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Neuroepithelial Interactions in Cancer

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Abstract

Nerves not only regulate the homeostasis and energetic metabolism of normal epithelial cells but also are critical for cancer, as cancer recapitulates the biology of neural regulation of epithelial tissues. Cancer cells rarely develop in denervated organs, and denervation affects tumorigenesis, in vivo and in humans. Axonogenesis occurs to supply the new malignant epithelial growth with nerves. Neurogenesis happens later, first in ganglia around organs or the spinal column and subsequently through recruitment of neuroblasts from the central nervous system. The hallmark of this stage is regulation of homeostasis and energetic metabolism. Perineural invasion is the most efficient interaction between cancer cells and nerves. The hallmark of this stage is increased proliferation and decreased apoptosis. Finally, carcinoma cells transdifferentiate into a neuronal profile in search of neural independence. The latter is the last stage in neuroepithelial interactions. Treatments for cancer must address the biology of neural regulation of cancer.

INTRODUCTION

Curiosity sparks science. In this case, perineural invasion (PNI) kindled curiosity, becoming the focus of research that changed the understanding of how nerves and cancer are related (1).

Even though PNI (**Figure 1*a***) is common in cancers and clinically significant when present, little was known about its biology. Described in German literature for more than 100 years (2),

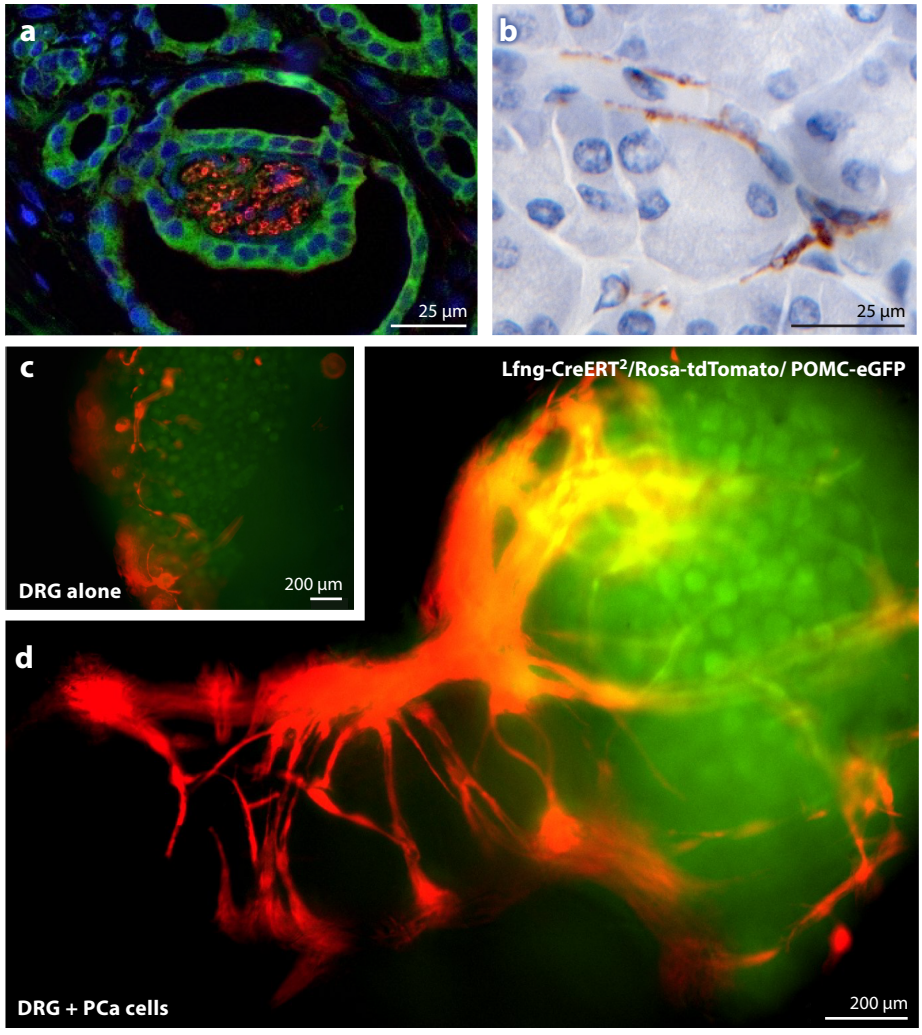


Figure 1

(*a*) Human tissues with fluorescent imaging showing perineural invasion. Nerve staining is shown in red, and the cancer cell (*green* cytoplasm, *blue* nuclei) can be seen wrapping around the nerve. (*b*) Immunohistochemistry with antibodies to protein gene product 9.5 in human pancreas shows a subepithelial plexus of nerves in direct contact with the epithelial cells. The plexus envelops the acinus. (*c*,*d*) Confocal imaging of the Lfng-CreERT^2/Rosa-tdTomato/POMC-eGFP lineage-tracing mice dorsal root ganglia (DRG) grown in the presence (*d*) and absence (*c*, as controls) of Du145 prostate cancer (PCa) cells. In this image, neuroblasts/immature neurons are shown in red. There is a significant quantitative difference in neurogenesis seen in the DRG in the presence of cancer cells, with not only neoneurons (*yellow*) but also nerves arising from them into the DRG neural plexus.

the only accepted fact was that it was not spread through lymphatics in the perineural space (3). At the beginning of this century, science had little understanding of the biology of PNI and the importance of the interaction among nerves, epithelial cells, and cancer. Early studies on the biology of PNI opened the doors for a new field in cancer research, creating a new paradigm.

Since the year 2000, understanding of the nature of this nascent field has deepened, spurred on by the identification and demonstration of extensive interactions between nerves and cancer, as well as normal epithelium. Understanding these neuroepithelial interactions enabled a new understanding of the biology and mechanisms of PNI, which led to identifying new concepts and mechanisms of cancer-induced axonogenesis and neurogenesis. The roles of differential nerve subtypes and neurotransmitters are as yet not fully understood, but recognition of these roles has led to a focus on how they influence carcinogenesis and metabolism, as well as a description of neuronal transdifferentiation.

More recently, we in the field have achieved a better comprehension of how nerves interact with normal epithelium, and this new understanding will guide research into nerves and cancer. Nerves regulate cancer in much the same way as they are responsible for the homeostasis and regulation of normal epithelium. Just as with angiogenesis, the involvement of nerves in cancer is a recapitulation of normal and repair biology. Without this understanding, the role of nerves in cancer is difficult to comprehend. Therefore, this article starts by addressing the role of nerves in the normal biology of epithelium and the repair biology of the host response.

NERVES REGULATE NORMAL EPITHELIUM

Nerve function is generally thought of as regulation of muscle movement and sensation. However, nerves also play a trophic function for epithelium, embryologically and into adulthood. Nerves are also involved in the development of epithelial organs and their morphology as well as functional maintenance.

Neural differentiation is a prerequisite for the embryonic regulation of tissues. The first elements to differentiate during embryonic development are neural. The most primitive stem cell has neural characteristics (4). The nervous system is the first to be formed and subsequently regulates the development of dermatomes and somatomes. Although many data are available, the concept that nerves regulate the embryologic formation of epithelial organs is still not widely accepted.

Nerve Regulation of Glandular Organogenesis

Cranial nerves shape craniofacial development in a way that goes beyond somatic muscular and sensitivity functions. Nerves are also involved in salivary glands, tooth epithelium, and cochlear morphogenesis. In the case of salivary glands, SRY-box transcription factor 2 (SOX2) regulates acinar formation, and SOX2 is regulated by acetylcholine signaling (5). Vasoactive intestinal polypeptide (VIP) nerves also participate in regulation of ductogenesis, duct elongation, and microlumen formation and fusion (6). Parasympathetic nerves accompany epithelial branching, using the wingless-related integration factor (WNT) signaling process. Nerve migration is mediated by neurotrophic factors such as neurturin (7) and semaphorins (8). The latter play a significant role in cancer-induced neurogenesis (9). Other secretory organs such as the breast and pancreas also require neural regulation of acinar and duct formation (10).

The influence of nerves in salivary glands persists into adulthood, with both sympathetic and parasympathetic nerves regulating secretion from these organs. As important as nerves are in embryogenesis, they are also required during adulthood. Adult denervated organs atrophy, particularly if parasympathetic support is removed (11–13). Stimulation of sympathetic adrenergic receptors via adrenergic mimetics has been shown to promote organ regeneration after

mechanical injury (14). Additionally, stimulation with an alpha-adrenergic agonist before radiation helps preserve the tissue (15). Conversely, pronerve therapies such as adrenergic mimetics or agonists or neurturin protect the salivary gland from damage in mechanical injury (14) or radiation (16).

Another secretory organ where organogenesis requires nerves is the prostate. The formation of the mouse pelvic area is dependent on the survival of a nucleus in the lower spinal cord (17). In the absence of androgens, this nucleus involutes and the embryo acquires the default female phenotype. In the presence of androgens, the nucleus remains and regulates the formation of the pelvic floor.

Experimental findings in prostate models show that nerve ablation affects organogenesis through a reduction of stem cells, aberrant epithelial branching, and organ atrophy, suggesting neural regulation of the stem cell niche (6). Neural regulation of the epithelial stem cell niche has also been observed in gastric cancer (18).

The regulatory process is bilateral. Not only do nerves regulate organogenesis but epithelia influence nerve formation. During embryogenesis, the male genitalia can also control neuronal cell proliferation during development of the central nervous system (19). The male genitalia can induce proliferation of neural precursors and peripherally induced central neurons (20). This process can be stopped by disrupting the male genitalia or the nerves connecting the ganglia to the primordia (21). Therefore, it is conceivable that neoplasms that arise from epithelium can induce axonal growth and neurogenesis.

Nerves and Regulation of Normal Epithelium

Studies have added to the knowledge of neural regulation of normal prostate epithelium (22). These are the first studies to hint at mechanisms within the cell over which nerves exercise regulation. The studies required the use of multiple prostate denervation methods, including excision of the rat major pelvic ganglion, spinal cord injury, or Botox® (neurotoxin) injection. All showed atrophic changes of the epithelium, as seen before (23, 24).

