VIEWPOINT

Lost in translation: applying 2D intercellular communication via tunneling nanotubes in cell culture to physiologically relevant 3D microenvironments

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Tunneling nanotubes (TNTs) are membranous conduits for direct cell-to-cell communication. Until the past decade, little had been known about their composite structure, function, and mechanisms of action in both normal physiologic conditions as well as in disease states. Now TNTs are attracting increasing interest for their key role(s) in the pathogenesis of disease, including neurodegenerative disorders, inflammatory and infectious diseases, and cancer. The field of TNT biology is still in its infancy, but inroads have been made in determining potential mechanisms and function of these remarkable structures. For example, TNTs function as critical conduits for cellular exchange of information; thus, in cancer, they may play an important role in critical pathophysiologic features of the disease, including cellular invasion, metastasis, and emergence of chemotherapy drug resistance. Although the TNT field is still in a nascent stage, we propose that TNTs can be investigated as novel targets for drug-based treatment of cancer and other diseases.

Introduction

The role of intercellular communication has been studied extensively for decades in both normal and abnormal tissue environments. Investigation of endogenous nano-sized delivery vehicles such as exosomes and microvesicles has further expanded our knowledge of delivery mechanisms for intercellular cross-talk [1–7]. Gap junctions and soluble diffusible signals such as cytokines are effective and sufficient for messages that need to be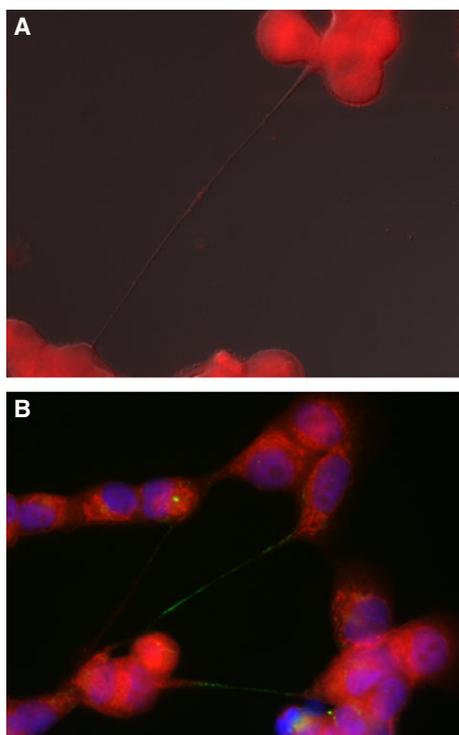 conveyed over relatively short distances [8]. However, for cells physically separated within a biological microenvironment, long-range forms of communication are necessary to literally 'bridge the gap' for such cross-talk to take place. For example, as cancer cells are dispersed throughout the tumor matrix with stromal components, the distance between them may significantly reduce their communication via chemokines, cytokines, or exosomes/microvesicles, especially in dense tumors with high interstitial pressures.

Abbreviation
TNTs, tunneling nanotubes.

Elucidating modes of intercellular communication: tunneling nanotubes as a new and distinct player in the field

Tunneling nanotubes (TNTs) represent a unique and highly efficient candidate to explain how intercellular communication takes places in a spectrum of disease processes, especially in cancer. Structurally, TNTs are membranous cellular extensions that vary in width from 50 to 1000 nm, and in length from a few to several hundred microns [9,10] (Fig. 1). However, these parameters may differ significantly between cell types. The function of TNTs is to act as intercellular conduits for exchange of cargo, including mitochondria, proteins, Golgi vesicles, viruses, microRNAs (miRNAs), and exosomes [9–24]. As such, TNTs serve as cellular 'nano-highways' for mediating transport of cellular organelles and cargo between connected but nonadjacent cells. Extensive use of inverted microscopic and confocal microscopic fluorescent time-lapse imaging has emerged as a staple for laboratories in identifying movement of cargo via TNTs and direct cell-to-cell transfer. Both unidirectional and bidirectional transport have been observed, and may depend on the width and length of TNTs in addition to cell types and cargo being transported [9,25–27].

