitro* and *in vivo* studies of various cancers showing anti-tumor effects of aprepitant. Could the authors explain their decision not to cite any of this literature?

We apologize for the oversight in not clarifying the current status of the field in the original manuscript. *In vitro* data for the use NK1R antagonists in breast cancer cell lines is mixed. For example, one study reports cytostatic effects of aprepitant on breast cancer cells (PMID: 32333097) while another report suggests that NK1R antagonism has no effect on cancer cell viability or growth (PMID: 23407059). Most importantly, we are not aware of any papers that demonstrate the *in vivo* use of aprepitant in inhibiting metastasis in breast cancer – the focus of the manuscript. However, we acknowledge the importance of citing these previous studies and have incorporated them in the revised manuscript.

6. Although I am not an expert in these types of experiments, the same measures between experiments seems to vary significantly with no explanation. For example, the reduction of the number of macromets with anti-Sp antibody is similar to the baseline with vehicle in the Aprepitant experiment. The tumor burden is very consistent throughout experiments - so this seems odd?

We thank the reviewer for their astute attention to detail. The anti-SP experiment (revised Fig. 2 h-j) is performed in NSG mice (immunocompromised) and consequently, the mice had higher baseline metastatic burden. In contrast, the aprepitant experiment (revised Fig. 5c) and most other experiments in the manuscript were performed in immunocompetent syngeneic mice whose immune systems more effectively restrain tumors. The strain of the host mice used is always listed in the corresponding schematic preceding the data for that experiment in the figures and in the corresponding figure legends.

Other comments:

1. Primary DRG cultures will have many non-neuronal cells, even after gradient purification, that divide and take over the cultures. This is usually mitigated by supplementing media with anti-mitotics. Since this can't be done here, how do the authors avoid this contamination?

As the reviewer pointed out, it is not possible to add anti-mitotics in our co-cultures due to the presence of cancer cells. The use of a Percoll-based density gradient allows us to minimize the number of non-neuronal cells in our cultures. At the end of three days in culture, we estimate close to 75-85% of cells within 3D collagen I to be β III-tubulin+. Since we do validate key aspects of our model, such as the requirement for SP, in assays that completely lack primary neurons, we believe that non-neuronal cells have little contribution to these conclusions. A more careful examination of the function of non-neuronal cells within the tumor microenvironment is out of the scope for this study.

[REDACTED]

[REDACTED]

3. The authors suggest the transplanted DRG are functional. I find this hard to believe. Normally these cells have their soma restricted to ganglia and make synaptic connections with the CNS. Functional proof of a higher burden then presented here is required.

In our revision, we have performed extensive characterization of DRG neurons co-transplanted with cancer cells that is consistent with their viability and functionality *in vivo*. Please see our response to your major point #1 above for more details.

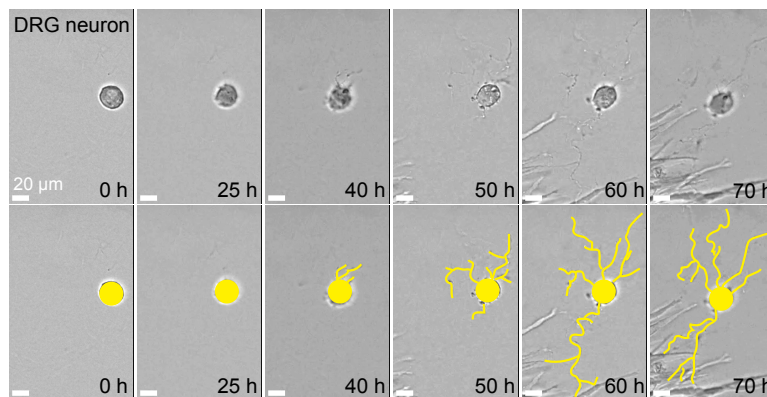
4. The authors claim to have a function blocking Substance P antibody that works in vivo but provide no evidence of the efficacy. Validation (and a dose response) is required.

We show that SP levels decrease post administration of anti-SP antibody (revised Extended Data Fig. 6d).

5. The images of the DRGs in the culture experiment suggest they may not be healthy. For example the image in Fig1F the soma is irregular with dense inclusions and the axon filaments look to be fragmented. Data showing what type of DRG neurons these are and that they are alive and functional is needed.

The images provided in Fig. 1f (now revised Fig. 1h) are from a timelapse movie. Here we show additional time-points from that exact movie showing that the neuron in culture continues to branch over time (Rebuttal Fig. #7). These are 3D co-cultures of neurons cultured along with 67NR spheroids. Given the 3D matrix, the focal plane of the neurons keeps changing, and manual focus is adjusted only every 12 hours. This is less important for cancer cell spheroids since their overall volume is much larger. This might explain what the reviewer considered to be irregularities within the soma of the neuron represented in Fig. 1h.

To lend further support to the health of neurons, we stained DRG neurons cultured by themselves or those cultured with 67NR cancer cell spheroids in 3D collagen. Unexpectedly, at the end of four days in culture, we found that DRG neurons co-cultured with cancer cells have fewer apoptotic neurons than those cultured alone (Extended Data Fig. 2a).



Rebuttal Fig. #7: Snapshots of a DRG neuron co-cultured with 67NR spheroids in 3D collagen I over time.

We are thankful to the reviewer for their detailed and constructive comments. Their input has been instrumental in strengthening our manuscript, as it motivated the extensive set of studies we performed.

Referee #3 (Remarks to the Author):

In their study entitled “Neuronal substance P drives breast cancer growth and metastasis via an extracellular RNA/TLR7 axis”, Padmanaban et al analyze mechanisms that underly the correlation of innervation of tumors and tumor growth as well as metastasis. The authors show that poorly metastatic tumors are less innervated. Using in vitro systems, in vivo models and patient derived material, the authors demonstrate that modulation of innervation or neuronal derived factors impacts tumor growth and metastasis and identify an SP/TACR1/ssRNA/TLR7 axis driving the observed effects. Based on these findings, the authors use an antinausea drug that targets TACR1 in two different in vivo models as well as in vitro 2D and 3D cultures.

The role of neurons in cancer progression and metastasis is increasingly recognized. However, mechanistic insight is still sparse. This study identified a potential mechanism how innervation or neuron derived factors affect cancer cells and increase their metastatic potential. The study provides interesting insight into a SP/TACR1/ssRNA/TLR7 axis driving tumor growth and metastasis together with a potential therapeutic intervention using Aprepitant to target TACR1. However, several questions and concerns remain to be addressed. The study is timely and the described results are novel and provide an interesting new perspective on the potential role of neurons in tumor growth and metastasis. The manuscript is well structured and the rationale for the performed experiments is clear. The used methodology is appropriate. References to previous literature is balanced and comprehensive.

We thank the reviewer for recognizing the significance of our findings. We have conducted extensive new experiments based on your thoughtful and constructive comments. Your input has been instrumental in strengthening our conclusions and expanding the scope of our manuscript.

Yet, there is much emphasis on in vitro systems especially for mechanistic insight that is not thoroughly validated in vivo. Numbers of animals for in vivo validation is low and there is no detailed evaluation of the in vivo data that would allow a more critical view on the role of the SP/TACR1/ssRNA/TLR7 axis in vivo. Therefore, the conclusions drawn in this manuscript should be better supported by thorough in vivo insight.

