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ORIGINAL ARTICLE

# Protruding membrane nanotubes: attachment of tubular protrusions to adjacent cells by several anchoring junctions

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**Abstract** Membrane nanotubes are a morphologically versatile group of membrane structures (some resembling filopodia), usually connecting two closely positioned cells. In this article, we set morphological criteria that distinguish the membrane nanotubes from filopodia, as there is no specific molecular marker known to date that unequivocally differentiates between filopodia and protruding nanotubes. Membrane nanotubes have been extensively studied from the morphological point of view and the transport that can be conducted through them, but little is known about the way they connect to the adjacent cell. Our results show that the nanotubes may connect to a neighboring cell by anchoring junctions. Among cell adhesion proteins, N-cadherin, β-catenin, nectin-2, afadin and the desmosomal protein desmoplakin-2 were immune-labeled. We found that N-cadherin and β-catenin are concentrated in nanotubes, while the concentrations of other junction-involved proteins are not increased in these structures. On the basis of data from transmission electron microscopy, we propose a model of the nanotube attachment where the connection of nanotubes is stabilized by several anchoring junctions, most likely adherens junctions that are formed when the nanotube is sliding along the target cell membrane.

**Keywords** Membrane nanotubes · Filopodia · T24 · Adherens junctions · N-cadherin · β-Catenin · Afadin

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## Introduction

Intercellular contact formation is one of the critical steps in formation of multicellular organisms, tissue organization and intercellular communication. In recent years, a new kind of intercellular communication was discovered, using tubular cell extensions called membrane nanotubes (Rustom et al. 2004). These membrane structures enable contact intercellular communication at fairly long distances of up to several 100 μm. Membrane nanotubes of various morphologies were discovered, mainly serving as tubes for conducting signals or cell compartments between cells (Önfelt et al. 2004; Gerdes et al. 2007; Davies and Sowinski 2008; Lokar et al. 2009). The same route can be used by the pathogens to be transferred between cells (Önfelt et al. 2006; Sherer and Mothes 2008; Sowinski et al. 2008; Gousset et al. 2009).

In our previous work, we divided the nanotubes mainly on the basis of the content of cytoskeleton into two types: those, which appear more frequently, shorter (approximately 30 μm) more dynamic and contain actin filaments were classified as type I, while longer, more stable nanotubes containing cytokeratins were denoted as type II. The type I nanotubes can be both protruding by growing out from the cell surface, they resemble filopodia or they can be retractive—pulled out from the cell after two already connected cells starts to move apart (Sherer and Mothes 2008). The protruding nanotubes are of high interest in the

studies of intercellular communication, during tissue regeneration or metastases propagation, because these nanotubes appear to actively seek for the contact with neighboring cells (Veranič et al. 2008).

The protruding nanotubes of type I share many similarities with classic filopodia and microspikes (review in Faix et al. 2009) that can be seen on most migratory cells. They both are tubular cell extensions, containing actin filaments and can attach to the neighboring cells or to the extracellular matrix (Vasioukhin et al. 2000; Faix et al. 2009).

The morphological similarities between type I protruding nanotubes and filopodia made us verify if the two structures share also the same mechanism for the attachment to the target cell. Interestingly, in spite of being crucial for the communicational function of nanotubes, there is no information available on the molecular mechanisms involved in attachment of nanotubes to the target cell.

For the filopodia, it has been well established that they are involved in the formation of one of the major anchoring type of intercellular junctions, the adherens junctions (Vasioukhin et al. 2000). The adherens junctions consist of two basic adhesive units: the cadherin/catenin complex and the nectin/afadin complexes. Cadherins and nectins form lateral homodimers that engage in intercellular homotetramer complex with the same homodimer of the neighboring cell. Cadherins need $Ca^{2+}$ for binding the cadherin of the other cell, while the nectins do not (reviewed by Sakisaka and Takai 2004; Takai et al. 2008; Niessen and Gottardi 2008). Cadherins bind α-catenin, and β-catenin and this molecular complex further associate with vinculin and other cytoskeletal proteins, resulting in the organization of adherens junction, or zonula adherens in polarized epithelial cells. The CAM protein nectins are bound to actin filaments by a linking protein afadin. Afadin additionally serves as an adaptor protein by further binding many scaffolding proteins and F-actin-binding proteins and contributes to the association of nectins with other cell–cell adhesion and intracellular signaling systems (Takai and Nakankishi 2003). Cadherins alone are not sufficient for the formation of adherence junctions; cooperation between nectins and cadherins is required for initiation of cell-to-cell contacts (Takai et al. 2008).