Gene expression analysis showed that the expression patterns of all three denervation methods were statistically similar. Genes that were downregulated as a result of denervation were associated with differentiated functions of the cancer cell. These included translation and translation initiation factor activity ribosomes and structural constituents of ribosomes and metabolic pathways. Upregulated genes in the denervated epithelium were consistent with an acute tissue repair response. Numerous growth factors were also upregulated including c-fos induced growth factor, connective tissue growth factor, and epidermal growth factor receptor. Fas ligand and tumor necrosis factor receptor superfamily member 1b were also upregulated. Additional upregulated genes included those associated with epithelial-to-mesenchymal transition events and stem cell properties.

Gene expression signature mapped to multiple mitochondrial pathways, suggesting an attenuation of cellular bioenergetic metabolism. We validated using targeted metabolic analyses of metabolites belonging to the glycolysis pathway, the pentose phosphate pathway, and the tricarboxylic acid (TCA) cycle. Levels of TCA metabolites, succinate, malate, and fumarate were significantly downregulated in Botox-treated tissues compared with controls. On the other hand, levels of glucose/fructose and ribose were significantly elevated upon Botox treatment. These findings suggest a shutdown in glucose utilization and potential dependence on gluconeogenesis upon Botox treatment (22).

Overall, these changes indicate a cell that is suffering from acute regulatory withdrawal from the nerves, affecting predominantly the energetic metabolism, and attempting to protect and heal,

until it undergoes apoptosis. In fact, we identified greater apoptosis in normal epithelial cells in denervated mice than controls. This biology does translate to human tissues, where apoptosis was increased in human nonneoplastic epithelium treated with Botox as a means of denervation versus controls (22).

All these data have been available previously, but the scientific community has yet to accept that nerves are needed for the embryologic formation of organs and the functional homeostasis of the epithelial component of adult organs. There is even an anatomic structure that supports this regulation. In sensory organs such as the retina and taste buds, the subepithelial neural plexuses fulfill this function. However, glandular and squamous epithelia also have a subepithelial plexus with nerve twiglet prolongations into the epithelium (**Figure 1*b***). The normal means and ranges of these intraepidermal nerves have been established. We know that the density of epidermal nerve fibers decreases with age and is lower in men compared with women (25).

These nerves regulate the energetic metabolism of cancer cells. Nerve subtypes that predominate in this subepithelial plexus are acetylcholinesterase parasympathetic, VIP (8–21), and neuropeptide Y (NPY). NPY nerves are needed for closing the epithelial component of the diabetic wound (26, 27). If they are decreased or absent, the wound will complete all faces of wound repair except reepithelialization, leaving the wound open. This may prove important in future treatments for diabetic wounds. Even endometriosis is innervated (28).

CANCER INDUCES AXONOGENESIS AND NEUROGENESIS

Cancer-Induced Axonogenesis

Cancer-induced axonogenesis can be understood as a predicted response to the increased epithelial volume that follows carcinogenesis (**Figure 2**). We find it as early as at the level of preneoplastic lesions in the prostate (9), as well as in pancreatic cancers (29). Cancer recapitulates the relationship between nerves and normal epithelium because this regulation is part of the inherent biology of epithelial cells, benign or malignant. To do so, it must induce the growth or hyperplasia of axons (axonogenesis). The new axons support the newly formed malignant epithelial cells. Axonogenesis is an acute phase phenomenon and occurs within the peripheral nervous system. Axons grow to support cancer growth. Both axon density and neurite count are increased in cancer compared with controls and are associated with more aggressive disease (9).

The next phase is neurogenesis or the formation of neoneurons (**Figure 2**). It is the chronic phase of this phenomenon that starts at the level of dorsal root ganglia (DRG), or ganglia around the primary organs, and continues with the migration of central nervous system neoneurons toward faraway tumors (9). These data also suggest a return to neural plasticity of a younger age, induced by cancer cells.

The process to prove neurogenesis began one more time with an observation, and proving it was long and complex, involving in vitro and human tissue studies. This resulted in the three-dimensional reconstruction of an entire prostate, to overcome the limitations of two-dimensional initial studies of angiogenesis.

The first model developed in our laboratory was the PNI in vitro model (1), composed of mouse DRG and human prostate cancer (PCa) cells embedded in Matrigel. We later adopted a more specific neurogenesis assay with the DRG isolated from Mapttm1(GFP)Klt/J mice. Only the nervous system is green fluorescent protein (GFP) positive. This permits visualization and quantitation of neurites using confocal microscopy. This model was also used with cell lines derived from cancers of many organs, again corroborating cancer-induced axonogenesis.

Human tissue studies followed, resulting in the identification of spatial and temporal associations between increased axon density and preneoplastic and neoplastic lesions of the human

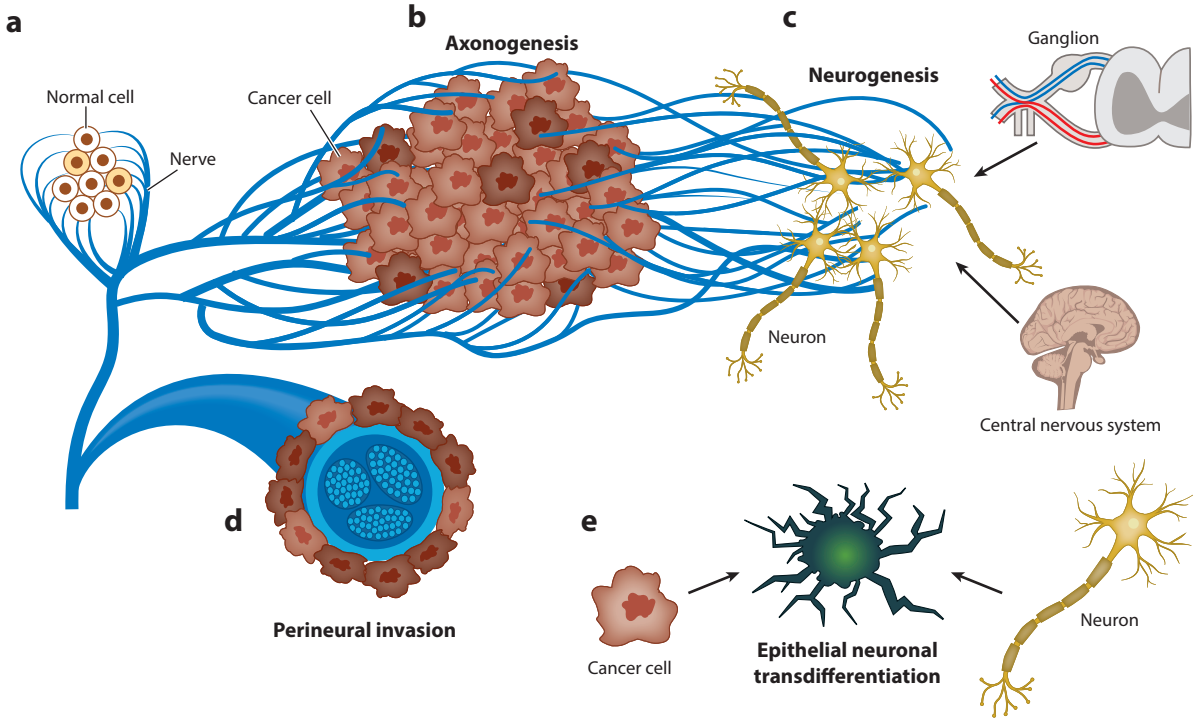


Figure 2

Graphical representation of the spectrum of neuroepithelial interactions. Cancer recapitulates the biology of neural regulation of epithelial tissues. (*a*) Nerves regulate the homeostasis and energetic metabolism of normal epithelial cells. (*b*) Axonogenesis from preexisting nerves happens to supply the new malignant epithelial growth. Cancer cells rarely develop in denervated organs, and denervation affects tumorigenesis, in vivo and in humans. (*c*) Neurogenesis happens later, first in ganglia around organs or the spinal column and subsequently through recruitment of neuroblasts from the central nervous system. The hallmark of this stage is regulation of homeostasis and energetic metabolism. (*d*) Perineural invasion is the most efficient interaction between cancer cells and nerves. The hallmark of this stage is increased proliferation and decreased apoptosis. (*e*) Finally, carcinoma cells transdifferentiate into a neuronal profile in search of neural independence.

prostate. Axon density is increased in cancer areas as well as in preneoplastic lesions when compared with controls. Two- and three-dimensional reconstructions of entire prostates confirmed axonogenesis in human tumors (9). Other authors have confirmed axonogenesis in several other cancer types (30). These include breast (31, 32), colon (33), head and neck (34), esophageal (35), and pancreatic (36) cancers.

Axonogenesis is also important clinically and has the potential of becoming a significant biomarker for cancer because it is correlated with features of aggressive PCa. The median area of the nerve immune marker protein gene product (PGP) 9.5 staining was higher in patients with a recurrence than in those without recurrence. More important, nerve density was also predictive of biochemical recurrence in a large cohort of patients (37). Increased nerve density correlated with increased proliferation of PCa cells. It also correlated with expression of proteins involved in survival pathways, hormonal regulation elements, and coregulators and repressors (7).

Nerve density is predictive of multiple clinical parameters used in colon cancer (38). Similar predictive data were identified in other organs including the breast (18, 31) and pancreas (39). Even within the glial tumors, a neurogenesis pattern is associated with adverse prognosis (40). These data indicate that axonogenesis is a clinically significant process in human PCa and confirm that

nerves are involved in the progression to aggressive cancers. For a thorough review on cancer-induced axonogenesis and neurogenesis beyond the scope of this article, readers are referred to an article by Schmitd et al. (30).

Mechanism Related to Axonogenesis

Transcriptomic studies derived from the PNI model identified candidate molecules that could be involved in axonogenesis. Among them was semaphorin 4F (S4F), a class IV transmembrane semaphorin. Semaphorins have multiple functions. They have the best-characterized roles in embryological axon guidance (41). Most cancer publications favor a protumorigenic role for S4F.

In addition, S4F plays a functional role in cancer-induced axonogenesis. PCa cells secrete S4F. PCa cell S4F overexpression using stable transfectants in the cancer cells induces overall axonogenesis in an N1E115 axonogenesis assay. S4F inhibition by small hairpin RNA blocks this effect. Using immunohistochemistry with a lab-developed antibody, we were able to show that patients with very high S4F expression in PCa were at higher risk of biochemical recurrence. These data establish a significant role for S4F in human PCa (42).

