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Commentary

Two routes of direct intercellular communication in brain cancer

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Glioblastoma is a particularly challenging disease characterized by the connection of tumor cells to functional multicellular networks that effectively resist therapies. In this issue of Biochemical Journal, Pinto et al. report the discovery of two distinct classes of intercellular membrane tube connections, tunneling nanotubes and tumor microtubes, in the same state-of-the-art culture model of patient-derived glioblastoma material. These findings contribute to our understanding of the heterogeneity of intercellular membrane tubes in health and disease, and pave the way for future functional studies on their various roles for disease progression and tumor resistance.

The discovery 16 years ago that thin and very long membrane tubes can interconnect individual cells has broadened our understanding of how normal and malignant tissues function [1–4]. In effect, these protrusions allow cells to convene to a communicating syncytial network that is capable of exchange of information, molecules, and partially even cell organelles in a complex and multitude manner [3,5]. While the (patho-)physiological importance of those intercellular membrane tube connections (ICMTCs) is not disputed any more, many questions remain, particularly regarding the coexistence of various subclasses of ICMTCs. A large number of names has been used to describe ICMTCs: tunneling nanotubes (TNTs) [1], membrane nanotubes (MNs) [6], tumor microtubes (TMs) [5], filamentous protrusions/networks [7], cytonemes [8], to name the most frequent. There is increasing evidence that this is not merely a Babylonian confusion of tongues for effectively the same thing, but that there are indeed subclasses of ICMTCs with different morphology and function [2].

In this issue of the *Biochemical Journal*, Pinto et al. [9] report for the first time the coexistence of two main ICMTCs in the very same model studied. By finding both TNTs and TMs in patient-derived tumor organoids, an *ex vivo* model for glioma research [10], the authors demonstrate that the two ICMTC classes (1) can occur in the same experimental system, (2) appear differentially regulated, and (3) might execute different functions in cancer biology. This has implications for the field of ICMTC research.

First, the authors describe the generation of two new glioblastoma stem cell lines from a more infiltrative tumor region of one (the same) patient: one from a region with lower cellularity/metabolic activity (C1), as determined by MRI spectroscopy prior to tumor resection, and one with higher cellularity/metabolic activity (C2). Intratumoral heterogeneity is a key feature of glioblastoma and other incurable brain tumors, and it is broadly accepted today that this heterogeneity between tumor regions and even single tumor cells of the same region greatly contributes to the development of tumor resistance over time, and is one of the main reasons why no targeted therapy has been demonstrated clinically efficacy in phase 3 clinical trials so far in this challenging disease [11]. That underlines the importance to better understand intratumoral heterogeneity, which is a strong starting point for the study. With respect to ICMTCs, intratumoral heterogeneity has only been reported for TMs so far. Here, only a subpopulation of glioblastoma cells that is characterized by more stem-like features is extending TMs to form a large multicellular syncytium that is capable of metabolic homeostasis and

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able to better resist radiotherapy, chemotherapy, even detect and repair damage to itself [5,12–14]. Thus, it is tempting to speculate that stem-like cancer cells have an increased capability to form ICMTCs than their non-stem-like counterparts. The extension of ICMTC-like structures has been described as a possible feature of nonmalignant stem/progenitor cells, e.g. during development, too [2,15]. However, with respect to ICMTCs, only cellular, not regional tumor heterogeneity has been studied so far, which is one strong aspect of the current study.

Second, Pinto et al. demonstrate that both newly generated glioblastoma stem-like cell lines are capable to form TNTs between them in a 2D culture system that, however, preserves stem-like conditions. Interestingly, the C2 cells which were obtained from a more established/metabolically active tumor region formed more TNTs than the C1 cells, providing support for the concept that ICMTCs increase with tumor progression [2]. TNTs were always positive for actin and negative for microtubules, consistent with their classification as classical TNTs. Moreover, these TNTs were functional, as determined by intercellular transfer of mitochondria via them. In line with the TNT formation data, acceptor cells containing donor-derived mitochondria were significantly more often found in C2 compared with C1.