In this work we identified cell adhesion proteins that are involved in the attachment of nanotubes that appear between human tumor urothelial cells (T24). It became evident that a similar mechanism takes place in attachment of membrane nanotubes as in the formation of adherens junctions by filopodia. The main difference is that the process of nanotubular attachment does not include drawing of the two opposing membranes together and clustering of adherens junction proteins that is known from filopodial formation of adherens junction. We propose a model of nanotube attachment to a neighboring cell by lateral attachment of the nanotube to the target cell and only after attachment an open-ended connection can be established.

## Materials and methods

### Urothelial cell cultures

Urothelial cell line T24 was cultured in a 1:1 mixture of advanced Dulbecco's modified Eagle's medium (Gibco, Invitrogen, Carlsbad, CA, USA) and Ham's F-12 (Sigma-Aldrich Corp., St. Luis, MO, USA), supplemented with 10% fetal calf serum (Gibco, Invitrogen, Carlsbad, CA, USA), 5 μg/ml insulin, 5 μg/ml transferrin, 100 mg/ml hydrocortisone and 5 ng/ml selenite (all Gibco, Invitrogen, Carlsbad, CA, USA), 1,800 U/ml cristacyclin (Pliva, Zagreb, Croatia) and 0.222 mg/ml streptomycin sulphate (Fatol Arzneilmittel GmbH, Schiffweiler, Germany). Cells were incubated at 37°C in a humidified incubator in an atmosphere of 5% $CO_2$. A day prior to experiment, cells were seeded onto sterile glass coverslips (Brand GmbH, Wertheim, Germany) at approximately 70–80% confluency and incubated overnight at 37°C.

### Antibodies

All the primary antibodies were purchased from AbCam Ld (Cambridge, UK), except those specially indicated. Mouse monoclonal antibodies were used for labeling of PAN-cadherin, clone CH-19 (Sigma-Aldrich Corp., St. Luis, MO, USA) and E-cadherin. Rabbit polyclonal antibodies were used for labeling the desmoplakin-2, desmoglein, N-cadherin, afadin and β-catenin. Nectin-2 was labeled with rat monoclonal antibodies.

As secondary antibodies, goat antimouse AlexaFluor™ 555 IgG (H + L) antibodies (Molecular Probes, Invitrogen, Carlsbad, CA, USA), goat antirabbit AlexaFluor™ 488 antibodies (Molecular Probes, Invitrogen, Carlsbad, CA, USA) and goat antirat AlexaFluor™ 555 were used, all at dilution of 1:500, or goat antirabbit TRITC (Sigma-Aldrich Corp., St. Luis, MO, USA) at a dilution of 1:100.

### Phase-contrast microscopy

Live cells were observed and analysed at 37°C with a phase-contrast objective on Axio-Imager Z1 microscope (Carl Zeiss MicroImaging GmbH, Heidelberg, Germany).

**Table 1** Morphological criteria to differentiate between filopodia and membrane nanotubes

| Tubular protrusion | Length | Location |
|---|---|---|
| Filopodium/ microspike | <10 μm | Originates from convexly curved region on the cell edge |
| Type I membrane nanotube | >10 μm | At the concave or straight region of cell rim, usually originates from the upper area of the cell, not from the cell edge; always directed to the target cell |

