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1 **Tunneling Nanotubes: The Fuel of Tumor Progression?**

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3

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10

11 **Keywords**

12 Tunneling nanotubes, cancer, cell-to-cell communication, glioblastoma

13

14 **Abstract**

15

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

27

28 **Cancer and Intercellular Communication**

29

30 Cancer is among the leading causes of mortality worldwide, responsible for 1 in 6
31 deaths, according to the World Health Organization. Over the past decades, many
32 therapeutic strategies have proven their effectiveness and the overall cancer death
33 rate has been reduced by 27% [1]. Several features of cancer cells make these
34 pathologies very aggressive and difficult to cure, such as their uncontrollable
35 proliferative capacity and their ability to get nourishment through neo-formed blood
36 vessels, to infiltrate healthy tissues forming metastasis, to evade the immune system
37 and, finally, to adapt to clinical treatments. In this context, intercellular
38 communication and particularly, cell-to-cell transfer of cellular material can contribute
39 to each of the aforementioned characteristics, including treatment resistance. Over
40 the past 20 years, numerous studies have shown that that exosomes and exo-
41 vesicles are able to carry malignant content (e.g. proteins, nucleic acids), likely
42 helping the recipient cells to express genes supporting proliferation, colonization,
43 immune evasion or to recover from damage provoked by treatment [2,3]. Recent
44 work has highlighted a new communication mechanism implemented by tumor cells:
45 tunneling nanotubes (TNTs), which are physical channels providing cytoplasmic
46 continuity between distant cells (Figure 1A). TNTs are thin, actin-based membrane
47 tubes that, in contrast to other cellular protrusions, listed in Table 1, are open-ended
48 at their extremities [4,5]. They allow the transfer of various-sized cargoes (Figure 1),
49 such as small molecules (e.g. Ca²⁺ ions), macromolecules (proteins, nucleic acids
50 etc.) and even organelles (vesicles, mitochondria, lysosomes, autophagosomes,
51 etc.) [6]. Several cells can be connected by TNTs, possibly leading to the formation
52 of a functional cellular network [7].

53 TNTs were first identified in 2004 by Rustom and colleagues in cultures of
54 pheochromocytoma PC12 cells [4]. Later, several other publications reported the
55 presence of “TNT-like structures”, (heterogenous intercellular connections, defined
56 on the basis of their morphology), in many other cell types in *in vitro* cultures,
57 including astrocytes [8], immune cells [9], as well as in tumor cancer cell lines, where
58 often their occurrence was correlated with more aggressive tumor phenotypes
59 [10,11]. Beyond tumors, TNT-like structures have been observed in early
60 developmental stages in various organisms [12] as well as in relation to stress-
61 induced responses, such as oxidative stress [8,13], allowing the discharge of cellular

62 waste or dangerous materials. Similarly, they can be used as a route for the
63 dissemination of pathogens such as HIV virus [14,15], bacteria [9], prions and
64 amyloids fibrils in the case of neurodegenerative diseases [16–21]. Although TNT-
65 like structures have been clearly identified as physical and functional entities in solid
66 tumors [22–26], the existence of these connections in whole healthy organs or
67 tissues is still a matter of debate. In this manuscript we review the studies on TNTs
68 and their heterogeneity in cancers and their possible role in tumor progression and
69 development of treatment -resistance, with a particular focus on glioblastoma (GBM).

70

71 **Detection of TNT-like structures *in vitro* and *in vivo* in cancer**

72

73 The first identification of TNTs occurred in PC12 cells, which are derived from a rare
74 rat tumor of adrenal gland tissue [4]. Subsequently, many other cancer cell lines
75 have been shown to form membranous connections bridging distant cells as
76 summarized in Table 1. Of importance for this review, TNT-like structures were also
77 observed in primary cells directly obtained from patients, for example in squamous
78 cell carcinoma [24,25], mesothelioma [10,22] and different forms of leukemia [27–
79 29]. Cancer cells can form heterotypic connections with cells of the tumor
80 microenvironment, including mesenchymal [30], endothelial [11] and immune cells
81 [31]. Cross-talk with the tumor microenvironment plays a significant role in sustaining
82 cancer progression, providing nutrients or buffering metabolic stress [32], and
83 interaction with immune cells can contribute to overcoming immunosurveillance [33].
84 While it is possible to identify TNT-like structures between the same or different cell
85 types in cell cultures using light microscopy [34], their identification in a more
86 complex context such as animal models or tumor resections is still very challenging.
87 This is because no specific marker for these structures has been identified yet, and
88 the optical resolution of classical microscopy doesn't allow for the morphological
89 characterization of these connections in a tissue environment [5,12]. Therefore, the
90 heterogeneity and lack of structural characterization of TNTs represent a major
91 problem for their investigation. Because of their morphological heterogeneity and
92 poor molecular and structural characterization, the intercellular connections
93 observed to-date have been named differently in different studies (nanoscale conduit
94 [11], tunneling nanotubes [22], intercellular bridges [12] or membranous tunneling
95 tubes [24]). This raised both confusion and skepticism in the field [35], and calls out

96 for both more rigorous definition and more accurate technical approaches to study
97 them. We propose to call TNTs only the connections that fulfill the following
98 characteristics: i) continuous membrane connections with the plasma membrane of
99 the connected cells, ii) non-adherent to substratum, iii) containing actin, iv) proven
100 cargo transport, and v) open-ended (see Table 1). On the other hand, we will refer to
101 “TNT-like” connections when one or more of these properties is not fulfilled or has
102 not been assessed. The first documentation of TNT-like structures *ex vivo* in solid
103 tumors was provided by the laboratory of Emil Lou in 2012, which described
104 mitochondria-containing connections in tissue sections of a mesothelioma resected
105 from a patient [22]. These observations were followed by others, showing various
106 intercellular connections in squamous cells carcinoma [24,25], in ovarian [23] and
107 pancreatic cancer [26] and in human glioblastoma cells engrafted into mice models
108 [36] (see Table 2). Little is known about the structural and functional features of
109 these connections *in vivo*. In some cases, however, the presence of mitochondria
110 and possibly other cargoes inside them supports the hypothesis that these structures
111 may be open-ended as canonical TNTs and may allow the transfer of cargoes.