As this area of research continues, we are confident that there will be involvement of other regulatory molecules in the process of cancer-induced axonogenesis, as nature favors multiple redundant regulatory mechanisms and pleiotropy. Neural growth factor neurotrophins (NGF) and proNGF have also been involved in cancer axonogenesis (43). Vascular endothelial growth factor was found to mediate axonogenesis in breast cancer (32). Carboxylated graphene oxide promoted axonal guidance growth by activating netrin-1/deleted in colorectal cancer (44). Some have suggested a role for exosomes in the regulation of cancer-induced adrenergic axonogenesis (45). Transforming growth factor β–induced expression of long noncoding lincRNA Platr18 controls breast cancer axonogenesis (46). Semaphorin 3F and ROBO1 have also been involved in this process (47).

Cancer Induces Neurogenesis

Some of the most significant and newest advances in biology are in the field of stem cell research. There are two preexisting populations of neural stem cells in adult mammals: one in the ependymal cell layer lining the ventricles (48) and one in the subventricular zone (49), each of which gives rise to glial cells and neurons. Neuronal stem cells are also present in the vascular niche, primarily in the endothelial cells (50). Furthermore, stem cells can be differentiated into peripheral neurons by coculturing them with stromal cells (51). Neurogenesis can also happen in DRG (52). It is also now well established that human and rodent bone marrow stromal cells can differentiate into cells bearing neuronal markers (53), a process called neuronal transdifferentiation, which will become important in later stages.

Early studies suggested that PCa induces neurogenesis in the human prostatic ganglia. The number of neurons in the prostate ganglia of patients with PCa was higher than in those without cancer, further supporting that hypothesis. Neural ganglia in mouse prostates with orthotopically implanted VCaP tumor PCa cells are larger than ganglia in control mice and have an increased number of cells within the ganglion.

To examine whether the increased number of cells originates from the newborn neurons, implanted mice were injected intraperitoneally with bromodeoxyuridine (BrdU). The prostates were harvested and the sections were stained with antibodies against BrdU and PGP 9.5. Double-labeled cells were identified within both ganglia and tumor-reactive stroma in prostates of orthotopic cancer cell–injected mice, but not in controls, suggesting that active neoneurogenesis in the DRG occurs as a result of cancer influence.

To precisely test the hypothesis that PCa induces neurogenesis, it is essential to identify primary neural stem cells (NSCs) that give rise to new neurons. NSCs are localized in the DRG but could not be precisely identified because the most commonly utilized mouse model to detect them, nestin-GFP, as well as other existing genetically modified mouse models (54), label not only the primary NSCs but also their immediate progeny as well as non–stem cells in the tissue, such as pericytes. Dr. Mirjana Maletic-Savatic of Baylor College of Medicine and colleagues (55) discovered that lunatic fringe (Lfng), a modifier of the transmembrane Notch receptors, is selectively expressed in NSCs and may be the most specific NSC marker reported to date. The new Lfng-CreERT^2/Rosa-tdTomato/POMC-eGFP lineage-tracing mice provided us with an excellent tool to study NSC biology in the context of PCa. Progenitor cell maintenance and neurogenesis in peripheral ganglia are regulated by Notch signaling (56), further supporting the utility of the Lfng-based mouse models for studies of NSCs in the DRG. To examine whether new neurons in the DRG form following exposure to cancer, Maletic-Savatic and colleagues crossed the Lfng-eGFP mice with the POMC-tdTomato mice, in which neuroblasts/immature neurons are labeled red. Thus, if new neurons are generated, we could visualize them easily.

We used DRG from these double-transgenic mice for the PNI in vitro model. After the addition of cancer cells, we observed a substantial difference in the number of newborn NSCs and the extension of the axons away from the DRG (**Figure 1*d***) than in controls (**Figure 1*c***). These data further indicate that PCa cells induce neurogenesis within DRG. Other researchers had previously shown that neurogenesis physiologically exists in DRG (57) but not in cancer-induced neurogenesis. This is likely to be the first part of the chronic neural response to cancer. These data corroborate the initial finding of neurogenesis at the DRG level (Y. Ding, P. Bu, M. Maletic-Savatic & G. Ayala, unpublished data).

To sustain neurogenesis, the primary pool of neural stem cells in the central nervous system must be recruited. Claire Magnon of INSERM, Paris, and colleagues (58) published a groundbreaking lineage tracing of neoneurons in prostate and breast cancer. Neurons migrate from the subventricular zone of the brain into the tumor bed, where they can differentiate into adrenergic neurons. They also found a higher density of neuronal progenitor cells within tumors (58). This mechanism seems to be the mature response to carcinogenesis and improves the capacity to maintain a cancer-supporting neural network.

NERVES ARE CRITICAL FOR CANCER

In patients with spinal cord injury, the risk of developing PCs is as much as 65% less than in noninjured patients (59). From this, it can be inferred that perhaps intact innervation is required for tumor growth. Indeed, prostate physical or chemical denervation before experimental orthotopic VCaP-luc injections in mice and rats resulted in reduced tumor incidence, reduced luciferase intensity, and decreased overall prostate weight (22). Chemical denervation was used to exclude vascular influence.

In a human neoadjuvant proof-of-principle study, four patients with bilateral Gleason $\leq$7 tumors received unilateral Botox injection and vehicle control injection into the contralateral lobe. A significant increase in apoptosis was observed in both nonneoplastic epithelium and PCa tissue at the Botox-treated site (22). Microvessel density was increased within tumor tissue, as expected since Botox is a vasodilator (60–62). These data indicate that nerves are critical for PCa tumorigenesis and aggressiveness (22).

Transcriptomic comparison of laser-captured human cancer cells from denervated rat prostates with cells from intact rat prostates shows that the top Gene Ontology categories for the down-regulated genes included translational elongation, ribosome, translation, cytosolic small and large ribosomal subunit, RNA binding, ribonucleoprotein complex, and protein binding. More

important, pathways that regulate cellular energy metabolism were also downregulated (mitochondrial pathways, mitochondrial ATP synthesis, mitochondrial proton-transporting ATP synthase complex, glycolysis, and electron transport chain). Evaluation of the gene expression profile of laser-captured cancer tissue obtained from a rare patient who developed PCa after traumatic spinal cord injury identified a gene expression pattern similar to that of cancer cells in denervated rat prostates.

Finally, transcriptomic analysis of Botox-treated tumors revealed a significantly similar denervation signature profile as identified in the denervated rat orthotopic human xenograft tumors (rho = 0.45) or the human prostate from the spinal cord injury patient (rho = 0.26). These data show that a similar gene expression signature can be recognized in mice and humans upon denervation through both chemical and physical methods, suggesting that the effect of nerves on cancer tissue is a fundamental phenomenon. A strong trans-species pattern becomes more evident. The principal signature is that of regulation of the energetic metabolism, similar to that found in normal epithelium.

The effects of denervation have been reproduced in other tumors. Denervation in breast cancer leads to volume regression by 70% (63). Genetic knockout of the muscarinic acetylcholine M3 receptor suppressed gastric tumorigenesis. Because the authors used transgenic models, they were able to identify suppression of stem cell expansion. This limited tumor formation, even in the presence of genetic hits in the stem cells. Vagotomy also reduced the risk of gastric cancer (18). Ablation of sensory neurons in a genetic model of pancreatic ductal adenocarcinoma slows initiation and progression of cancer (64). These findings indicate a basis for developing new cancer therapies (65).

NEUROPEPTIDES AND NEUROTRANSMITTERS: MEDIATORS OF NEURAL INTERVENTION IN CARCINOGENESIS

Nerves use multiple molecules to communicate with target cells, namely neuropeptides and neurotransmitters. They use multiple structures to deliver these molecules, including synapses and/or secretion of neuroendocrine granules. To date, how nerves connect with cancer cells remains unknown.

Reductionist approaches lead us to search for the simplest answer—a single molecule that can be modified for a clinical effect. Yet the complex world of neurobiology precludes the simple. It is likely that multiple nerve subtypes and neurotransmitters/neuropeptides will have a panoply of effects on cancer cells. Such is the nature of biology, redundant and pleiotropic. The true effect and mechanisms of nerve influence in cancer will not be understood until we develop complex multiparametric relational scientific methods that consider the effect of multiple neurotransmitters and neuropeptides.

Neuropeptide Y

Initial studies to identify nerve subtypes involved in cancer-induced neurogenesis identified increases in three different nerve subtypes, NPY, adrenergic, and vasointestinal polypeptide (66). NPY with the strongest trans-species generic denervation profile was associated with metabolic regulation and proved a good place to start experiments. NPY influences food intake and energy metabolism, stress, and immune response. It is involved in tumor progression, cell proliferation, matrix invasion, metastasis, and angiogenesis.

Studies indicate that NPY is a key regulator of PCa apoptosis, motility, energetic metabolism, and radiation therapy resistance. NPY nerve density correlates with aggressive disease. NPY is a critical regulator of nerve–PCa interactions affecting pathogenesis and therapy resistance.

Taking this a step further, NPY in vitro inhibition, using an NPY1 receptor (NPY1R) antagonist BIBP-3226, results in greater apoptosis in both LNCaP and Du145 cells. These studies suggest that NPY plays a critical role in cell survival. NPY inhibition also resulted in changes in the migration of Du145/PC-3 cells. NPY inhibition with NPY1R small interfering RNA (siRNA) resulted in a delay in wound closure (scratch assay), suggesting that NPY is also involved in cancer cell migration.

Two NPY denervated groups (LNCaP siNPY1R versus siNS; LNCaP treated with NPY1R antagonist BIBP-3226 versus vehicle controls) were compared using laser-captured microdissection and transcriptomic analysis. Metabolomic profiles showed robust differences in response to NPY denervation. Seventy pathways were enriched between the two metabolite-level responses in LNCaP cells. Of these, 53 were metabolism and catabolism pathways, including purine, pyrimidine, amino sugar, pyruvate, and fructose. The remaining 17 were pathways related to electron transport, cellular respiration, the TCA cycle, glycolysis and gluconeogenesis, and amino acid synthesis. These findings suggested that NPY could be the most significant molecule that influences the generic denervation profiles identified in both mouse and human studies. Additionally, we identified nuclear factor kappa B (NFκB) as a potential downstream effector (66), as in PNI.

NPY is also a biomarker for aggressive PCa. Elevated levels of NPY1R were predictive of PCa-specific death. However, the most predictive information was for NPY nerve density, where the number of nerves was independently predictive of both biochemical recurrence and PCa-specific death. A patient with a high NPY nerve count has five times greater risk of dying of PCa (66).