## Immunofluorescence labeling and microscopy

For desmoplakin, pan-cadherin, N-cadherin and β-catenin labeling, cells were washed with PBS and fixed for 30 minutes in 2% paraformaldehyde (Merck KGaA, Darmstadt, Germany). Paraformaldehyde solution was warmed to 37°C. After washing with PBS, the coverslips were incubated in ice-cold 0.25% Triton X-100 in PBS for 6 minutes at room temperature, washed three times in PBS and incubated in 0.33 M sucrose in 0.2 M cacodylate buffer for 30 minutes. Afterwards, samples were blocked in 2% bovine serum albumin (BSA, Sigma-Aldrich Corp., St. Luis, MO, USA) in 0.2% $NaN_3$ (Fluka, Chemika) in PBS for 30 minutes at room temperature. Coverslips with cells were incubated in primary antibodies for 1 h at 37°C or overnight at 4°C, then washed in PBS for 10 minutes and incubated in secondary antibodies for 30 minutes at 37°C. Actin labeling in 16.7 μg/ml phalloidin (phalloidin-FITC) (Sigma-Aldrich Corp., St. Luis, MO, USA) in 20% methanol (Carlo Erba Reagenti, Rodano, MI, Italy) or AlexaFluor™ 350 at dilution 1:5 in 1% BSA in PBS for 30 minutes was performed after secondary antibody incubation and 10 minutes washing in PBS. Afterwards, coverslips were decanted and embedded in Vectashield-{4′,6-diamidino-2-phenylindole} (Vectashield-DAPI) (Vector Laboratories, Peterborough, UK), mounted on objective glass and analysed in a fluorescence microscope Eclipse T300 (Nikon Corp., Tokyo, Japan) or Axio-Imager Z1 microscope (Carl Zeiss MicroImaging GmbH, Heidelberg, Germany).

## Transmission electron microscopy

Cells were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde (Merck KGaA, Darmstadt, Germany) in cacodylate buffer for 2 h, washed overnight in 0.33 M sucrose in 0.1 M cacodylate buffer, dehydrated in a graded series of ethanol (Carlo Erba Reagenti, Rodano, MI, Italy), and embedded in 1:1 mixture of epon and propylene oxide

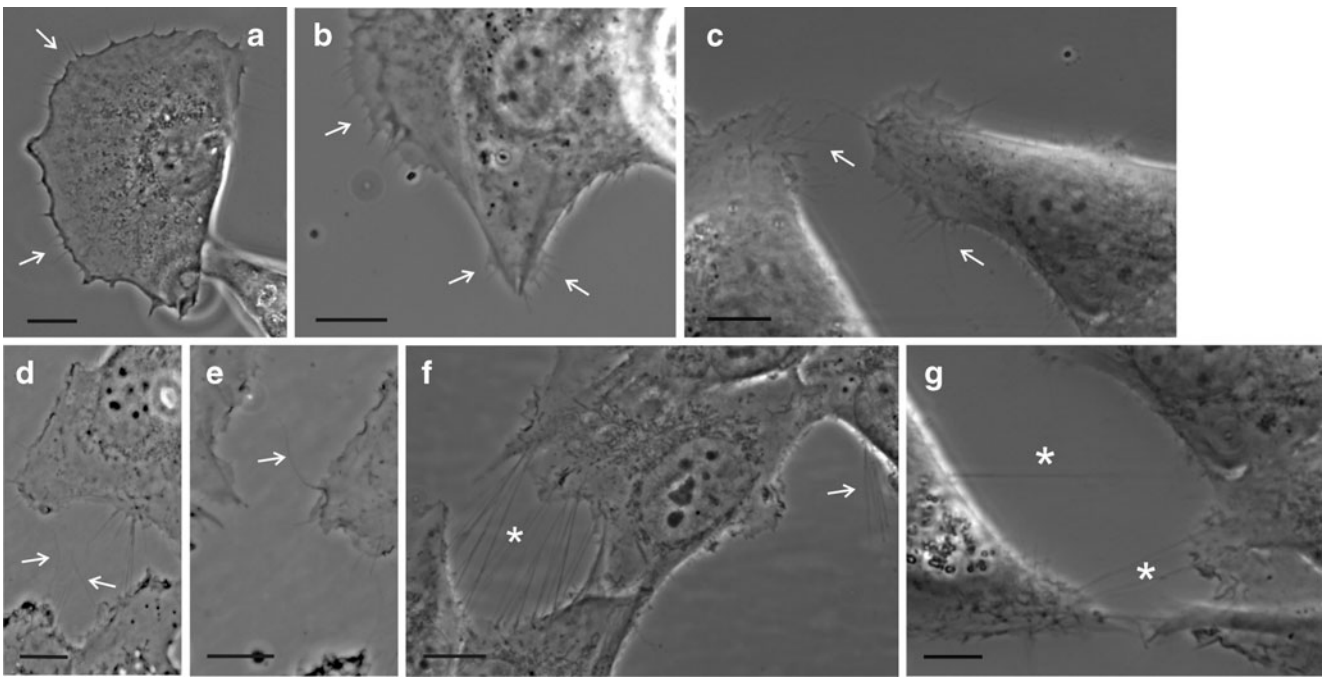


**Fig. 1** **a**–**c** Examples of filopodia (*arrows*) formed by T24 cells. **d**–**g** Examples of membrane nanotubes: protruding (*arrows* in **d**, **e**, **f**) and connecting (*asterisk* in **f** and **g**) type I nanotubes. *Bars*: 10 μm