112

113 **Morphology and structure of TNTs**

114

115 Despite the lack of a specific marker, TNTs can be identified in cell culture by
116 fluorescent labelling of the plasma membrane and cytoskeleton components and
117 observed by using light microscopy, (Figure 1B). However, specific fixation protocols
118 are needed to preserve their delicate and fragile nature [34], and functional assays
119 have to be performed in addition to morphological studies to fulfill the definition of
120 TNTs (see above). TNTs exhibit high variability in their morphology, in terms of
121 length and thickness, and in cytoskeleton content, specifically regarding the
122 presence/absence of microtubules [34]. Nevertheless, they always appear as actin-
123 based connections and their presence and functionality can be affected by inhibitors
124 of actin polymerization (e.g. latrunculin, cytochalasin) (Table 2). In cancer cellular
125 models, the observed connections can range from tens to several hundreds of
126 microns [10,11,25]. In some tumor tissues, exceptional connections longer than 500
127 μm were observed [24,36]. Whereas, in most of the studies *in vitro*, the diameter of
128 the connections in tissues was in the nanoscale ($<1 \mu\text{m}$), microscale connections (>1
129 μm) [24,36] were also present. However, these long and thick connections are fitting

130 best with the definition of tumor microtubes rather than TNTs (Table 1). At present,
131 we do not know whether TNTs display different morphologies *in vitro* or *in vivo* (e.g.
132 filopodia, mitotic bridges) (e.g. filopodia, mitotic bridges) or whether nanoscale
133 connections are detectable in the complexity of the tissue. The thickness of TNTs
134 also correlates with their cytoskeleton content, with protrusions containing
135 microtubules having larger diameters [9]. However, some cancers seem to present
136 both types of connections: those containing only actin and those with actin and
137 microtubules [11,25].

138 Few studies have addressed the ultrastructure of TNTs in cancer models with the
139 use of electron microscopy [37,38]. A deeper structural analysis of TNTs, using a
140 combination of cryo-fluorescence microscopy with cryo-electron microscopy, was
141 conducted recently. This study used a catecholaminergic-a-differentiated (CAD) cell
142 line, established from a brain tumor in a transgenic mouse, and SH-SY5Y cells,
143 isolated from a neuroblastoma patient [5]. By using experimental conditions set up
144 to better preserve TNT structure, this study has shown that in these two types of
145 neuronal cell lines, TNTs can be composed of multiple individual tubes (named
146 iTNTs) held together by N-cadherin-positive structures and often open-ended at their
147 tips [5]. Nonetheless, whether iTNTs exist in different cell types and tumors, and/or
148 *in vivo* remains an open question.

149

150 **Functional approaches**

151

152 The distinguishing characteristic of TNTs with respect to other cellular extensions
153 (e.g. filopodia, mitotic bridges, see Table 1) is their ability to transfer cellular material.
154 Some works provided qualitative evidence of cargoes inside TNT-like structures
155 observed in different cancers [22,37], without proving that actual transfer had
156 occurred and without excluding cell division as the mechanism by which the cellular
157 material was shared. To exclude the latter possibility, membrane vesicles or
158 organelles such as mitochondria or lysosomes can be labelled in a population of
159 cells defined as donors. This population is then co-cultured with an acceptor
160 population (differently labelled) to further detect and quantify the cargoes transferred
161 in the acceptors by fluorescence microscopy (in fixed or live condition) or flow
162 cytometry [34]. The co-culture has to be performed placing the two populations in
163 direct physical contact at an appropriate cell density that favors the formation and

164 detection of TNTs. In order to evaluate secretion as a possible mechanism of
165 transfer, the two populations can be separated by a filter which allows the transfer of
166 secreted material, or they can be grown in different dishes and the acceptor
167 population challenged with the supernatant from donor cells [34]. The weakness of
168 this approach is that it only allows the direct transfer (cell contact-mediated) of the
169 labelled cargo to be determined. It does not consider other materials that could be
170 transported through the same connections, including the ones that could be shared
171 in the opposite direction. To overcome this limit, other approaches such as mass
172 spectrometry [38] and transcriptomic analysis [11] have been recently applied to
173 detect alterations at the proteome and transcriptome levels. In these examples, the
174 acceptor population acquired pro-tumoral features correlated to the transfer of
175 proteins or miRNA involved in cell survival, drug response or cellular reprogramming.
176 All these approaches show how TNTs might be differently exploited in various types
177 of cancer (Table 2). However, we still don't know if the variability observed at the
178 TNT level in the various studies and in the various cancers corresponds to different
179 roles for TNTs in the cancers or just to the different questions addressed. Few
180 approaches have studied the dynamics and transfer ability of these structures *in*
181 *vivo*. Using multiphoton microscopy, connections between human tumor cells were
182 detected in mouse xenografts [36] (Table 3); while the transfer between human and
183 murine cells was quantified by amplification of species-specific DNA sequences or
184 detection of labelled material by flow cytometry [11,28,29]. Although powerful and of
185 great interest, these approaches have made it possible to monitor the transfer
186 without specifically identifying its mechanism, in particular without excluding the
187 secretion mechanism. In conclusion, due to the limitations of the *in vivo* models (e.g.
188 TNT preservation and observation), the field needs to pursue the study of these
189 fragile structures in cellular models, as much as possible representative of the
190 tumoral tissue (eg, patient-derived cells), which allow to address more easily specific
191 questions on the mechanism and content of the transfer and its impact on the
192 receiving cells. In parallel, new tissular models recapitulating the tumoral context as
193 tumor-derived organoid cultures, need to be implemented in the field. Finally,
194 additional efforts need to be made to overcome the technical limitations of the *in vivo*
195 study of TNTs to finally unravel their role in physio-pathological contexts beyond their
196 morphological diversities.