NPY and its receptors are very significant in pediatric diseases. They have been involved in the regulation of neuroblastoma (26, 27) and Ewing's sarcoma (28), albeit with different receptors (NPY2R and NPY5R). They have also been involved in liver (67), colon (68, 69), head and neck (70), pancreatic (71), and breast (72, 73) cancers. The expression of NPY in many cases correlates with some features of the aggressive disease, having been associated with metastasis, PNI, advanced stages, and/or poor prognosis.

Of interest, NPY and NPY1R have been proposed to improve tumor imaging. Biodegradable bioluminescent nanobubbles (74), NPY analogs conjugated to gold nanocages (75), NPY analog labeled with ^{99m}Tc (68, 76), Ga-labeled NPY short analogs (77), and truncated peptides (78) have all been proposed. These delivery methods are of great interest as they could be used to deliver treatments to the cancer itself. In one experiment, an NPY analog was used as targeting molecule and two methotrexate molecules were used as acting molecules. These conjugates had higher potency than methotrexate alone and could overcome therapy resistance (79). Although this study is very preliminary, it addresses not just treatment of the cancer cell but also the potential to disconnect the cancer cell from its neural microenvironment.

Adrenergic Axis

Receptors for adrenergic signaling have been identified in tumors of the cervix (80), ovary (81), lung (82), colon (83), breast (84, 85), and prostate (86). Many mechanisms have been associated with adrenergic effects in various solid tumors. Among them are blocking of the PI3K/AKT/mTOR pathway in cervical cancer, mediated by alpha receptors (80); activation of an angiometabolic switch in PCa; modulation of AMP kinase autophagy in gastric cancer (87); dormancy of tumor cells through upregulation of GAS6 in PCa (88); suppression of Rap1B prenylation to promote the metastatic phenotype in breast cancer cells (89); downregulation of PPARγ to promote angiogenesis and growth in breast cancer (90); an adrenergic-neurotrophin loop in pancreatic cancer (91); targeting of the Sonic hedgehog-Gli1 signaling activation in PCa; promotion of proliferation in lung cancer via the ERK1/2/CREB pathway (92); and increased metastasis in ovarian cancer via increased PGE2 synthesis (93).

In general, there is agreement that adrenergic nerves and the presence of adrenergic receptors are associated with cancer aggressiveness and increased proliferation. This is likely done through activation of intracellular pathways. The question is whether adrenergic antagonists can be used to affect cancer.

There are ample laboratory data on surrogate endpoints in vitro and in vivo. Also, biologic endpoints have been completed on samples from a randomized placebo-controlled biomarker trial. Surrogate endpoints of perioperative beta-blockade for breast cancer were improved over controls (94). In a retrospective study examining perioperative beta-blocker use and cancer recurrence and metastases (1,029 patients), β2-adrenergic receptor block inhibition reduces triple-negative breast cancer brain metastases (95).

There is ample evidence of the beta-blockade effect on cancer survival, but the data remain controversial. Beta-blockade was associated with decreased cancer recurrence for stage II patients (95). A meta-analysis on the effect of beta-blockers in breast cancer concluded that the data were promising but larger and better-defined studies were needed. Longer disease-free survival time was identified, but no effects on overall survival were found (84).

Anil Sood of the University of Texas MD Anderson Cancer Center and colleagues (96), in a multi-institutional retrospective chart review of patients with epithelial ovarian cancer treated with platinum-based chemotherapy, found a longer median overall survival among users of a nonselective beta-blocker when compared with nonusers. These studies were later contested by others. Another study of multiple myeloma patients found that the use of a selective beta-blocker improved the disease specific survival versus nonusers (97).

In a Scandinavian population study, beta-blocker use was associated with lower liver cancer mortality. Again, nonselective earlier generation beta-blockers had the best results (98). A Veterans Affairs study found no improvement in overall survival in patients with PCa (99). Use of beta-blockers is associated with decreased distant metastases and potentially improved disease-specific survival in small cell carcinoma of the lung (100).

Additionally, α1 adrenergic receptor antagonists have been involved in cancer risk reduction. A study found a 1.46 times lower relative risk and a 31.7% lower attributable risk of developing PCa in men who received adrenergic receptor antagonist therapy versus those who did not (101). A Finnish screening trial did not confirm these findings but did find a decrease in high-grade tumors (102).

Cholinergic Axis

The influence of acetylcholine on cancer cells remains less studied. Parasympathetic neurogenesis correlates with an adverse prognosis in pancreatic ductal adenocarcinoma (39). Increased activity of cholinergic and adrenergic receptors can lead to changes of cell behavior and defects of cell genome in non-small cell lung cancer (103). It is also thought that parasympathetic nerves promote the progression of colorectal cancer through α9 nicotinic acetylcholine receptor (104).

In our experience, the identification of parasympathetic nerves is exceedingly difficult, due to background stain in the reactive stroma of tumors. However, it is also possible that acetylcholine is produced by the reactive stroma myofibroblast and that it acts as an early syncytial nervous system, needed in urgent repair response. Further studies are needed.

PERINEURAL INVASION

We first started investigating PNI because it was the most obvious relationship between nerves and cancer. But PNI is not the start of that relationship, although it is the most successful. Initially, secreted factors from the cancer cells induce axonogenesis and neurogenesis in tumors. This

process results in the formation of a tumor neural plexus that provides regulatory control of the homoeostasis and energetic metabolism of the cancer cells. The net effect of this interaction is that cancer cells become more efficient energetically and aggressive. Subsequently, cancer cells migrate along the nerve twiglets to larger nerves, usually at the periphery of the organ, where they establish PNI (**Figure 2**).

While the hallmark of nerve function in axonogenesis is homeostatic regulation, the hallmark of PNI is growth advantage and suppression of apoptosis (105). PNI is the most efficient relationship between nerves and cancer. It represents the pinnacle of the interaction between cancer cells and nerves. The result of this interaction is survival advantage for both cancer cells and nerves. It is as if cancer cells, already connected to small nerve twiglets within the tumor, race to connect to larger nerves to commence the ultimate neuroepithelial interaction.

As PNI is established, the success of the interaction can be measured by a clinical biomarker, PNI diameter. PNI diameter measures the diameter of cancer cells around the nerve, perpendicular to the long axis of the nerve. PNI diameter is one of the strongest predictors for survival in PCa. The larger the diameter, the lower the survival. Also, the larger the PNI diameter, the larger the overall tumor volume (37). The latter is a demonstration of the hallmark growth survival advantage found in cancer cells in PNI.

PNI is an interactive symbiotic process between nerves and cancer cells (1). The cancer grows more in the presence of nerves, and nerves grow more in the presence of cancer, later translating into axonogenesis. PCa cells in PNI exhibit a significantly reduced rate of apoptosis and an increased rate of proliferation. Furthermore, two mechanisms might be involved in this survival advantage. The identification of an epithelial pathway (106) was based upon upregulation of NFκB and its downstream targets PIM-2 and DAD-1 in the cancer cells when nerves were present. All components are involved in antiapoptosis signaling cascades (107). The profile identified recapitulates an inflammatory type response, rather than an increase in genetic instability. A stromal regulatory pathway based upon the secretion of caveolin by the nerves in the perineural space was also identified (108). This likely represents an injury response. These studies were reproduced in pancreatic (109, 110) and colon (111) cancer.

Neural cell adhesion molecule was found upregulated in PNI cancer cells (112). We also found that bystin was upregulated in PNI cells using transcriptomic comparative studies of the models and controls, and corroborated on tissues (113). Of interest, bystin is an adhesion molecule that the placenta uses in the early stages of adhesion. Yet it was not until Memorial Sloan Kettering Cancer Center's model advances under the leadership of Richard Wong that the understanding of the dynamics of PNI cancer migration along the nerve and the role of Schwann cells became possible (114). For an excellent review of PNI, readers are referred to an article by Bakst & Wong (115). The clinical significance of PNI in most solid tumors needs no review.

The entire spectrum of neuroepithelial interactions—cancer proliferation, axonogenesis, and PNI—is enhanced in the presence of stromal cells (116). The reactive stromal host response seems to play a critical role in establishing axonogenesis and then neurogenesis. Concordantly, it is understandable that significant alterations in processes related to neurogenesis and axonogenesis were identified in the reactive stroma transcriptome (117).

NEURONAL TRANS DIFFERENTIATION OF CANCER: INDEPENDENCE AND AGGRESSION

The functional role of the nerves and cancer initiation progression are now better understood. Nerves are required for the regulation of normal epithelium and in support of carcinogenesis and tumor aggression. Denervation affects tumorigenesis. It seems that if nerves are so

critical for cancer, cancer would attempt to become neural independent. A process of neuronal transdifferentiation achieves that by allowing a mature, fully differentiated cell to transform to a different phenotype. It happens physiologically and in cancer. The best-known example is epithelial mesenchymal transformation.

Much attention has been given to the neuroendocrine differentiation of tumors, particularly in the prostate and in the lung. Yet, most solid organs have neuroendocrine tumors that we call, generically, small cell carcinomas. These are diagnosed and classified on the basis of the presence of structural proteins of the neuroendocrine granule, such as chromogranin and synaptophysin. Yet we lack an understanding of the ontology of this process. It is important to stress that neuroendocrine cells are a subtype of neurons with a different mechanism of communication through release of granules rather than synapses.

Prostate LNCAP cancer cells under stress, using any method, acquire a characteristic morphology that resembles neuronal differentiation. Similar morphology has been identified under starvation in HCT116, MCF-7, H1299, ZR-75-1, and PC-3 cells, to varying degrees. On the basis of this observation, silicone experimentation to compare a transcriptomic brain profile with PCa of different levels of progression was undertaken. As PCa approaches metastatic and androgen resistance, a statistically significant profile-related brain is recognized. Two hundred seventy-four of 988 upregulated genes in metastatic hormone-resistant PCa statistically significantly overlapped with the brain signature. Gene ontology studies demonstrated that the most significantly upregulated biological processes encompass nervous system development and neuronal morphogenesis (118). These were not endocrine gene products or secreted peptide ligands with cognate receptors. Some of these genes were validated in human tissue studies. One of these was Bassoon cytomatrix presynaptic protein, a structural scaffolding protein involved in the presynaptic cytoskeleton. We observed linear membranous intercellular structures similar to connective structures (118). Presynaptic proteins also have been described previously in pulmonary squamous cell carcinoma (SCC) (119).