Third, the authors generate 'tumor organoids' in which resected tumor specimen from patients are directly cultured under floating stem-like conditions, thus maintained in a 3D environment containing a more realistic matrix. These type of culture conditions allow to maintain tumor organoids over weeks, and are particularly apt to maintain tumor cell heterogeneity. In organoids from both C1 and C2 regions, not only TNTs were detected, again meeting the typical staining characteristics and characterized by mitochondria transfer, but now a second ICMTC type: TMs. In line, they found that neuronal growth-associated protein 43 (GAP-43), a main molecular driver of TM formation [5], was 12-fold higher expressed in the C2 cells in organoids. Consistenly, this was particularly the case in organoids from C2 cells, where many TMs stained positive for GAP-43; an enrichment of GAP-43 in TMs, including their neurite growth cone-like tips, has been reported before [5].

Finally, Pinto et al. subjected C1 and C2 glioblastoma stem-like cells growing adherently (which under this specific culture condition can form TNTs but not TMs) to radiotherapy. Here, C2 cells, but not C1 cells, reacted to the cytotoxic stress with a transient increase in TNT formation. In contrast, C1 cells even reduced mitochondria transfer, which is remarkable since the vast majority of published *in vitro* data suggested that cellular stress quite consistently increased TNT formation and mitochondrial transfer [16]. This again (1) stresses the importance of intratumoral heterogeneity where apparently some tumor regions can better adapt to therapeutic stress than others, or at least maintain their putative resistance mechanisms, and (2) provides a compelling case that if human diseases are studied, ICMTC biology should preferentially be investigated in state-of-the-art *ex vivo* and *in vivo* models that optimally recapitulate the situation in the human organism.

While all this data supports the increasing notion that intravital or at least advanced *ex vivo* studies of ICMTCs, which includes their existence and biological functions, is of crucial importance to further advance the field, considerable methodological challenges remain. While the thicker and most likely longer-lived TMs are easier to detect, track and interrogate, most stringently so far in brain tumors implanted under a chronic cranial window in mice that allows to track individual cancer cells and their TMs over months [5,17], only a handful of groups has mastered to visualized TNTs in live tissue so far [18]. However, there have been remarkable recent advances: interpericyte open-ended TNTs were found to regulate neurovascular coupling in the mouse retina, with intercellular calcium waves (that have been well described for TMs in tumor cell networks [5]) conducted via these TNTs and serving as conduit for communication [19].

ICMTC research bears many exciting opportunities to better understand cellular communication, homeostasis, stemness, movement, proliferation, resistance, and the function and structure of multicellular networks that allow cells to function as one coordinating syncytium which is the basis of every supracellular structure like tissues, organs, and organisms. Today we are just at the beginning to unravel the multiple structures and biological functions of ICMTCs in normal physiology and in many diseases [2,3,16,20,21]. Figure 1 illustrates a similar coexistence of TNTs and TMs in glioblastoma stem-like cells seeded in a novel, refined 2D Monolayer Assay — demonstrating that keeping cancer cells under strict stem-like, non-differentiating conditions can, under certain circumstances that still need to be developed for various cell types, even allow to study various ICMTCs in experimental assays that are considerably easy to handle and to investigate. Similar but also different functions of TMs and TNTs are highly likely, and need to be better explored in the future. While the relevance for cellular homeostasis and resistance of both TMs and TNTs is undisputed, the fact that TMs are not open-ended, but — as far as we know today — consistently separated by connexin 43 gap junctions (which can, however, also occur in some TNT-like structures, at least according to some published data and concepts)