197

198 **Tumoral context might favor TNT connectivity**

199

200 Since their discovery, TNTs have been described as a mechanism of adaptive
201 response to cellular stress. Interestingly, several cancer-related environmental
202 conditions have been shown to stimulate their formation. Reactive oxygen species
203 (ROS), known to be intensively produced by cancer cells [39], have been shown to
204 induce TNT formation in different contexts, including cancer [8,13,20,29,40] (Table
205 1). Moreover, treatments such as chemo and radiotherapy induce ROS production
206 [41]. Hypoxia, typical of the denser tumor regions, has been found to be a TNT
207 inducer in ovarian [42] and prostate cancers [43]. Interestingly, other conditions
208 mimicking the tumor microenvironment *in vitro* stimulate TNT-mediated
209 communication, such as acidic pH, hyperglycemia, serum deprivation [22,43] and
210 exposure to TNF- α normally produced during inflammation [44]. Finally, different
211 signaling pathways that are often dysregulated in cancer have been shown to be
212 involved in TNT formation, like PI3K/Akt/mTor [37,40,42,43], K-RAS [45] and p53
213 [13,40]. These signaling cascades could activate downstream proteins, such as M-
214 Sec in the case of immune cells [46], which are involved in actin remodeling and
215 polymerization and have been shown to induce TNT formation [47]. Altogether, these
216 findings suggest that the tumor context, globally experienced as a stress by cells,
217 provides the conditions that favor TNT formation and communication. In turn we can
218 speculate that this route for intercellular communication allowing cells to share
219 material may result in a beneficial effect for the connected cancer cells, as described
220 in the following sections.

221

222 **Roles of TNT in cancer progression**

223

224 The ability of cancer cells to interconnect among themselves is correlated to more
225 aggressive cancer phenotypes. For example, in ovarian and breast cancers, highly
226 malignant and metastatic cells are more prone to interconnect in tumor networks
227 than their less aggressive counterparts [10,11]. Also, in gliomas, where for the first
228 time tumors have been described as a functional inter-communicating network, there
229 is a correlation between extended interconnectivity and the most aggressive grades
230 of tumors and their poorer therapeutic outcome in response to radiotherapy [36].
231 However, the mechanisms of treatment resistance have not been fully elucidated

232 yet. Different cancers could be applying different strategies to protect themselves
233 from the therapeutic attempts and eventually a unique mechanism may be
234 determined. Below we review the possible roles of TNT-like connections in different
235 types of cancer and how they affect cancer progression. We then focus on the
236 specific example of GBM.

237

238 **TNT-mediated transfer can promote aggressive features**

239

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

245 In the case of breast cancer, TNT-mediated contacts from cells of the tumoral
246 environment such as macrophages seem to drive the acquisition of an invasive
247 phenotype in the cancer cells [31]. Although it is not clear how this contact could
248 induce this phenotypic switch, mitochondria appear to be good candidates for
249 transferred cargo that could induce invasiveness. In fact, breast cancer cells have
250 been shown to be able to receive mitochondria from mesenchymal cells (MSCs)
251 through TNT-like structures [30]. Further, the uptake of isolated mitochondria derived
252 from MSCs, by a protocol defined as MitoCeption, was able to induce migratory
253 ability and cellular proliferation [48]. Many studies have shown TNT-mediated
254 mitochondria transfer to be possible [24,25,30,49]; although the possibility that
255 mitochondria could be transferred through the supernatant should be considered, as
256 a few reports have suggested that the mitochondria could be released and taken up
257 by neighboring cells [50]. Transfer of mitochondria has also been found to restore
258 tumorigenic potential in cells devoid of mitochondrial DNA [51,52], although these
259 studies did not address the mechanism of mitochondrial transfer. Furthermore, TNT-
260 mediated traffic of mitochondria was correlated to increased invasiveness in bladder
261 cancer [37]. Here, different cancer cell lines in co-culture could exchange functional
262 mitochondria with each other, possibly stimulating the migratory capacity of the
263 acceptor cells, as assessed by *in vitro* assays. Further, their ability to form larger
264 tumors with a higher vascularization index was stimulated when implanted in nude
265 mice. In a second study, additional evidence suggests that the acquisition of these

266 pro-tumor properties might be due to TNT-mediated transfer of miRNA from the most
267 aggressive to the least aggressive cells leading to the activation of the Deptor-mTor
268 signaling pathway, an important downstream mediator of cancer cell proliferation and
269 motility [53].

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

285

286 **TNTs can support therapy resistance**

287

288 Intercellular communication through TNT-like structures and resistance to therapies
289 appear to be tightly correlated. As for the other cancer features that might be driven
290 by contact-mediated transfer of cargoes, TNT-like structures may provide a way for
291 distributing harmful substances and cellular wastes, or sharing defensive tools
292 against treatment, such as mitochondria, miRNA and specific factors (Figure 2).
293 TNT-mediated communication appears to be stimulated by radiotherapy, which
294 causes free radical production, known to be TNT-inducers [41], radiofrequency
295 treatment [56] and chemotherapy [43]. A recent study in prostatic cancer has shown
296 that the chemotherapeutic blockage of the androgen receptor, which induces
297 metabolic stress, enhances TNT-like structure formation [43]. Disrupting these
298 connections by cytochalasin D sensitizes prostatic cancer cells to treatment-induced
299 cell death, suggesting that the presence of this stress-induced network favors cancer

300 cell survival upon treatment. In this study, lysosomes, mitochondria and stress-
301 induced chaperones were observed inside the TNT-like structures. Therefore, it is
302 possible that transferring these cellular components may benefit stressed cells.
303 Conversely, TNT-like structures could be used as a way to get rid of damaged
304 organelles or autophagosomes [25] and possibly other dangerous substances, such
305 as ROS, produced in response to treatments, or the drugs themselves (Figure 2C).
306 Transfer of a soluble drug via TNT-like structures has also been observed in both
307 pancreatic and ovarian cancer cellular models [26]. Here, multidrug-resistant cell
308 lines use TNT-like connections to redistribute doxorubicin from chemo-resistant
309 toward chemo-sensitive cells, leading to cell death of the latter and enrichment of the
310 therapy-resistant population. Although the possibility of using TNT-like structures as
311 a drug outflow pathway must be considered, there are currently no quantitative data
312 supporting the actual relevance of this mechanism *in vivo*. Also, this work raises
313 questions on the specificity of the transferred materials through TNTs, and whether
314 this occurs through an active or passive mechanism of redistribution.