Colin Collins from the Vancouver Prostate Center and colleagues (120) have also identified neuronal phenotypes in metastatic hormone-resistant PCa. Ralph Buttyan from the VCH Research Institute and colleagues (121) have shown that PCa cells can reprogram (transdifferentiate) to an intermediate cell with neural/neural crest stem cell–like cells, before continuing to neuroendocrine differentiation. Additionally, a subset of gastric cancer stem cells can produce neurons (122).

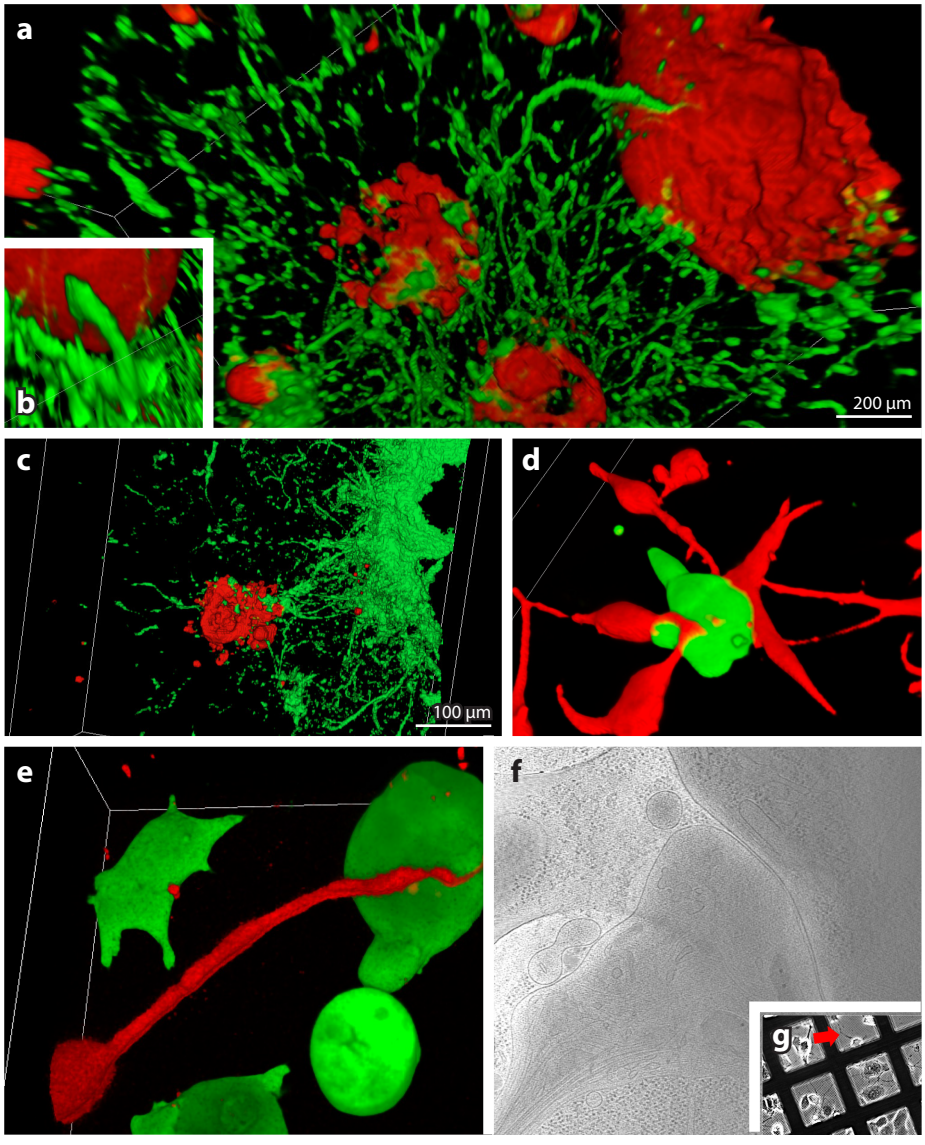
True neurons transmit action potential. Previous studies, some dating back to the 1980s, have shown that SCC and small cell carcinomas of the lung express voltage-dependent calcium, potassium, and sodium channels and that the cancer cells can transmit action potential (119, 123, 124). It cannot be claimed that these PCa cells convert to fully functional neurons; rather, they acquire neuronal features that may potentiate independence from the neural axis. Further studies are necessary to substantiate this conclusion. However, it opens up the possibility of utilizing neurotoxins as a novel treatment approach to not only inhibit neuroepithelial interactions but also deter the cell survival advantage conferred by neuronal transdifferentiation.

It is only through the understanding that nerves regulate normal epithelium that neural regulation of cancer becomes rational, and only through the understanding of the critical nature of nerves in cancer development and aggressiveness can we begin to understand the transformation of cancer cells into a neuronal profile in search of neural independence. Just as epithelial–mesenchymal transformation is critical for acquiring metastatic potential, epithelial neuronal transdifferentiation is critical for acquiring independence from the host. Cancer cells acquire neuronal features that permit autocrine regulation.

NEUROEPITHELIAL CONNECTIONS

To date, there is no known structural connection between nerves and epithelial cells other than those found in sensory epithelium. While the functional effects of nerves on normal and malignant epithelial cells are apparent, how these nerves communicate with epithelial cells remained a mystery. To unravel the mystery, a series of experiments was designed to confirm these connections. We used two models for confocal microscopy, one with DRG and the other with individual neurons in culture.

In the first, we cocultured human PCa cells, stained with CellTracker Deep Red, and DRG from the previously described Mapttm1(GFP)Klt/J mice in Matrigel matrix. The findings were revealing. Neurites initially sprung from the DRG and directed their growth to the cancer colonies (**Figure 3*c***). Later, a neural network was formed between the cancer colonies, with neurites



(*Caption appears on following page*)

Figure 3 (*Figure appears on preceding page*)

Images from confocal microscopy of cocultured human prostate cancer cells and dorsal root ganglia (DRG) from Mapttm1(GFP)Klt/J mice. Neurites (*green*) initially sprung from the DRG and directed their growth to the cancer colonies (*red*) (*c*). Neurites would grow preferentially from the DRG to the cancer colonies but would bend to reach the colonies (*a*). The neurites directedly entered the cancer colonies (*b*). Confocal microscopy images of the coculture of human prostate cancer Du145 cells expressing green fluorescent protein (Du145-GFP) and the 50B11 immortalized rat DRG neuronal cells are shown. Colors are inverted in this experiment: Cancer cells are green, and the neurons are red. Neurons project neurites toward the cancer cells and establish direct contact with the cancer cells (*e*), while at the same time neurons establish a network between them (*d*). Electron cryotomography images are shown of a coculture of Du145 cells growing in a grid and DRG placed outside but close to the grid. Only neurites entered the grid, such that in the grid we have only cancer cells and neurites. On day 2, dendrites could be seen entering the grid and contacting cancer cells (*g*, *red arrow*). Neurites carried neural microtubules and granules in the apical surface. Examination of this area showed that neurites come in direct contact with the cancer cell, in a mushroom-type structure, similar to a synapse (*f*). The membranes between the cancer cell and the neurite were appositional, but we were not able to distinguish gap junctions or an established synapse.

crossing in between them. Neurites would grow preferentially from the DRG to the cancer colonies but would bend to reach the colonies (**Figure 3*a***). The neurites directedly entered the cancer colonies (**Figure 3*b***) (Y. Ding, P. Bu & G. Ayala, unpublished data).

The second model consisted of human PCa Du145 cells expressing GFP (Du145-GFP) and immortalized rat DRG neuronal cell line 50B11 stained with CellTracker Deep Red, cocultured in 100 μl of Matrigel. Colors are inverted in this experiment: Cancer cells are green, and the neurons are red. Neurons project neurites toward the cancer cells, and the cancer cells approach these neurites with lamellipodia. Neurites establish direct contact with the cancer cells (**Figure 3*e***), while at the same time neurons establish a network between them (**Figure 3*d***) (Y. Ding, P. Bu & G. Ayala, unpublished data).

These images are the first actual visualization of direct contact between neurons and cancer cells and the establishment of neural networks with cancer colonies. This contact was further explored using electron cryotomography on grids in collaboration with Dr. Irina Serysheva. Du-145 cells were grown on grids in Matrigel, and the DRG was placed outside the grid, such that the only cells in the grid were cancer cells. At day 3, images were taken in collaboration with the Electron Cryo-Microscopy Core Facility at the McGovern School of Medicine and Dr. Serysheva (Y. Ding, P. Bu, I. Serysheva & G. Ayala, unpublished data).

On day 2, we could see dendrites entering the grid and contacting cancer cells (**Figure 3*g***). The grid only had cancer cells and neurites. Neurites carried neural microtubules and granules in the apical surface. Examination of this area showed that neurites come in direct contact with the cancer cell, in a mushroom-type structure, similar to a synapse (**Figure 3*f***). The membranes between the cancer cell and the neurite were appositional.

CONCLUSION

We traveled through a maze of neuroepithelial interactions. The neural regulation of normal epithelium seems to be the basis of all neuroepithelial interactions. All the interactions between nerves and cancer cells recapitulate this natural regulatory mechanism.

Cancer cells induce axonogenesis to supply the growth of new malignant epithelial cells, beginning at the level of preneoplastic lesions. Axonogenesis is the initiating phenomena that permits other interactions between nerves and cancer cells. Eventually, the supply of neurons is insufficient to support new axonal growth, and neurogenesis begins. First it happens at the local level, in the DRG, and subsequently the neuroblast-rich regions of the brain are recruited. Neuroblasts travel from the brain to the cancer and complete the formation of a tumor-supporting neural

network. The hallmark of this period is regulation of basic cellular functions within the cancer cells. The major emphasis is on the energetic metabolism of cancer cells, as happens in normal epithelium (**Figure 2**). Axonogenesis and neurogenesis also serve as permissive factors for PNI. PNI results in a symbiotic growth advantage for the cancer cells and nerves, as well as metastatic selection pressures. The hallmark of this stage of interactions is inhibition of apoptosis and explosive growth and aggressiveness. Finally, malignant epithelial cells transdifferentiate into quasi neurons, acquiring independence from neural regulation, and develop even further enhanced aggressiveness. Transdifferentiation also allows cancer cells to grow in environments that are poor in nerves (**Figure 2**). Therefore, understanding the specific mechanism of this carcinoma/nerve interaction is key to developing new and effective therapies for cancer. Without addressing this new biology, cancer treatment will continue to be limited in efficacy.

