Trends in Cancer

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Review

Tunneling Nanotubes: The Fuel of Tumor Progression?

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Tunneling nanotubes (TNTs) are thin membrane tubes connecting remote cells and allowing the transfer of cellular content. TNTs have been reported in several cancer *in vitro, ex vivo*, and *in vivo* models. Cancer cells exploit TNT-like connections to exchange material between themselves or with the tumoral microenvironment. Cells acquire new abilities (e.g., enhanced metabolic plasticity, migratory phenotypes, angiogenic ability, and therapy resistance) via these exchanges, contributing to cancer aggressiveness. Here, we review the morphological and functional features of TNT-like structures and their impact on cancer progression and resistance to therapies. Finally, we discuss the case of glioblastoma (GBM), in which a functional and resistant network between cancer cells in an *in vivo* model has been described for the first time.

Highlights

TNTs are physical membranous channels of communication between cells.

TNTs transfer mitochondria, nucleic acids, lysosomes, autophagosomes, protein-containing vesicles, and drugs.

Different types of TNT-like structure have been shown in several cancer cell lines, murine xenograft models, and in tumor sections from patients

TNT-mediated transfer of material can promote invasiveness, angiogenic ability, proliferation, metabolism plasticity, and therapy resistance

In gliomas, a functional network, comprising different types of intercellular connection, including TNTs, drives a more aggressive phenotype

Cancer and Intercellular Communication

Cancer is among the leading causes of mortality worldwide, responsible for 1 in 6 deaths, according to the World Health Organization. Over the past decades, many therapeutic strategies have proven their effectiveness and the overall cancer death rate has been reduced by 27% [1]. Several features of cancer cells make these pathologies very aggressive and difficult to cure, such as their uncontrollable proliferative capacity and their ability to obtain nourishment through neoformed blood vessels, to infiltrate healthy tissues forming metastasis, to evade the immune system, and, finally, to adapt to clinical treatments. In this context, intercellular communication, particularly, cell-to-cell transfer of cellular material, can contribute to each of the aforementioned characteristics, including treatment resistance. Over the past 20 years, numerous studies have shown that exosomes and exovesicles are able to carry malignant content (e.g., proteins and nucleic acids), likely helping the recipient cells to express genes supporting proliferation, colonization, and immune evasion, or to recover from damage provoked by treatment [2,3]. Recent work highlighted a new communication mechanism implemented by tumor cells, tunneling nanotubes (TNTs), which are physical channels providing cytoplasmic continuity between distant cells (Figure 1A). TNTs are thin, actin-based membrane tubes that, by contrast to other cellular protrusions, listed in Table 1, are open-ended at their extremities [4,5]. They allow the transfer of various-sized cargoes (Figure 1), such as small molecules (e.g., Ca^{2+} ions), macromolecules (proteins, nucleic acids, etc.), and even organelles (vesicles, mitochondria, lysosomes, autophagosomes, etc.) [6]. Several cells can be connected by TNTs, possibly leading to the formation of a functional cellular network [7].

TNTs were first identified in 2004 by Rustom and colleagues in cultures of pheochromocytoma PC12 cells [4]. Later, several other publications reported the presence of 'TNT-like structures' (heterogenous intercellular connections, defined on the basis of their morphology) in many other cell types in *in vitro* cultures, including astrocytes [8], immune cells [9], as well as in tumor cancer cell lines, where their occurrence was often correlated with more aggressive tumor phenotypes [10,11]. Beyond tumors, TNT-like structures have been observed in early developmental stages in various organisms [12] as well as in relation to stress-induced responses, such as oxidative stress [8,13], allowing the discharge of cellular waste or dangerous materials. Similarly,

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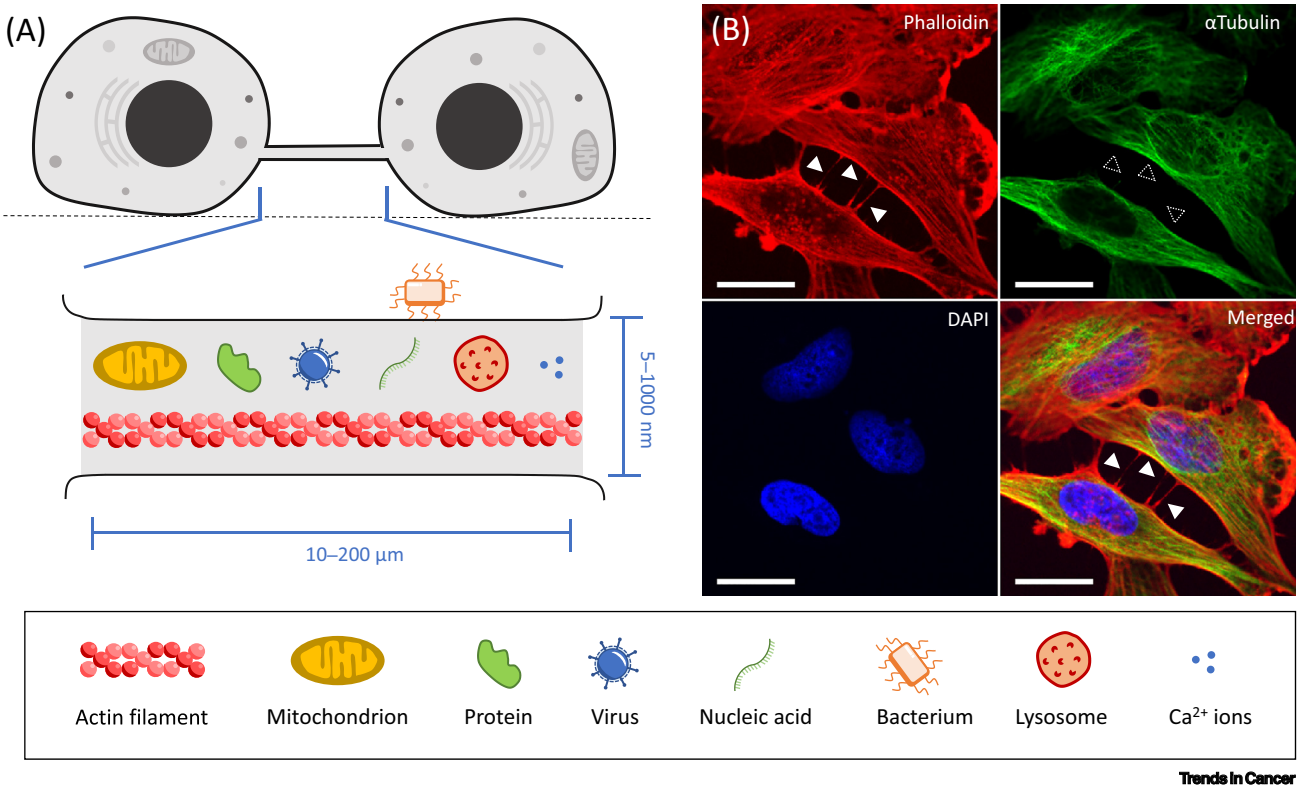