We appreciate the reviewer's thoughtful comments and concerns regarding the level of *in vivo* validation in our manuscript. We agree that *in vivo* evidence is essential for robust conclusions. Although it may appear that we have used a relatively small number of animals (~10-20) per study, the observed effect sizes are substantial and statistically significant.

However, we understand the importance of more comprehensive *in vivo* validation. Our original data along with those completed during the course of this revision have used multiple orthogonal *in vivo* approaches to define the role of each component of the SP/ TACR1/ ssRNA/ TLR7 axis. These efforts are summarized below:

(1) **Sensory neurons:** We used *in vivo* co-transplantation (Fig. 1 k-o) and denervation (Fig. 1 p-r) studies to show the requirement for sensory neurons in tumor growth, invasion, and metastasis.

(2) **SP:** Using a function-blocking antibody (Fig. 2 h-j) and a genetic knockout model (Fig. 2 k-m), we find that neuronal SP drives breast cancer metastasis.

(3) **TACR1:** Using lentiviral knockdown (Extended Data Fig. 6 o-q) and small molecule inhibition of TACR1 using aprepitant (Fig. 5 a-e), we find that neuronal SP signals via tumoral TACR1 receptors.

(4) **ssRNA40:** Intraductal RNase A treatments significantly inhibit tumor growth and metastasis (Fig. 3 g-i). Additionally, we find that pre-treatment of cancer cells with DRG-CM+RNase A strongly inhibits the metastasis-promoting effects of DRG-CM (Fig. 3 j-l).

(5) **TLR7:** Knockdown of Tlr7 significantly inhibits tumor growth and metastasis in immune-competent (Fig. 4 f-h) and immune-compromised models (Extended Data Fig. 9 i-k).

(6) We find that RNase A treatment of tumors depleted of Tlr7 does not any further reduce tumor growth, suggesting that they signal through a common pathway (Extended Data Fig. 9 l-n).

1. The authors show that the highly metastatic 4T1 line shows higher innervation than the low metastatic variant 67NR and also demonstrate in other models and patient samples that higher innervation correlates with increased metastasis. It remains unclear, which properties of cancer cells induce tumor innervation and thereby trigger interactions between neurons and cancer cells that promote tumor growth and metastasis.

We thank the reviewer for encouraging us to perform additional experiments that led to very unexpected and exciting results. There already exists a body of literature characterizing the release of neurotrophic factors by cancer cells (reviewed in PMIDs: 30944117 and 31974491). We instead wanted to test a bold hypothesis that the tumor microenvironment could be a regulator of tumor innervation. We recently discovered that the endothelium of metastatic tumors secretes Slit2 and that endothelial-Slit2 is required for metastasis (Nature, 2020; PMID: 32999457). In its traditional role, Slit2 is an axon-guidance molecule. Consequently, we hypothesized that endothelial-Slit2 could

regulate tumor innervation. To test this, we orthotopically transplanted 4T1 tumors into mice with an endothelial-specific depletion of Slit2 and immunostained these tumors for the pan-neuronal marker (β III-tubulin) and a sensory nerve marker (CGRP). We found that both the total innervation and sensory-specific innervation were significantly decreased in tumors lacking endothelial-Slit2 (Fig. 4l, m). These data demonstrate a requirement of a pro-metastatic endothelial-derived signal (Slit2) that promotes innervation of mammary tumors.

2. In patient cohorts, the authors describe higher rates of lymph node metastasis. In the mouse model, the authors quantify lung metastasis. Throughout the manuscript, the general term of metastasis is used. However, based on the provided data it is not clear whether the SP/TACR1/ssRNA/TLR7 axis leads to a general increase in metastasis or whether preferential organ-tropism can be observed. What about other metastatic sites breast cancer is prone to metastasize to including bone, liver or brain? The experiment shown in Figure 3 i-k could be extended by other injections routes than intravenous injection to analyze effects on bone, liver and brain metastasis. Can the authors provide insight from the patient cohorts regarding the occurrence of organ-specific metastasis?

We thank the reviewer for this important question. In our original manuscript, we only characterized the effects of sensory innervation on lung metastasis since most of the mouse models of breast cancer preferentially colonize the lung when injected orthotopically. To overcome this, as the reviewer suggested, we performed intra-cardiac injections of mCherry+ 67NR cancer cells that were pre-treated with either tumor-CM or DRG-CM. We found that pre-treatment with DRG-CM significantly increased metastatic colonization to the lung, liver, brain, and ovaries (Extended Data Fig. 7 i-k). None of the mice had bone metastases.

Unfortunately, we do not have any metastatic site data with the human samples. Most of our samples are collected at the time of tumor removal (commonly a mastectomy). At this point, most of the staging is performed based on the primary tumor size and nodal status.

3. When co-implanting DRGs together with 67NR cells, the authors observe increased tumor growth, higher numbers of CTCs as well as increased number of micrometastases. The authors state that 67NR tumor cells can intravasate but then a high proportion of cancer cells fails to extravasate. Does that mean, that experimentally induced innervation by co-implantation of DRGs locally affects 67NR tumor cells and increases their capacity in intravasate but the effect on invasiveness is lost within the circulation and cells fail to extravasate and colonize the target organ?

We apologize for not providing a clearer explanation in the original text. 67NR cells are widely accepted as a poorly metastatic cell line and it is believed (although never directly shown) that this is due to an intravasation defect. We find that 67NR cancer cells seem to get to the lung frequently but remain trapped in the lung endothelium, suggesting an extravasation defect (Extended Data Fig. 4e).

When we co-transplant 67NR cancer cells with DRG neurons, we still see many cells within the lung endothelium but a greater number of cells successfully extravasate to form micro-metastases. This is the quantification provided in Fig. 1o. This increase in metastatic colonization could be a combinatorial increase observed due to an increase in local invasion, intravasation, and extravasation.

Alternatively, one could assume that in naturally occurring innervation as observed in the 4T1 model, innervation is also observed at the metastatic site? That might mean that cancer cells that are able to induce innervation at the primary site can also induce innervation at the metastatic site. Whereas 67NR even with co-implantation of DRGs fail to accomplish this at the metastatic site. Analysis of innervation at the metastatic site is critical to further address this point.

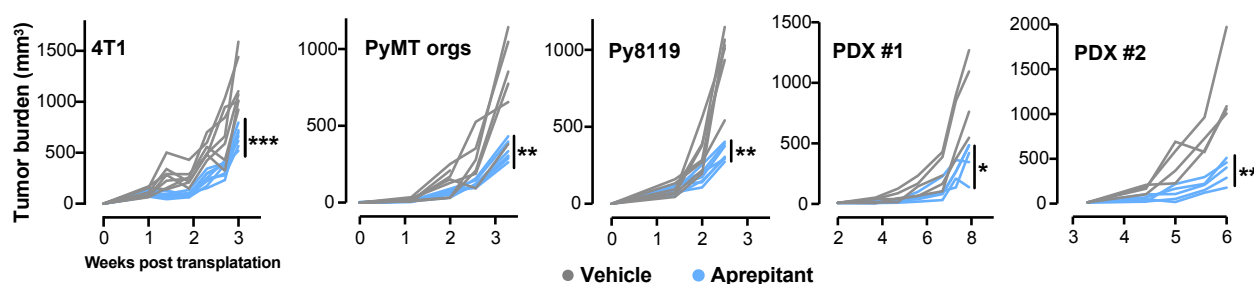
Thank you for this thoughtful comment. We carefully looked at the metastatic sites (lung and liver) of mice bearing orthotopic 67NR or 4T1 tumors. We were able to find evidence of innervation within metastases arising from 4T1 tumors (representative example is in Extended Data Fig. 1c). Unfortunately, metastases arising from 67NR tumors are very rare and no larger than 5-10 cells, making any inferences regarding innervation impossible. It remains possible that for a cancer cell to survive at the metastatic site, it needs to be able to induce innervation but directly testing that hypothesis is out of the scope of this study.

