Cancer exosomes induce tumor innervation

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Patients with densely innervated tumors suffer with increased metastasis and decreased survival as compared to those with less innervated tumors. We hypothesize that in some tumors, nerves are acquired by a tumor-induced process, called axonogenesis. Here, we use PC12 cells as an in vitro neuronal model, human tumor samples and murine in vivo models to test this hypothesis. When appropriately stimulated, PC12 cells extend processes, called neurites. We show that patient tumors release vesicles, called exosomes, which induce PC12 neurite outgrowth. Using a cancer mouse model, we show that tumors compromised in exosome release are less innervated than controls. Moreover, in vivo pharmacological blockade of exosome release similarly attenuates tumor innervation. We characterize these nerves as sensory in nature and demonstrate that axonogenesis is potentiated by the exosome-packaged axonal guidance molecule, EphrinB1. These findings indicate that tumor released exosomes induce tumor innervation and exosomes containing EphrinB1 potentiate this activity.
nnervated tumors are more aggressive than less innervated
tumors. For instance, in prostate cancer, recruitment of nerve
fibers to cancer tissue is associated with higher tumor pro-
iferative indices and a higher risk of recurrence and metastasis.9,10
Denervation studies in mouse cancer models support a functional
contribution of nerves in disease progression.11,12 Such studies
strongly indicate that the nervous system is not merely a bystander but
instead an active participant in carcinogenesis and cancer pro-
gression. However, a mechanistic understanding of how tumors
obtain their neural elements remains unclear. Tumors may acquire
innervation by growing within innervated tissues; in other
words, nerves are already present within the microenvironment
and the tumor acquires them by default. However, the clinical
findings that some tumors of the same tissue are more innervated
than others indicate instead an active, tumor-initiated process,
similar to angiogenesis and lymphangiogenesis. The possibility
that tumors invoke their own innervation, termed axon formation,
has not been extensively explored.13,14

Extracellular release of neurotrophic factors [e.g., nerve growth
factor (NGF)] by tumor cells can contribute to cancer
progression.15,16 While such a mechanism likely contributes to
tumor innervation, tumors release additional components that
may also directly promote axon formation. Among these are
extracellular vesicles such as exosomes. Exosomes are 30–150 nm
vesicles that package a rich cargo (proteins, DNA, RNA, and
lipids). Because they are generated by invagination of endocytic
vesicles, the topology of exosomal transmembrane proteins is
preserved as is, presumably, their biological activity. Exosomes
are released into the extracellular milieu by most, if not all, cells17
and function as vehicles of intercellular communication.18
Mounting evidence supports the hypothesis that tumor-released
exosomes promote disease progression through a number of
mechanisms, including the induction/promotion of metastasis
and tumor tolerance.19,20 We hypothesized that such a mechanism
utilized by cancer cells to promote disease progression is the
induction of tumor innervation. Here, we show that tumor
released exosomes mediate axon formation in cancer and that this
innervation is sensory in nature.

We utilize PC12 cells, a rat pheochromocytoma cell line, as an
in vitro screen for axonogenic activity. When appropriately
stimulated, PC12 cells extend neurites and morphologically
resemble neurons. Our data with human samples indicate that
exosomes from head and neck cancer patient tumors and
matched blood induce significantly more neurite outgrowth from
PC12 cells than exosomes from non-cancer control blood and
tissue. To gain further mechanistic insight into this phenomenon,
we turned to a murine model of human papillomavirus induced
(HPV+) oropharyngeal squamous cell carcinoma (OPSCC). This
model consists of oropharyngeal epithelial cells from C57Bl/6
mice that stably express HPV16 viral oncogenes, E6 and E7, H-
Ras and luciferase (mEE RL) cells.21 We show that exosomes
released from conditioned media of these cells induce neurite
outgrowth of PC12 cells. When we compromise exosome release
of mEE RL cells by CRISPR/Cas9 genomic modification of Rab27A/
B, we find a significant attenuation of this activity. Moreover,
mice implanted with the Rab27A/B modified tumors are sparsely
innervated. As an additional test of this hypothesis, when mEE RL
bearing mice are treated with the exosome release inhib-
itor, GW4869, tumor innervation is significantly reduced com-
pared to that occurring in vehicle-treated mice.

Many molecules contained as cargo in exosomes are potential
candidates for exosome-induced axonogenesis. We experimentally
excluded NGF and focused on EphrinB1 as its increased expression
leads to aggressive disease in different human cancers as well as in
the mEE RL mouse model.22–24 EphrinB1 is a single pass trans-
membrane protein ligand that binds and activates the Eph receptor
tyrosine kinases; furthermore, EphrinB1 itself becomes phos-
phorylated and initiates its own signaling.25 During development,
EphrinB1 functions as an axonal guidance molecule.26 Similar to
other ephrins, EphrinB1 is frequently associated with growth
cone collapse and inhibition of axon outgrowth,27,28 potentially
re-directing axonal trajectory. We wondered if the contribution
of EphrinB1 to disease progression extends beyond its signaling
capabilities to include its axonal guidance properties. Here,
we show that tumor released exosomes with a high EphrinB1
content potentiate neurite outgrowth of PC12 cells in vitro
while compromised EphrinB1 expression or function significantly
attenuates it. Consistent with these findings, mEE RL tumors
over-expressing EphrinB1 are significantly more innervated
than those with basal EphrinB1 expression. Taken together, these
data indicate that tumor released exosomes contribute to axono-
genesis and that exosomal EphrinB1 potentiates this activity.

Results

Head and neck squamous cell carcinomas are innervated.
Immunohistochemical (IHC) staining of formalin-fixed paraffin
embedded human head and neck squamous cell carcinomas
(HNSCCs) for β-III tubulin, a neuron specific tubulin isoform,
demonstrates these tumors are innervated; individual β-III
positive fibers are coursing throughout the tumor tissue
(Fig. 1a, “Nerve twigs”). These “nerve twigs” should not be
confused with perineural invasion (PNI)29,30. PNI refers to tumor
invading into nerves along the perineural space; axonogenesis
does not refer to nerves invading into tumor. Within the perineural
sheath, β-III tubulin positive fibers are tightly packed together in
a very organized manner (Fig. 1b, “nerve bundle”). The β-III
positive nerve fibers we identified are instead coursing as
individual, unorganized twigs lacking a perineural sheath (Fig. 1a
“nerve twigs”). Additional IHC staining shows that HNSCCs are
negative for Tyrosine Hydroxylase (TH, sympathetic marker) and
Vasoactive Intestinal Polypeptide (VIP, parasympathetic marker)
but positive for Transient Receptor Potential Vanilloid type 1
channel (TRPV1, sensory marker) (Fig. 1b) indicating sensory
innervation. Double IHC characterization of these neural “twigs”
demonstrates co-localization of β-III tubulin (pink, arrows) and
TRPV1 channels (brown, arrowheads) (Fig. 1c). Interestingly,
some positively labeled fibers appeared to be nucleated, though
their identity remains unclear. Regardless of their identity, we
proceeded to quantify positively stained (nucleated) fibers
using stereology. We found that irrespective of whether tumors
were densely or sparsely innervated, most of the β-III tubulin
positive fibers were also immuno-positive for TRPV1 (Fig. 1d).
On average, 83.7% ± 6.5% of β-III tubulin positive fibers were also
TRPV1 positive. These data are consistent with sensory tumor
innervation.