Figure 1. Tunneling Nanotubes (TNTs) in Cell Culture. (A) Schematic of two cells connected by a TNT in cell culture. The connection floats above the adhesion surface (dashed line). The lower part shows a magnification of the TNT and possible cargoes traveling along it. The range of TNT diameters and lengths is indicated. (B) Representative fluorescence images of TNTs between cells in culture. U-251 glioblastoma cells were plated at a density of 20 k cells/cm^2 for 24 h, fixed with PFA 4%, and permeabilized in 0.2% Triton-X100. Actin filaments (in red), microtubules (in green), and nuclei (in blue) were stained with phalloidin-rhodamine (1/500 Invitrogen R415), anti-αTubulin (1/1000 Sigma-Aldrich T9026), and DAPI (Sigma-Aldrich D9542), respectively. White-filled arrowheads point to TNTs positive for actin staining. Dashed arrowheads indicate the absence of tubulin staining. Confocal images acquired with Spinning Disk Yokogawa CSU-X1. Scale bars 20 µm.

they can be used as a route for the dissemination of pathogens, such as HIV [14,15], bacteria [9], and prions and amyloid fibrils in the case of neurodegenerative diseases [16–21]. Although TNT-like structures have been clearly identified as physical and functional entities in solid tumors [22–26], the existence of these connections in whole healthy organs or tissues is still a matter of debate. Here, we review studies on TNTs and their heterogeneity in cancers and their possible role in tumor progression and development of treatment resistance, with a particular focus on GBM.

Detection of TNT-like Structures *In Vitro* and *In Vivo* in Cancer

The first identification of TNTs occurred in PC12 cells, which are derived from a rare rat tumor of adrenal gland tissue [4]. Subsequently, many other cancer cell lines have been shown to form membranous connections bridging distant cells, as summarized in Table 1. Of importance for this review, TNT-like structures were also observed in primary cells directly obtained from patients, for example, from squamous cell carcinoma [24,25], mesothelioma [10,22], and different forms of leukemia [27–29]. Cancer cells can form heterotypic connections with cells of the tumor microenvironment (TME), including mesenchymal [30], endothelial [11], and immune cells [31]. Crosstalk with the TME has a significant role in sustaining cancer progression, providing nutrients or buffering metabolic stress [32], and interaction with immune cells can contribute to overcoming immunosurveillance [33]. While it is possible to identify TNT-like structures between

Table 1. Types of Cellular Projection

Name	Description	Actin/microtubule content	Membrane fusion with target cell?	Function	Refs
Cilia	Large protuberances emerging from cell body	Actin and microtubules	No	Environment sensing, coordination of signaling pathways	[83]
Stereocilia	Thin specialized cell protrusions on apical surface	Actin	No	Cellular polarity, transduction of mechanic stimuli	[84]
Lamellipodia and ruffles	Dynamic veil-shaped cell protrusions	Actin	No	Leading edge in cell migration	[85]
Filopodia	Finger-like, dynamic, thin membrane protrusions	Actin	No	Cell adhesion, environment sensing	[86]
Cytonemes/specialized filopodia	Finger-like, dynamic, thin membrane protrusions extending to target cell	Actin	No	Morphogen delivery by direct contact with target cells	[87]
Mitotic bridges	Thin bridges between daughter cells after mitosis	Actin	Yes	Reminiscent of cellular division, can share material	[88]
Neurites	Large extensions from cell body of neurons	Actin and microtubules	No	Neurotransmitter release/reception and propagation of action potential	[89]
Tumor microtubes	Thick membrane extensions containing GAP junctions, either connecting two cells or finger-like protrusions	Actin and microtubules	Yes/No	Transmission of intercellular ion fluxes, cell invasion, formation of neuron–glioma synapses	[67,72,73,90]
TNTs	Thin membrane connections, open-ended	Actin, sometimes microtubules	Yes	Exchange of cellular cargo between cells	[6]
Invadopodia	Finger-like membrane protrusions	Actin	No	Matrix degradation	[91]
Podosomes	Dynamic membrane-bound microdomains	Actin	No	Adhesion, mechanosensing, and matrix degradation	[92]

the same or different cell types in cell cultures using light microscopy [34], their identification in a more complex context, such as animal models or tumor resections, is still challenging. This is because no specific marker for these structures has been identified yet, and the optical resolution of classical microscopy does not allow for the morphological characterization of these connections in a tissue environment [5,12]. Therefore, the heterogeneity and lack of structural characterization of TNTs represent major problems for their investigation. Given their morphological heterogeneity and poor molecular and structural characterization, the intercellular connections observed to date have been named differently in different studies (nanoscale conduit [11], tunneling nanotubes [22], intercellular bridges [12] ,or membranous tunneling tubes [24]). This has raised both confusion and skepticism in the field [35], and calls out for both more rigorous definition and more accurate technical approaches to study them. We propose that 'TNT' should only refer to the connections that fulfill the following characteristics: (i) continuous membrane connections with the plasma membrane of the connected cells; (ii) nonadherent to substratum; (iii) containing actin; (iv) proven cargo transport; and (v) open-ended (Table 1). By contrast, we refer to 'TNT-like' connections when one or more of these properties is not fulfilled or has not been assessed.