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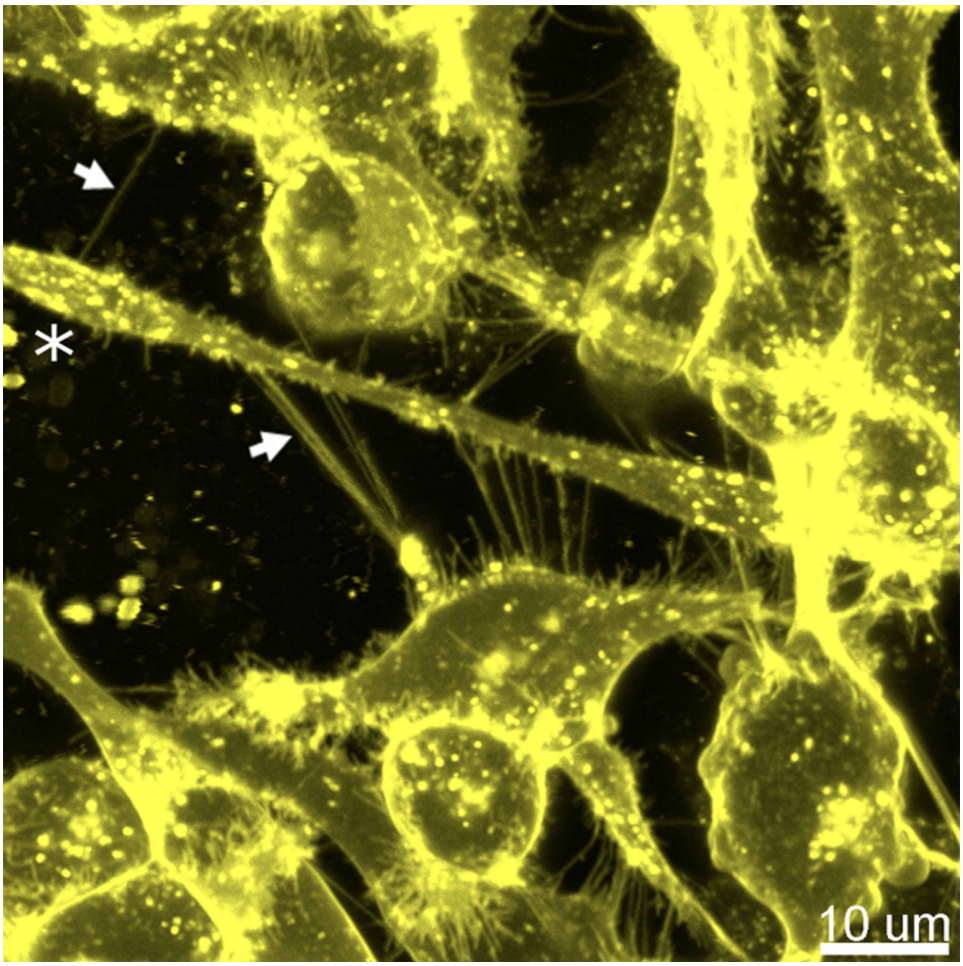

Figure 1. Thicker tumor microtubes (TMs) and thinner tunneling nanotube (TNT)-like structures in primary glioblastoma cells from patients.

Confocal images of gliblastoma cells *in vitro* using the LipiLight 488 membrane staining (Scale bar, 10 μm). Asterisk shows a TM; Arrows denote TNT-like structures.

makes for example TM-mediated intercellular transfer of mitochondria, other cell organelles, and large molecules highly unlikely and has indeed not been found so far [2,5].

Therefore, with respect to the similarities and differences of TMs vs TNTs, many important points remain to be investigated: (1) Do TNTs and TMs interact with each other, jointly providing mechanism of resistance to cellular stress which includes current antitumor therapies? (2) Do already known TM drivers (particularly neurodevelopmental genes like GAP43 or Ttyh1) drive TNTs, too? What about the few known molecular TNTs drivers [22] in TM biology? (3) Can TMs, at least partially, also convene to one 'multi-cable' structure where many TMs with potential different functions run in parallel to each other, as convincingly shown for TNTs by the group of Chiara Zurzolo? [23]. (4) Do TNTs exist in glioblastoma *in vivo*, and is there a suitable methodology to visualize and functionally study them? (5) If different molecular mechanisms exist, will only a future co-targeting of TMs and TNTs lead to maximum antitumor and resistance-breaking therapeutic effects? (6) Is there a similar or fundamentally different network structure of TM- and TNT-mediated multicellular networks?

The answers to these questions will provide important insights into fundamental mechanisms of cell biology and disease mechanisms alike. For glioblastoma and other incurable brain tumors, it can help to better understand their mechanisms of progression and resistance, and at the same time serve as a much-needed input for the generation of novel treatment concepts.

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Competing Interests

F.W. reports the patent (WO2017020982A1) "Agents for use in the treatment of glioma". F.W. is a co-founder of DC Europa Ltd (a company trading under the name Divide & Conquer) that is developing new medicines for the treatment of glioma, particularly against ICMTCs. Divide & Conquer also provides research funding to F.W.'s lab under a research collaboration agreement.

Abbreviations

GAP-43, growth-associated protein 43; ICMTCs, intercellular membrane tube connections; TMs, tumor microtubes; TNTs, tunneling nanotubes.

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