315 As mentioned above, TNT-based networking allows the exchange of “defensive
316 tools” against treatment (Figure 2A). The transfer of mitochondria has been shown to
317 modulate the response to treatments in a beneficial manner for the recipient cells
318 [49,57,58], impacting on their cellular metabolism [48,58], rescuing their aerobic
319 respiration [59] and providing metabolic support against treatment-related stress
320 [58]. This was first observed in PC12 cells, where delivery of healthy mitochondria
321 through TNT-like structures from untreated to UV-injured cells protected the latter
322 from apoptosis [60]. This rescue mechanism is also applied by MSCs to
323 chemotherapy treated ECs [55]. Both MSCs and ECs have been found to transfer
324 mitochondria to cancer cells of different origins, resulting in an improved resistance
325 to doxorubicin in the cells that received the transfer [30]. This mechanism appears to
326 be critical in different forms of leukemia. Leukemic cells, engrafted in mice bone
327 marrow, were able to obtain and receive mitochondria from stromal cells with an
328 impact on cancer cell metabolism [27,28,58], cell proliferation [58,61] and
329 chemoresistance [27]. The disruption of this transfer increased the sensitivity of the
330 cancer cells to various chemotherapies [27]. This suggests that MSCs could play a
331 protective role toward tumor cells by eliminating the damaged mitochondria they
332 receive, thereby stabilizing the homeostasis of the cancer population, and possibly
333 providing metabolic support. Moreover, chemotherapy-induced ROS production can

334 enhance mitochondria transfer [28], again suggesting that mitochondrial transfer may
335 be a mechanism for adaptation to treatment. Interestingly, the inhibition of CD38,
336 previously described to promote mitochondrial release from astrocytes [50], could
337 prevent the contact-mediated mitochondria transfer from MSCs to leukemic cells,
338 resulting in an increased apoptosis of the leukemic cells and improved mice survival
339 [29]. This opens the possibility of specifically targeting mitochondria transfer at the
340 clinical level. Following this evidence, others have assessed the communication
341 between stroma and leukemic cancer cells. By using mass spectrometry they
342 revealed the transfer of specific factors, like stress-induced chaperons, together with
343 cellular vesicles, with a potential role in survival and adaptation [38]. Other cargoes
344 such as miRNA can be transferred between cells leading to the acquisition of
345 therapy resistance. Thayanithy and collaborators [23] have shown that the transfer of
346 miR-19 and miR-199a occurred in heterotypic connections between different cancer
347 cell lines of the same tumor, osteosarcoma and ovarian cancer, respectively.
348 Specifically, miR-199a appears to be differentially expressed in chemo-sensitive and
349 chemo-resistant cells, suggesting that the transfer of this particular miRNA could
350 drive treatment-resistant features in the receiving cells. Altogether, TNTs could be a
351 beneficial feature for cancer cells and the ability to exploit this efficient route of
352 communication may be positively selected during treatment.

353

354 **Glioblastoma: an “exemplary” or “peculiar” case of TNT-like networks?**

355

356 Among the deadliest types of cancer, GBM stands out for its aggressiveness and
357 resilience in response to treatment. GBM is the most undifferentiated and invasive
358 cancer within the gliomas and it is classified as a IV grade tumor. Surgery followed
359 by chemo and radiotherapy was insufficient to eradicate completely cancer cells from
360 the brain, although the mean survival of patients increased from less than one year
361 to about 15 months [62,63]. Currently no treatment is effective in preventing cancer
362 relapse and the reasons for therapy failure are poorly understood. Some studies
363 correlate the occurrence of relapse with elevated intra-tumoral heterogeneity: distinct
364 molecular profiles coexist and exhibit differential therapeutic responses [64]. In
365 particular, glioblastoma stem cells (GSCs) have been found to be the most resistant
366 to treatments and likely are at the origin of relapses [65]. Moreover, treatments can

367 positively modulate tumor heterogeneity by inducing cellular plasticity and trans-
368 differentiation [66].

369 As outlined above, in the last few years, various studies have supported the
370 possibility that intercellular communication through cell-to-cell connections might be
371 a critical mechanism for treatment failure and tumor relapse. GBM is the first case
372 where a functional and resistant network among cancer cells has been described in
373 an *in vivo* model [36]. Specifically, GSCs, coming from patients with different grades
374 of glioma, were implanted in nude mice brains, where they developed a multicellular
375 and communicative network. In this study, Winkler and collaborators demonstrated
376 that highly interconnected tumors, which corresponded to higher malignant grades of
377 the original tumor, were found to be more resistant to irradiation [36]. Cancer cells
378 were shown to be able to propagate ion fluxes by long and thick membrane
379 protrusions, containing both actin and microtubules, which the authors termed tumor
380 microtubes (TMs), see Table 1. Moreover, the same authors suggested that TMs
381 could be essential for driving the repopulation of a surgically resected area in GBM
382 mouse models [67] (Figure 3). The formation of TMs appears to be dependent on the
383 expression of connexin 43 (Cx43), a monomeric component of GAP-junctions, and
384 growth-associated protein 43 (GAP-43), a crucial protein for neurite formation,
385 regeneration, and plasticity. When Cx43 or GAP43 were knocked down, the number
386 of TMs decreased and the sensitivity to radiotherapy increased. Cx43 is a known
387 regulator of the intracellular concentration of Ca^{2+} [68] and it has been also described
388 to play a critical but controversial role in GBM progression, acting both as tumor
389 suppressor and tumor inducer, promoting growth, cell migration and resistance to
390 apoptosis [69]. Interestingly, Gerdes and colleagues [70] reported earlier that a
391 subset of TNTs observed in kidney-derived cells contained Cx43 forming a hemi-
392 connexon or a GAP-junction at their tip. It was also proposed that GAP-junctions
393 could mediate the transfer of electrical signals in electrically coupled TNTs [6].
394 Nonetheless, the presence of GAP-junctions along TNT-connections would not allow
395 the transfer of any cargo of a size superior to their pore size (1 kDa) [71], like
396 organelles or macromolecules. In the case of TMs the authors did not report transfer
397 of conventional TNT-cargoes, such as mitochondria or vesicles, within their lumen,
398 although they did observe nuclei travelling along these connections from a healthy
399 cell to a cell damaged by the treatment [36]. In addition, TMs display neurite-like
400 features, as they have been described to be post-synaptic targets for the