Whether TNTs form between different cells may ultimately depend on cell types, combinations thereof, and in what context. For example, in our early work, we cocultured cancerous cells from mesothelioma (a malignancy of the pleural lining of the lung cavity) with noncancerous mesothelial cells, and found no communication via TNTs between the two different cell populations (although each cell type formed numerous TNTs among themselves) [9]. However, our subsequent studies did detect TNT formation between different cell types, including cocultures of osteosarcoma (bone cancer) and osteoblasts, and between ovarian cancer and ovarian epithelial cells [24]. Numerous studies in noncancer biology have readily detected TNT formation between different cell types, including cardiomyocyte and mesenchymal stem cells that led to rescue of damaged cells via transfer of mitochondria [28–30]. This finding has been reproduced in several studies and serves as one of the prime examples in the field of how TNT interactions may be time- and context-dependent. Interestingly, while TNTs may form transiently in cell culture over minutes to hours, their stability is variable. In fact, they are highly sensitive to stress factors such as prolonged exposure to light, excessive movement (e.g., shaking of culture plates), and other mechanical stresses [9,25,31]. This sensitivity highlights the difficulty in evaluating the effects of intercellular transfer of cargo mediated by these cellular extensions.

It is probable that future studies will more clearly elucidate the role of TNTs as critical effectors in the pathophysiology of disease(s). Thus, it is reasonable to postulate that disrupting or preventing TNT-mediated communication by targeted drugs or other means will present a novel therapeutic strategy [22,32,33]. However, it would also be critical to identify mechanisms and genetics that underlie TNT biogenesis. To date, there have been few such studies which have been reported in the literature.

Fig. 1. Representative example of tunneling nanotubes connecting cancer cells (malignant pleural mesothelioma). Images were taken using a Leica TCS SP5 microscope with 40 ×/1.25 NA oil objective lenses. (A) Two malignant mesothelioma cells (MSTO-211H biphasic mesothelioma cell line) connected by a long thin tunneling nanotube that is transporting mitochondria. The mitochondrial cargo is noted halfway along the nanotube as a visible bulge, stained using fluorescent MitoTracker Red. (B) Tunneling nanotubes connecting malignant mesothelioma cells and transporting mitochondria (red stain; MitoTracker Red) as well as Golgi vesicles (green; GM130 antibody). Blue = DAPI staining of nuclei).

The importance of cell cycle in the genesis of TNTs has yet to be explored. Work from our group investigating how TNTs form between cancer cells has shown that factors stimulating or suppressing TNT formation are not entirely dependent on the rate of cellular proliferation [9,34]. Subtypes of TNTs have been described, including most commonly those that extend between cells located at short or long distances. One type forms between cells that are already adjacent or in very close proximity to each other; these cells then track in different directions but leave TNTs (or TNT-like intercellular bridges) behind and intact for a finite amount of time [35]. This is not unlike the abscission that occurs during the process of cytokinesis, which results in intercellular bridges between dividing cells, and/or long connections formed during early cellular development. To distinguish TNTs from these processes, the use of time-lapse imaging is particularly helpful for confirming the provenance of TNTs that form at long-range between distant cells or groups of cells in culture.

Making the transition: moving toward evaluating TNTs *in vivo*

TNTs are novel candidates to explain how direct cell-to-cell communication occurs [22]. Initial experiments from our group consistently demonstrated TNTs *in vitro*, and also in freshly resected and intact malignant mesothelioma and lung adenocarcinoma tumors from human patients [9]. We and others have subsequently demonstrated that TNTs are not exclusive to these two malignancies, but can form between malignant cells from a wide variety of histologic cancers, regardless of the site of origin. Key supportive evidence for TNTs *in vivo* comes also from the field of immunology, where TNTs were imaged in an inflammatory cornea animal model [36].

Current approaches to assessing tumors for clinical evaluation depend on 2D histopathologic evaluation. One of the main questions in the field of TNTs is the physiologic and pathological relevance of these structures. Despite increasing data supporting the role for TNTs as conduits for intercellular transfer of molecular cargo, many existing studies have only visualized TNTs *in vitro*. To demonstrate their presence *in vivo*, we developed detailed protocols for imaging of putative TNTs in human tumors, based in part on our main research focus in the cell biology of highly invasive cancers. The approach fundamentally rested on the need to image these extracellular protrusions as a three-dimensional structure. We propose that reproducible and consistent imaging techniques will be essential to characterizing the role of these structures in cancer, as well as normal tissue.

History, perspectives, and future directions on 3D imaging of TNTs and TNT-like cellular protrusions: progress since identification of TNTs in 2004

Following the identification and report of TNTs in isolated PC12 (pheochromocytoma) cells by Rustom *et al.* more than a decade ago [25], nearly all published studies of TNTs have involved examination of their function in cell culture. Chinnery *et al.* [36] provided the first evidence of membrane nanotubes by using an animal model of inflammation to image dendritic cell nanotubes in an *ex vivo* model of corneal disease. To that point, nanotubes had not been imaged in tumors or in any other context other than in cell culture. Thus, we sought to confirm our *in vitro* findings of TNT formation by imaging these cellular connections in malignant tumors. In 2012, we reported the first evidence of nanotube connections in tumors from human patients with cancer, with initial studies focusing on cancers of the lung and of pleural lining (mesothelioma) [9].