The first documentation of TNT-like structures *ex vivo* in solid tumors was provided by the laboratory of Emil Lou in 2012, which described mitochondria-containing connections in tissue sections of a mesothelioma resected from a patient [22]. These observations were followed by others, showing various intercellular connections in squamous cell carcinoma [24,25], ovarian

Table 2. Tumor Cell Models Used for the Study of TNT-Mediated Communication *In Vitro*

Tumor model	Cargo	TNT function	TNT regulators[a]	Year of publication	Refs
Rat pheochromocytoma cell lines	Lysosomes, soluble and membrane markers	n.d.	n.d.	2004	[4]
HeLa (cervical cancer)	Calcium	n.d.	M-Sec	2009	[46]
Mesothelioma cell lines and primary human mesothelioma cells	Golgi vesicles, mitochondria, fluorescent proteins	n.d.	Low serum (+), hyperglycemic (+), acidic medium (+), EMT-inducing cytokines (+), metformin (–), everolimus (–), latrunculin A (–)	2012	[22]
Ovarian and breast cancer cell lines	Cytoplasmic content, mitochondria	Mitochondria transfer from stromal cells promotes chemoresistance	n.d.	2013	[30]
Osteosarcoma and ovarian cancer cell lines	miRNA	Spreading of genetic and oncogenic material between tumoral–tumoral and tumoral–stromal cells	Low serum and hyperglycemic medium (+)	2014	[23]
Mesothelioma cell lines	n.d.	TNT correlates with more aggressive phenotype and expression of genes related to invasion and metastasis	Low serum and hyperglycemic medium (+), migrastatin (–)	2014	[10]
Head and neck squamous cell carcinoma primary cells	Mitochondria and nucleic acids	Electrical coupling	n.d.	2014	[24]
Primary rat astrocytes and glioma cell line	Mitochondria	Support in glioma cell proliferation	H_2O_2 (+), latrunculin A (–)	2015	[13]
Metastatic breast cancer cell lines	miRNA	Transfer of miRNA alters phenotype of receiving endothelial cells. TNT correlates with more aggressive phenotype	Docetaxel (–), latrunculin A (–), cytochalasin D (–)	2015	[11]
Pancreatic adenocarcinoma cell lines	Electron-dense particles	n.d.	Radiofrequency treatment (+)	2015	[56]
Rat pheochromocytoma cell lines	Mitochondria	Rescued UV-treated apoptotic cells	Cytochalasin B (–)	2015	[60]
Ovarian cancer cell lines (different chemoresistances)	Mitochondria	Adaptation mechanism to hypoxia in chemoresistant cells	Hypoxia (+)	2016	[42]
Head and neck squamous cell carcinoma cell lines	Lysosomes, mitochondria, autophagosomes	n.d.	MMP2, FAK	2017	[25]
Bladder cancer cell lines	Mitochondria	Mitochondria transfer promotes invasiveness	n.d.	2017	[37]
Acute myeloid leukemia primary cells	Mitochondria	Mitochondria transfer from bone marrow supports cancer cell metabolism and promotes stress-adaptative response	NOX2	2017	[28]
Pancreatic adenocarcinoma and ovarian cancer cell lines	Doxorubicin	Redistribution of drug	Doxorubicin (+)	2018	[26]
Acute lymphoblastic leukemia cell lines and human primary T leukemic cells	Mitochondria	Mitochondria transfer promotion of chemoresistance	Cytochalasin D (–), MTX (-)	2018	[27]
Colon cancer cell lines	n.d.	Transfer of oncogenic protein (mutated KRAS) and activation of Erk pathway in acceptor cells	KRAS	2019	[45]
Breast cancer cell lines	Membrane and/or vesicles	Transfer between macrophages and tumor cells inducing invasiveness	M-Sec	2019	[31]

Table 2. (continued)

Tumor model	Cargo	TNT function	TNT regulators[a]	Year of publication	Refs
Prostate cancer cell lines	Lysosomes, mitochondria, stress-induced chaperones	Adaptation mechanism therapeutic stress	Chemotherapy by androgen receptor blockade (+), low serum, hyperglycemic, acidic medium (+), hypoxia (+), cytochalasin D (–)	2019	[43]
Chronic myeloid leukemia cell lines	Protein-containing vesicles	Protein transfer from stromal cells provides protection to leukemic cells	n.d.	2019	[38]
Patient bone marrow cells and multiple myeloma-derived cell lines	Mitochondria	Mitochondria transfer from bone marrow supports cancer cell metabolism and promotes stress-adaptative response	CD38, Chemotherapy by bortezomid (+), cytochalasin B (–)	2019	[29]
Bladder cancer cell lines	miRNA	Induction of invasive and proliferative phenotype	n.d.	2019	[53]
GBM cancer cell line	Functionalized liposomes	Delivery of nanoparticles	n.d.	2019	[76]

[a](+), induced; (–), inhibited; n.d., not described.

[23] and pancreatic cancer [26], and human glioblastoma (GBM) cells engrafted into mice models [36] (Table 2). Little is known about the structural and functional features of these connections *in vivo*. In some cases, however, the presence of mitochondria and possibly other cargoes inside them supports the hypothesis that these structures are open-ended and, thus, are canonical TNTs and allow the transfer of cargoes.

Morphology and Structure of TNTs

Despite the lack of a specific marker, TNTs can be identified in cell culture by fluorescent labeling of the plasma membrane and cytoskeleton components, observed by using light microscopy (Figure 1B). However, specific fixation protocols are needed to preserve their delicate and fragile nature [34], and functional assays have to be performed in addition to morphological studies to fulfill the definition of TNTs (see earlier). TNTs exhibit high variability in their morphology, in terms of length, thickness, and cytoskeleton content, specifically regarding the presence/absence of microtubules [34]. Nevertheless, they always appear as actin-based connections and their presence and functionality can be affected by inhibitors of actin polymerization (e.g., latrunculin or cytochalasin) (Table 2). In cancer cellular models, the observed connections can range from tens to several hundreds of microns [10,11,25]. In some tumor tissues, exceptional connections >500 µm have been observed [24,36]. Although in most *in vitro* studies, the diameter of the connections in tissues was on the nanoscale (<1 µm), microscale connections (>1 µm) [24,36] were also present. However, these long and thick connections fit best with the definition of tumor microtubes rather than of TNTs (Table 1). At present, we do not know whether TNTs display different morphologies *in vitro* or *in vivo* or whether nanoscale connections are detectable in the complexity of the tissue. The thickness of TNTs also correlates with their cytoskeleton content, with protrusions containing microtubules having larger diameters [9]. However, some cancers appear to present both types of connection: those containing only actin and those with both actin and microtubules [11,25].