overnight and for 2 h in epon. Epon was left to polymerize in thermostat for 5 days (a day at 35°C, 45°C, 60°C, 70°C and 80°C). Cells embedded in epon were separated from a cover glass in liquid nitrogen. Ultrathin sections were examined with a PHILIPS CM 100 transmission electron microscope.

Measurements of tubular protrusions

Lengths of tubular protrusions were measured with Axio Vision software ver. 4.8 (Carl Zeiss MicroImaging GmbH, Heidelberg, Germany).

## Results and discussion

In subconfluent cultures the cells have the ability to freely migrate, searching for counterparts to whom they bind or communicate. Forming thin dynamic filopodial-like membrane protrusions (membrane nanotubes) to actively explore the surroundings appears to be an efficient way for intercellular communication.

Filopodium/microspike or a membrane nanotube?

From a morphological perspective and lack of specific markers, it is hard to differentiate between tubular protrusions that cells extend when exploring its surroundings. The most obvious difference between these protruding structures is the length of the tubular protrusions. The filopodia and microspikes (although different by their morphological properties, we do not distinguish between these two entities, since they fall into the same category) usually do not exceed several micrometers in length and extend from lamellipodia or slightly curved regions of plasma membrane (Faix et al.

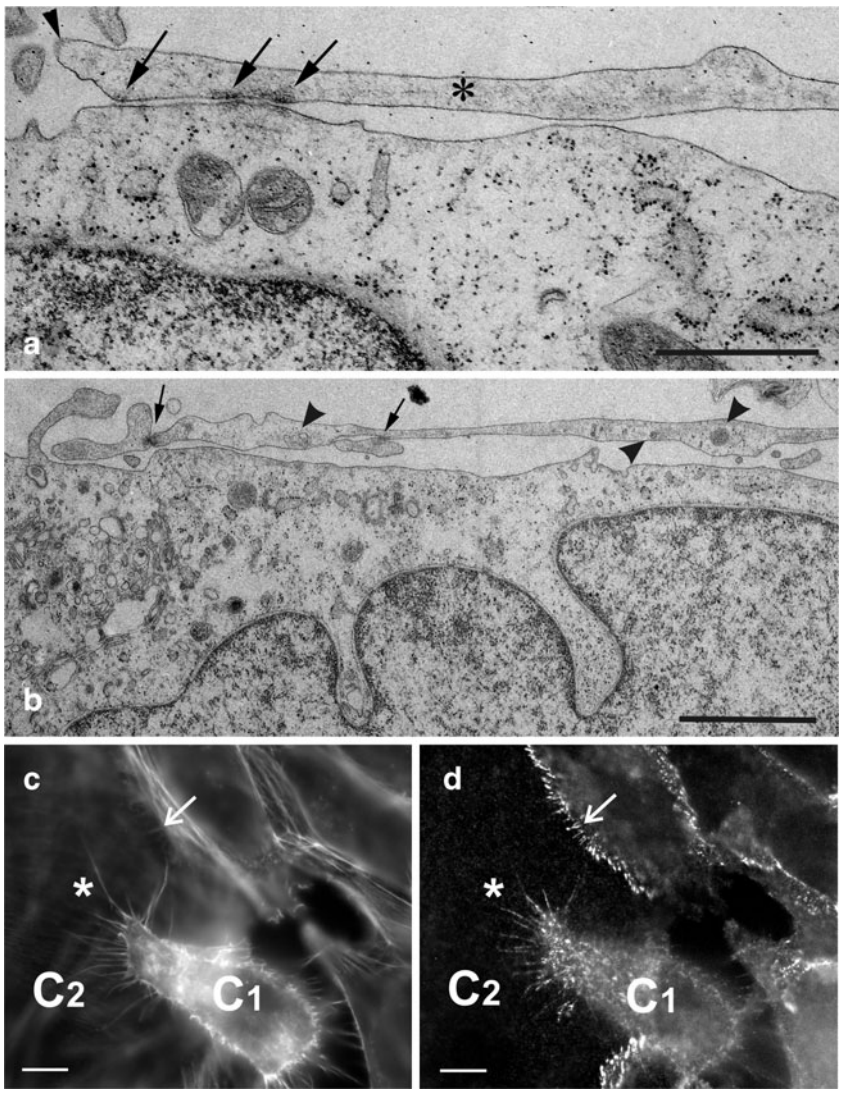


**Fig. 2** **a**, **b** TEM micrographs of a tubular protrusion (*asterisk*) attached to a targeted cell by anchoring junctions (*arrows*) forming between the target cell and the lateral side of the nanotube. The tip of the protrusion is still free (*arrowhead*). In **b**, the protrusion is connected to the plasma membrane of the target cell. The protrusion contains membranous structures indicating that it is a nanotube. *Bars*: 200 nm (**a**); 500 nm (**b**). T24 cells stained for actin filaments (**c**) and pan-cadherin (**d**). Cell C1 is crawling upon cell C2. Cadherins are present at cell–cell contacts (*arrows*) and actin containing nanotubes (*asterisk*), even though not at the very tips of the nanotubes, indicating that intercellular contacts are forming along protruding type I nanotubes. *Bars*: 10 μm