We subsequently detected TNTs in tumors from patients with ovarian cancer [24] and also identified and reported TNTs in murine models of osteosarcoma [24]. Other groups have adopted these imaging techniques to successfully image TNTs in tumors, including laryngeal carcinomas and ovarian tumors, further broadening our understanding of the potential relevance of these structures *in vivo* [37,38]. A recent and elegant study provided the first demonstration of membrane tubes (termed tumor microtubes due to their increased thickness and width compared to nanotubes) in a live, *in vivo* animal model of malignant brain tumors [39]. Furthermore, they also provided clear evidence linking the finding of tumor microtubes to clinical prognosis, as they correlated microtubes to established prognostic factors in gliomas such as the presence or absence of deletions of chromosomes 1p and 19q. A subsequent study used similar confocal techniques for visualizing membrane nanotubes connecting macrophages in muscle tissue *in vivo* [40]. Collectively, these studies provided a body of growing evidence that confocal techniques for visualizing TNTs can be adopted for investigations into the function of TNT-mediated intercellular communication *in vivo* and in real time.

TNTs in cancer: understanding the niche for TNTs in the complex and heterogeneous tumor–stromal matrix

Intercellular communication between cancer cells is crucial to the progression of invasive cancers, but the mechanisms by which communication occurs between distant and proximal cells in a tumor matrix remain poorly understood. Stromal cells may comprise as much as 90% of a malignant tumor, underscoring the need to identify modes of transport between malignant cells not located in immediate proximity [41,42]. Furthermore, cell–cell junctions are disrupted upon epithelial–mesenchymal transition, a potential precursor to metastasis [43,44] (presumably) making intercellular communication via these junctions impossible for separated cells. Thus, in the context of cancer, there is a major gap in knowledge of how intercellular communication occurs between distant and proximal cells in the tumor matrix of malignant tumors [45,46].

Exosomes, microvesicles, and microparticles are increasingly explored as purveyors of long-distance messages, but reliance on diffusion does not allow for a high rate of specificity and certainty in reaching potential target cells. This all occurs in the physiologic context of a 3D tumor matrix that is dynamic and constantly changing. Performing confocal imaging of tumors allows for a 'snapshot' of this dynamic process and serves as a foundation for using similar techniques for *in vivo* imaging to observe intercellular communication occurring in real time. The tools and techniques published to date and reviewed briefly here provide proof-of-principle evidence that nanotubes can be imaged in intact tumors. One next step to determining the pathophysiologic role of TNTs in disease is to determine how to correlate these 3D findings to etiology, progression, and response of these diseases to current modalities of treatment.

Establishing the role of TNTs in human pathophysiology and other diseases

Globally, research on TNTs has encompassed a wide variety of noncancer cell types, including immune-derived cells (dendritic cells and monocytes [47,48], mature macrophages [49,50], T cells [51–53], B cells [26], and neutrophils [54]), other cells of mesodermal origin (kidney cells [55], mesothelial cells [56], endothelial progenitor cells [21], mesenchymal stromal cells [19,57], cardiomyocytes [17]), and cells of

ectodermal origin (neuronal cells [27]), among others. Thus, while TNTs may play a natural role in cell physiology and are thus not specific to cancer, TNTs also represent a newly recognized form of intercellular communication and transfer of signals in cancer [9,22]. Indeed, there has been a steep rise in the number of research and review articles on these structures over the past several years, indicating a growing level of interest in investigating and elucidating their mechanisms, functions, and their general role(s) in the pathogenesis of human disease as well as normal human physiology (Fig. 2).

Investigating roles and therapeutic targeting of TNTs in cancer and other diseases in the context of clinically relevant scenarios