A few studies have addressed the ultrastructure of TNTs in cancer models with the use of electron microscopy [37,38]. A deeper structural analysis of TNTs, using a combination of cryo-fluorescence microscopy with cryo-electron microscopy, was conducted recently. This study used a catecholaminergic differentiated (CAD) cell line, established from a brain tumor in a transgenic mouse, and SH-SY5Y cells, isolated from a patient with neuroblastoma [5]. By using

experimental conditions set up to better preserve TNT structure, this study showed that, in these two types of neuronal cell line, TNTs can comprise multiple individual tubes (named iTNTs) held together by N-cadherin-positive structures and often open-ended at their tips [5]. Nonetheless, whether iTNTs exist in different cell types and tumors and/or *in vivo* remain open questions.

Functional Approaches

The distinguishing characteristic of TNTs with respect to other cellular extensions (e.g., filopodia or mitotic bridges; Table 1) is their ability to transfer cellular material. Some research has provided qualitative evidence of cargoes inside TNT-like structures observed in different cancers [22,37], without proving that actual transfer had occurred and without excluding cell division as the mechanism by which the cellular material was shared. To exclude the latter, membrane vesicles or organelles, such as mitochondria or lysosomes, can be labeled in a population of cells defined as donors. This population is then co-cultured with an acceptor population (differently labeled) to further detect and quantify the cargoes transferred in the acceptors by fluorescence microscopy (in fixed or live condition) or flow cytometry [34]. The co-culture has to be performed placing the two populations in direct physical contact at an appropriate cell density that favors the formation and detection of TNTs. To evaluate secretion as a possible mechanism of transfer, the two populations can be separated by a filter that allows the transfer of secreted material, or they can be grown in different dishes and the acceptor population challenged with the supernatant from donor cells [34]. The weakness of this approach is that it only allows the direct transfer (cell contact mediated) of the labeled cargo to be determined. It does not consider other materials that could be transported through the same connections, including those that could be shared in the opposite direction. To overcome this limit, other approaches, such as mass spectrometry [38] and transcriptomic analysis [11], have been recently applied to detect alterations at the proteome and transcriptome levels. In these examples, the acceptor population acquired protumoral features correlated with the transfer of proteins or miRNA involved in cell survival, drug response, or cellular reprogramming. All these approaches show how TNTs might be differently exploited in various types of cancer (Table 2). However, we still do not know whether the variability observed at the TNT level in the various studies and in the various cancers corresponds to different roles for TNTs in the cancers or just to the different questions addressed.

Few approaches have studied the dynamics and transfer ability of these structures *in vivo*. Using multiphoton microscopy, connections between human tumor cells were detected in mouse xenografts [36] (Table 3), while the transfer between human and murine cells was quantified by amplification of species-specific DNA sequences or detection of labeled material by flow cytometry [11,28,29]. Although powerful and of interest, these approaches make it possible to monitor the transfer without specifically identifying its mechanism, in particular without excluding the secretion mechanism.

In conclusion, due to the limitations of the *in vivo* models (e.g., TNT preservation and observation), the field needs to pursue the study of these fragile structures in cellular models that are representative as much as possible of the tumoral tissue (e.g., patient-derived cells); this would enable researchers to address more easily specific questions on the mechanism and content of the transfer and its impact on the receiving cells. In parallel, new tissular models recapitulating the tumoral context as tumor-derived organoid cultures need to be implemented in the field. Finally, additional efforts need to be made to overcome the technical limitations of the *in vivo* study of TNTs to finally unravel their role in physiopathological contexts beyond their morphological diversities.

Tumoral Context Might Favor TNT Connectivity

Since their discovery, TNTs have been described as a mechanism of adaptive response to cellular stress. Interestingly, several cancer-related environmental conditions have been shown to

Table 3. Evidence of TNT-like Communication in Tissue

Cancer	Model	Labeling	Year of publication	Refs
Malignant pleural mesothelioma and lung adenocarcinoma	Patient tissue	Mitochondria	2012	[22]
Ovarian cancer	Patient tissue	Mitochondria	2014	[23]
Osteosarcoma	Murine orthotopic model of osteosarcoma	Mitochondria	2014	[23]
Head and neck squamous cell carcinoma	Patient tissue	F-actin, mitochondria	2014	[24]
Glioma	Mouse tumor xenograft from primary stem cells	Cytosolic GFP expression	2015	[36]
Head and neck squamous cell carcinoma	Patient tissue	Actin, tubulin	2017	[25]
	Mouse tumor xenograft from cell line	Actin, tubulin	2017	[25]
Acute myeloid leukemia	Mouse tumor xenograft from human leukemic cells	Mitochondria	2017	[28]
Glioma	Mouse tumor xenograft from primary stem cells	Cytosolic GFP expression	2017	[67]
Pancreatic adenocarcinoma	Patient tissue	Mitochondria	2018	[26]
Developing human telencephalon and human GBM	Patient tissue	Collagen IV	2018	[54]
Multiple myeloma	Mouse tumor xenograft from cell line	Mitochondria	2019	[29]

stimulate their formation. Reactive oxygen species (ROS), known to be intensively produced by cancer cells [39], have been shown to induce TNT formation in different contexts, including cancer [8,13,20,29,40] (Table 1). Moreover, treatments such as chemo and radiotherapy induce ROS production [41]. Hypoxia, typical of the denser tumor regions, has been found to be a TNT inducer in ovarian [42] and prostate cancers [43]. Interestingly, other conditions mimicking the TME *in vitro* stimulate TNT-mediated communication, such as acidic pH, hyperglycemia, serum deprivation [22,43], and exposure to tumor necrosis factor (TNF)-α normally produced during inflammation [44]. Finally, different signaling pathways that are often dysregulated in cancer have been shown to be involved in TNT formation, such as PI3K/Akt/mTOR [37,40,42,43], K-RAS [45] ,and p53 [13,40]. These signaling cascades could activate downstream proteins, such as M-Sec in the case of immune cells [46], which are involved in actin remodeling and polymerization and have been shown to induce TNT formation [47]. Altogether, these findings suggest that the tumor context, globally experienced as a stress by cells, provides the conditions that favor TNT formation and communication. In turn, we can speculate that this route for intercellular communication allowing cells to share material may result in a beneficial effect for the connected cancer cells, as described in the following sections.