The cancer/nerve nexus presents possible therapeutic options and conundrums. How do we manage the influence of nerves in cancer without affecting other critical regulatory nerve functions in the body? Tumor nerve ablation, although feasible, would cause massive functional disarray. Data from pancreatic cancer ganglia ablation are inconclusive (125). Significant data on the effects of tumor denervation in humans can be found in gastric cancer studies. In an epidemiologic study, the incidence of gastric cancer in patients who had undergone supraselective vagotomy for ulcers was 1.5 for the first 10 years and 0 in the subsequent 10 years (126). While these data are only suggestive, they point to the significant potential of incorporating nerve-targeted therapies for cancer.

These data lead to considerations of localized therapies. Our basic approach of using Botox as a denervating agent let us demonstrate as proof of principle that the biology of neuroepithelial interactions exists in humans. Yet its practical applicability will likely be limited to attempting to induce regression in surface in situ lesions. It is also possible that neoadjuvant uses, through local injections before radiation or chemotherapy, have clinical potential. Radiation therapy, while effective at killing cancer cells, induces an NPY reactive neurogenesis (66). NPY axonogenesis could explain radiation therapy resistance. Neutralizing this side effect could help patient survival.

It is also of note that chemotherapy regimens are limited by the side effects they produce, the most significant of which is lasting nerve damage. Chemotherapy stops when we affect the neural regulatory system needed for cancer growth. If we can target nerves concomitantly, without destroying them, we have the potential of greatly improving chemotherapy effects. In both cases, nerve-targeted therapies would act as neoadjuvant or adjuvant forces that improve clinical efficacy of current therapies.

The key to nerve-targeted therapies needs to be specificity and geography. Early studies targeting neural receptors associated with chemotherapeutic agents have been performed (79). These studies, however, use the neural regulatory target as a guidance molecule. The ultimate approach would target the neural support of the tumor using nanomolecules covered with tumor-specific antibodies and carrying substances that target specific neural pathways that permit cancer growth and aggressiveness.

What neural-targeted therapies do we have available? Fortunately, neurotoxins abound in nature and in chemical warfare. Neurotoxins from these sources can be repurposed as cancer therapies, with organ and tumor specificity and minimal organ and body side effects. While the side effects are not likely to be minimal, we must use an approach similar to chemotherapy and rescue when the effects are most toxic and not permanent. Many of these substances, mainly snake venoms, are already being experimented with, but using a different rationale (127–131).

Other areas of great significance have not been addressed in this article but should not be forgotten. Such areas include neural regulation of stem cells, both in the bone marrow space and within epithelia, and neural regulation of the immune system.

What started as an attempt to understand PNI became the means to discovery of a new biology for cancer. Nerves are critical for cancer development and aggressiveness (132). We must learn how to target them, by embracing biologic complexity.

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LITERATURE CITED

1. Ayala GE, Wheeler TM, Shine HD, Schmelz M, Frolov A, et al. 2001. In vitro dorsal root ganglia and human prostate cell line interaction: redefining perineural invasion in prostate cancer. *Prostate* 49:213–23
2. Ernst P. 1905. Uber das Wachstum und die Verbreitung Bostariger eshwulste insbesondere des Krebes in den Lymphbahnen der Nerven. *Beitr. Pathol. Anat.* 7:29–51
3. Larson DL, Rodin AE, Roberts DK, O'Steen WK, Rapperport AS, Lewis SR. 1966. Perineural lymphatics: myth or fact. *Am. J. Surg.* 112:488–92
4. Vogel G. 1999. Harnessing the power of stem cells. *Science* 283:1432–34
5. Emmerson E, May AJ, Nathan S, Cruz-Pacheco N, Lizama CO, et al. 2017. SOX2 regulates acinar cell development in the salivary gland. *eLife* 6:e26620
6. Sudiwala S, Knox SM. 2019. The emerging role of cranial nerves in shaping craniofacial development. *Genesis* 57:e23282
7. Knosp WM, Knox SM, Hoffman MP. 2012. Salivary gland organogenesis. *Wiley Interdiscip. Rev. Dev. Biol.* 1:69–82
8. Andrew DJ. 1998. Regulation and formation of the Drosophila salivary glands. *Ann. N. Y. Acad. Sci.* 842:55–69
9. Ayala GE, Dai H, Powell M, Li R, Ding Y, et al. 2008. Cancer-related axonogenesis and neurogenesis in prostate cancer. *Clin. Cancer Res.* 14:7593–603
10. Vining KH, Lombaert IMA, Patel VN, Kibbey SE, Pradhan-Bhatt S, et al. 2019. Neurturin-containing laminin matrices support innervated branching epithelium from adult epithelial salispheres. *Biomaterials* 216:119245
11. Zhang SE, Su YX, Zheng GS, Liang YJ, Liao GQ. 2014. Reinnervated nerves contribute to the secretion function and regeneration of denervated submandibular glands in rabbits. *Eur. J. Oral. Sci.* 122:372–81
12. Kyriacou K, Garrett JR, Gjorstrup P. 1988. Structural and functional studies of the effects of sympathetic nerve stimulation on rabbit submandibular salivary glands. *Arch. Oral. Biol.* 33:271–80
13. Kyriacou K, Garrett JR. 1988. Morphological changes in the rabbit submandibular gland after parasympathetic or sympathetic denervation. *Arch. Oral Biol.* 33:281–90
14. Boshell JL, Pennington C. 1980. Histological observations on the effects of isoproterenol on regenerating submandibular glands of the rat. *Cell Tissue Res.* 213:411–16
15. Norberg LE, Lundquist PG. 1988. An ultrastructural study of salivary gland radiosensitivity after alpha-adrenergic stimulation. *Auris Nasus Larynx* 15:1–17
16. Ferreira JNA, Zheng C, Lombaert IMA, Goldsmith CM, Cotrim AP, et al. 2018. Neurturin gene therapy protects parasympathetic function to prevent irradiation-induced murine salivary gland hypofunction. *Mol. Ther. Methods Clin. Dev.* 9:172–80
17. Bitoh Y, Shimotake T, Sasaki Y, Iwai N. 2002. Development of the pelvic floor muscles of murine embryos with anorectal malformations. *J. Pediatr. Surg.* 37:224–27
18. Zhao CM, Hayakawa Y, Kodama Y, Muthupalani S, Westphalen CB, et al. 2014. Denervation suppresses gastric tumorigenesis. *Sci. Transl. Med.* 6:250ra115
19. Nishi R. 2003. Target-mediated control of neural differentiation. *Prog. Neurobiol.* 69:213–27
20. Stewart RR, Spergel D, Macagno ER. 1986. Segmental differentiation in the leech nervous system: the genesis of cell number in the segmental ganglia of *Haemopis marmorata*. *J. Comp. Neurol.* 253:253–59
21. Baptista CA, Gershon TR, Macagno ER. 1990. Peripheral organs control central neurogenesis in the leech. *Nature* 346:855–58

22. Coarfa C, Florentin D, Putluri N, Ding Y, Au J, et al. 2018. Influence of the neural microenvironment on prostate cancer. *Prostate* 78:128–39
23. Wang JM, McKenna KE, McVary KT, Lee C. 1991. Requirement of innervation for maintenance of structural and functional integrity in the rat prostate. *Biol. Reprod.* 44:1171–76
24. Doggweiler R, Zermann DH, Ishigooka M, Schmidt RA. 1998. Botox-induced prostatic involution. *Prostate* 37:44–50
25. Goransson LG, Mellgren SI, Lindal S, Omdal R. 2004. The effect of age and gender on epidermal nerve fiber density. *Neurology* 62:774–77
26. Cho NR, Yu Y, Oh CK, Ko DS, Myung K, et al. 2021. Neuropeptide Y: a potential theranostic biomarker for diabetic peripheral neuropathy in patients with type-2 diabetes. *Ther. Adv. Chronic Dis.* **https://doi.org/10.1177/20406223211041936**
27. Pradhan L, Nabzdyk C, Andersen ND, LoGerfo FW, Veves A. 2009. Inflammation and neuropeptides: the connection in diabetic wound healing. *Expert Rev. Mol. Med.* 11:e2
28. Tokushige N, Markham R, Russell P, Fraser IS. 2006. Nerve fibres in peritoneal endometriosis. *Hum. Reprod.* 21:3001–7
29. Stopczynski RE, Normolle DP, Hartman DJ, Ying H, DeBerry JJ, et al. 2014. Neuroplastic changes occur early in the development of pancreatic ductal adenocarcinoma. *Cancer Res.* 74:1718–27
30. Schmitd LB, Perez-Pacheco C, D'Silva NJ. 2021. Nerve density in cancer: Less is better. *FASEB Bioadv.* 3:773–86
31. Zhao Q, Yang Y, Liang X, Du G, Liu L, et al. 2014. The clinicopathological significance of neurogenesis in breast cancer. *BMC Cancer* 14:484
32. Han H, Yang C, Zhang Y, Han C, Zhang G. 2021. Vascular endothelial growth factor mediates the sprouted axonogenesis of breast cancer in rat. *Am. J. Pathol.* 191:515–26
33. Rademakers G, Vaes N, Schonkeren S, Koch A, Sharkey KA, Melotte V. 2017. The role of enteric neurons in the development and progression of colorectal cancer. *Biochim. Biophys. Acta Rev. Cancer* 1868:420–34
34. Amit M, Takahashi H, Dragomir MP, Lindemann A, Gleber-Netto FO, et al. 2020. Loss of p53 drives neuron reprogramming in head and neck cancer. *Nature* 578:449–54
35. Griffin N, Rowe CW, Gao F, Jobling P, Wills V, et al. 2020. Clinicopathological significance of nerves in esophageal cancer. *Am. J. Pathol.* 190:1921–30
36. Zhang L, Tao L, Guo L, Zhan J, Yuan C, et al. 2018. G-CSF associates with neurogenesis and predicts prognosis and sensitivity to chemotherapy in pancreatic ductal adenocarcinoma. *Cancer Manag. Res.* 10:2767–75
37. Olar A, He D, Florentin D, Ding Y, Ayala G. 2014. Biologic correlates and significance of axonogenesis in prostate cancer. *Hum. Pathol.* 45:1358–64
38. Albo D, Akay CL, Marshall CL, Wilks JA, Verstovsek G, et al. 2011. Neurogenesis in colorectal cancer is a marker of aggressive tumor behavior and poor outcomes. *Cancer* 117:4834–45
39. Zhang L, Guo L, Tao M, Fu W, Xiu D. 2016. Parasympathetic neurogenesis is strongly associated with tumor budding and correlates with an adverse prognosis in pancreatic ductal adenocarcinoma. *Chin. J. Cancer Res.* 28:180–86
40. Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, et al. 2006. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell* 9:157–73
41. de Wit J, Verhaagen J. 2003. Role of semaphorins in the adult nervous system. *Prog. Neurobiol.* 71:249–67
42. Ding Y, He D, Florentin D, Frolov A, Hilsenbeck S, et al. 2013. Semaphorin 4F as a critical regulator of neuroepithelial interactions and a biomarker of aggressive prostate cancer. *Clin. Cancer Res.* 19:6101–11
43. Renz BW, Takahashi R, Tanaka T, Macchini M, Hayakawa Y, et al. 2018. β2 adrenergic-neurotrophin feedforward loop promotes pancreatic cancer. *Cancer Cell* 34:863–67
44. Liu M, Jia Z, Xiao X, Zhang Z, Li P, et al. 2018. Carboxylated graphene oxide promoted axonal guidance growth by activating Netrin-1/deleted in colorectal cancer signaling in rat primary cultured cortical neurons. *J. Biomed. Mater. Res. A* 106:1500–10
45. Silverman DA, Martinez VK, Dougherty PM, Myers JN, Calin GA, Amit M. 2021. Cancer-associated neurogenesis and nerve-cancer cross-talk. *Cancer Res.* 81:1431–40