401 surrounding neurons. Indeed axons can dock onto TMs and generate synchronized
402 calcium transients in glioma networks via AMPA receptors [72,73], Further,
403 depolarization of the post-synaptic glioma cells promoted TM proliferation [73] and
404 invasion [72].

405 Overall, the nature of TMs and the mechanisms at stake in this cellular network still
406 need to be unraveled. As for their morphological appearance and physical properties
407 TMs are very different from TNTs because they are not open-ended, they are much
408 thicker (1.7 μm on average), more stable in time [74] and contain both actin and
409 microtubules, thus resembling more of a neuritic extension than TNTs [75] (see
410 Table 1). Nevertheless, direct cell-to-cell communication seems to play a key role in
411 the resistance to treatments in GBM and growing evidence suggests that the transfer
412 of cargo mediated by open connections could contribute to tumor progression as
413 shown previously in other cancer forms. A few *in vitro* studies suggest that GBM
414 cells are capable of transferring cellular material through thinner TNT-like structures.
415 U-87 and U-251 cell lines, common GBM cellular models, can form TNT-like
416 structures [76–78] (see Figure 1), and their formation can be increased in response
417 to external stimuli, such as protein aggregate uptake or cocaine administration
418 [77,78]. Moreover, preliminary studies show that communication between astrocytes
419 and glioma cells, known to facilitate cancer progression [79] can occur through TNT-
420 like structures [13,76] and the transfer of mitochondria seems to modulate GBM cell
421 abilities in favor of a more proliferative [13] and drug-resistant state [80]. However,
422 the study of intercellular exchange of material needs to be elevated in more complex
423 and representative tumor models. The fact that GBM cells were able to form a
424 network in mice xenografts, but failed in forming connections when cultured *in vitro*
425 [67], suggests that TMs may exist only in the *in vivo* condition. It is possible that
426 GBM networks are composed of several types of connections that vary in size and
427 properties: open-ended TNTs, synaptic-like connections and/or thick GAP-junction-
428 linked protrusions such as TMs (Figure 3).

429

430 **Concluding Remarks and Future Perspectives**

431

432 In the last decade, growing evidence has supported the existence and the
433 importance of intercellular communication based on TNT-like connections in various
434 tumors. Several cancer cell types have been shown to grow such connections and

435 communicate through them in culture, and similar structures have been found in
436 tumor sections [22], proving their existence in real tumors. Different studies have
437 described TNT-like structures with diverse morphologies and characteristics;
438 therefore, the ability to transfer cellular material has been used to define them
439 functionally rather than structurally. Cancer is one of the few contexts where TNTs
440 have been functionally described, whereby the transfer of cellular cargoes has been
441 shown to have an impact on the behavior of the recipient cells and lead to further
442 development of the disease. However, fundamental questions still remain regarding
443 the structural diversity of the different protrusions, as well as the molecular
444 determinants and the signaling pathways that would stimulate their growth in cancer
445 cells, compared to non-cancer cells.

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

468 To address the complexity of the real pathology and also the diversity of TNT-like
469 connections, the use of models representative of the tumor environment is required.
470 Many of the studies reported here were carried out in cell lines *in vitro*. Only more
471 recent work has addressed the study of TNT-like structures with the use of patient-
472 derived xenografts in mice. Based on current knowledge it appears that blocking
473 TNT-like connectivity could be a promising strategy to fight cancer, eventually
474 hindering cancer progression and sensitizing tumor cells toward treatments. A
475 couple of drugs have been described as able to specifically inhibit TNT formation in
476 cell culture [81,82], but these need to be tested in cancer mouse models.
477 Conversely, TNTs have also been used as a route to diffuse therapeutics, like drugs
478 [26] and nanoparticles [76], affecting predominantly the network of connected cancer
479 cells. Certainly, a deeper understanding of TNT-based communication is critical for a
480 better comprehension of cancer progression and treatment resistance, and in future
481 years this knowledge could lead to the development of new, more effective
482 therapies.

483

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485

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492

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- 714

715 **Table 1.** Types of Cellular Projections

716

Name	Description	Actin / microtubules content	Membrane fusion with a target cell	Function	Refs
Cilia	Large protuberance emerging from the cell body	Actin and microtubules	No	Environment sensing, coordination of signaling pathways	[83]
Stereocilia	Thin specialized cell protrusion on the 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 a target cell	Actin	No	Morphogens-delivery by direct contact to the 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 the cell body of neurons	Actin and microtubules	No	Neurotransmitter release/reception and propagation of the action potential	[89]
Tumor microtubes	Thick membrane extensions containing GAP-junctions, either connecting cells either finger-like	Actin and microtubules	Yes/No	Transmission of intercellular ion fluxes, cell invasion, formation of neuron-glioma synapses	[67,72,73,90]
Tunneling nanotubes	Thin membrane connections, open-ended	Actin, sometimes microtubules	Yes	Exchange of cellular cargoes between cells	[6]
Invadopodia	Finger-like membrane protrusions	Actin	No	Matrix degradation	[91]
Podosomes	Dynamic membrane-bound microdomain	Actin	No	Adhesion, mechanosensing and matrix degradation	[92]

717

718 **Table 2.** Tumor cell models used for the study of TNT-mediated communication *in*