It is now well-established that two-dimensional *in vitro* evaluation of cancer cells is inadequate for accurately assessing cellular behavior in tumors, whether for early stages of cancer, later stages of metastatic cell seeding, and even for testing response during drug screening. Spheroid, organoid, and other 3D models of tumors are under active investigation, and it is imperative that TNT researchers also adapt and develop methods for both *in vitro* and *in vivo* assessment of TNTs. Nonetheless, despite the relative inadequacies of standard 2D *in vitro* culture, critical information can in fact be gleaned regarding TNT formation and behavior in cancer. As basic and simple as it is, the classic scratch or wound-healing assay can be used not only to assess cancer cell invasion but also reveal that TNT formation takes place along the
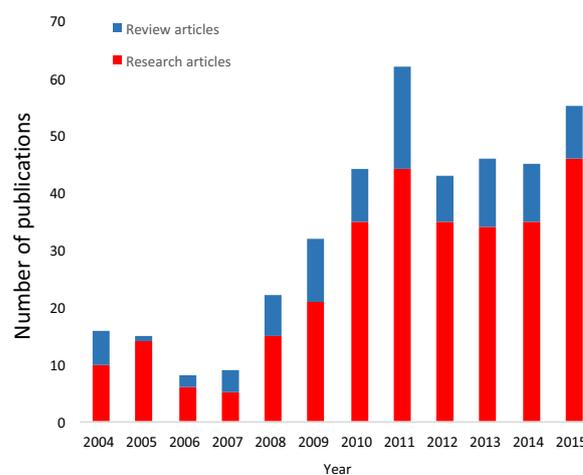
Fig. 2. Number of tunneling nanotube (TNT) or related publications (research and review articles) published since the initial identification of TNTs by Rustom *et al.* [25].

leading invasive edge of malignant cells. Our work using time-lapse imaging discovered this form of cell–cell communication as malignant mesothelioma cells migrated toward each other and proliferated while moving to fill an empty space (Lou *et al.*, Movie S1) [9]. Extending that to real-life clinical scenarios to cancer, we can imagine that the 3D version of this would in fact be microscopic remnant and scattered cells left behind following surgical resection of a malignant tumor. Thus, the findings from a simple scratch assay allowed us to propose three hypotheses: first, TNTs may facilitate progression at the leading edge of invasive tumors. Second, residual scattered malignant cells that remain following definitive surgical resection and chemotherapy treatment may produce TNTs that coordinate cell activity and stimulate recurrence of the treated tumor. Third, malignant cells may harness TNTs as conduits for propagation of resistance to chemotherapeutic agents, via transfer of cellular chemoresistance factors or cargo that increases drug efflux (e.g., p-glycoprotein [58]). Thus with this perspective in mind, we postulate that one strategy for disrupting TNT-mediated intercellular communication would be to use TNT-specific inhibitors in the perioperative period (i.e., for a short period leading up to, during, and immediately following surgical resection of a malignant tumor) [32]. At the present time, the use of continuous peri-operative drug-based treatment is not a routine standard of care.

The maturation of methods for studying TNTs has broad implication outside of cancer research as well. Indeed, it is important to acknowledge that while there are likely heterogeneity and differences in TNTs across various cell types, information gleaned from one form of cells or disease type can potentially be applied to TNTs in other settings. As one example, TNTs have been shown to induce antibiotic resistance in bacteria [59] via acquisition of conjugated plasmids. This finding has a parallel scenario in cancer: cancer cells may also be capable of acquiring genes that induce resistance to chemotherapy through this same, or a very similar, form of TNT-mediated horizontal transfer. An additional example comes from the formation of membrane nanotubes in natural killer (NK) cells, from studies which were done nearly a decade ago. In this case, nanotubes connected NK cells to target cells, facilitating distant target cell lysis via facilitation of contact and movement of target cells toward NK cells [60]. Studies which have demonstrated the propagation of signals for apoptosis, such as caspase proteins [61], via TNTs support the idea that other similar-sized signals could be transmitted as well.

Tunneling nanotubes: a novel drug target for cancer-directed therapy

TNTs are not exclusive to cancer, and are a cellular entity in 'normal', nonmalignant cells. Just as cellular proliferation is increased in cancer—perversion of a normal cellular and physiologic process—TNTs may be significantly upregulated in the setting of malignant tumors [9,10,34]. Thus, it will be important to establish standard procedures and methods for quantitating TNTs in conditions that are similar to the tumor microenvironment *in vivo*. For the purposes of *in vitro* examination of TNTs, we discovered that a low-serum, hyperglycemic medium was useful for stimulating increased formation of TNTs, up to fivefold compared to standard passage medium for the cells we were using (10% FCS, 25 mM glucose RPMI). While TNTs are not specific or unique to cancer, and while nonmalignant cells also form TNTs, the potential formation of TNTs—and establishment of TNTs between malignant and nonmalignant cells—is an active area of exploration in our laboratory. While we initially observed that malignant mesothelioma did not form TNTs to mesothelial cells when cocultured, we have found other instances of malignant–stromal cell interaction via TNTs in other cancers (E. Lou, P. O'Hare, V. Thayanithy, S. Subramanian & C. J. Steer, unpublished data and [24]).