Roles of TNT in Cancer Progression

The ability of cancer cells to interconnect among themselves is correlated with more aggressive cancer phenotypes. For example, in ovarian and breast cancers, highly malignant and metastatic cells are more prone to interconnect in tumor networks than their less aggressive counterparts [10,11]. Also, in gliomas, where for the first time tumors have been described as a functional intercommunicating network, there is a correlation between extended interconnectivity and the most aggressive grades of tumors and their poorer therapeutic outcome in response to radiotherapy [36]. However, the mechanisms of treatment resistance have not been fully elucidated yet. Different cancers could be applying different strategies to protect themselves from the

therapeutic attempts and eventually a unique mechanism may be determined. Here, we review the possible roles of TNT-like connections in different types of cancer and how they affect cancer progression. We then focus on the specific example of GBM.

TNT-Mediated Transfer Can Promote Aggressive Features

TNTs appear to drive the acquisition of aggressive features in the receiving cells through the transfer of different cellular materials. As we will see, cells may use TNTs as a route to remove dangerous material (Figure 2A,C). Another possibility is that the uptake of cellular material, such as miRNA, mitochondria, or other sets of proteins, might drive phenotypic modifications of the recipient cells (Figure 2A,B).

In breast cancer, TNT-mediated contacts from cells of the TME, such as macrophages, appear to drive the acquisition of an invasive phenotype in the cancer cells [31]. Although it is not clear how

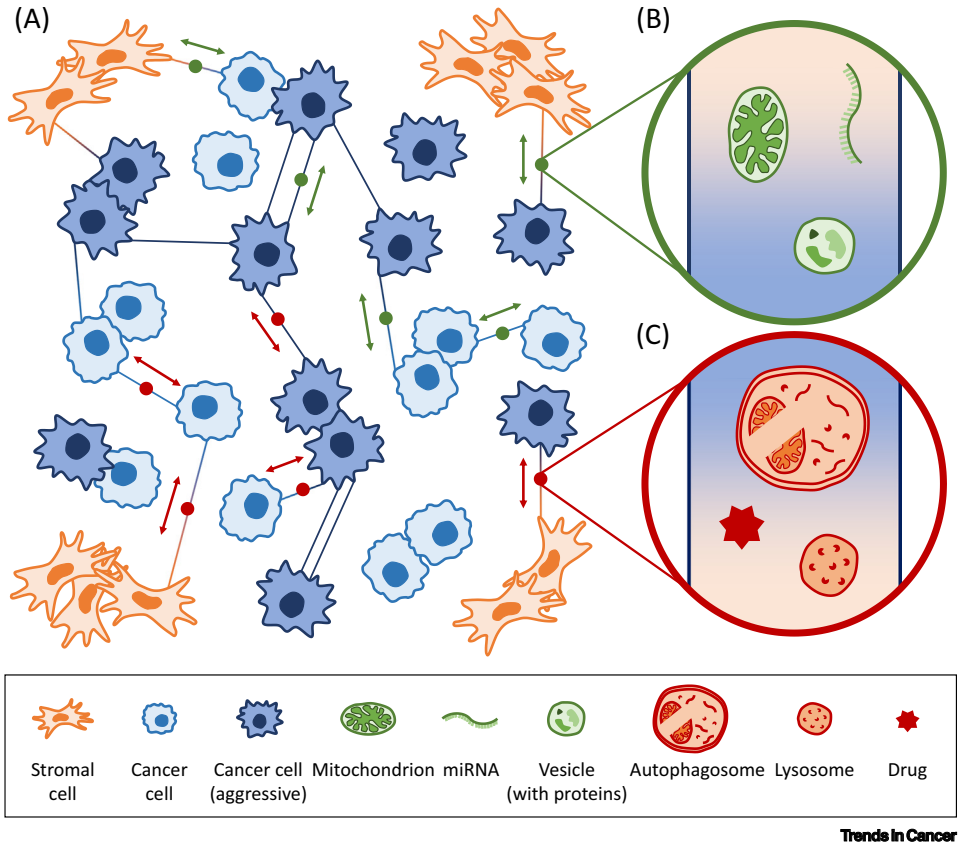

Figure 2. Schematic of a Tunneling Nanotube (TNT)-Based Network in Cancer. (A) Cancer cells with different states of aggressiveness coexist and interact via TNTs. Aggressive cancer cells (dark blue) display higher interconnection rates than their less aggressive counterparts (light blue). Cancer cells are surrounded by stromal cells (red) to which they also communicate through TNTs. The homotypic or heterotypic connections between these cell types can be used to share oncogenic content (green circle) or to remove material to degrade (red circle). (B) Magnification of oncogenic cargoes traveling along the connection providing protumoral features in the receiving cell and healthy lysosomes. Acquisition of mitochondria can promote chemoresistance and invasiveness and provide metabolic help in stress-induced conditions. Transfer of miRNA can drive modifications in the phenotype of recipient cells, leading to a more aggressive phenotype. Moreover, cellular vesicle content can impact the proteomic profile of the receiving cells and change their ability to respond to treatments. (C) Different materials discarded by a cell through TNTs. Organelles used for degradation, such as autophagosomes and lysosomes, might be transferred via TNTs as a clearing mechanism. TNTs could also be used as a route for the redistribution of drugs, which would otherwise be toxic in high concentration.