4. In vivo trials in this study are designed to analyze only short-term effects. Growth kinetic is only evaluated up to 4 weeks after injection. To monitor the growth kinetic, tumor growth should be followed longer and survival experiments should be performed. This is particularly important for experiments with the TACR1 antagonist. Here it looks like that Aprepitant treatment slows tumor growth for 1 week followed by tumor growth at similar rates compared to the control group. Furthermore, representation of growth curves of individual mice instead of mean values would better reflect the actual effects.

ACUC processes vary considerably by country and even by institution. The approach followed by the Rockefeller University ACUC is to anchor the criteria for maximum extent of cancer progression in the health and behavior of the mice and we are expected to sacrifice our animals when they show any signs of distress including change in activity, mobility, breathing pattern, or unexplained weight change. The relevant sentence from our ACUC states “It would take roughly 3-4 weeks to develop clinical symptom from the illness such as appetite loss. At the time the tumor reaches at 1500 mm³ or significant clinical symptom are observed, the mice would be euthanized in the way defined in this proposal.” Therefore, we are at the limit for how long we can conduct these experiments in mice.

However, we took your criticism seriously and extended our analyses to two independent PDX models of triple negative breast cancer (Jackson Lab #TNM00096 and 98). Across both models, aprepitant significantly decreased tumor growth (Fig. 5d, e).

As per your suggestion, we have re-generated the graphs for all of the aprepitant experiments such that you are able to visualize the variability in tumor sizes (Rebuttal Fig. #8). For aprepitant-treated 4T1 tumors, although the primary growth rate increased over time, the mice had significantly fewer metastases (Fig. 5c, tumors were size-matched at endpoint). Other cell lines (e.g., Py8119) tested in the manuscript continued to have a slower tumor growth rate over time when treated with aprepitant (Extended Data Fig. 11c).



Rebuttal Fig. #8: Tumor growth curves of aprepitant-treated tumors across multiple mouse models of breast cancer

The *in vivo* trials are performed with low replicate numbers. Metastatic burden is only represented as number of micro- or macrometastases. Looking at the provided representative gross overviews of the lungs it appears that the size of the individual metastasis is almost similar while numbers of lesions changes. Can the authors add information of the metastasis burden based on cumulative metastatic load and size of individual tumors.

We thank the reviewer for raising this important point. We repeated the quantifications for our metastasis assays to now reflect the percentage of metastatic lung area (area of metastases/ total lung area*100). Of note, these results are consistent with those in the original manuscript (Figs. 1r, 2j, 2m, 3i, 4h, 5c).

5. The authors use *ex vivo* spheroid assays for critical mechanistic insight. While the use of such systems is valuable to gain first mechanistic insight, more thorough *in vivo* validation should be done. This is particularly important for effects where biodistribution or availability of factors is limited in a *in vivo* setting. In this study, there is lack of detailed *in vivo* validation of the observed effects.

As alluded to above, we have worked very hard to extend all the key findings of this manuscript *in vivo*. We have used orthogonal approaches to define the requirement for sensory nerves, neuronal SP, tumoral TACR1, ssRNA, and tumoral TLR7 for breast cancer growth and metastasis across multiple models of breast cancer.

In the revised manuscript, we have also added a key experiment that further strengthens our conclusions *in vivo*. We orthotopically transplanted either 4T1 or EO771 LM2 cells into syngeneic host mice that lacked the *Tac1* gene (*Tac1-null*). *Tac1* is the gene that encodes substance-P. We found that loss of host-derived substance-P significantly inhibits tumor growth and metastasis in both these models of breast cancer (Fig. 2 k-m, Extended Data Fig. 6 h-j). We hope that the referee agrees that the current manuscript has a substantial amount of *in vivo* data with multiple orthogonal models.

We thank the referee for these insightful comments and suggestions, which have strengthened our manuscript.

Referee #4 (Remarks to the Author):

In this manuscript, Padmanaban et al. discovered that sensory innervation in tumor lesions promotes cancer growth and metastasis via substance P and single-stranded RNA (ssRNA). First, the authors showed that aggressive tumors tend to be innervated more in mouse and human cancers. Then they found that the sensory neuron innervation promotes tumor growth and invasive capacity by developing a 3D co-culture assay system. Substance P produced by neurons is likely to serve as a mediator between neuron-tumor interactions. Moreover, the authors identified that ssRNA is secreted when the tumor cells are co-cultured with neuronal cells, and the ssRNA in turn activates the transcriptional program to induce invasion and proliferation in a TLR7-, but not TLR3-, dependent manner. Interestingly, the authors further showed the efficacy of a clinically available inhibitor of substance P receptor. Taken together, the authors identified the novel link between neuron-tumor interactions to facilitate tumor progression.

Although this study is potentially interesting, there are various weaknesses that need to be addressed. In particular, the molecular mechanisms of how substance P promotes ssRNA secretion are not fully addressed, and the contribution of TLR7 needs to be more profoundly analyzed. Specific comments are following:

Thank you for your insightful comments. We have worked diligently to revise our manuscript to address all your concerns which have directly led to exciting new findings that have significantly increased the scope of the manuscript. First, we define a mechanism by which neuronal substance-P causes cell death in a small number of cancer cells to release ssRNA that act in a paracrine manner on tumoral TLR7 receptors to drive metastasis (Fig. 3 m-o, see detailed response to point 5). Next, we built upon our original findings to show that the ssRNA activates TLR7 *via* a MyD88-independent, non-canonical signaling pathway (Extended Data Fig. 10 a-c). Furthermore, we also show that TLR7 depletion significantly inhibits metastasis in an immune-independent manner (Extended Data Fig. 9 i-k).

Major comments:

1) In several figures, the authors utilized the tumor cell injection into the tail vein as a model to evaluate metastasis. However, this is not a suitable model for metastasis since the tumor cells do not need to leave the primary tumor lesion, which is a key event in metastasis. Rather, it would be better to examine macro-metastasis using the method that generates primary lesions such as intraductal injections.

We apologize for not explicitly clarifying in the text but almost all our metastasis assays are orthotopic injections or intraductal injections. Both methods involve the delivery of cancer cells into the mammary gland that results in the formation of a mammary tumor. Specifically, metastasis assays in Figs. 1k-o, 1p-r, 2h-j, 2k-m, 3g-i, 4f-h, 5a-e, Extended Data Figs. 3g-i, 4c-d, 5e-f, 6h-j, 6o-q, 9i-k, 9l-n, 11b-d are all orthotopic experiments.