46. Grelet S, Frereux C, Obellianne C, Noguchi K, Howley BV, et al. 2022. TGFβ-induced expression of long noncoding lincRNA Platr18 controls breast cancer axonogenesis. *Life Sci. Alliance* 5(2):e202101261
47. Chedotal A. 2007. Chemotropic axon guidance molecules in tumorigenesis. *Prog. Exp. Tumor. Res.* 39:78–90
48. Johansson CB, Momma S, Clarke DL, Risling M, Lendahl U, Frisen J. 1999. Identification of a neural stem cell in the adult mammalian central nervous system. *Cell* 96:25–34
49. Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. 1999. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97:703–16
50. Shen Q, Goderie SK, Jin L, Karanth N, Sun Y, et al. 2004. Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. *Science* 304:1338–40
51. Pomp O, Brokhman I, Ben-Dor I, Reubinoff B, Goldstein RS. 2005. Generation of peripheral sensory and sympathetic neurons and neural crest cells from human embryonic stem cells. *Stem Cells* 23:923–30
52. Geuna S, Borrione P, Fornaro M, Giacobini-Robecchi MG. 2000. Neurogenesis and stem cells in adult mammalian dorsal root ganglia. *Anat. Rec.* 261:139–40
53. Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR. 2000. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. *Science* 290:1779–82
54. Semerci F, Maletic-Savatic M. 2016. Transgenic mouse models for studying adult neurogenesis. *Front. Biol.* 11:151–67
55. Semerci F, Choi WT, Bajic A, Thakkar A, Encinas JM, et al. 2017. Lunatic fringe-mediated Notch signaling regulates adult hippocampal neural stem cell maintenance. *eLife* 6:e24660
56. Tsarovina K, Schellenberger J, Schneider C, Rohrer H. 2008. Progenitor cell maintenance and neurogenesis in sympathetic ganglia involves Notch signaling. *Mol. Cell. Neurosci.* 37:20–31
57. Muratori L, Ronchi G, Raimondo S, Geuna S, Giacobini-Robecchi MG, Fornaro M. 2015. Generation of new neurons in dorsal root Ganglia in adult rats after peripheral nerve crush injury. *Neural. Plast.* 2015:860546
58. Mauffrey P, Tchitchek N, Barroca V, Bemelmans AP, Firlej V, et al. 2019. Progenitors from the central nervous system drive neurogenesis in cancer. *Nature* 569:672–78
59. Barbonetti A, D'Andrea S, Martorella A, Felzani G, Francavilla S, Francavilla F. 2018. Risk of prostate cancer in men with spinal cord injury: a systematic review and meta-analysis. *Asian J. Androl.* 20:555–60
60. Neumeister MW. 2010. Botulinum toxin type A in the treatment of Raynaud's phenomenon. *J. Hand. Surg. Am.* 35:2085–92
61. Iorio ML, Masden DL, Higgins JP. 2012. Botulinum toxin A treatment of Raynaud's phenomenon: a review. *Semin. Arthritis Rheum.* 41:599–603
62. Merritt WH. 2015. Role and rationale for extended periarterial sympathectomy in the management of severe Raynaud syndrome: techniques and results. *Hand. Clin.* 31:101–20
63. Kappos EA, Engels PE, Tremp M, Sieber PK, von Felten S, et al. 2018. Denervation leads to volume regression in breast cancer. *J. Plast. Reconstr. Aesthet. Surg.* 71:833–39
64. Saloman JL, Albers KM, Li D, Hartman DJ, Crawford HC, et al. 2016. Ablation of sensory neurons in a genetic model of pancreatic ductal adenocarcinoma slows initiation and progression of cancer. *PNAS* 113:3078–83
65. Chen D, Ayala GE. 2018. Innervating prostate cancer. *N. Engl. J. Med.* 378:675–77
66. Ding Y, Lee M, Gao Y, Bu P, Coarfa C, et al. 2021. Neuropeptide Y nerve paracrine regulation of prostate cancer oncogenesis and therapy resistance. *Prostate* 81:58–71
67. Dietrich P, Wormser L, Fritz V, Seitz T, De Maria M, et al. 2020. Molecular crosstalk between Y5 receptor and neuropeptide Y drives liver cancer. *J. Clin. Investig.* 130:2509–26
68. Wang X, Chen X, Zhou H, Qian Y, Han N, et al. 2019. The long noncoding RNA, LINC01555, promotes invasion and metastasis of colorectal cancer by activating the neuropeptide, neuromedin U. *Med. Sci. Monit.* 25:4014–24
69. Kasprzak A, Adamek A. 2020. The neuropeptide system and colorectal cancer liver metastases: mechanisms and management. *Int. J. Mol. Sci.* 21(10):3494
70. Misawa K, Imai A, Mochizuki D, Misawa Y, Endo S, et al. 2017. Genes encoding neuropeptide receptors are epigenetic markers in patients with head and neck cancer: a site-specific analysis. *Oncotarget* 8:76318–28

71. Waldmann J, Fendrich V, Reichert M, Hecker A, Bartsch DK, et al. 2018. Expression of neuropeptide Y and its receptors Y1 and Y2 in pancreatic intraepithelial neoplasia and invasive pancreatic cancer in a transgenic mouse model and human samples of pancreatic cancer. *J. Surg. Res.* 223:230–36
72. Gutierrez S, Boada MD. 2018. Neuropeptide-induced modulation of carcinogenesis in a metastatic breast cancer cell line (MDA-MB-231^{LUC+}). *Cancer Cell Int*. 18:216
73. Dawoud MM, Abdelaziz KK, Alhanafy AM, Ali MSE, Elkhouly EAB. 2021. Clinical significance of immunohistochemical expression of neuropeptide Y1 receptor in patients with breast cancer in Egypt. *Appl. Immunohistochem. Mol. Morphol.* 29:277–86
74. Li J, Tian Y, Shan D, Gong A, Zeng L, et al. 2017. Neuropeptide Y Y_1 receptor-mediated biodegradable photoluminescent nanobubbles as ultrasound contrast agents for targeted breast cancer imaging. *Biomaterials* 116:106–17
75. Avvakumova S, Galbiati E, Sironi L, Locarno SA, Gambini L, et al. 2016. Theranostic nanocages for imaging and photothermal therapy of prostate cancer cells by active targeting of neuropeptide-Y receptor. *Bioconjug. Chem.* 27:2911–22
76. Cardoso ME, Tejeria E, Rey Rios AM, Teran M. 2020. Development and characterization of a ^{99m}Tc-labeled neuropeptide Y short analog with potential application in breast cancer imaging. *Chem. Biol. Drug Des.* 95:302–10
77. Cardoso ME, Tejeria E, Zirbesegger K, Savio E, Teran M, Rey Rios AM. 2021. Development and characterization of two novel ^{68}Ga-labelled neuropeptide Y short analogues with potential application in breast cancer imaging. *Chem. Biol. Drug Des.* 98:182–91
78. Zhang C, Pan J, Lin KS, Dude I, Lau J, et al. 2016. Targeting the neuropeptide Y1 receptor for cancer imaging by positron emission tomography using novel truncated peptides. *Mol. Pharm.* 13:3657–64
79. Bohme D, Krieghoff J, Beck-Sickinger AG. 2016. Double methotrexate-modified neuropeptide Y analogues express increased toxicity and overcome drug resistance in breast cancer cells. *J. Med. Chem.* 59:3409–17
80. Wang W, Guo X, Dan H. 2020. α2A-adrenergic receptor inhibits the progression of cervical cancer through blocking PI3K/AKT/mTOR pathway. *Onco. Targets Ther.* 13:10535–46
81. Huang T, Tworoger SS, Hecht JL, Rice MS, Sood AK, et al. 2016. Association of ovarian tumor β2-adrenergic receptor status with ovarian cancer risk factors and survival. *Cancer Epidemiol. Biomarkers Prev.* 25:1587–94
82. Yazawa T, Kaira K, Shimizu K, Shimizu A, Mori K, et al. 2016. Prognostic significance of β2-adrenergic receptor expression in non-small cell lung cancer. *Am. J. Transl. Res.* 8:5059–70
83. Ogawa H, Kaira K, Motegi Y, Yokobori T, Takada T, et al. 2020. Prognostic significance of β2-adrenergic receptor expression in patients with surgically resected colorectal cancer. *Int. J. Clin. Oncol.* 25:1137–44
84. Wang T, Li Y, Lu HL, Meng QW, Cai L, Chen XS. 2015. β-Adrenergic receptors: new target in breast cancer. *Asian Pac. J. Cancer Prev.* 16:8031–39
85. Caparica R, Richard F, Brandao M, Awada A, Sotiriou C, de Azambuja E. 2020. Prognostic and predictive impact of beta-2 adrenergic receptor expression in HER2-positive breast cancer. *Clin. Breast Cancer* 20:262–73.e7
86. Magnon C, Hall SJ, Lin J, Xue X, Gerber L, et al. 2013. Autonomic nerve development contributes to prostate cancer progression. *Science* 341:1236361
87. Zhi X, Li B, Li Z, Zhang J, Yu J, et al. 2019. Adrenergic modulation of AMPK-dependent autophagy by chronic stress enhances cell proliferation and survival in gastric cancer. *Int. J. Oncol.* 54:1625–38
88. Decker AM, Decker JT, Jung Y, Cackowski FC, Daignault-Newton S, et al. 2020. Adrenergic blockade promotes maintenance of dormancy in prostate cancer through upregulation of GAS6. *Transl. Oncol.* 13:100781
89. Wilson JM, Lorimer E, Tyburski MD, Williams CL. 2015. β-Adrenergic receptors suppress Rap1B prenylation and promote the metastatic phenotype in breast cancer cells. *Cancer Biol. Ther.* 16:1364–74
90. Zhou J, Liu Z, Zhang L, Hu X, Wang Z, et al. 2020. Activation of β2-adrenergic receptor promotes growth and angiogenesis in breast cancer by down-regulating PPARγ. *Cancer Res. Treat.* 52:830–47
91. Am. Assoc. Cancer Res. 2018. An adrenergic-neurotrophin feedforward loop promotes pancreatic cancer. *Cancer Discov.* 8:138