Validation of exosome purification. Prior to testing the con-
tribution of exosomes to axonogenesis, we validated our differ-
ential ultracentrifugation exosome purification technique.
Exosomes were purified from human plasma; exosomes from
human tissue samples were similarly purified from conditioned
media collected after 48 h in culture. Scanning electron micro-
scopic analysis of exosome preparations purified from normal
donor plasma yielded vesicles consistent in shape and size
(30–150 nm) with exosomes (Fig. 2a). Atomic force microscopy
confirms a 63–105 nm size (Fig. 2b) and nanoparticle tracking
analysis (for counting and sizing exosomes) (Fig. 2c) likewise
indicates a size distribution consistent with exosomes.31,32. These
data indicate that our purification method yields vesicles con-
sistent in size and shape with exosomes. We further characterized
exosomes by western blot analysis for the exosome markers, CD9
and CD81. We found that exosomes purified from both control and patient plasma were positive for one or both of these markers as were normal tonsil tissue and tumor released exosomes (Fig. 2d, Supplementary). The tonsil serves as control tissue since the majority of HPV + OPSCCs arise there.

**Cancer patient exosomes induce neurite outgrowth.** To test our hypothesis that tumor released exosomes induce axonogenesis, we utilized PC12 cells, a rat pheochromocytoma cell line, as an in vitro assay. When stimulated with NGF (100 ng/ml), PC12 cells differentiate into neuron-like cells extending neurites. To test whether exosomes harbor neurite outgrowth activity, PC12 cells were stimulated with 3 µg of exosomes that were harvested from either 10 ml of blood or the conditioned media of the matched tumor tissue from eight head and neck cancer patients (Pt1-Pt8). We similarly collected blood from three healthy volunteers (N1-N3) and adult tonsils from two non-cancer donors (TL1, 2). PC12 cells were fixed 48 h later and immunostained for β-III tubulin. Neurite outgrowth was quantified and the number of neurites compared. Given the time to procure these matched (blood and tumor) human samples, patient exosomes were not tested at the same time or on the same plate and thus are graphed separately (Fig. 3a, b). Consistent with the literature, we found that untreated PC12 cells extend few β-III tubulin positive neurites while those stimulated with NGF do so robustly. Exosomes from patients (both plasma and tumor) stimulated significant neurite outgrowth of PC12 cells while control plasma and tonsil exosomes had minimal neurite outgrowth activity (Fig. 3a, b). All comparisons can be found in Supplementary Tables 1–3. These data indicate that exosomes from head and neck cancer patients harbor neurite outgrowth activity that is absent in the exosomes of healthy controls. Western blot analysis of exosomes for EphrinB1 suggests that EphrinB1 is not required for neurite outgrowth activity in this assay (Supplementary Figure 1).

**mEERL tumors are innervated.** To determine whether the murine model of HNSCC we use is innervated similar to its human counterpart, mice were injected with mEERL cells in the hind limb (1 × 10^6 cells/mouse), tumors harvested at endpoint, fixed, embedded and IHC stained for β-III tubulin, TH, VIP, and TRPV1. Similar to patient HNSCCs, mEERL tumors harbored β-III tubulin positive nerve twigs that were sensory in nature (TRPV1 positive) (Fig. 4a). Recent reports indicate that stress experienced by cancer cells (e.g. low oxygen) can induce their expression of β-III tubulin. To address this possibility, serial sections of mEERL tumors were IHC stained for cytokeratin (epithelial marker) and β-III tubulin (neuronal marker) and found to display starkly different staining patterns (Fig. 4b), confirming that tumor cells and β-III tubulin positive fibers are distinct entities. To further characterize the nature of mEERL tumor innervation, we performed triple immunofluorescent labeling for β-III tubulin, TRPV1 and Tau (another neuronal marker) on formalin fixed paraffin embedded tumors. We found that β-III tubulin positive fibers were also positive for TRPV1 and Tau (merge, Fig. 4c). Taken together, these data are consistent with sensory innervation of mEERL tumors similar to the innervation in patient HNSCC tumors.

**mEERL exosomes induce neurite outgrowth.** Prior to testing neurite outgrowth activity of mEERL released exosomes, we characterized these exosomes as follows: exosomes purified from mEERL conditioned media were analyzed by electron microscopy (Supplementary Figure 2a), atomic force microscopy (Supplementary Figure 2b) and nanoparticle particle analysis (Supplementary Figure 2c) and, similar to their human counterparts, were consistent in size and shape with exosomes. Recent published studies suggest that more stringent methods are critical for eliminating other vesicles and cellular debris from exosome purifications. One such method requires the addition of density gradient centrifugation. To test whether this more stringent methodology purifies exosomes with neurite outgrowth activity, conditioned media from mEERL cells stably over-expressing EpherinB1 (mEERL EphrinB1) was collected, subjected to differential ultracentrifugation and subsequently to density gradient centrifugation. Fifteen fractions were collected and fractions 4–13 were analyzed by western blot for the exosomal markers CD9 and CD81. Consistent with the published literature, CD9+ and CD81+ vesicles were present in fraction 8. Exosomes purified by differential ultracentrifugation alone (“crude” sample) were also CD9+/CD81+ (Supplementary Figure 2d). To determine which fractions induce neurite outgrowth, fractions 4, 5, 8, 13, and
“crude” exosomes were tested on PC12 cells. While fraction 8 and “crude” exosomes demonstrate neurite outgrowth activity, CD9 negative fractions 4, 5, and 13 lacked this activity (Supplementary Figure 2e; all comparisons found in Supplementary Table 7). These data indicate that inclusion of density gradient centrifugation concentrates CD9+/CD81+ exosomes to a single fraction that retains neurite outgrowth activity. Interestingly, CD9+/CD81+ fractions are also positive for EphrinB1 (Supplementary Figure 2d) indicating that neurite outgrowth activity is retained in EphrinB1 positive CD9+/CD81+ exosomes.