We used tail vein injections of cancer cells for only two experiments that allowed us to draw conclusions relevant to the later stages of metastasis: (1) Co-injection with DRG neurons promotes metastasis in an experimental metastasis assay (also performed orthotopically; Extended Data Fig. 4 g-i). (2) Pre-treatment of cancer cells with DRG-CM significantly promotes metastatic colonization. Furthermore, RNase treatment inhibits the metastasis-promoting effects of DRG-CM (Fig. 3 j-l) This experiment could not be performed orthotopically since the effect of DRG-CM could not be tested for prolonged times.

As a part of the revisions, we performed intra-cardiac injections of 67NR cancer cells pre-treated with tumor or DRG-CM. Since most orthotopic models of breast cancer metastasize preferentially to the lung, intra-cardiac injections allowed us to quantify effects on multiple distant organ sites. Specifically, we found that pre-treatment with DRG neurons significantly increases metastasis to the lung, liver, and brain. (Extended Data Figure 7 i-k).

2) In Figure 1n, the authors showed that the denervating agent capsaicin inhibits breast cancer metastasis. However, a previous study has shown that this compound rather promotes metastasis using a similar animal model, which contradicts the authors' findings (PMID: 15161056). Therefore, the authors need to reconcile this discrepancy.

Thank you for raising this important point. We apologize for not discussing this in the original manuscript. We are familiar with this paper and there are several critical differences to our work:

- (1) Route of capsaicin administration: Subcutaneous injections used in the referenced paper deliver capsaicin into the blood which likely has systemic effects. In contrast, we perform intraductal administrations which restrict the neurotoxin to the ductal epithelium.
- (2) Frequency of capsaicin administration: Capsaicin was only administered twice 7-21 days prior to inoculation with cancer cells in the referenced paper. In contrast, we perform continued capsaicin administration after cancer cell inoculation to maintain a sustained denervated state.

- (3) Dose of capsaicin: Capsaicin was used at two doses – 50 mg/ kg (no difference in metastatic outcome) or 125 mg/ kg (increase in metastases) in the referenced paper. In contrast, we consistently used 75 mg/ kg in our study.
- (4) The authors do not show evidence for if/ how capsaicin altered innervation of the mammary gland/ tumor. We show that intraductal administration of capsaicin potently ablates sensory innervation within the mammary gland (Extended Data Fig. 5a).

3) In Extended Figure 3d, the authors should test if the human neurons similarly promote invasion or proliferation capacity in human tumor cells, instead of using mouse DRG neurons. This is important for extrapolating their findings into clinics.

Unfortunately, we couldn't find any cell line/ commercially available sources for human DRG neurons. However, we propose a mechanism by which neuronal substance-P drives breast cancer growth, invasion, and metastasis. Substance-P is a 11-amino acid long, highly conserved member of the tachykinin peptide family; suggesting that this axis could be conserved in humans. Consistently, we find that substance-P and ssRNA drive invasiveness of human breast cancer cell spheroids (Extended Data Fig. 8a, b), Aprepitant significantly reduced the invasiveness of human breast cancer spheroids (Extended Data Fig. 11a).

To further address your important point, in the revised manuscript, we tested the ability of aprepitant to inhibit the growth of PDX tumors. For these experiments, we used two independent models of triple negative breast cancer and delivered aprepitant intra-peritoneally once the mice had palpable tumors. In both PDX models, we observed a significant reduction in tumor growth (Fig. 5d, e).

4) Figure 3A lacks proper negative control experiments (e.g. without any treatment). The authors should repeat this experiment in a more appropriate manner.

We thank the reviewer for pointing out this ambiguity. The untreated control for Fig. 3a (revised Fig. 3c) is depicted in Fig. 2b. These experiments were done in parallel. They are represented independently to allow general flow in text and figures. We have edited the legends of the corresponding figure to clarify this point.

Importantly, several lines of evidence suggested that some RNases can reduce tumor cell viability and/or metastatic capacity (PMID: 21194552, 23832341). Therefore, it would be meaningful to confirm that the RNase they used does not affect tumor invasiveness.

We thank the reviewer for pointing our attention to this body of work – we have now cited them in our manuscript.

As included in the original manuscript, treatment with RNase A, III, or T1 did not alter the invasiveness of cancer cell spheroids cultured in tumor-CM (Fig. 3c, top panels). In the revised manuscript, we repeated these experiments using cancer cell spheroids cultured in regular growth medium (as opposed to conditioned medium). We did not observe any change in the invasiveness of these spheroids upon treatment with any of the RNases (Extended Data Fig. 7f).

5) Although the authors' model suggests that substance P promotes the ssRNA secretion from tumor cells, this notion is not fully supported by experimental evidence. The authors should evaluate the amount of secreted ssRNA in spheroid culture conditions with and without substance P by using 4T1 and 67NR cell lines.

We agree with the reviewer that it is important to evaluate the amount of RNA released by cancer cells upon co-culture with DRG neurons and upon SP treatment. During the revisions, we measured the amount of extracellular RNA in the culture medium from (i) 67NR cancer spheroids only, (ii) 67NR-DRG co-cultures, or (iii) 67NR+SP cultures. We found that the amount of RNA in the culture medium was significantly higher upon DRG co-culture or SP treatment (Fig. 3o). These data, along with those in the original manuscript, directly implicate extracellular RNA released in response to neuronal substance-P as the mediator of breast cancer invasion and metastasis.

Furthermore, in response to the referee's astute idea in point #7, we found the source of extracellular RNA to be apoptosis in a small fraction of cancer cells (please see response to point #7 below for more details (Fig. 3m). Importantly, we saw a significant decrease in the amount of extracellular RNA in 67NR+DRG co-cultures after treatment with z-VAD-FMK, a pan-caspase inhibitor (Fig. 3o).

6) The authors used ssRNA40 and poly I:C to stimulate TLR7 and TLR3, respectively. However, methods for the treatment were not written. Given TLR7 is expressed in the endosome, ssRNA should be complexed with cationic lipids such as DOTAP to activate the cells. It is not clear if the authors used cationic lipids for the treatment.

Thank you for raising this concern. ssRNA40 is an oligonucleotide that is already complexed with the cationic lipid LyoVec to prevent degradation and promote uptake. Therefore, it was added as-is to the culture medium. A sentence describing this has been added to the Methods section.

Manufacturer's website for additional information: <https://www.invivogen.com/ssrna40-lv>

The authors should use other synthetic TLR7 ligands such as imidazoquinolines, too.

We thank the reviewer for this great suggestion. When cultured in the presence of R837 (imiquimod) or R848 (resiquimod), the invasiveness of 67NR cancer cell spheroids significantly increased, although R837 had a much stronger effect (Extended Data Fig. 9e). Interestingly, R837 is a TLR7-specific agonist while R848 is a TLR7/8 dual-agonist.

Similarly, the concentrations of poly I:C used in the study may not be sufficient to activate TLR3, if they simply added it to the media.

For our culture experiments with 67NR spheroids, we used poly I:C concentrations ranging from 0.5-5 µg/ ml and saw no effect on cancer cell invasion (Extended Data Fig. 7g). Published reports from various groups, including ours (PMIDs 23035017, 16423036, 31058074, 32999457) have used Poly (I:C) at these concentrations to activate TLR3. Importantly, we knocked down Tlr3 in 67NR cancer cell spheroids and co-cultured them with DRG neurons. DRGs were able to promote cancer cell invasiveness in a TLR3-independent manner (Extended Data Fig. 9 b-d).