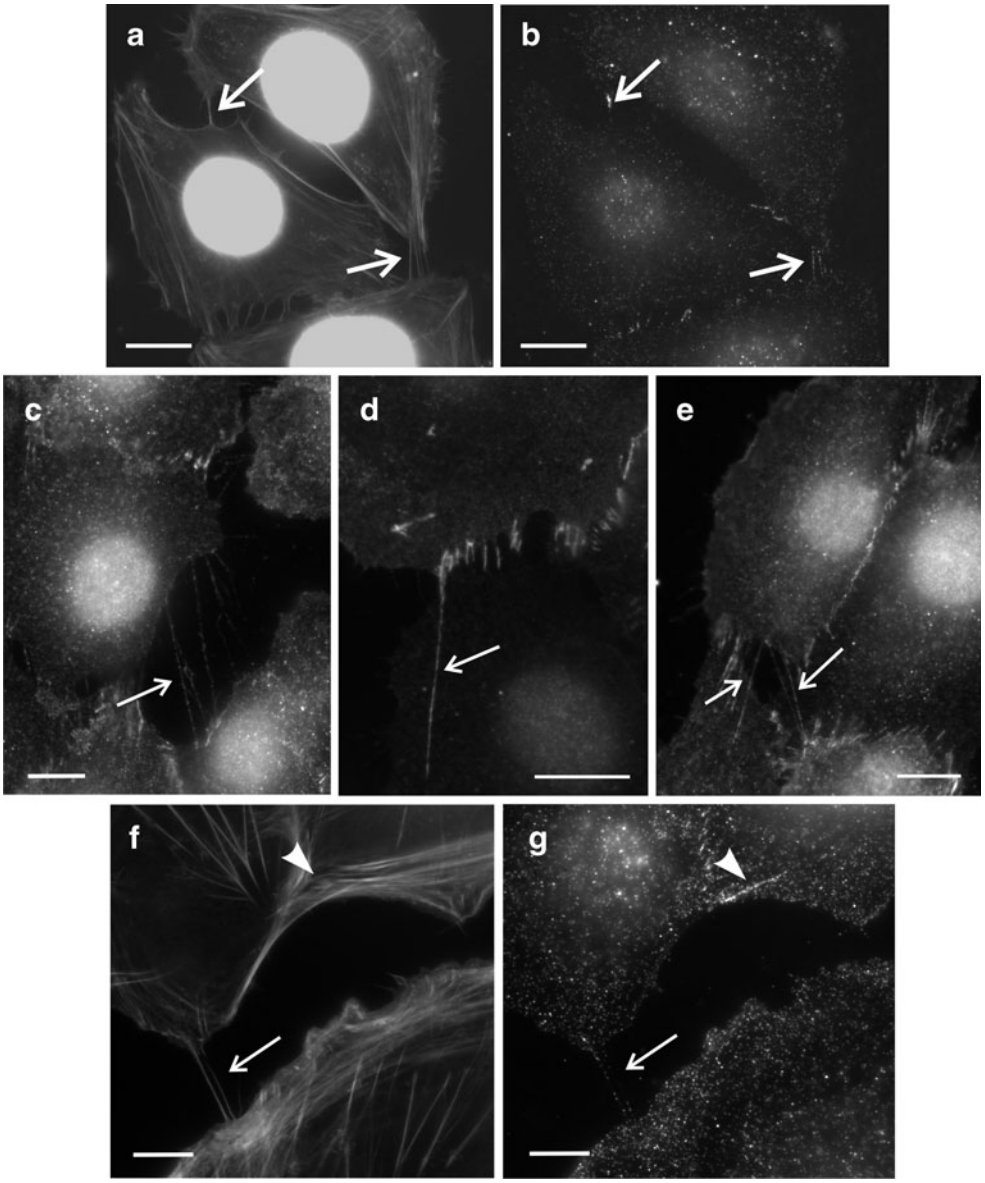


**Fig. 3** Cells stained for actin (**a**, **f**) and N-cadherin (**b**), β-catenin (**c**, **d**, **e**) and afadin (**g**). N-cadherin is present along the entire length of protruding nanotubes, as well as in nanotubes already connected to a cell close by (**a**, **b**, *arrows*). In **e**, one cell forms the type I nanotubular protrusion and some already formed nanotubular bridges (*arrows*). The anchoring junction plaque protein β-catenin is concentrated along entire length of the protruding type of nanotube (*arrows* in **d**, **e**) as well in those which are connecting two separated cells (*arrows* in **e**). Afadin (**g**) is found in the protruding type of nanotubes (*arrowhead*) as well as in the nanotubes connecting two adjacent cells (*arrow*). *Bars*: 10 μm

2009 and references therein, Medalia et al. 2007), while membrane nanotubes may reach up to 30 μm. To differentiate between filopodia and type I membrane nanotubes, we set the morphological criteria to define which structures are filopodia/microspikes and which are the membrane nanotubes. The morphological criteria are given in Table 1 and examples are given in Fig. 1.

Membrane nanotube may begin growing as filopodium, but usually originates from the upper area of the cell, not from the cell edge. For the formation of a membrane nanotube, a presence of a target cell close by is required (Fig. 1d–g) since no membrane nanotubes were observed on individual cells at distances larger than 100 μm from their neighbors. On the other hand, the existence of filopodia does not appear to depend on the presence of

