J. Cell. Mol. Med. Vol 8, No 3, 2004 pp. 388-396

Vesicle traffic through intercellular bridges in DU 145 human prostate cancer cells

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Received: September 6, 2004; Accepted: September 20, 2004

Abstract

We detected cell-to-cell communication via intercellular bridges in DU 145 human prostate cancer cells by fluorescence microscopy. Since DU 145 cells have deficient gap junctions, intercellular bridges may have a prominent role in the transfer of chemical signals between these cells. In culture, DU 145 cells are contiguous over several cell diameters through filopodial extensions, and directly communicate with adjacent cells across intercellular bridges. These structures range from 100 nm to 5 μm in diameter, and from a few microns to at least 50-100 μm in length. Time-lapse imagery revealed that (1) filopodia rapidly move at a rate of microns per minute to contact neighboring cells and (2) intercellular bridges are conduits for transport of membrane vesicles (1-3 μm in diameter) between adjacent cells. Immunofluorescence detected alpha-tubulin in intercellular bridges and filopodia, indicative of microtubule bundles, greater than a micron in diameter. The functional meaning, interrelationship of these membrane extensions are discussed, along with the significance of these findings for other culture systems such as stem cells. Potential applications of this work include the development of anticancer therapies that target intercellular communication and controlling formation of cancer spheroids for drug testing.

Keywords: intercellular bridges - filopodia - membrane vesicles - intercellular communication - prostate cancer

Introduction

Intercellular communication is essential to the development and function of multicellular organisms. Chemical signals are transmitted between cells via intricate structures such as gap junctions and chemical synapses [1]. Another mechanism of intercellular communication has recently been proposed that involves intercellular bridges [2, 3]. Much of the information on the mode of cellular exchange across these tubular structures comes from normal cells and germ cells in particular [3–5]. While it is recognized that some cancer cells can assemble intercellular bridges [6, 7], the structure and function of these membrane extensions are poorly understood in neoplastic cultures.

During carcinogenesis, there are significant changes in intercellular communication, altering the balance between cell proliferation and death. To date, much of the research on this topic has focused on gap junctions. This mechanism is defective in many cancer cells [8]. LNCaP, DU 145 and PC 3 human

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prostate cancer cells are representative of this trend [9, 10]. Infecting these cells with an adenovirus vector encoding connexin 26, a gap junction protein, suppressed cell growth and induced cell-cycle arrest and apoptosis [11]. Restoration of appropriate intercellular communication distorted by carcinogenesis may be a novel approach to cancer treatment.

In cancer cells with deficient gap junctions, intercellular bridges may have a prominent role in intercellular communication and, as such, may be useful targets for anti-cancer therapies. In the present study, we begin to characterize this mechanism of communication in cancer cells, with DU 145 as a model cell line [12]. We describe bridge structures containing microtubules that facilitate vesicle traffic between cells.

Materials and methods

Cell culture

Human prostate cancer cells DU 145 (HTB 81) were purchased from American Type Culture Collection (Manassas, VA), and cultures were propagated at 37ºC, 5% CO_2 and 95% relative humidity in GTSF-2 complete medium [13] containing 7% fetal bovine serum (pH 7.4). Stock cultures of this cell line were maintained in T-flask and subcultured by trypsinization.

DU 145 cells were harvested from stock cultures by scraping and transferred to LabTek chambered cover glass (Nalge Nunc International, Rochester, NY) to facilitate visualization of cellular structures. Chambers were either pretreated with an attachment-limiting substrate, agar (liquid-overlay culture), or used without a coating (monolayer culture). For the former, the chambers were covered with a thin layer (1 mm) of 0.25% agar (Invitrogen, Carlsbad, CA) prepared in serum-free GTSF-2 medium [14]. The inoculum for these cultures consisted of 2.0 $\times 10^4$ cells/cm^2 in 2 ml of complete GTSF-2.

Fluorescence microscopy

For fluorescent labeling of cell membranes, DU 145 cells from stock cultures were suspended at a density of 1x10^6/ml in serum-free medium. The lipophilic stain Vybrant® DiI (Molecular Probes Inc., Eugene, OR) was added at a concentration of 5 μL per milliliter cell suspension and mixed by gentle pipetting. After 20-min incubation in the dark at 37°C, the labeled suspension was centrifuged at 1500 rpm for 5 min at 37°C, and the supernatant was removed. Cell samples were gently resuspended in complete medium at 37°C, washed again, and seeded in triplicate in chambered cover glass (liquid-overlay and monolayer cultures). In a second type of experiment, triplicate samples of DU 145 cells were divided in halves and each half separately labeled with DiI and another fluorescent dye, Vybrant® DiO (Molecular Probes), using the same procedure. The labeled cells were then combined to form the culture inocula. Cells were examined with phase-contrast and fluorescence microscopy every 4 h for a total of 48 h. At select times, time-lapse images were acquired at 30 s intervals. Image analysis was performed by Image-Pro Plus and Scope-Pro software (Media Cybernetics, Silver Spring, MD).