7) The molecular mechanisms of how substance P promotes the secretion of ssRNA is unclear and should be clarified. Is the apoptotic process activated to secrete as DAMPs? Alternatively, are there any secretory mechanisms that transport ssRNA specifically?

Thanks to your comment, along with those of reviewer #1, we worked hard to further define the mechanism of ssRNA release upon culture with DRGs or SP. As you alluded to in the comment above, we see a significant increase in apoptosis in cancer cells upon culture with DRG neurons and upon SP treatment (Fig. 3m). Importantly, apoptosis was restricted to <1% of the cancer cell spheroid (Fig. 3m). Addition of a pan-caspase inhibitor, z-VAD-FMK, blocked the DRG/ SP-mediated increase in cancer cell invasiveness (Fig. 3n). These data demonstrate that neuronal SP causes apoptosis in a subset of cancer cells, releasing ssRNA that binds neighboring cancer cells to promote breast cancer metastasis.

8) In Figure 4k, the authors did not treat the cells with substance P or ssRNA, which should be necessary for the activation of invasive capacity in their model.

We apologize for the confusion. RNA-sequencing was performed in 4T1 (highly metastatic) cells which are invasive into collagen even in the absence of SP or ssRNA. Knockdown of Tlr7 in these cells significantly decreases their invasive and proliferative capacity *in vitro* (Extended Data Fig. 9g) and tumor growth and metastasis *in vivo* (Fig. 4 f-h) —revealing signaling through this receptor. Because there is some degree of cell death in culture, released ssRNAs are likely causing a baseline activation of TLR7 signaling. We should also clarify that addition of either SP or ssRNA40 further increased the invasiveness of 4T1 spheroids (Extended Data Fig. 8). Therefore, since we find a requirement for TLR7 in 4T1 cancer cells for metastasis, we performed the RNA-seq experiment in these cells upon Tlr7 depletion.

To further evaluate the role of substance P in TLR7 signaling, the authors should repeat this experiment by culturing control and Tlr7-deficient cells with or without substance P. By doing so, the authors can check if invasive programs are activated upon the treatment with substance P, and this effect is abrogated under Tlr7 deficiency.

This is a great idea! As per the reviewer's suggestions, we treated control or TLR7 depleted cancer cell spheroids with substance-P. We found that while SP drives invasion of cancer cells in the control condition, this effect is blocked upon TLR7 loss (Fig. 4d). These data provide additional support to our model in which substance-P acts upstream of TLR7 in this neuro-cancer axis.

9) The TLR7 signaling pathway is known to activate type I interferon signatures as well as inflammation. In this regard, the pathways differentially regulated by the depletion of TLR7 (Figure 4l) are surprising. Does this mean that a non-canonical signaling pathway is activated in this setting? The authors can analyze the question by knocking out MyD88, an adaptor protein critical for the canonical TLR7 signaling.

We thank the reviewer for motivating us to do additional experiments since they directly led to new findings that strengthened our model. First, we depleted MyD88 in cancer cell spheroids and co-cultured them with DRG neurons.

We found that MyD88 depletion did not alter the ability of DRG neurons to drive invasion (Extended Data Fig. 10 a-c). This contrasts with the requirement for tumoral TLR7 in DRG-mediated regulation of cancer cell invasion (Fig. 4 a-c). These data suggest that neuronal activation of TLR7 signaling occurs *via* a MyD88-independent pathway. Second, we analyzed the immune microenvironment in tumors depleted of TLR7. There were no changes in the number of CD45+, CD3+, nor NK1.1+ immune cells upon loss of TLR7. And finally, we orthotopically transplanted TLR7 depleted cancer cells into the mammary fat pad of NSG mice. Even in the immunocompromised background, TLR7 depletion significantly decreased metastasis. Taken together, these revision experiments have revealed that DRG-mediated TLR7 signaling occurs in a non-canonical, MyD88-independent, immune-independent manner to drive breast cancer metastasis.

Minor comment:

1) The authors need to describe what CTC stands for.

We have edited the text to state “circulating tumor cells”.

We are grateful for the referee's thoughtful comments on our manuscript, as they have significantly reinforced our findings and expanded the scope.

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors are to be commended for the very thorough revision, which addresses all my comments. This paper is an important contribution to the field in general and to Cancer Neuroscience in particular. It will be of interest to a broad multidisciplinary readership.

Referee #2 (Remarks to the Author):

The authors have produced a major revision and provided new data to address each question that we and the other reviewers raised. In most cases, the new experiments align perfectly with their hypotheses. The manuscript has been rewritten to address the initial logical flaws and somewhat improve references to the previous literature. Although I have no additional experimental issues to raise, many of my original points stand and I admit to remaining skeptical about the complex model proposed involving SP>ssRNA>TLR7. However, the data clearly point to a role for sensory neuron derived neuropeptides in metastasis. It will be up to future studies to confirm and potentially extend these observations.

Referee #3 (Remarks to the Author):

The authors added a considerable amount of new experimental data in the revised manuscript. The additional data supports the proposed model on the role neurons in driving metastasis. The majority of my concerns and comments were addressed. Overall, the data now show a complete picture of a novel mechanism how neuron-tumor interactions lead to increased metastasis. In my opinion, there is only one piece of the puzzle missing.

In my previous comments I asked for tumor cell characteristics that induce increased innervation of tumors with high vs low metastatic potential. The authors refer to published data that show the release of neurotrophic by cancer cells. However, those references are more general and don't provide insight into the models used in this study. The finding that endothelial-derived Slit2 drives innervation is very intriguing. But now leads to the question, whether tumors with low metastatic potential (e.g. 67NR, EO771 or HCC1806) exhibit less endothelial slit 1 compared to the counterparts with higher metastatic potential (4T1, EO771 LM2 and HC1806 LM2). Addition of such data would nicely complete the proposed mechanism.

Overall, this is a well conducted study that provides novel insight into a fascinating aspect of cellular interactions that drive cancer metastasis.

Referee #4 (Remarks to the Author):

In the revised manuscript, the authors have made significant improvements in addressing the critical comments. However, some questions have been raised by the newly generated data.

- 1) The authors show that SP induces cell death in a small fraction of cancer cells. It is not clear why certain cells are susceptible to SP while others are not. It would be beneficial for the authors to provide insight into the potential mechanisms underlying this heterogeneity.
- 2) The authors' new data indicate that endothelial cells promote innervation into tumor tissue via SLIT2. I am curious if cancer cells themselves produce SLIT2. If so, it would be intriguing to investigate whether cancer-derived SLIT2 contributes to enhanced neuronal activity and SP secretion.
- 3) The authors propose that MyD88-independent mechanisms downstream of TLR7 confer pro-invasive properties. It would be valuable if the authors could suggest potential mediators for this signaling pathway.

Minor points:

- 1) Please clarify what scSLIT2 stands for.