92. Hu P, He J, Liu S, Wang M, Pan B, Zhang W. 2016. β2-adrenergic receptor activation promotes the proliferation of A549 lung cancer cells via the ERK1/2/CREB pathway. *Oncol. Rep.* 36:1757–63
93. Nagaraja AS, Dorniak PL, Sadaoui NC, Kang Y, Lin T, et al. 2016. Sustained adrenergic signaling leads to increased metastasis in ovarian cancer via increased PGE2 synthesis. *Oncogene* 35:2390–97
94. Shaashua L, Shabat-Simon M, Haldar R, Matzner P, Zmora O, et al. 2017. Perioperative COX-2 and β-adrenergic blockade improves metastatic biomarkers in breast cancer patients in a phase-II randomized trial. *Clin. Cancer Res.* 23:4651–61
95. Choy C, Raytis JL, Smith DD, Duenas M, Neman J, et al. 2016. Inhibition of β2-adrenergic receptor reduces triple-negative breast cancer brain metastases: the potential benefit of perioperative β-blockade. *Oncol. Rep.* 35:3135–42
96. Watkins JL, Thaker PH, Nick AM, Ramondetta LM, Kumar S, et al. 2015. Clinical impact of selective and nonselective beta-blockers on survival in patients with ovarian cancer. *Cancer* 121:3444–51
97. Hwa YL, Shi Q, Kumar SK, Lacy MQ, Gertz MA, et al. 2017. Beta-blockers improve survival outcomes in patients with multiple myeloma: a retrospective evaluation. *Am. J. Hematol.* 92:50–55
98. Udumyan R, Montgomery S, Duberg AS, Fang F, Valdimarsdottir U, et al. 2020. Beta-adrenergic receptor blockers and liver cancer mortality in a national cohort of hepatocellular carcinoma patients. *Scand. J. Gastroenterol.* 55:597–605
99. Posielski NM, Richards KA, Liou JI, Borza T, Abel EJ, et al. 2021. Beta-adrenergic antagonists and cancer specific survival in patients with advanced prostate cancer: a Veterans Administration cohort study. *Urology* 155:186–91
100. Chaudhary KR, Yan SX, Heilbroner SP, Sonett JR, Stoopler MB, et al. 2019. Effects of β-adrenergic antagonists on chemoradiation therapy for locally advanced non-small cell lung cancer. *J. Clin. Med.* 8:575
101. Harris AM, Warner BW, Wilson JM, Becker A, Rowland RG, et al. 2007. Effect of α1-adrenoceptor antagonist exposure on prostate cancer incidence: an observational cohort study. *J. Urol.* 178:2176–80
102. Murtola TJ, Tammela TL, Maattanen L, Ala-Opas M, Stenman UH, Auvinen A. 2009. Prostate cancer incidence among finasteride and alpha-blocker users in the Finnish Prostate Cancer Screening Trial. *Br. J. Cancer* 101:843–48
103. Buzgan NG, Semenenko OF, Dorosevich AE. 2016. [Influence of acetylcholine and adrenergic receptors on development and progression of non-small cell lung cancer]. *Vopr. Onkol.* 62:694–700 (In Russian)
104. Zhou H, Shi B, Jia Y, Qiu G, Yang W, et al. 2018. Expression and significance of autonomic nerves and α9 nicotinic acetylcholine receptor in colorectal cancer. *Mol. Med. Rep.* 17:8423–31
105. Olar A, He D, Florentin D, Ding Y, Wheeler T, Ayala G. 2014. Biological correlates of prostate cancer perineural invasion diameter. *Hum. Pathol.* 45:1365–69
106. Ayala GE, Dai H, Ittmann M, Li R, Powell M, et al. 2004. Growth and survival mechanisms associated with perineural invasion in prostate cancer. *Cancer Res.* 64:6082–90
107. Dai H, Li R, Wheeler T, Diaz de Vivar A, Frolov A, et al. 2005. Pim-2 upregulation: biological implications associated with disease progression and perinueral invasion in prostate cancer. *Prostate* 65:276–86
108. Ayala GE, Dai H, Tahir SA, Li R, Timme T, et al. 2006. Stromal antiapoptotic paracrine loop in perineural invasion of prostatic carcinoma. *Cancer Res.* 66:5159–64
109. Dai H, Li R, Wheeler T, Ozen M, Ittmann M, et al. 2007. Enhanced survival in perineural invasion of pancreatic cancer: an in vitro approach. *Hum. Pathol.* 38:299–307
110. He D, Manzoni A, Florentin D, Fisher W, Ding Y, et al. 2016. Biologic effect of neurogenesis in pancreatic cancer. *Hum. Pathol.* 52:182–89
111. Albo D, Akay CL, Marshall CL, Wilks JA, Verstovsek G, et al. 2011. Neurogenesis in colorectal cancer is a marker of aggressive tumor behavior and poor outcomes. *Cancer* 117(21):4834–45
112. Li R, Wheeler T, Dai H, Ayala G. 2003. Neural cell adhesion molecule is upregulated in nerves with prostate cancer invasion. *Hum. Pathol.* 34:457–61
113. Ayala GE, Dai H, Li R, Ittmann M, Thompson TC, et al. 2006. Bystin in perineural invasion of prostate cancer. *Prostate* 66:266–72
114. Deborde S, Gusain L, Powers A, Marcadis A, Yu Y, et al. 2022. Reprogrammed Schwann cells organize into dynamic tracks that promote pancreatic cancer invasion. *Cancer Discov.* 12(10):2454–73

115. Bakst RL, Wong RJ. 2016. Mechanisms of perineural invasion. *J. Neurol. Surg. B Skull Base* 77:96–106
116. Cornell RJ, Rowley D, Wheeler T, Ali N, Ayala G. 2003. Neuroepithelial interactions in prostate cancer are enhanced in the presence of prostatic stroma. *Urology* 61:870–75
117. Dakhova O, Ozen M, Creighton CJ, Li R, Ayala G, et al. 2009. Global gene expression analysis of reactive stroma in prostate cancer. *Clin. Cancer Res.* 15:3979–89
118. Farach A, Ding Y, Lee M, Creighton C, Delk NA, et al. 2016. Neuronal trans-differentiation in prostate cancer cells. *Prostate* 76:1312–25
119. Benatar M, Blaes F, Johnston I, Wilson K, Vincent A, et al. 2001. Presynaptic neuronal antigens expressed by a small cell lung carcinoma cell line. *J. Neuroimmunol.* 113:153–62
120. Lapuk AV, Wu C, Wyatt AW, McPherson A, McConeghy BJ, et al. 2012. From sequence to molecular pathology, and a mechanism driving the neuroendocrine phenotype in prostate cancer. *J. Pathol.* 227:286–97
121. Nouri M, Caradec J, Lubik AA, Li N, Hollier BG, et al. 2017. Therapy-induced developmental reprogramming of prostate cancer cells and acquired therapy resistance. *Oncotarget* 8:18949–67
122. Lu R, Fan C, Shangguan W, Liu Y, Li Y, et al. 2017. Neurons generated from carcinoma stem cells support cancer progression. *Signal. Transduct. Target. Ther.* 2:16036
123. Blandino JK, Viglione MP, Bradley WA, Oie HK, Kim YI. 1995. Voltage-dependent sodium channels in human small-cell lung cancer cells: role in action potentials and inhibition by Lambert-Eaton syndrome IgG. *J. Membr. Biol.* 143:153–63
124. Johansson S, Rydqvist B, Swerup C, Heilbronn E, Arhem P. 1989. Action potentials of cultured human oat cells: whole-cell measurements with the patch-clamp technique. *Acta Physiol. Scand.* 135:573–78
125. Wong GY, Schroeder DR, Carns PE, Wilson JL, Martin DP, et al. 2004. Effect of neurolytic celiac plexus block on pain relief, quality of life, and survival in patients with unresectable pancreatic cancer: a randomized controlled trial. *JAMA* 291:1092–99
126. Rabben HL, Zhao CM, Hayakawa Y, Wang TC, Chen D. 2016. Vagotomy and gastric tumorigenesis. *Curr. Neuropharmacol.* 14:967–72
127. Urra FA, Araya-Maturana R. 2020. Putting the brakes on tumorigenesis with snake venom toxins: new molecular insights for cancer drug discovery. *Semin. Cancer Biol.* 80:195–204
128. McCullough D, Atofanei C, Knight E, Trim SA, Trim CM. 2020. Kinome scale profiling of venom effects on cancer cells reveals potential new venom activities. *Toxicon* 185:129–46
129. Perez-Peinado C, Defaus S, Andreu D. 2020. Hitchhiking with nature: snake venom peptides to fight cancer and superbugs. *Toxins* 12(4):255
130. Montoya-Gomez A, Montealegre-Sanchez L, Garcia-Perdomo HA, Jimenez-Charris E. 2020. Cervical cancer and potential pharmacological treatment with snake venoms. *Mol. Biol. Rep.* 47:4709–21
131. Derakhshani A, Silvestris N, Hajiasgharzadeh K, Mahmoudzadeh S, Fereidouni M, et al. 2020. Expression and characterization of a novel recombinant cytotoxin II from *Naja naja oxiana* venom: a potential treatment for breast cancer. *Int. J. Biol. Macromol.* 162:1283–92
132. Servcik K. 2019. How the body's nerves become accomplices in the spread of cancer. *Science*, Sep. 12. **https://doi.org/10.1126/science.aaz4612**