To prepare DU 145 cells for in situ immunofluorescence of alpha-tubulin, culture chambers were aspirated to remove conditioned medium and washed (2 x 1 min) with pre-warmed PBS (pH 7.4). Chamber cover glass slides were subsequently processed at ambient temperature. Culture triplicate samples were first fixed for 10 min with 3.7% methanol-free formaldehyde (Sigma Chemical Co., St. Louis, MO) in PBS, washed (3 x 1 min) in PBS, permeabilized by 5-min exposure to 0.1% Triton X-100 (Sigma) in PBS containing 1% BSA, and then washed (3 x 5 min) in PBS + 1% BSA. Anti-alpha-tubulin mouse IgG_1 monoclonal antibody and fluorescent anti-mouse IgG antibody were purchased from Molecular Probes. Culture samples were immunostained by incubation for 30 min with 5 μl of primary antibody per milliliter PBS in the culture chamber from a stock solution of 1 μg/ml PBS, permeabilized in 0.1% Triton X-100 in PBS + 1% BSA, and washed for 5 min in PBS + 1% BSA. This was followed by incubation in the dark for 30 min with the secondary antibody at 2 μl/ml PBS from a stock of 4μg/ml PBS and finally washed for 5 min with PBS + 1% BSA. Cover glass slides were air-dried and mounted in glycerin for analysis.

Results

On an attachment-limiting substrate, agar, DU 145 cells emit elongated filopodia and intercellular bridges of different dimensions. These membrane extensions were detected by labeling DU 145 cells with the lipophilic fluorescent dye DiI after 16 h in liquid-overlay culture (Figs. 1, 2, 4). Cells extended one or multiple filopodia simultaneously (Figs.