719 *vitro*

TUMOR MODEL	CARGO	TNT FUNCTION	TNT REGULATORS ^a	YEAR	Refs
Rat pheochromocytoma cell lines	Lysosomes, soluble and membrane marker	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 the expression 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 ₂ O ₂ (+), Latrunculin A (-)	2015	[13]
Metastatic breast cancer cell lines	miRNA	Transfer of miRNA and alter the phenotype of the receiving endothelial cells. TNT correlates with more aggressive phenotype	Docetaxel (-), LatrunculinA (-), Cytochalasin D (-)	2015	[11]
Pancreatic adenocarcinoma cell lines	Electron-dense particles	n.d.	Radiofrequency treatment (+)	2015	[56]
Rat pheochromocytoma cell lines	Mitochondria	Rescued of 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 (AML) primary cells	Mitochondria	Mitochondria transfer from the bone marrow supports cancer cell metabolism and promotes stress-adaptative response	NOX2	2017	[28]
Pancreatic adenocarcinoma and ovarian cancer cell lines	Doxorubicin	Redistribution of the 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]
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 the 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]
Glioblastoma cancer cell line	Functionalized liposomes	Delivery of nanoparticles	n.d.	2019	[76]

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722 ^a(+), induced; (-), inhibited; n.d.,not described.

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727

728 **Table 3.** Evidence of TNT-like communication in tissue

CANCER	MODEL	LABELLING	YEAR	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]
Head and neck squamous cell carcinoma	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]

729

730 **Figure 1.** TNT in cell culture. (A) Schematic of two cells connected by a TNT in cell
731 culture. The connection floats above the adhesion surface (dashed line). The lower
732 part shows a magnification of the TNT and possible cargoes travelling along it. The
733 range of TNT diameters and lengths is indicated. (B) Representative fluorescence
734 images of TNTs between cells in culture. U-251 glioblastoma cells were plated at a
735 density of 20 k cells/cm² for 24 h, fixed with PFA 4% and permeabilized in 0.2%
736 Triton-X100. Actin filaments (in red), microtubules (in green) and nuclei (in blue)
737 were, respectively, stained with phalloidin-rhodamine (1/500 Invitrogen R415), anti-
738 α Tubulin (1/1000 Sigma-Aldrich T9026) and DAPI (Sigma-Aldrich D9542). White-
739 filled arrowheads point to TNTs positive for actin staining. Dashed arrowheads
740 indicate the absence of tubulin staining. Confocal images acquired with Spinning
741 Disk Yokogawa CSU-X1. Scale bars 20 μ m.