Author Rebuttals to First Revision:

Summary of changes in the second revision:

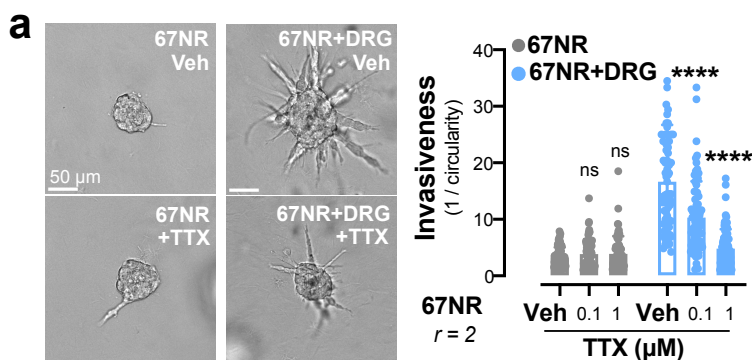
We are grateful to all referees who praised our revised draft. Two referees asked for a few experiments that expand upon the studies requested in the previous round. In response, we provide the following in our revised manuscript:

1) We show that the SLIT2 is overexpressed in the endothelium of highly metastatic tumors (4T1, EO771 LM2, HCC1806 LM2) relative to the endothelium of respective isogenic, poorly metastatic tumors. This is consistent with our model in which endothelial SLIT2 drives innervation of metastatic breast tumors.

(2) As a reminder, we had previously shown that neuronal substance-P (SP) induces cell death in a small subset of cancer cells, causing release of ssRNA that activates TLR7 receptors on neighboring cancer cells to drive pro-metastatic phenotypes. We now present evidence that neuronal SP causes cell death in a subpopulation of cancer cells that over-express the SP receptor—TACR1. Depletion of TACR1 prevents SP-induced cancer cell death.

(3) We have further characterized the sensory-neuron induced non-canonical, MyD88-independent TLR7 signaling axis in cancer cells. We show that TLR7 activation in cancer cells results in downstream AKT/PI3K signaling. Inhibition of PI3K significantly impairs the ability of DRG neurons to promote cancer spheroid invasion in 3D co-culture assays. This finding provides a direct molecular link between neuronal activity and a key oncogenic signaling pathway.

(4) Finally, we further build on our model to demonstrate that the pro-metastatic effects of sensory neurons are neuronal activity dependent. Treatment with tetrodotoxin, a sodium channel blocker that inhibits action potentials, significantly impairs the ability of DRG neurons to drive cancer spheroid invasion (Rebuttal Fig. 1a).



Rebuttal Fig. 1 (a) Left: Representative micrographs of 67NR spheroids co-cultured with DRG neurons in the presence of a sodium channel blocker, tetrodotoxin (TTX). Scale bar, 50 μm. Right: TTX blocks the ability of DRG neurons to drive cancer spheroid invasion in a dose-dependent manner. ns $p > 0.9999$, **** $p < 0.0001$ by Kruskal-Wallis test. Also Extended Data Fig. 7e.

Referee #1 (Remarks to the Author):

The authors are to be commended for the very thorough revision, which addresses all my comments. This paper is an important contribution to the field in general and to Cancer Neuroscience in particular. It will be of interest to a broad multidisciplinary readership.

We thank the reviewer for their recognition of the significance of our study and for their insightful comments in the first round of revision that led to significant improvements in the manuscript.

Referee #2 (Remarks to the Author):

The authors have produced a major revision and provided new data to address each question that we and the other reviewers raised. In most cases, the new experiments align perfectly with their hypotheses. The manuscript has been rewritten to address the initial logical flaws and somewhat improve references to the previous literature. Although I have no additional experimental issues to raise, many of my original points stand and I admit to remaining skeptical about the complex model proposed involving SP>ssRNA>TLR7. However, the data clearly point to a role for sensory neuron derived neuropeptides in metastasis. It will be up to future studies to confirm and potentially extend these observations.

We thank the reviewer for their comments in the previous round of revision. As the referee states, we have performed all the experiments they requested. We are pleased to learn that the referee has no additional experimental suggestions and that they recognize our data to 'clearly point to a role for sensory neuron derived neuropeptides in metastasis'.

Referee #3 (Remarks to the Author):

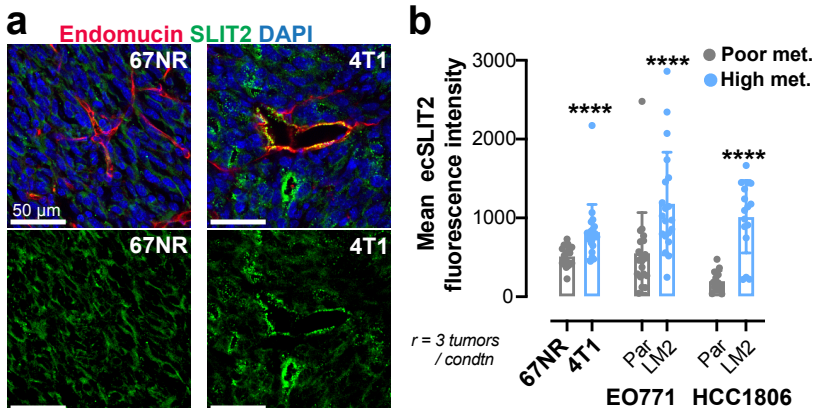
The authors added a considerable amount of new experimental data in the revised manuscript. The additional data supports the proposed model on the role neurons in driving metastasis. The majority of my

concerns and comments were addressed. Overall, the data now show a complete picture of a novel mechanism how neuron-tumor interactions lead to increased metastasis.

We sincerely thank the reviewer for sharing our excitement about our findings and for providing insightful comments that have undoubtedly enhanced the quality of our manuscript.

In my opinion, there is only one piece of the puzzle missing. In my previous comments I asked for tumor cell characteristics that induce increased innervation of tumors with high vs low metastatic potential. The authors refer to published data that show the release of neurotrophic by cancer cells. However, those references are more general and don't provide insight into the models used in this study. The finding that endothelial-derived Slit2 drives innervation is very intriguing. But now leads to the question, whether tumors with low metastatic potential (e.g. 67NR, EO771 or HCC1806) exhibit less endothelial slit 1 compared to the counterparts with higher metastatic potential (4T1, EO771 LM2 and HC1806 LM2). Addition of such data would nicely complete the proposed mechanism.

Thank you for this important question. A previous study from our group reported that SLIT2 is highly expressed in the endothelium of highly metastatic tumors relative to poorly metastatic tumors across models of melanoma, lung, and breast cancers, including the 67NR/ 4T1 model (Tavora *et al.*, *Nature*, 2020). We have now extended this finding to all the syngeneic models used in the current study. Immunofluorescence analysis revealed that endothelium of highly metastatic breast tumors (4T1, EO771 LM2, HCC1806 LM2) express significantly higher levels of SLIT2 relative to isogenic poorly metastatic tumors (67NR, EO771, or HCC1806 respectively; Rebuttal Fig. 2a, b).



Rebuttal Fig. 2. (a) Representative micrographs of poorly metastatic 67NR tumors (left) or highly metastatic 4T1 tumors (right) stained for SLIT2 and endomucin (endothelial-specific marker). (b) Highly metastatic breast tumors exhibit a higher mean fluorescence intensity of SLIT2 within the endothelium compared to poorly metastatic tumors. **** $p < 0.0001$ by Mann-Whitney test.

Also Extended Data Fig. 10e, f.