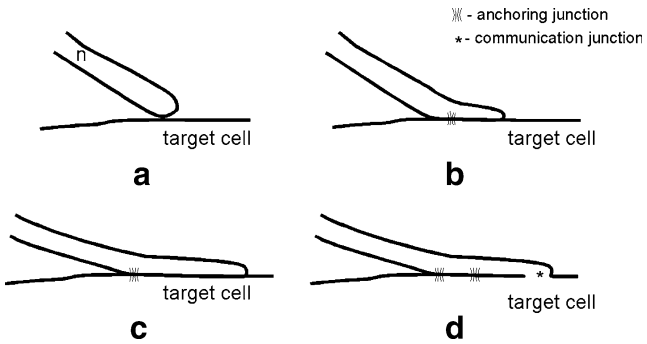


**Fig. 4** Schematic representation of the type I nanotube (*n*) attachment to the adjacent cell. When the nanotube reaches the adjacent cell (**a**), it anchors to the plasma membrane (**b**) with the nanotube tip sliding upon the membrane, forming additional anchoring junctions to prevent being swept away from the plasma membrane of the target cell. When the nanotube finds an appropriate site on the plasma membrane, a cytosolic connection (*asterisk*) is established

other cells (Fig. 1a). Another striking difference is the ability of nanotubes to conduct intercellular signals or organelles and thus enable intercellular cytosolic communication by being open ended (Önfelt et al. 2006; Veranič et al. 2008), which has never been observed for filopodia.

Membrane nanotube may attach to adjacent cell. On the basis of transmission electron microscopy, we show that the nanotubes become attached to the plasma membrane of the neighboring cell by an anchoring type of intercellular junctions forming between a patch of plasma membrane of the target cell and the tip of the nanotube (Fig. 2a, b). After the initial junction is formed, the nanotube seems to slide along the plasma membrane, initiating additional junctions at the side where the nanotube approaches the membrane of the target cell. It appears that the nanotube requires several anchoring junctions to stabilize the attachment of the nanotube to the target cell before it forms an open-ended connection that allows intercellular communication (Fig. 2a) (Veranič et al. 2008).