742

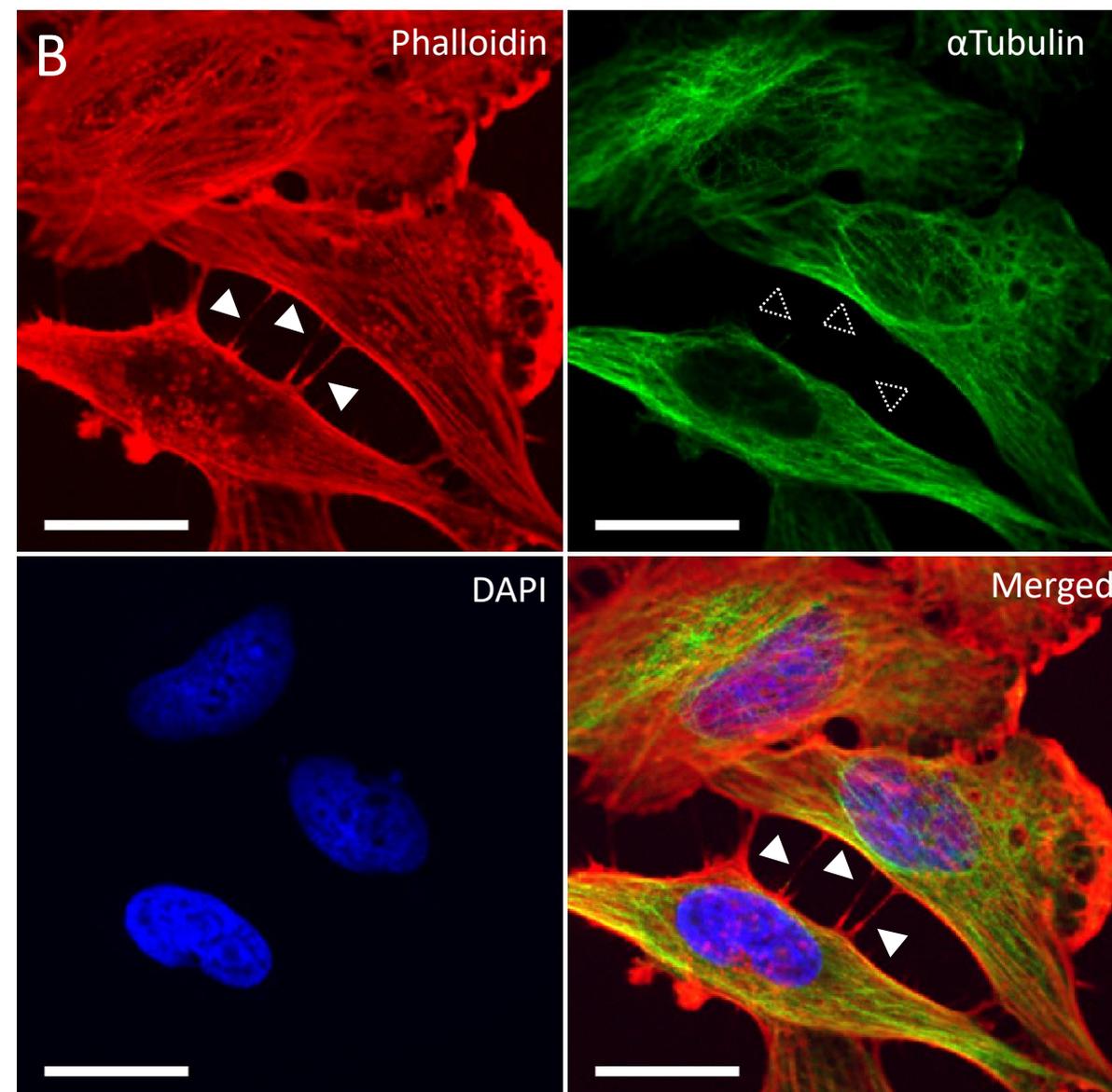
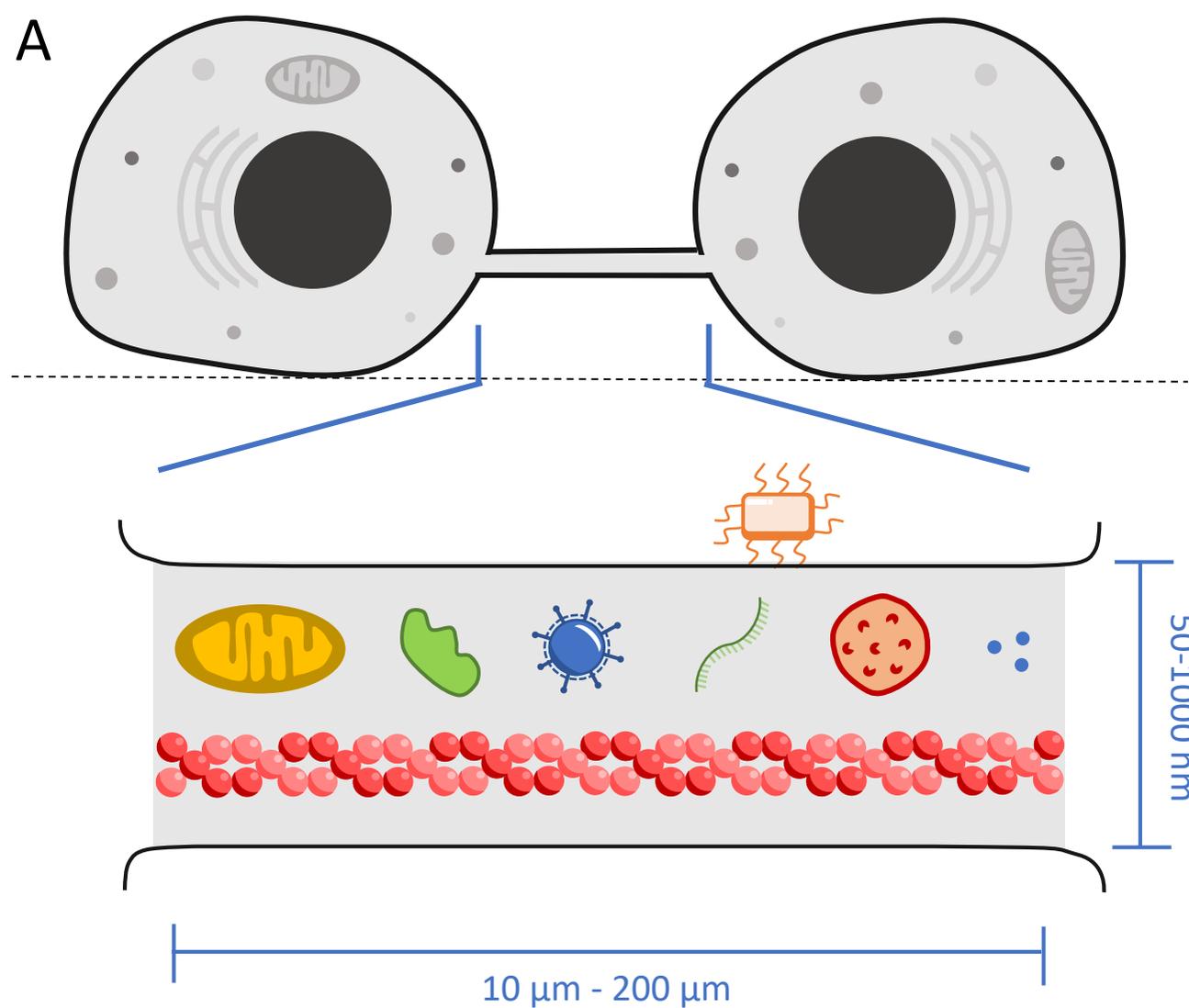
743 **Figure 2.** Schematic of TNT-based network in cancer. In (A), cancer cells with
744 different states of aggressiveness coexist and interact via TNTs. Aggressive cancer
745 cells (in dark blue) display higher interconnection rates than their less aggressive
746 counterparts (in light blue). Cancer cells are surrounded by stromal cells (red) to
747 which they also communicate through TNTs. The homotypic or heterotypic
748 connections between these cell types can be used to share oncogenic content
749 (green circle) or to get rid of material to degrade (red circle). (B) shows a
750 magnification of oncogenic cargoes travelling along the connection providing pro-
751 tumoral features in the receiving cell and healthy lysosomes. Acquisition of
752 mitochondria can promote chemoresistance, invasiveness and provide metabolic
753 help in stress-induced conditions. Transfer of miRNA can drive modifications in the
754 phenotype of the recipient cells, leading to a more aggressive phenotype. Moreover,

755 cellular vesicle content can impact on the proteomic profile of the receiving cells and
756 change their ability to respond to treatments. (C) shows different materials discarded
757 by a cell through TNTs. Organelles used for degradation of damaging content, such
758 as autophagosomes and lysosomes, might be transferred via TNTs as a clearing
759 mechanism. TNTs could also be used as a route for the redistribution of drugs, which
760 would otherwise be toxic in high concentration.