**Inhibition of exosome release attenuates innervation.** To test the hypothesis that tumor released exosomes induce tumor innervation, we utilized CRISPR/Cas9 to genetically modify Rab27A and Rab27B in mEERL parental cells. These two small GTPases contribute to exosome release and their knock-down compromises release of CD9+/exosomes38,39. The clone generated is heterozygous for Rab27A and homozygous deleted for Rab27B (mEERL Rab27A+/−Rab27B−/−). For characterization and testing of this clone, exosome samples were normalized to producing cell number. Exosomes isolated from Rab27A+/−Rab27B−/− cells displayed less CD9 signal than parental mEERL exosomes; this is consistent with previous studies demonstrating a decreased capacity to release CD9 positive exosomes by cells compromised in Rab27A/B expression (Supplementary Figure 3a)40. Exosomes were further analyzed by nanoparticle tracking analysis (Supplementary Figure 3b) and membrane permeant fluorescein labeling (CFDA-SE) (Supplementary Figure 3c) indicating decreased exosome release by the Rab27A+/−Rab27B−/− mutant cells.

To test whether compromised exosome release affects neurite outgrowth of PC12 cells, mEERL parental and mEERL Rab27A+/−Rab27B−/− cells were cultured in vitro, exosomes purified from conditioned media, normalized to producing cell number and equivalent volumes applied to PC12 cells (n = 4 wells; condition; experiment repeated at least 3 times). The neurite outgrowth activity of mEERL Rab27A+/−Rab27B−/− exosomes was significantly attenuated compared to that of exosomes from mEERL parental cells (Fig. 5a; all comparisons found in Supplementary Table 4). To test whether compromised exosome release alters innervation in vivo, C57Bl/6 mice (n = 7 mice/group) were implanted with mEERL parental or Rab27A+/−Rab27B−/− cells; tumors were harvested and 15 μg of whole tumor lysate quantified by western blot for β-III tubulin, TRPV1 and Tau (Fig. 5b). Consistent with our hypothesis, Rab27A+/−Rab27B−/− tumors were significantly decreased in β-III tubulin, TRPV1 and Tau as compared to mEERL parental tumors (Fig. 5c-e). These data support our hypothesis that tumor released exosomes contribute to tumor innervation. Notably, Rab27A+/−Rab27B−/− tumors also grew significantly slower than mEERL parental tumors (Supplementary Figure 3d).

Importantly, in vitro proliferation assays show that cell doubling time was not significantly different between mEERL parental and Rab27A+/−Rab27B−/− cells (Supplementary Figure 3e).

Fig. 2 Validation of human exosome purification. a Scanning electron micrograph of exosomes purified from human plasma. Scale bar, 200 nm. n = 4 biological samples. b Atomic force microscopy amplitude trace of exosomes purified from human plasma. Size range, 63-105 nm. Scale bar, 500 nm. n = 7 biological samples. c Representative nanoparticle tracking analysis of exosomes purified from human plasma. N = 4 biological samples. d Western blot analysis of control (Cntl) and patient (Pt) exosomes (exos). Western blots have been cropped for clarity and conciseness. Nl1-2 plasma from healthy volunteers, TL 1 normal adult tonsil. n = 3 technical replicates.
vehicle-treated control mice (Fig. 5g). Representative photomicrographs of IHC stained tumor tissue for β-III tubulin show single fibers similar to those identified in human and mEERL tumors (Figs 5h, 1a).

Exosome-mediated neurite outgrowth does not require NGF. Previous reports indicate that neurotrophic factors secreted by tumor cells can induce tumor innervation9,10. mEERL cells express NGF but not BDNF, NT-3, NT-4/5 or GDNF (Geo Accession GSE68935)43. Thus, we wondered whether release of NGF by mEERL cells contributes to neurite outgrowth activity. To test this, conditioned media from mEERL parental and EphrinB1 cells was incubated with neutralizing anti-NGF antibody for 24 h and tested on PC12 cells. While NGF neutralization robustly attenuated neurite outgrowth of recombinant NGF, it had no effect on neurite outgrowth stimulated by conditioned media from either mEERL parental or EphrinB1 cells indicating that soluble NGF is not required for neurite outgrowth activity in this assay (Fig. 6a; all comparisons found in Supplementary Table 4). It remains possible that NGF is packaged as cargo within exosomes and mediates neurite outgrowth in this way. To test this, whole cell lysates and purified exosomes from mEERL parental and EphrinB1 cells were analyzed by western blot for NGF. We confirmed that mEERL parental and EphrinB1 over-expressing cells produce NGF, as indicated by the presence of two bands corresponding to the processed forms of NGF (13.5 and 16.5 kD) in the whole cell lysate (WCL)44. These bands are
EphrinB1 potentiates exosome neurite outgrowth activity. We wondered what exosomal cargo was necessary and sufficient for neurite outgrowth activity. During development, EphrinB1 functions as an axonal guidance molecule. Moreover, EphrinB1 has been identified as exosome cargo in other systems (Exocarta.org) and significantly contributes to disease progression in HNSCC and HCC. Thus, to test its contribution in axonogenesis, we generated EphrinB1 modified mEERL cell lines. Stable overexpression of wild-type EphrinB1 is referred to as mEERL EphrinB1. CRISPR/Cas9 engineered mEERL cell lines compromised in EphrinB1 function or expression are denoted as follows: mEERL EphrinB1 Null1, or Null2 (two independent clones in which EphrinB1 is deleted) and extracellularly truncated EphrinB1 cells are denoted mEERL EphrinB1ΔECD. The characterization of these CRISPR lines is presented in Supplementary Figures 4–5.

To determine the contribution EphrinB1 to neurite outgrowth, exosomes were purified from the conditioned media of the various mEERL cell lines and tested on PC12 cells. Exosomes from mEERL parental cells significantly induced neurite outgrowth of PC12 cells while over-expression of EphrinB1 potentiated this activity (Fig. 6c; all comparisons found in Supplementary Table 5). MEERL EphrinB1ΔECD, Null1 and Null2 exosomes retained neurite outgrowth activity indicating that mEERL released exosomes promote neurite outgrowth and that EphrinB1 is not required for this activity, but significantly potentiates it.

Given that exosomes purified from mEERL EphrinB1 cells potentiated neurite outgrowth of PC12 cells, we tested whether it is packaged as exosome cargo. Consistent with the published literature, EphrinB1 was indeed packaged within exosomes as assessed by western blot analysis (Fig. 6d) (Exocarta.org). Moreover, while the extracellular domain of EphrinB1 was absent in mEERL EphrinB1ΔECD exosomes, the intracellular domain remained as cargo running at a similar molecular weight as (endogenously) proteolytically processed EphrinB1 (Fig. 6d).