this contact could induce this phenotypic switch, mitochondria appear to be good candidates for transferred cargo that could induce invasiveness. In fact, breast cancer cells have been shown to be able to receive mitochondria from mesenchymal cells (MSCs) through TNT-like structures [30]. Furthermore, the uptake of isolated mitochondria derived from MSCs, by a protocol defined as MitoCeption, was able to induce migratory ability and cellular proliferation [48]. Many studies have shown TNT-mediated mitochondria transfer to be possible [24,25,30,49]; however, the possibility that mitochondria could be transferred through the supernatant should be considered, given that research has suggested that the mitochondria could be released and taken up by neighboring cells [50]. Transfer of mitochondria has also been found to restore tumorigenic potential in cells devoid of mitochondrial DNA [51,52], although these studies did not address the mechanism of mitochondrial transfer. Furthermore, TNT-mediated traffic of mitochondria was correlated with increased invasiveness in bladder cancer [37]. Here, different cancer cell lines in co-culture could exchange functional mitochondria with each other, possibly stimulating the migratory capacity of the acceptor cells, as assessed by *in vitro* assays. Furthermore, their ability to form larger tumors with a higher vascularization index was stimulated when implanted in nude mice. In a second study, additional evidence suggested that the acquisition of these protumor properties is due to TNT-mediated transfer of miRNA from the most aggressive to the least aggressive cells, leading to the activation of the Deptor-mTOR signaling pathway, an important downstream mediator of cancer cell proliferation and motility [53].

Endothelial cells (ECs) have a critical role in physiological and tumoral vascularization and their angiogenic potential might be regulated by TNT-mediated interactions. TNT-like connections sprouting from ECs or pericytes have been identified in sections of developing human cerebral cortex and human GBM, two contexts in which the process of vascularization is intensively active [54]. Moreover, ECs experiencing chemotherapy stress are able to receive mitochondria from MSCs via TNT connections and this transfer could rescue the damaged cells, promoting cell proliferation and restoring migratory and angiogenic abilities [55]. Furthermore, elegant work by Connor and colleagues [11] showed that TNT-mediated transfer from metastatic cancer cells to ECs can induce an alteration of the miRNA profile of the receiving cells. This work showed for the first time TNTs as a route for the dissemination of oncogenic material that resulted in reprogramming of the ECs. Altogether, the current evidence suggests that TNT-mediated transfer of mitochondria and mRNA stimulates invasiveness, proliferation, and angiogenic ability.

TNTs Can Support Therapy Resistance

Intercellular communication through TNT-like structures and resistance to therapies appear to be tightly correlated. As for the other cancer features that might be driven by contact-mediated transfer of cargoes, TNT-like structures may provide a way for distributing harmful substances and cellular wastes, or sharing defensive tools against treatment, such as mitochondria, miRNA, and specific factors (Figure 2). TNT-mediated communication appears to be stimulated by radiotherapy, which causes free radical production, known to be a TNT inducer [41], and by radiofrequency treatment [56], and chemotherapy [43]. A recent study in prostatic cancer showed that chemotherapeutic blockage of the androgen receptor, which induces metabolic stress, enhanced TNT-like structure formation [43]. Disrupting these connections by cytochalasin D sensitized prostatic cancer cells to treatment-induced cell death, suggesting that the presence of this stress-induced network favors cancer cell survival upon treatment. In this study, lysosomes, mitochondria, and stress-induced chaperones were observed inside the TNT-like structures. Therefore, it is possible that transferring these cellular components benefits stressed cells. Conversely, TNT-like structures could be used as a way to remove damaged organelles or autophagosomes [25] and possibly other dangerous substances, such as ROS, produced in response to treatments, or the drugs themselves (Figure 2C). Transfer of a soluble drug via

TNT-like structures has also been observed in both pancreatic and ovarian cancer cellular models [26]. Here, multidrug-resistant cell lines use TNT-like connections to redistribute doxorubicin from chemoresistant toward chemosensitive cells, leading to cell death of the latter and enrichment of the therapy-resistant population. Although the possibility of using TNT-like structures as a drug outflow pathway must be considered, there are currently no quantitative data supporting the actual relevance of this mechanism *in vivo*. Also, this work raises questions over the specificity of the transferred materials through TNTs, and whether this occurs through an active or passive mechanism of redistribution.

As mentioned earlier, TNT-based networking allows the exchange of 'defensive tools' against treatment (Figure 2A). The transfer of mitochondria has been shown to modulate the response to treatments in a beneficial manner for the recipient cells [49,57,58], impacting their cellular metabolism [48,58], rescuing their aerobic respiration [59], and providing metabolic support against treatment-related stress [58]. This was first observed in PC12 cells, where delivery of healthy mitochondria through TNT-like structures from untreated to UV-injured cells protected the latter from apoptosis [60]. This rescue mechanism is also applied by MSCs to chemotherapy-treated ECs [55]. Both MSCs and ECs have been found to transfer mitochondria to cancer cells of different origins, resulting in an improved resistance to doxorubicin in the cells that received the transfer [30]. This mechanism appears to be critical in different forms of leukemia. Leukemic cells, engrafted in murine bone marrow, were able to obtain and receive mitochondria from stromal cells with an impact on cancer cell metabolism [27,28,58], cell proliferation [58,61], and chemoresistance [27]. The disruption of this transfer increased the sensitivity of the cancer cells to various chemotherapies [27]. This suggests that MSCs have a protective role toward tumor cells by eliminating the damaged mitochondria they receive, thereby stabilizing the homeostasis of the cancer population, and possibly providing metabolic support. Moreover, chemotherapy-induced ROS production can enhance mitochondria transfer [28], again suggesting mitochondrial transfer as a mechanism for adaptation to treatment. Interestingly, the inhibition of CD38, previously described to promote mitochondrial release from astrocytes [50], could prevent the contact-mediated mitochondria transfer from MSCs to leukemic cells, resulting in increased apoptosis of the leukemic cells and improved mouse survival [29]. This opens the possibility of specifically targeting mitochondria transfer at the clinical level. Following this evidence, others have assessed the communication between stroma and leukemic cancer cells. Mass spectrometry was used to reveal the transfer of specific factors, such as stress-induced chaperones, together with cellular vesicles, with a potential role in survival and adaptation [38]. Other cargoes, such as miRNA, can be transferred between cells, leading to the acquisition of therapy resistance. Thayanithy and collaborators [23] showed that the transfer of miR-19 and miR-199a occurred in heterotypic connections between different cancer cell lines of the same tumor: osteosarcoma and ovarian cancer, respectively. Specifically, miR-199a appears to be differentially expressed in chemosensitive and chemoresistant cells, suggesting that the transfer of this particular miRNA drives treatment-resistant features in the receiving cells. Thus, TNTs could be a beneficial feature for cancer cells, and the ability to exploit this efficient route of communication may be positively selected during treatment.