761

762 **Figure 3.** Schematic of GBM network and different types of intercellular connections.
763 GBM cells (in blue) interconnect forming a functional network composed of different
764 types of connections. Thick ($>1 \mu\text{m}$) protrusions (TMs) connect GBM cells and
765 contain both Cx43 and GAP43 which regulate Ca^{2+} flux along the network. Thinner
766 ($<1 \mu\text{m}$) TNT-like connections are present between GBM cells and may allow
767 transfer of material. GBM cells also form TMs that do not contact other cells and are
768 able to drive cell invasion in a GAP43-dependent manner. Pre-synaptic neurons (in
769 orange) extend axons which appose onto the TMs and regulate the Ca^{2+} flux along
770 the GBM network, promoting cell invasion and cell proliferation. Astrocytes (in
771 yellow) of the tumoral brain environment can communicate with GBM cells through
772 TNT-like connections and transfer mitochondria to the tumoral cells, eventually
773 affecting the behavior (e.g., proliferation and response to treatments) of the receiving
774 cells.

Figure 1



Actin filament



Mitochondrion



Protein



Virus



Nucleic acid



Bacteria



Lysosome



Ca²⁺ ions

Figure 2

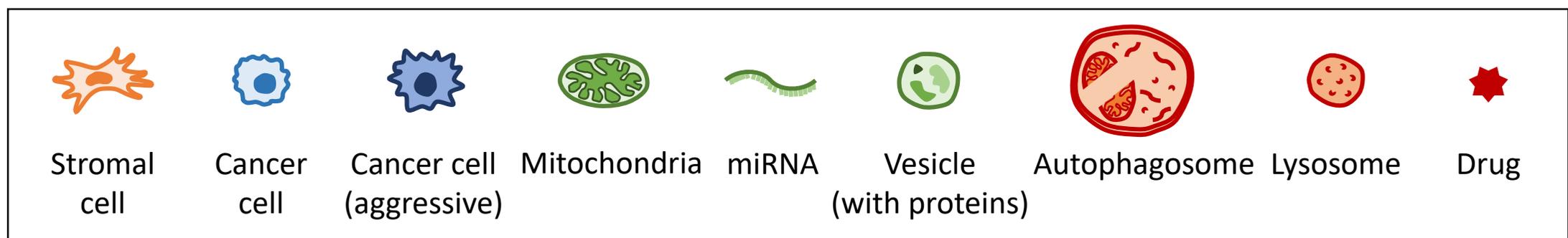
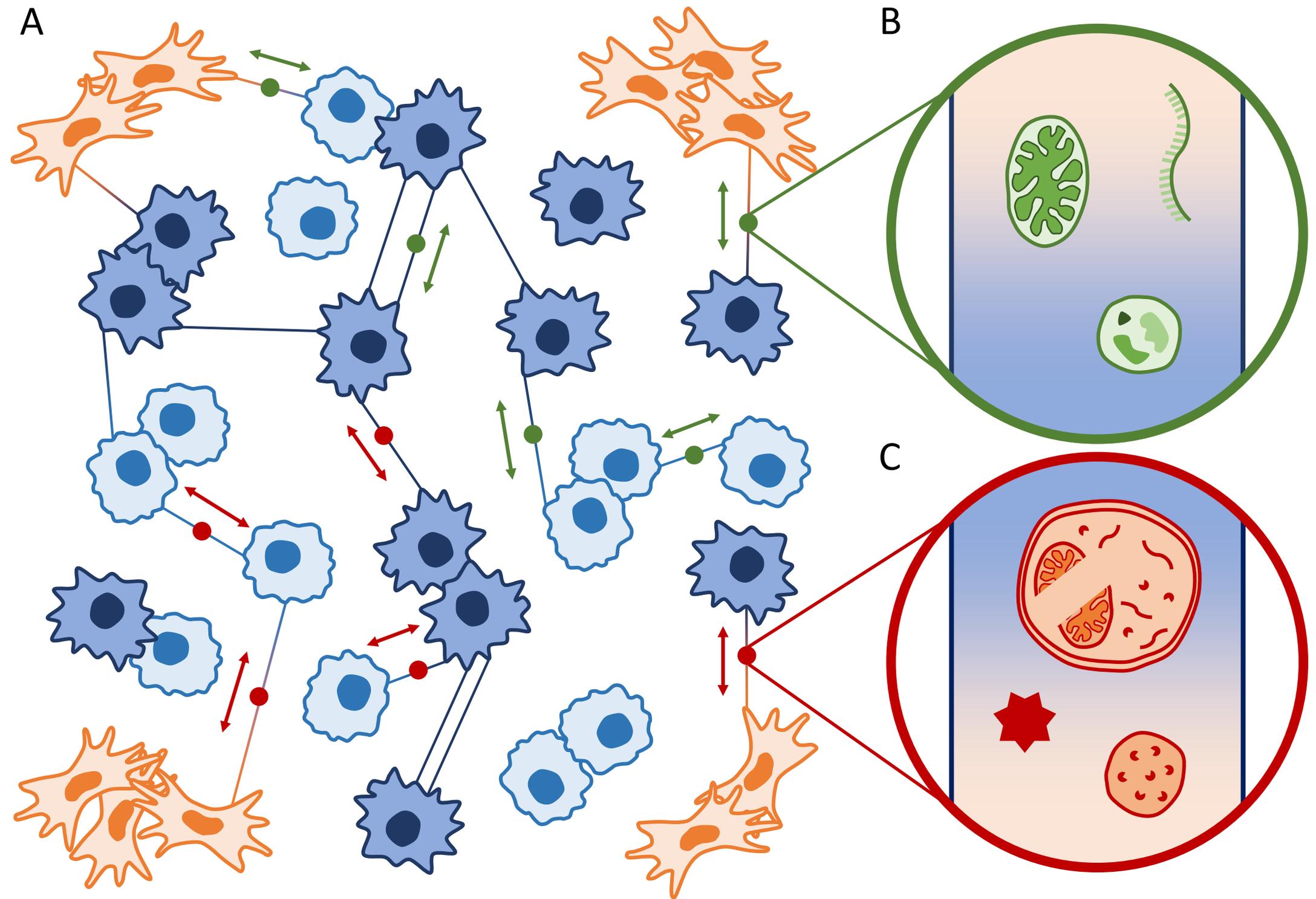


Figure 3