Overall, this is a well conducted study that provides novel insight into a fascinating aspect of cellular interactions that drive cancer metastasis.

Thank you.

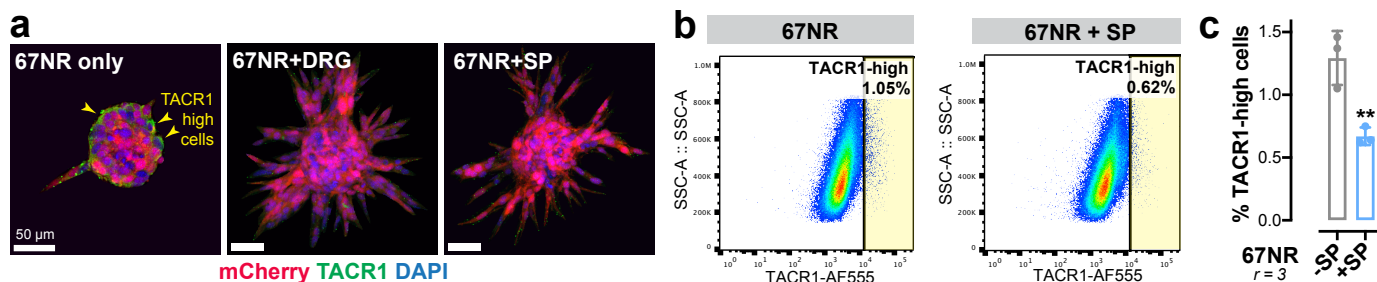
Referee #4 (Remarks to the Author):

In the revised manuscript, the authors have made significant improvements in addressing the critical comments. However, some questions have been raised by the newly generated data.

We are pleased to learn that we have effectively addressed all of the reviewer's comments from the first round of revisions. We thank the reviewer for raising the important points below since they have further strengthened our model and expanded its scope.

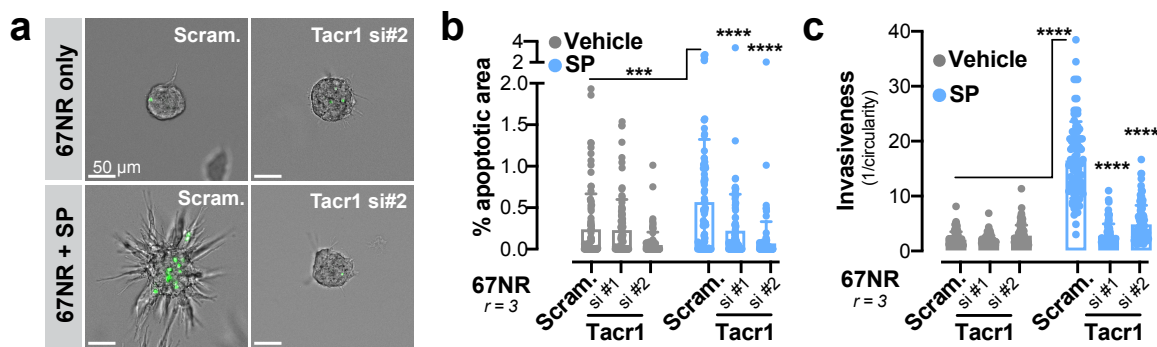
1) The authors show that SP induces cell death in a small fraction of cancer cells. It is not clear why certain cells are susceptible to SP while others are not. It would be beneficial for the authors to provide insight into the potential mechanisms underlying this heterogeneity.

Thank you for this important question. Using immunofluorescence and flow cytometry analysis, we find that a small sub-population of cancer cells express high levels of SP's receptor, TACR1. Therefore, we hypothesized that these TACR1-high cells would be most susceptible to cell death upon SP treatment. Consistent with this hypothesis, we found a ~40% decrease in the number of TACR1-high cancer cells after co-culture with DRG neurons or SP treatment (Rebuttal Fig. 3 a-c). These findings suggest that the heterogeneity in the expression of SP's receptor, TACR1 contributes to the heterogeneity in breast cancer cell death upon SP exposure.



Rebuttal Fig. 3. (a) Representative images showing a small number of mCherry+ cancer cells expressing high levels of TACR1 (green). TACR1-high cells are absent upon DRG co-culture or SP treatment. Scale bar, 50 μ m. (b-c) Flow cytometry analysis of TACR1 expression in 67NR cancer cells shows a significant reduction in the percentage of TACR1-high cells upon SP treatment. ** $p = 0.0089$ by two-tailed t-test. **Also Extended Data Fig. 9 b-d.**

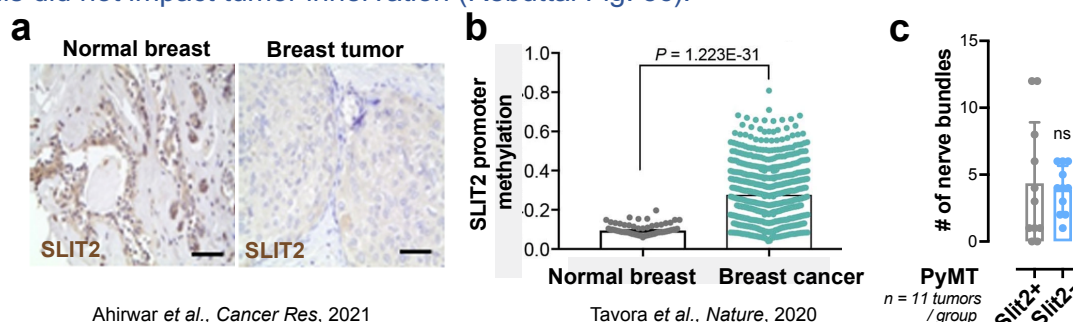
To more directly determine whether SP-induced cancer cell death is dependent on TACR1 expression, we depleted TACR1 from cancer cell spheroids cultured in the presence of exogenous SP. We observed that TACR1 expression in cancer cells is required for SP-mediated cell death (Rebuttal Fig. 4a, b) and invasion (Rebuttal Fig. 4a, c).



Rebuttal Fig. 4. (a) Representative micrographs of 67NR spheroids depleted for TACR1 cultured with SP, in the presence of a membrane-permeable caspase 3/7 substrate that fluoresces (green) in response to caspase activity. Scale bar, 50 μ m. (b) SP significantly increase the percentage of apoptotic area within 67NR spheroids. TACR1 depletion blocks SP-mediated cancer cell death. Mean $\pm$ SD. *** $p = 0.0003$ by Mann-Whitney test, **** $p < 0.0001$ by Kruskal-Wallis test. (c) Depletion of TACR1 in cancer cells prevents SP-induced increase in spheroid invasion. Mean $\pm$ SD. **** $p < 0.0001$ by Kruskal-Wallis test. **Also Extended Data Fig. 9 e-g.**

2) The authors' new data indicate that endothelial cells promote innervation into tumor tissue via SLIT2. I am curious if cancer cells themselves produce SLIT2. If so, it would be intriguing to investigate whether cancer-derived SLIT2 contributes to enhanced neuronal activity and SP secretion.