The data indicate that exosomal EphrinB1 is not required for neurite outgrowth activity, but does potentiate it. Thus, we wondered if exosomal EphrinB1 activates known signaling pathways in PC12 cells that are important for neuritogenesis. One such pathway is the MAP Kinase pathway. To test this, PC12 cells were stimulated for 5 minutes with either PBS (negative control), NGF (50 ng/ml, positive control), or exosomes purified from either mEERL parental, EphrinB1 or Null2 conditioned media. PC12 cells were then harvested and whole cell lysate analyzed by western blot. Stimulation with NGF induced phosphorylation of Erk1/2 while control (PBS) did not activate the MAP Kinase pathway. Interestingly, stimulation with mEERL parental, EphrinB1 and Null2 exosomes also induced a similar activation of this signaling pathway (Fig. 6c). These data suggest that binding of EphrinB1 to PC12 expressed receptor(s) is not required for initiation of MAP kinase signaling. The PC12 neurite outgrowth data with exosomes purified from these cell lines (Fig. 6c) indicate that exosomes from mEERL EphrinB1 cells potentiate neurite outgrowth of PC12 cells. It remains possible that EphrinB1 exosomes promote sustained MAP kinase signaling in PC12 cells and that this prolonged signaling results in potentiation of neurite outgrowth. Such a mechanism would be consistent with the published literature.

High-risk HPV E6 and neurite outgrowth. We have thus far tested neurite outgrowth activity from mEERL cells or their derivatives, all of which are HPV positive. We wondered whether exosome mediated neurite outgrowth activity extended to
exosomes from non-HPV transformed cells. To test this, exosomes from two HPV negative human squamous cell carcinoma cell lines (UM-SCC1 and UM-SCC19, referred to as SCC1 and SCC19) and those from two HPV positive cell lines (UM-SCC47 and 93-VU-147T-UP-6, referred to as SCC47 and 147T) were tested on PC12 cells. Interestingly, though HPV negative exosomes harbored significantly less neurite outgrowth activity than HPV positive exosomes, they still induced neurite outgrowth from PC12 cells suggesting that HPV infection is not required for neurite outgrowth activity of exosomes (Fig. 7a; all comparisons found in Supplementary Table 5).

Since our data indicate that EphrinB1 potentiates neurite outgrowth activity of exosomes, we wondered if merely over-expressing EphrinB1 in an HPV negative squamous cell carcinoma cell line would increase neurite outgrowth activity of its exosomes. Thus, we stably over-expressed EphrinB1 in SCC1 cells and tested their exosomes on PC12 cells. Importantly, SCC1 cells express endogenous EphrinB1 albeit at a low level (Supplementary Figure 7c). Exosomes from SCC1-EphrinB1 cells induced significantly more neurite outgrowth than those from the SCC1 parental cells (Fig. 7b; all comparisons found in Supplementary Table 6). These data suggest that HPV negative head and neck cancer patients that harbor elevated EphrinB1 expression may also harbor elevated tumor innervation potential. To test this concept in vivo, immune incompetent NOD SCID mice were implanted with either SCC1 or SCC1-EphrinB1 cells (n = 4 mice/group). Tumor innervation was assessed 10 days later. Whole tumor lysates (n = 4 mice/group) were analyzed by western blot (Fig. 7c). SCC-1 EphrinB1 tumors had significantly more β-III tubulin and Tau by western blot than SCC1 parental
enhanced tumor growth in vivo (Supplementary Figure 7a). In the present study, over-expression of EphrinB1 significantly increased neurite outgrowth activity (Fig. 7f). Although not directly relevant to the main theme of the present study, we focused on them. HPV16 induces OPSCC and is thus considered a high risk HPV,53 low risk HPVs rarely cause cancer. One important difference between high and low risk HPVs is found in their E6 proteins; only high risk E6 contains a C-terminal PDZ binding motif (PDZBM) which contributes to oncogenic transformation54,55. In fact, E6–PDZBM mediates interactions with a number of different proteins that contribute to oncogenic transformation56–61. To test if these interactions also contribute to exosome mediated neurite outgrowth activity, we tested exosomes from primary human tonsil epithelia (HTE), exosomes from cells stably expressing HPV16 E6 and E7 (HTE E6E7) and exosomes from cells in which the E6 PDZBM is deleted (HTE E6ΔE7). Expression of full length E6 and E7 was sufficient to induce tumors (Fig. 7d, e) suggesting increased tumor innervation; tumor expression of TRPV1 was not significantly different (Fig. 7f). Although not directly relevant to the main theme of the present study, over-expression of EphrinB1 significantly enhanced tumor growth in vivo (Supplementary Figure 7a). Individual tumor growth curves are presented in Supplementary Figure 7b. Taken together, these data indicate that exosomes from both HPV positive and HPV negative squamous cell carcinoma cell lines induce tumor innervation. Consistent with our other data, EphrinB1 potentiates this activity.

We were intrigued by the apparent contribution of HPV infection on exosome-mediated neurite outgrowth activity (Fig. 7a). Since E6 and E7 are the main viral oncoproteins, we