In order to identify which proteins are involved in the formation of the anchoring junctions, we screened by immunofluorescence labeling for several candidates of cell junction proteins. Of the transmembrane proteins directly involved in the intercellular connections, we labeled the cells for pan-cadherin, E-cadherin, N-cadherin, nectin-2 and desmoplakin. Additionally, the proteins responsible for linking those transmembrane proteins to the cytoskeleton were labeled. Therefore, antibodies against β-catenin, desmoplakin and afadin were used for proving a possible attachment to the actin filaments, which were labeled with phalloidin-FITC or phalloidin-AlexaFluor$^{TM}$ 350. We found that in the nanotubes, attached to the bottom of the plastic dish and nanotubes already connected to adjacent cells, N-cadherins (Fig. 3a, b) and β-catenins (Fig. 3c–e) are concentrated, while other proteins of anchoring junctions were found equally distributed in both nanotubes and other regions of the cell (Fig. 3g). We thus predict that only the proteins of adherens junctions are responsible for the attachment of type I nanotubes to the target cells. These findings are in accordance to the reports of the attachment of similar types of cellular extensions as are filopodia in epithelial cells during cell–cell adhesion formation (Vasioukhin et al. 2000) and the *processes adhaerentes* found in mesenchymal stem cells (Wuchter et al. 2007).

Most of the ongoing research is focused on the morphology of nanotubes and transport of cellular components employing both electron and fluorescence microscopy (reviewed in Gerdes et al. 2007; Davies and Sowinski 2008; Lokar et al. 2009), but little is known about their dynamics, stability, and how the connections are initiated and formed by these membrane structures. The growth and the transport through them, once a connection is present, involves the integrity and the remodeling of the actin cytoskeleton (Veranič et al. 2008; Gurke et al. 2008; Bukoreshtliev et al. 2009; Lokar et al. 2009). It has been shown that the contact between the nanotube and the cell may or may not provide cytosolic continuity (review in Lokar et al. 2009). The biochemical data on the nature of the contacts are not known: there are speculations that the connection is mediated by an anchoring junction (Veranič et al. 2008; Sherer and Mothes 2008). We have shown that the components of adherence junctions (namely N-cadherins, β-catenins, afadin) are present in the membrane nanotubes, both in protruding nanotubes as well as in connecting nanotubes, while other cell-junction forming proteins are absent from them.

Based on our observations (TEM, immunofluorescence, time-lapse data), we propose the following model of the nanotube attachment and cytosolic connection to the target cell. Cells form filopodial-like protrusions by which they search their surroundings. When they find an adjacent cell (Fig. 4a), protruding nanotubes slide upon the surface of the plasma membrane searching for the appropriate docking site to establish a communication junction (Fig. 2c, d). In the process, the lateral side of the nanotube (the side in contact with the plasma membrane of target cell) becomes transiently anchored to the plasma membrane (Fig. 4b) via cadherin system by multiple anchoring points (Figs. 2d, 4c), following the line where the nanotube has searched the surface of target cell. This prevents the nanotube to be swept away from the cell surface by eternal flows. In favor of this model is the observation that at the tips of the nanotubes, no cell-anchoring proteins are found with only the actin cytoskeleton, and presumably membrane moulding, driving the elongation of the nanotube upon plasma membrane surface (Fig. 2c). Multiple points of a nanotube attachment also confirm the observation that the attached part of the nanotube tends to be straight (Fig. 2d), while the sliding, unattached, part of the nanotube may have a rather curved line (Fig. 2c). Once the nanotube finds an appropriate patch of membrane, a communication junction and cytoplasmic connection may be formed between the nanotube and a target cell, enabling the transport of cellular components via this cytoplasmic bridge (Fig. 4d).

Our results indicate that the nanotubes of type I attach to the target cell by adherens junctions at multiple points, strengthening the connection between the sliding nanotube and a target cell membrane. Cell adhesion proteins that mediate this connection belong to the cadherin family; in T24 cell line, the anchoring proteins are N-cadherin and its adaptor protein β-catenin. In nanotubular structures, also afadin is found, concentrated in type I protruding nanotubes (Fig 3g), suggesting that nectin–afadin complex may also be involved in attachment of the nanotubes.

It still remains to be elucidated which are the factors that direct the movement of the nanotube upon plasma membrane

surface and which are the specific regions on the target cell where a communication junction can be formed.

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