To this end, determining a higher ratio of TNTs in cancer cells compared to stromal cells in the microenvironment would make them a viable target (a 'therapeutic index'). It would be important to determine how increased 'intercellular trafficking' of cellular cargo via TNTs lead to or promotes cell transformation and tumor formation. As an example, previous work from our laboratory established the concept of a 'nanotube index,' in which ratios could be calculated quantifiably demonstrating differences in rate of TNT formation over time among various cancer and stromal cell lines [10,34]. For example, we showed that malignant mesothelioma cells formed significantly more TNTs than noncancerous cells [10]. However, with ovarian cancer cells, there was notable heterogeneity in the rate of TNT formation among cancer cell lines and benign ovarian epithelial cells. Although platinum-chemoresistant cells (C200 and SKOV3 cell lines) were increased over chemosensitive and normal epithelial cells (A2780 and IOSE, respectively), the mean number of TNTs formed per cell was also highest in these latter cell populations [34]. This result could have been due to the sheer number of chemoresistant cells, leading to crowding in culture conditions that simply did not provide enough

room for TNT growth. Most notably, TNTs proliferate most readily in subconfluent cultures. In addition to forming TNTs in coculture, IOSE cells when cultured alone showed significantly more TNTs/cell than SKOV3 cells [34]. These findings supported our hypothesis that TNTs initiate distal tumor–stroma cell interactions, with potential variability as to the source of the initiating cell. Additional studies are needed to identify the underlying mechanism responsible for TNT formation and how it is regulated between cell types.

A novel strategy for cancer therapy and inflammatory diseases: severing the lines of TNT-mediated communication

It will be imperative to identify structural components associated with, or more ideally specific to TNTs in order to identify pharmacologic inhibitors of those components. To date, however, investigation has relied on actin-destabilizing agents as a fundamental structural backbone of TNTs. For *in vitro* study of TNT disruption, commonly available agents such as cytochalasin D or B, latrunculin A, and others have been used [53,62,63]. However, none of these agents is TNT-specific and it will be important to identify potential TNT-specific inhibitors that make pharmacologic sense in the context of each disease. The development of a specific inhibitor that could target TNTs across all cell types with high affinity would, of course, be optimal. For suppression of cancer TNTs, our studies have demonstrated that blocking the mTOR pathway with direct pathway (everolimus) or indirect pathway (metformin) inhibitors was an effective approach in both mesothelioma and ovarian cancer *in vitro* [9,34]. Another pharmacologic approach that efficaciously suppressed TNT formation in mesothelioma was inhibiting the actin-bundling protein fascin with a derivative of the drug migrastatin [9,10,34]. The use of these drugs highlights just a few examples of medically and clinically relevant therapeutics that could be used to block TNT-mediated communication in disease, but which would require further extensive investigation *in vivo*. Agents that disrupt TNT-mediated communication would play a potential role in adjunct treatment of cancer, such as in the postsurgical setting when microscopic remnant cells reconnect with each other in order to reform the malignant tumor(s) [32]. Scenarios in which interruption of such communication would be critical to success include tumor invasion, tumor recurrence, and development of chemotherapy resistance [32].

Conclusions and perspectives

The mechanisms by which cells communicate with one another in the tumor microenvironment are not well understood [2,64,65]. Published and ongoing work in this emerging field challenge the paradigm that gap junctions, exosomes, or cytokines, and other diffusible chemical signals are exclusive modes by which cells mediate intercellular communication. Tunneling nanotubes are a natural biologic conduit for intercellular signaling and transport of cellular cargo. At this time, there appear to be more questions than answers in terms of what the mechanisms and functions of these unique cellular protrusions are in various cell types. However, the maturation of methods for studying TNTs has broad implications for cancer research and also other fields of medical biology. In oncology, it is worth asking the question: if cells can harness nanotubes for apoptosis, why not for proliferation of malignancies? Future work in this field may examine transfer of genetic materials, such as DNA, mRNA, and small RNAs. In at least one published study, mRNA-containing organelles were documented to transfer via what seem to be TNTs (stable cytoplasmic bridges, also called ring canals) joining rat spermatids [66]. Blockade of such transfer would have potential implications for cancer therapy [22]. Our group has demonstrated TNT-mediated intercellular transfer of miRNAs implicated in chemoresistance, including transfer between malignant and stromal cells as well as between malignant cells [24]. Thus, blockade of such transfer would have strong potential implications as a new and likely complementary approach to targeted cancer therapy at the cellular level [22]. The role of TNTs in the pathogenesis of cancer is both exciting and notably will be investigated as the next generation of prognostic markers and novel targets for cancer treatment. We anticipate that the level of enthusiasm for investigation of TNTs will be matched by researchers investigating other forms of disease as well.

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Author contributions

EL wrote the initial draft of the manuscript. All authors edited and approved the manuscript prior to submission.

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