**Fig. 6** mEERL exosomes induce neurite outgrowth without NGF. a PC12 cells were stimulated with 100 ng/ml of recombinant NGF (NGF), mEERL parental (parental) or mEERL EphrinB1 (EphrinB1) conditioned media with or without neutralizing anti-NGF antibody (+ Ab); 24 h later, cells were fixed and stained for β-III tubulin expression which was then quantified. Statistical analysis by two-way ANOVA with post hoc Fisher’s Least Significant Difference (LSD) test. LSD p values reported; *p<0.006, ns, not significant. The variance between groups compared is similar. n = 3 technical replicates/condition; experiment repeated at least twice with biological replicates. Central value used was the mean. All comparisons and LSD p values found in Supplementary Table 4. b Western blot analysis of exosomes (Exo) or whole cell lysate (WCL) from the indicated mEERL cell lines. Western blot repeated at least n = 2 times with biological replicates. c Neurite outgrowth quantification of PC12 cells following exosome treatment from the indicated sources. Statistical analysis by one-way ANOVA with post hoc Fisher’s Least Significant Difference (LSD) test. LSD p values reported; *p<0.02; ns, not significant. n = 5 technical replicates/condition; experiment repeated at least twice with biological replicates. Central value used was the mean. The variance between groups compared is similar. All comparisons and LSD p values found in Supplementary Table 5. d Western blot analysis of exosomes purified from the indicated mEERL cell lines. EphrinB1-Ex, EphrinB1 extracellular epitope antibody. EphrinB1-In, EphrinB1 intracellular epitope antibody. Western blot repeated at least n = 2 times with biological replicates. e Western blot analysis of PC12 lysate following stimulation for 5 min with PBS, NGF (50 μg/ml) or exosomes purified from mEERL parental, EphrinB1, or Null2 conditioned media. Experiment repeated at least three times with biological replicates. All error bars are standard deviation. Western blots have been cropped for clarity and conciseness.
**Fig. 7** HPV status and EphrinB1 contribute to neurite outgrowth. a Quantified neurite outgrowth of PC12 cells following exosome stimulation with HPV negative (−) or positive (+) human cell lines; statistical analysis by one-way ANOVA with post hoc Tukey test for differences between HPV- and + groups; *p<0.001. The variance between groups compared is similar. n = 3 technical replicates/condition; experiment repeated at least two times. Central value was the mean. Comparisons between individual groups were analyzed by one-way ANOVA with post hoc Fisher’s Least Significant Difference (LSD) test. All comparisons and LSD p values found in Supplementary Table 5. b Quantified neurite outgrowth of PC12 cells following exosome stimulation from HPV(−) SCC1 parental cells or SCC1(−) EphrinB1 over-expressing cells [SCC1(−) EphrinB1]. Statistical analysis by one-way ANOVA with post hoc Fisher’s Least Significant Difference (LSD) test; LSD p values reported, *p<0.02. The variance between groups compared is similar. n = 4 technical replicates/condition; experiment repeated twice. Central value used was the mean. All comparison and LSD p values found in Supplementary Table 6. c Western blot analysis of whole tumor lysates from mice bearing SCC1 or SCC1 EphrinB1 tumors. Western blots repeated at least n = 4 times with technical replicates. Western blots have been cropped for clarity and conciseness. Densitometric quantification of western blot in panel c for d β-III tubulin, e Tau, and f TRPV1. Statistical analysis by unpaired, two-tailed Student’s t-test; *p<0.03; ns not significant. The variance between groups compared is similar. Central value was the mean. g Quantified neurite outgrowth of PC12 cells following stimulation with exosomes from HTE, human tonsil epithelia; HTE E6ΔE7, cells expressing HPV16 E6 deleted of its PDZBM(Δ) and E7; HTE E6E7, cells expressing HPV16 E6 and E7. Statistical analysis by one-way ANOVA with post hoc Fisher’s Least Significant Difference (LSD) test, LSD p values reported, *p<0.0001; ns, not significant. The variance between groups compared is similar. Central value was the mean. n = 3 replicates/condition; experiment repeated twice. All comparisons and LSD p values found in Supplementary Table 6. All error bars are standard deviation.
neurite outgrowth activity while deletion of E6 PDZBM abrogated this effect (Fig. 7g; all comparisons found in Supplementary Table 6). These data suggest that HPV16 E6 PDZBM modulates exosome content or quality thereby affecting neurite outgrowth activity. In addition and consistent with our other findings, we found that these human cell lines express EphrinB1 to varying degrees and that expression did not appear to correlate with PC12 neuritogenesis (Supplementary Figure 7c). These data are consistent with our findings suggesting that EphrinB1 is not required for exosome-mediated neurite outgrowth activity. To gain further insights on the contribution of EphrinB1 to tumor innervation, C57Bl/6 mice were implanted with either mEERL parental, EphrinB1, ΔECD, Null1, or Null2 cells (n = 10 mice/group). Fourteen days post-implantation, animals were sacrificed and tumors harvested; n = 5 tumors/group were utilized for whole tumor lysate and western blot analysis for β-III tubulin, Tau and TRPV1. Signals were normalized to GAPDH and quantified by densitometry. We found that mEERL EphrinB1 tumors harbored significantly more β-III tubulin, TRPV1 and Tau positive fibers as compared to mEERL parental, Null1 and Null2 tumors (Supplementary Figure 8a–d; all comparisons found in Supplementary Tables 6–8), consistent with our other data indicating that EphrinB1 potentiates tumor innervation. Interestingly, the extent of innervation (β-III tubulin and Tau) was similar between mEERL EphrinB1 and mEERL EphrinB1ΔECD tumors (Supplementary Figure 8b, c), however mEERL EphrinB1ΔECD tumors possessed significantly less TRPV1 expression (Supplementary Figure 8d). These data indicate that while mEERL EphrinB1ΔECD tumors are as highly innervated as mEERL EphrinB1 tumors (having the same extent of β-III tubulin and Tau expression), this increased innervation is not all TRPV1 positive. Thus, the data suggest that the type of innervation may be different in the mEERL EphrinB1ΔECD tumors as compared to the mEERL EphrinB1 tumors. Tumor growth curves are presented in Supplementary Figure 8e. Consistent with the published data, over-expression of EphrinB1 results in increased tumor growth.37 We tested if these neural proteins were expressed by mEERL cells themselves via western blot analysis of whole cell lysates. Supplementary Figure 8f show that while whole tumor lysate (mEERL EphrinB1) was positive for β-III tubulin, Tau and TRPV1, whole cell lysates from mEERL parental cells and its derivative cell lines were negative for these neuronal markers indicating that the cancer cells obtain nerves in vivo.

**Exosomes from other cancers induce PC12 neurite outgrowth.** While the laboratory focus is HPV-induced HNSCC, we wondered whether our findings extended to other types of cancers. Thus, we harvested exosomes from conditioned media of CT26 (colorectal cancer cell line), B16 (melanoma cell line) and 4T1 (breast cancer cell line) tumors, verified they expressed CD9 (Fig. 8a) and tested their ability to induce neurite outgrowth of PC12 cells. Similar to human and mouse HNSCC, exosomes from these murine cancer cell lines induced significant neurite outgrowth of PC12 cells (Fig. 8b; all comparisons found in Supplementary Table 6). Consistent with our findings with mEERL and human exosomes, EphrinB1 was not required for exosome-induced neurite outgrowth as only B16 exosomes packaged EphrinB1 (Supplementary Figure 9a). Interestingly, B16 released exosomes also demonstrated the most robust neurite outgrowth activity (Fig. 8b). These data extend our findings and suggest that tumor released exosomes may mediate tumor innervation in a wide variety of solid tumors though this hypothesis requires extensive testing.