GBM: An 'Exemplary' or 'Peculiar' Case of TNT-Like Network?

Among the deadliest types of cancer, GBM stands out for its aggressiveness and resilience in response to treatment. GBM is the most undifferentiated and invasive cancer within the gliomas and is classified as a grade IV tumor. Surgery followed by chemo and radiotherapy is insufficient to eradicate completely cancer cells from the brain, although the mean survival of patients increases from less than 1 year to ~15 months [62,63]. Currently, no treatment is effective in preventing cancer relapse and the reasons for therapy failure are poorly understood. Some

studies correlate the occurrence of relapse with elevated intratumoral heterogeneity: distinct molecular profiles coexist and exhibit differential therapeutic responses [64]. In particular, GBM stem cells (GSCs) have been found to be the most resistant to treatments and likely are at the origin of relapses [65]. Moreover, treatments can positively modulate tumor heterogeneity by inducing cellular plasticity and transdifferentiation [66].

As outlined earlier, during the past few years, various studies have supported the possibility that intercellular communication through cell–cell connections are a critical mechanism for treatment failure and tumor relapse. GBM is the first case where a functional and resistant network among cancer cells has been described in an *in vivo* model [36]. Specifically, GSCs from patients with different grades of glioma were implanted in nude mouse brains, where they developed a multicellular and communicative network. In this study, Winkler and collaborators demonstrated that highly interconnected tumors, which corresponded to higher malignant grades of the original tumor, were more resistant to irradiation [36]. Cancer cells were able to propagate ion fluxes by long and thick membrane protrusions, containing both actin and microtubules, which the authors termed 'tumor microtubes' (TMs) (Table 1). Moreover, the same authors suggested that TMs are essential for driving the repopulation of a surgically resected area in GBM mouse models [67] (Figure 3). The formation of TMs appears to be dependent on the expression of connexin 43 (Cx43), a monomeric component of GAP junctions, and growth-associated protein 43 (GAP-43), a crucial protein for neurite formation, regeneration, and plasticity [36]. When Cx43 or GAP-43 were knocked down, the number of TMs decreased and the sensitivity to radiotherapy increased. Cx43 is a known regulator of the intracellular concentration of Ca^{2+} [68] and it has been also described to have a critical but controversial role in GBM progression, acting both as tumor suppressor and tumor inducer, promoting growth, cell migration, and resistance to apoptosis [69]. Interestingly, Gerdes and colleagues [70] reported earlier that a subset of TNTs observed in kidney-derived cells contained Cx43 forming a hemi-connexon or a GAP junction at their tip. It was also proposed that GAP junctions could mediate the transfer of electrical signals in electrically coupled TNTs [6]. Nonetheless, the presence of GAP junctions along TNT connections would not allow the transfer of any cargo of a size superior to their pore size (1 kDa) [71], such as organelles or macromolecules. In the case of TMs, the authors did not report the transfer of conventional TNT cargoes, such as mitochondria or vesicles, within their lumen, although they did observe nuclei traveling along these connections from a healthy cell to a cell damaged by the treatment [36]. In addition, TMs display neurite-like features, because they have been described to be postsynaptic targets for the surrounding neurons. Indeed, axons can dock onto TMs and generate synchronized calcium transients in glioma networks via AMPA receptors [72,73], Furthermore, depolarization of the postsynaptic glioma cells promoted TM-dependent proliferation [73] and invasion [72].

Overall, the nature of TMs and the mechanisms at stake in this cellular network still need to be unraveled. As for their morphological appearance and physical properties, TMs are very different from TNTs because they are not open-ended, they are much thicker (1.7 μm on average), more stable in time [74], and contain both actin and microtubules, thus resembling more of a neuritic extension than TNTs [75] (Table 1). Nevertheless, direct cell–cell communication appears to have a key role in the resistance to treatment in GBM and growing evidence suggests that the transfer of cargo mediated by open connections contributes to tumor progression, as shown previously in other cancer forms. A few *in vitro* studies suggest that GBM cells are capable of transferring cellular material through thinner TNT-like structures. U-87 and U-251 cell lines, common GBM cellular models, can form TNT-like structures [76–78] (Figure 1), and their formation can be increased in response to external stimuli, such as protein aggregate uptake or cocaine administration [77,78]. Moreover, preliminary studies show that communication between