Several studies have shown that SLIT2 is a tumor suppressor whose expression is lost across multiple cancers (PMIDs: 12384551, 14735202, 12881718), including breast cancer (Rebuttal Fig. 5a). A previous study from our group showed that the promoter of SLIT2 is hypermethylated in breast cancers (Tavora *et al.*, *Nature*, 2020; Rebuttal Fig. 5b). Therefore, SLIT2 is poorly expressed by cancer cells and unlikely to impact the neuro-cancer axis described in our manuscript. Consistently, genetic deletion of SLIT2 within cancer cells did not impact tumor innervation (Rebuttal Fig. 5c).

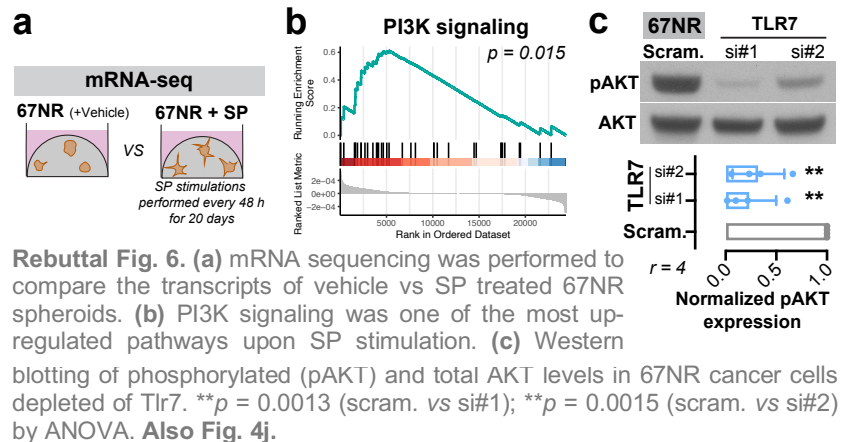


Rebuttal Fig. 5. (a) Representative images showing reduced expression of SLIT2 in breast tumors compared to normal breast tissue. Images are from Ahirwar *et al.*, *Cancer Res*, 2021. (b) SLIT2 promoter methylation in normal breast tissues and invasive

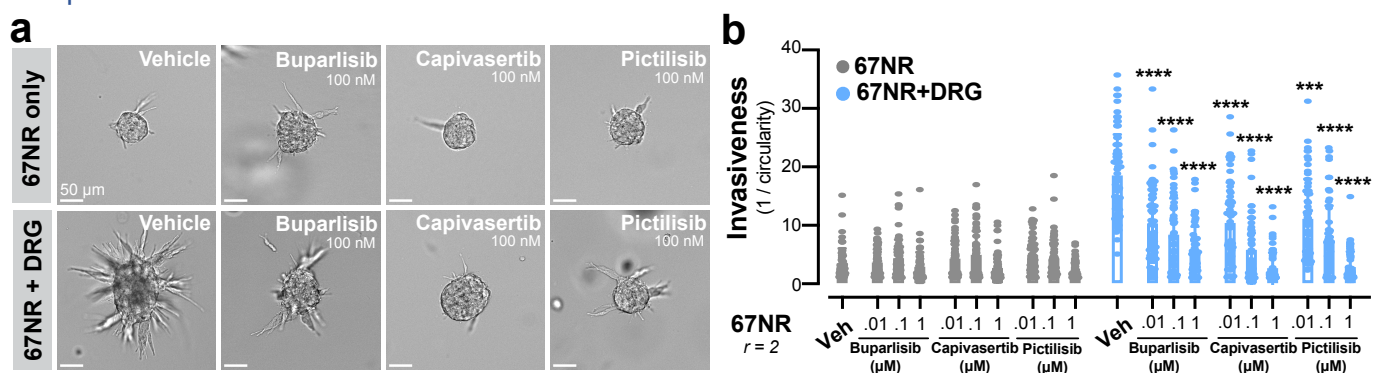
breast carcinomas, reproduced from the Human Cancer database (MethHC). Dot plot represents the mean *SLIT2* promoter methylation $\pm$ s.e.m. Breast tissue, $n = 92$; Breast cancer, $n = 735$. This data is from Tavora *et al.*, *Nature*, 2020. (c) Genetic deletion of *SLIT2* in cancer cells was performed in *Slit2 fl/fl*; MMTV-PyMT; MMTV-Cre mice. Loss of *Slit2* within the cancer cell compartment did not alter the number of NFL+ nerve bundles within tumors. $^{ns}p = 0.6414$ by Mann-Whitney test. **Also Extended Data Fig. 10f.**

3) The authors propose that MyD88-independent mechanisms downstream of TLR7 confer pro-invasive properties. It would be valuable if the authors could suggest potential mediators for this signaling pathway.

We thank the reviewer for this question since it led us to some very exciting findings. Two lines of experiments led us to investigate PI3K/ AKT signaling downstream of TLR7 activation: (i) mRNA-sequencing of cancer cell spheroids depleted for TLR7 revealed a downregulation of PI3K signaling (Fig. 4i), (ii) prolonged exposure of cancer cells spheroids to SP also promoted PI3K signaling (Rebuttal Fig. 6a, b).



These data suggested that PI3K signaling was activated downstream of neuronal activation of tumoral TLR7 receptors. Consistent with this model, we found significantly lower levels of phosphorylated AKT (pAKT) in breast cancer cells upon TLR7 depletion (Rebuttal Fig. 6c). Importantly, three independent small molecule inhibitors of PI3K signaling prevent DRG neuron-induced cancer spheroid invasiveness in a dose-dependent manner (Rebuttal Fig. 7a, b). All three PI3K inhibitors also prevent SP-induced cancer spheroid invasiveness (Extended Data Fig. 10c). Activation of PI3K signaling downstream of TLR receptors has been observed in other cell types (macrophages, PMID: 12616480) or disease contexts (myocardial ischemia, PMID: 20421349). Together, these data present PI3K signaling as a MyD88-independent mechanism downstream of TLR7 in breast cancer cells that confers pro-invasive phenotypes in cancer cells. This provides a molecular link between neuronal activity and a key oncogenic signaling pathway in an epithelial tumor.



Rebuttal Fig. 7. (a) Representative micrographs of 67NR cancer cell spheroids co-cultured with DRG neurons in the presence of three independent small molecule inhibitors of PI3K signaling. Scale bar, 50 μ m. (b) Inhibition of PI3K signaling prevents DRG neuron driven increase in cancer spheroid invasiveness in a dose-dependent manner. $^{***}p = 0.0005$, $^{****}p < 0.0001$ by Kruskal-Wallis test. **Also Fig. 4k, l.**

Minor points:

1) Please clarify what scSLIT2 stands for.

ecSLIT2 stands for endothelial cell derived SLIT2. We have clarified this in the manuscript.

We thank the reviewer for these carefully considered comments and suggestions. Collectively they were very effective in stimulating us to conduct important new experiments and analyses. Our manuscript has benefitted significantly from your time and expertise.

Reviewer Reports on the Second Revision:

Referees' comments:

Referee #3 (Remarks to the Author):

In the previous round of review, I had one remaining open question that was now addressed in the revised version. I have no further comments or concerns. Overall, the study provides thorough and comprehensive insight into an exciting aspect of cancer metastasis biology.

Referee #4 (Remarks to the Author):

Following two rounds of revision, substantial enhancements have been implemented in the article, further solidifying the understanding of the neuronal role in propelling metastasis. Consequently, the article is now ready for publication.