**Discussion**

Our findings propose a new mechanism for tumor-induced, exosome-mediated axonogenesis. We show that human and mouse HPV + HNSCCs are innervated de novo by TRPV1, Tau, β-III tubulin positive sensory nerves. Moreover, while mEERL tumors secrete NGF, NGF is not required for neurite outgrowth activity in our in vitro assay nor is it packaged within exosomes. Mechanistically, packaging of full length EphrinB1 as exosome cargo significantly potentiates neurite outgrowth in vitro and tumor innervation in vivo and its deletion reverses the EphrinB1 mediated potentiation. While the PC12 assay would have predicted that mEERL EphrinB1ΔECD tumors to be sparsely innervated in vivo, we instead found that they were as innervated (based on β-III tubulin and Tau) as mEERL EphrinB1 tumors (Supplementary Figure 8a–d). These data emphasize the complexity of the tumor microenvironment that cannot be replicated in the PC12 in vitro assay as exosomes have the potential to act on multiple cell targets to induce axogenesis. Despite this, both systems indicate that EphrinB1 potentiates tumor innervation and, perhaps more importantly, that full-length EphrinB1 is not required for tumor innervation. In addition, we show that compromising release of CD9 + exosomes results in significantly decreased innervation in vivo, further implicating tumor released exosomes as mediating tumor innervation. These pre-clinical studies are supported by findings with human HNSCC samples.

**Fig. 8** Exosomes from multiple cancer cell lines harbor neurite outgrowth activity. **a** Western blot analysis of whole cell lysate (WCL) or exosomes (Exo) from the indicated cell lines. Western blots have been cropped for clarity and conciseness. **b** Quantification of neurite outgrowth of PC12 cells following exosome stimulation from the indicated cell lines. Statistical analysis by one-way ANOVA with post hoc Fisher’s Least Significant Difference (LSD) test. LSD p values reported; *p<0.01. All comparisons and LSD p values found in Supplementary Table 6. The variance between the groups statistically compared is similar. Central value used was the mean. n = 4 technical replicates/condition; experiment repeated at least 3 times with biological replicates. Error bars, standard deviation.
where matched HNSCC patient plasma and tumor exosomes harbor neurite outgrowth activity. Taken together, these data indicate that CD9 + exosomes released by HPV-positive tumor cells promote tumor innervation in vivo. We also provide in vitro evidence that exosomes from other cancer cell lines also harbor neurite outgrowth activity suggesting that tumor innervation may be induced by tumor released exosomes in other solid tumors.

Our findings also indicate that other exosomal cargo including DNA, RNA, miRNA, and lipids should be examined for their axonogenic activity. Whatever the combination of factors involved, it is clear that interventions targeting tumor exosome release or blocking the ability of nerves to respond to exosomes may be of therapeutic value. While this concept requires rigorous testing, should it be proven valid, there would be great interest in its clinical translation.

Among the questions that require consideration is what advantage does innervation bestow on the tumor? Nerves generally bundle along with blood vessels, providing ready access to required nutrients. Thus, it is possible that some tumors induce their own innervation to provide a rich blood supply and promote tumor growth. Our data support this hypothesis. Consistent with this, EphrinB1 possesses proangiogenic properties. Alternatively, tumor innervation may regulate the local immune response which in many solid cancers, particularly head and neck cancers, critically contributes to disease progression. Importantly, neuro-immune interactions are evolutionarily conserved and critical for homeostasis. Thus, tumors may promote their own innervation as a means to dampen immune responses, promote tumor tolerance, disease progression, and dissemination. Evaluating the relationship between tumor innervation, vascularization and immune infiltrates in the tumor will help distinguish between these possibilities.

**Methods**

### Antibodies utilized for western blot analysis

- anti-CD9 (Abcam, ab29726, 1:1,000), anti-CD81 (clone B-11, sc-16602, 1:1,000, Santa Cruz), anti-EphrinB1 (ECD epitope, AF473, R&D Systems, 1:500), anti-EphrinB1 (ICD epitope, LifeSpan Biosciences, LS-C100001, 1:500), anti-NFγ (Abcam, ab49205, 1:500), anti-β-III Tubulin (ab81207, Abcam, 1:1,000), anti-GAPDH (ThermoFisher, AM4300, 1:5,000), anti-β-actin (1:500, A2282, Sigma), anti-Tau (1:500, Abcam, ab75714), anti-Phosphorylated Erk1/2 (1:500, #9106 s, Cell Signaling), anti-Erk1/2 (1:500, AB544, Millipore), anti-TRPV1 (Novus, NB100-98897, 1:500), HRP-coupled secondary antibodies purchased from Thermofisher. Citations for these validated antibodies are listed in Supplementary Table 9.

### Antibodies utilized for IHC

- anti-β-III Tubulin (2G10, ab78078, 1:250, Abcam), anti-Tyrosine Hydroxylase (Ab112, 1:750, Abcam), anti-TRPV1 (cat# ACC-030, 1:100, Alomone labs), anti-VIP (ab22736, 1:100, Abcam), anti-cytokeratin (Abcam, ab1068, 1:200).

### Antibodies utilized for immunofluorescence

- anti-Tau (MAB3420, 1:200, Millipore), anti-β-III tubulin (AB9354, 1:1,500, Millipore), anti-TRPV1 (cat# ACC-030, 1:100, Alomone labs). Secondary antibodies: Goat anti chicken IgY-αβγ (1:1,500, Alexafluor, Cat # A-11041), Goat anti mouse IgG2a-488 (1:1,500, Alexafluor, Cat # A-21131), Goat anti Rabbit IgG-647 (1:1,500, Alexafluor, Cat # A-21247).

### Antibody utilized for quantification of neurites

- anti-β-III tubulin (Millipore, AB9354, 1:1,000).

### Antibody for NFγ neutralization

- anti-NFγ (ThermoFisher, PA1-18378, 6 μg/ml).

### Cell lines

All human cell lines were authenticated by short tandem repeat profiling (Bio synthesis, Lewisville, TX or Genetica DNA Laboratories, Cincinnati, OH). In addition, all cell lines were confirmed mycoplasma free as per Uphoff and Drexler. UM-SCC1, UM-SCC19, and UM-SCC47 cell lines maintained with DMEM with 10% fetal calf serum and 1% penicillin/streptomycin. The SCC1-EphrinB1 cell line was generated by transfection of pDNA3.1/Zeo-EphrinB1 using Lipofectamine 2000 (ThermoFisher, #11668019) as per manufacturer’s instructions. Following transfection, cells were treated with 250 μg/ml Zeocin (ThermoFisher #R282005) until control cells were dead (7 days). Clones were isolated and expanded from 150 mm dishes seeded with approximately 50 cells using cloning cylinders (ThermoFisher #190-552-22) and screened by western blot for EphrinB1 expression (R&D Systems AF473).