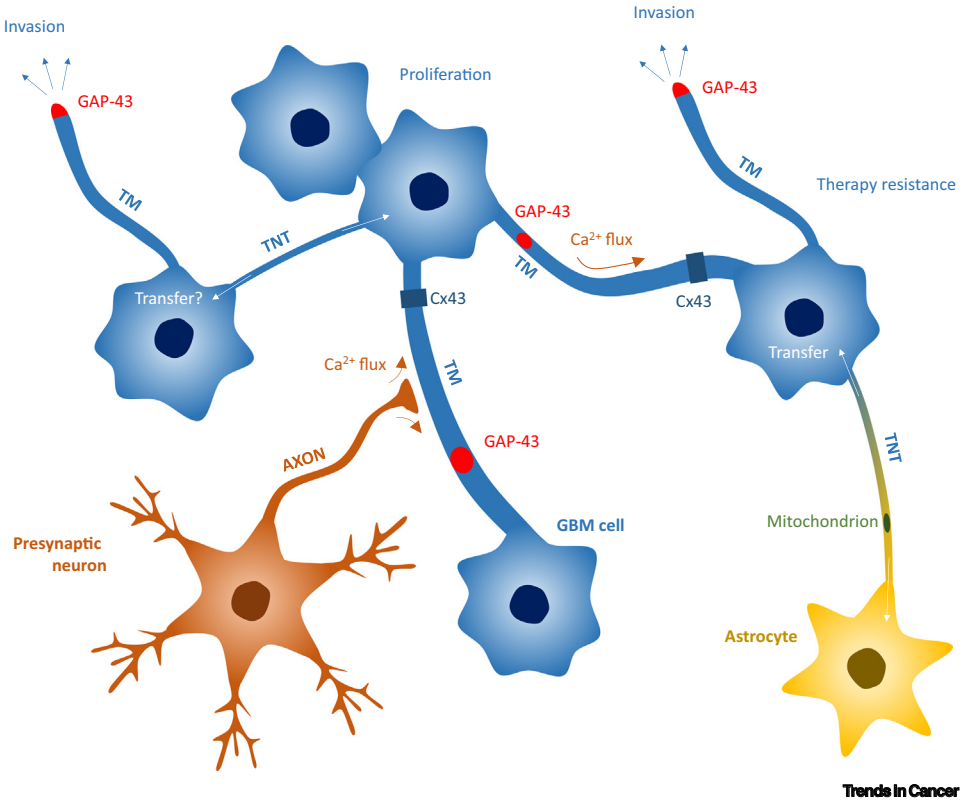

Figure 3. Schematic of a Glioblastoma (GBM) Network and Different Types of Intercellular Connection. GBM cells (blue) interconnect forming a functional network comprising different types of connection. Thick (>1 μm) protrusions (tumor microtubes; TMs) connect GBM cells and contain both Connexin 43 (Cx43) and growth-associated protein 43 (GAP-43), which regulate Ca^{2+} flux along the network. Thinner (<1 μm) TNT-like connections are present between GBM cells and may allow the transfer of material. GBM cells also form TMs that do not contact other cells and are able to drive cell invasion in a GAP-43-dependent manner. Presynaptic neurons (orange) extend axons that appose onto TMs and regulate the Ca^{2+} flux along the GBM network, promoting cell invasion and cell proliferation. Astrocytes (yellow) of the tumoral brain environment can communicate with GBM cells through TNT-like connections and transfer mitochondria to the tumoral cells, eventually affecting the behavior (e.g., proliferation and response to treatments) of the receiving cells.

astrocytes and glioma cells, known to facilitate cancer progression [79], can occur through TNT-like structures [13,76] and the transfer of mitochondria appears to modulate GBM cell abilities in favor of a more proliferative [13] and drug-resistant state [80]. However, the study of intercellular exchange of material needs to be elevated in more complex and representative tumor models. The fact that GBM cells were able to form a network in mice xenografts, but failed in forming connections when cultured *in vitro* [67], suggests that TMs exist only in the *in vivo* condition. It is possible that GBM networks comprise several types of connection that vary in size and properties: open-ended TNTs, synaptic-like connections ,and/or thick GAP junction-linked protrusions, such as TMs (Figure 3).

Concluding Remarks and Future Perspectives

Over the past decade, growing evidence has supported the existence and importance of intercellular communication based on TNT-like connections in various tumors. Several cancer cell types have been shown to grow such connections and communicate through them in culture, and similar structures have been found in tumor sections [22], proving their existence in real tumors. Different studies have described TNT-like structures with diverse morphologies and characteristics; therefore, the ability to transfer cellular material has been used to define them functionally rather than structurally.

Outstanding Questions

Are TNT-like structures a common feature in all cancers?

Does the structural diversity observed in TNT-like structures *in vitro* and *in vivo* correspond to different roles in cell–cell communication?

What other cellular materials are transferred through TNTs beyond those detected by specific labeling?

What are the molecular mechanisms that drive phenotypic modification following transfer of cellular content?

Cancer is one of the few contexts where TNTs have been functionally described, whereby the transfer of cellular cargoes has been shown to have an impact on the behavior of the recipient cells and lead to further development of the disease. However, fundamental questions remain regarding the structural diversity of the different protrusions, as well as the molecular determinants and the signaling pathways that would stimulate their growth in cancer cells compared with noncancer cells.

Until now, the outcome of the transfer has been more often addressed as impacting predetermined features. For example, studies have investigated whether the unilateral transfer of a specific tagged cargo affected the migratory capacity or angiogenesis of the recipient cells. The observation of a specific cargo transfer does not necessarily implicate a role for that specific cargo, since other material, not detected because it is nonlabeled, could be transported through the connections and lead to changes in the partner cells. Few studies have addressed the question globally, designing experiments to study the alteration induced by the transfer in the receiving cells at the transcriptomic [11] or proteomic [38] level. Even less work, if any, has addressed the changes under the assumption that bilateral transfer could occur and modify the fate of each one of the two connected cells. Moreover, the mechanisms by which the transfer of cargoes mediated by TNTs impacts the migratory or angiogenic ability of the cell remain largely unknown. In the case of resistance to treatments, the acquisition of cargoes, such as mitochondria and miRNA, could be the direct cause of enhanced regrowth potential [51,52] or transcriptomic reprogramming [23], respectively, leading to the establishment of a more resistant phenotype. In other cases, the treatment itself appears to induce TNT-mediated communication, which probably acts as the mechanism in response to the induced stress [29,55], protecting the cells from the induced damage. Overall, the ability of certain cancer cells to exploit TNTs as mechanisms of communication might be positively selected during treatment, favoring such cells to become the majority (see Outstanding Questions).

To address the complexity of the real pathology and also the diversity of TNT-like connections, the use of models representative of the tumor environment is required. Many of the studies reported here were carried out in cell lines *in vitro.* Only more recent work has addressed the study of TNT-like structures with the use of patient-derived xenografts in mice. Based on current knowledge, it appears that blocking TNT-like connectivity could be a promising strategy to fight cancer, eventually hindering cancer progression and sensitizing tumor cells toward treatments. A couple of drugs have been described as being able to specifically inhibit TNT formation in cell culture [81,82], but these need to be tested in cancer mouse models. Conversely, TNTs have also been used as a route to diffuse therapeutics, such as drugs [26] and nanoparticles [76], affecting predominantly the network of connected cancer cells. Certainly, a deeper understanding of TNT-based communication is critical for a better comprehension of cancer progression and treatment resistance, and, in future years, this knowledge could lead to the development of new, more effective therapies.

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