Primary human tonsil epithelia were collected under an approved IRB protocol and maintained with KGM ( Gibco, cat # 10724-011). HTE E6a/E7 and HTE E6a/E7 were generated by retroviral transduction and maintained in E-media as described below. Human squamous cell carcinoma cell (SCC) lines 1, 19 and 47 were a generous gift from Dr. Douglas Trask (University of Iowa, Iowa City, IA). These human cell lines were generated by the Head and Neck SPORE Translational Research Group at the University of Michigan (UM-SCCs; Ann Arbor, MI); the University of Michigan is where Dr. Trask originally obtained the cell lines. Completed genotyping of 73 UM-SCC cell lines has been published. Continued efforts to genotype remaining and newly generated cell lines are posted on the UM Head and Neck SPORE Tissue Core website (http://www2.med.umich.edu/cancer/hnsopore/core-tissue.cfm). The human 93-VU-147T-Up 6 squamous cell carcinoma cell line (referred to as 93-VU in the text and figures) was a generous gift from Dr. Peter Snijders (Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam) and originated from the primary tumor of a patient that had HPV16 positive oral squamous cell carcinoma. This cell line was maintained in the same medium as the other human SCC cell lines.

The CT26 cell line is a murine colorectal cancer cell line that was purchased from ATCC (CT26.WT, ATCC CRL-2638). The B16 cell line is a murine melanoma cell line that was purchased from ATCC (B16-F0, ATCC CRL-6322).

The CT1 cell line is a murine breast cancer cell line that was purchased from ATCC (ATCC CRL-2539).

mEERL cells (parental and all derivatives) were maintained with E-medium: DMEM (Corning, cat# 10-017-CV)/Ham’s F12 (Corning, cat#10-080-CV), 10% exosome depleted fetal calf serum, 0.5 μg/ml hygromycin, 8.4 μg/ml chloramphenicol, 5 μg/ml transferrin, 5 μg/ml insulin, 1.36 ng/ml tri-iodo-thyronine, and 5 ng/ml EGF.

The mEERL EphrinB1 CRISPR clones were generated using two distinct strategies. One strategy employed simultaneous double-targeting to remove a large portion of gDNA spanning exons 1-5 (as in ref. 69) and one utilized single-targeting to produce frame-shift causing indels leading to early termination. Target selection and guide sequence cloning were carried out using the tools and protocol of Ran et al.80. PCR assays for the double targeting strategy employed primers external to (1-5A Ext.) or within (1-5A Int.) the predicted deletion site. The external assay should yield a total of 10,485 bp wt amplicon and a 229 bp Δ amplicon while the internal assay produces a 330 bp wt amplicon and no Δ amplicon. Single target screening utilized PCR to amplify a region surrounding the target site followed by restriction digest with BglII, the recognition site of which should be destroyed when double strand breaks are incorrectly repaired. The primers sequence was as follow: -5A Ext. FWD sequence: 5′-ATCTGGAATTGACTCTT GGCC-3′, 1-5A Ext. REV sequence: 5′-TAGGCTACTGAGGACAGG-3′, 1-5A Int. FWD sequence: 5′-TGGCCCTTACGATCCGACG-3′, 1-5A Int. REV sequence: 5′- TCCAGCGGCTATGTAGTTG-3′.

The mEERL EphrinB1/AEDC clone was generated from the double-targeting strategy. PCR assays show the predicted deletion product using primers external to the targeted region and lack of an amplicon using primers within the deletion (Supplementary Figure 4B). The sequence data shows that EphrinB1Δ and a sequence of exon 3 shared by EphrinB1Δ clone resulted from a strategy for double knock out of RAB27A and RAB27B in which sgRNA's targeted to exon 4 of RAB27A, exon 4 of RAB27B, and a sequence of exon 3 shared by RAB27A and B were co-transfected. PCR revealed a heterozygous, truncated deletion product for RAB27Δ (Supplementary Figure 4B) and the expected homozygous deletion amplicon for RAB27Δ (Supplementary Figure 6B). RAB27Δ sequence data indicated distinct repair products at the deletion site; although one allele exhibits an immediate stop codon, it is unclear where the other might terminate. However, western blotting confirms lack of detectable protein (data not shown).

PC12 cells were purchased from ATCC and maintained with DMEM with 10% horse serum (Gibco, cat # 26050-088) and 5% fetal calf serum. When used for neurite outgrowth assays, PC12 cells were maintained with DMEM with 1% horse serum and 0.5% fetal calf serum.

**Electron microscopy.** Exosome samples were processed and analyzed by the Microscopy and Cell Analysis Core at Mayo Clinic: http://www.mayo.edu/research/core/services/microscopy-cell-analysis-core/overview.

**Atomic force microscopy (AFM).** Purified exosomes were diluted 1:10 in deionized water, added to a clean glass dish, and allowed to air-dry for 2 hours before depositing a gentle streak of hydrophobic exosomes deposited on glass dish were characterized using an Atomic Force Microscope (Model: MFP-3D BIO™, Asylum Research, Santa Barbara, CA). Images were acquired in AC mode in air using a
silicon probe (AC240TS-R3, Asylum Research) with a typical resonance frequency of 70 kHz and spring constant of 2 Nm⁻¹. Height and amplitude images were recorded simultaneously at 512 × 512 pixels with a scan rate of 0.6 Hz. Image processing was performed using Igor Pro 6.34 (WaveMetrics, Portland, OR) and analyzed with Image J.

Immunohistochemistry (IHC). Tissues were fixed in 10% neutral buffered formalin and processed on a Leica 300 ASP tissue processor. All tissues were sectioned at 5 μm thick on a Leica RM2255 microtome. Sections were stained with Viscoprep, VIP, and TRPVI, and n = 30 mEERL tumors were stained for β-III tubulin. N = 12 human HNSCC tumors were processed for double IHC to simultaneously label β-III tubulin and TRPVI. Double IHC was performed using MACH 2 Double stain 1 Polymer detection kit (Biocare, #MIRC7523) as per manufacturer’s instructions. The slides were placed on a pre-warmed automated slide staining system (Ventana Medical Systems, Inc.) was used for the optimization and staining. The Ventana View DAB detection kit was used as the chromogen and the slides were counterstained with hematoxylin. Omission of the primary antibody served as the negative control. Stained sections were analyzed on an Olympus BX51 upright microscope equipped with an Olympus DP71 color CCD camera; 20×/0.75 UPlanSApo and 40×/0.90 UPlan SApo objectives were used.

β-III tubulin/TRPVI positive fibers were quantified by stereology on an Olympus BX50 upright microscope using Micronbright Field Stereo Investigator (version 11.02) using the Cavalieri estimator. Omission of the primary antibody served as the negative control.

Western blot analysis. For western blot analysis of neuritogenesis signaling in PC12 cells, 35 mm tissue culture dishes were coated with collagen (Sigma #23225) overnight at room temperature. 35 mm dishes were washed in PBS while the negative control consisted of 100 μL PBS and stored at −80°C.

Exosome purification from human plasma. Twenty ml of whole blood were pipetted directly onto Ficol-loaded Leucosep tubes and centrifuged at room temperature for 30 min at 800 X g with the brake off. Exosomes were isolated from the recovered plasma by differential ultracentrifugation as described.

BCA protein assay of exosomes. The standard BCA protein assay was utilized with modifications to accommodate the low protein yield from exosome preparations. Briefly, 5 μl of 10% TX-100 (Thermo Scientific) were added to an aliquot of 50 μl of purified exosomes and incubated 10 min at room temperature. A working ratio of 1:11 was used and incubated in a 96 well plate for 1 h at 37°C. Absorbance at 562 nm was then measured (SpectraMax Plus 384) and protein concentration estimated from a quartic model fit to the BSA standard curve.

### ARTICLE

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**Differential ultracentrifugation/optiprep density gradient.** Following differential ultracentrifugation as described above, a discontinuous iodixanol gradient was utilized similar to Van Deen et al. with some modifications. Solutions of 5, 10, 20, and 40% iodixanol were made by mixing appropriate amounts of a homogenization buffer [0.25 M sucrose, 1 mM EDTA, 10 mM Tris–HCL, (pH 7.4)] and an iodixanol solution. This solution was prepared by combining a stock solution of Optiprep® (60% w/v) aqueous iodixanol solution, Sigma) and a solution buffer [0.25 M sucrose, 6 mM EDTA, 60 mM Tris–HCL, (pH 7.4)]. The gradient was formed by layering 4 ml of 40%, 4 ml of 20%, 4 ml of 10% and 3 ml of 5% solutions on top of each other in a 15.5 ml open top polyallomer tube (Beckman Coulter). Four hundred microlitre of crude exosomes (isolated by differential ultracentrifugation) were overlaid onto the top of the gradient which was then centrifuged for 18 h at 100,000g and 4°C (SureSpin 630/17 rotor, ThermoScientific® Sorvall®). Gradient fractions of 1 ml were collected from the top of the self-forming gradient, diluted to 14 ml in PBS and centrifuged for 3 h at 100,000 × g and 4°C. The resulting pellets were re-suspended in 100 μL PBS and stored at −80°C.

**Exosome purification from human plasma.** Twenty ml of whole blood were pipetted directly onto Ficol-loaded Leucosep tubes and centrifuged at room temperature for 30 min at 800 X g with the brake off. Exosomes were isolated from the recovered plasma by differential ultracentrifugation as described.

**BNA protein assay of exosomes.** The standard BCA protein assay was utilized with modifications to accommodate the low protein yield from exosome preparations. Briefly, 5 μl of 10% TX-100 (Thermo Scientific) were added to an aliquot of 50 μl of purified exosomes and incubated 10 min at room temperature. A working ratio of 1:11 was used and incubated in a 96 well plate for 1 h at 37°C. Absorbance at 562 nm was then measured (SpectraMax Plus 384) and protein concentration estimated from a quartic model fit to the BSA standard curve.

**Western blot analysis.** Sample protein concentrations were determined by BCA protein assay as described. Equal total protein was separated by SDS-PAGE, transferred to PVDF membranes (Immobilon- P, Millipore), blocked with either 5% Bovine Albumin Fraction V (Millipore) or 5% milk (Carnation instant non-fat dry milk) and re-suspended in 200 μL of 10% TX-100 (Thermo Scientific) and re-suspended in 200 μL of 10% TX-100 and 4 °C. The resulting pellets were re-suspended in 100 μL PBS and stored at −80°C.

**Exosome purification from human plasma.** Twenty ml of whole blood were pipetted directly onto Ficol-loaded Leucosep tubes and centrifuged at room temperature for 30 min at 800 X g with the brake off. Exosomes were isolated from the recovered plasma by differential ultracentrifugation as described.

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**Western blot analysis.** Sample protein concentrations were determined by BCA protein assay as described. Equal total protein was separated by SDS-PAGE, transferred to PVDF membranes (Immobilon- P, Millipore), blocked with either 5% Bovine Albumin Fraction V (Millipore) or 5% milk (Carnation instant non-fat dry milk) and re-suspended in 200 μL of 10% TX-100 (Thermo Scientific) and re-suspended in 200 μL of 10% TX-100 and 4 °C. The resulting pellets were re-suspended in 100 μL PBS and stored at −80°C.

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Descriptive statistics are presented as mean ± standard deviation (see Figure legends). Two-tailed Student’s t-test, one-way or two-way ANOVA were utilized for statistical analysis as indicated in the figure legends. Post hoc Fisher’s Least Significant Difference (LSD) or Tukey test were used as noted in the figure legends. All comparisons and LSD p values can be found in Supplementary Tables 1–8. PC12 assays utilizing exosomes from cell lines were run with three-five technical replicates (specific number of replicates is noted for each experiment in the relevant figure legends) for each condition and experiments were repeated at least 2 times (biological replicates). PC12 assays utilizing exosomes from human samples (plasma or tumor) were treated differently as these samples were limited. Thus, exosomes for each human sample were tested in triplicate; experiments repeated two times.

For animal studies, a statistical power analysis for a two-sample t-test using R software (version 3.4.1) was performed for sample size estimation as follows. For animal studies with n = 4 mice/group, we have more than 80% power to detect an expected effect size of 2.38 with an alpha = 0.05. For animal studies with n = 7 mice/group, we have more than 80% power to detect an expected effect size of 1.63 with an alpha = 0.05. For animal studies with n = 10 mice/group, we have more than 80% power to detect an expected effect size of 1.32 and an alpha = 0.05. Tumor growth curves were analyzed by two-tailed Student’s t-test. Error bars are standard deviation or standard error of the mean (as indicated in figure legends).

For all datasets, qq plots were utilized to analyze normality. Any data points that were at least 1.5 interquartile ranges below the first quartile (Q1), or at least 1.5 interquartile ranges above the third quartile (Q3) of the data were considered outliers and were excluded from the analysis.

Tumor groups were assigned arbitrarily. Once tumors were initiated, mice were randomized to two groups. Randomization was performed by using the = Rand() function in Excel to generate a random number associated with each mouse; the lowest numbered mice were designated for one group (e.g., tumor growth analysis) and the higher numbered animals were utilized for another group (e.g. generating tumor lysate for western blot analysis). Two animal studies (NOD SCID mice for growing human SCC tumors, Fig. 7d-g, and GW4869 mouse experiment, Fig. 5 f–h) were utilized only for generating tumor for innervation analysis (whole tumor lysate and FFPE for IHC). Thus in these expirng of sets, tumor types were assigned arbitrarily to mice but there was no randomization since all mice were sacrificed for the same purpose.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions
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