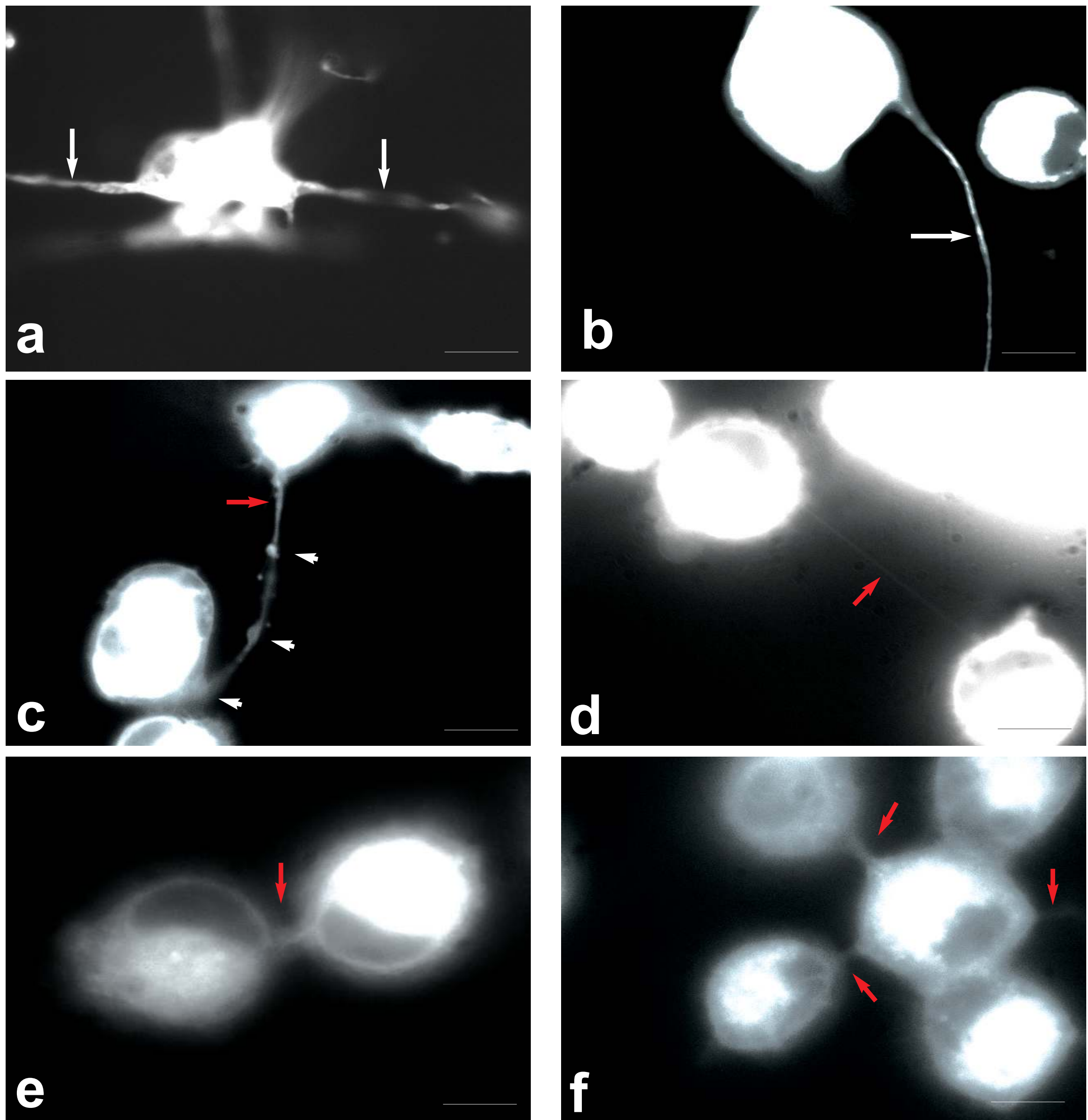

Fig. 1 Filopodia (white arrows) and intercellular bridges (red arrows) in DU 145 liquid-overlay culture 16 h after inoculation. Cell membranes were labeled with fluorescent DiI: a) cell with numerous filopodia; b) long-thin filopodium extending from cell surface; c) long-thin filopodia with bead-like lipid bulge (arrowheads, ~1-3 mm diameter) and adhesion pad (arrowhead) in contact with another cell; d) long-thin (~150 nm diameter) intercellular bridge; e) short-wide (~ 4 mm diameter) intercellular bridge between two cells; f) multiple short-wide (~2 mm diameter) intercellular bridges between a group of cells forming a nascent spheroid. Scale bar: 10 µm.

1a,b) that sometimes contained bead-like membrane bulges (1 to 3 mm in diameter) and an adhesion pad on the outermost tip (Fig. 1c). Filopodia and intercellular bridges range in diameter between 100 to 200 nm for thin extensions (Fig. 1d) and 1 to 5 µm (Figs. 1e,f) for wider structures. Their length is between a few microns (Figs. 1e,f) and at least 50 to 100 µm (Figs. 1b,c). Pairs or groups of cells

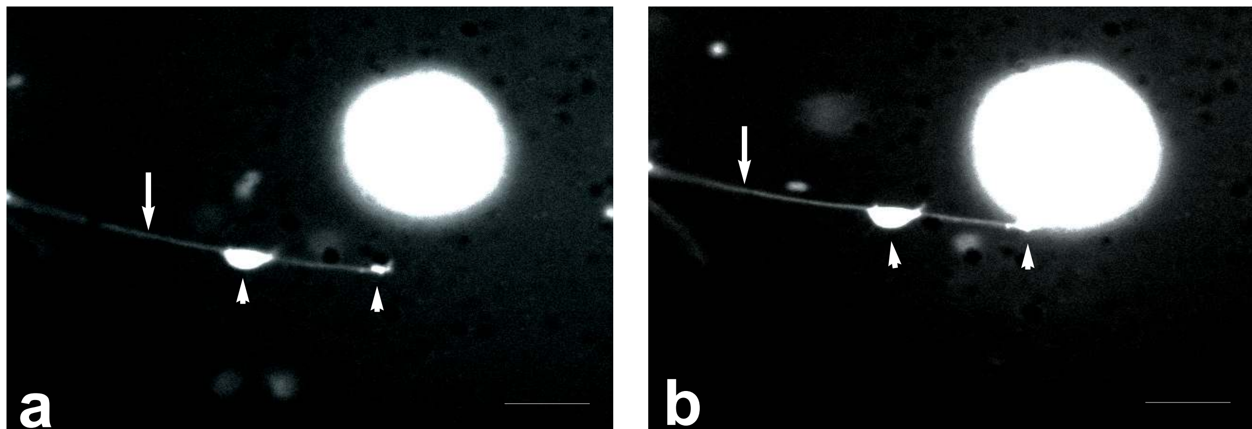

Fig. 2 Dynamics of filopodia (arrows) interactions between DU 145 cells labeled with DiI in liquid-overlay culture. Time-lapse images were acquired at 30 s intervals. A mobile filopodium with lipid bulge (arrowhead) reaches towards neighboring cell (a) and contacts the target cell (b) with the adhesion pad (arrowhead) at the tip of the extension. Data was acquired 16 h after inoculation. Scale bar: 10 µm.

appear to form chains interlinked by intercellular bridges (Figs. 1e,f).

Time-lapse imagery revealed that filopodia are dynamic structures, rapidly extending several microns over a period of 30 s to make contact with a neighboring cell (Fig. 2ab). Filopodia and intercellular bridges are not limited to liquid-overlay culture, but are also evident in monolayer cultures of the same cell line attached to glass (Fig. 3). Chains of cells interlinked by intercellular bridges are abundant in monolayer culture (Figs. 3b,c). Immunofluorescence detected alpha-tubulin in intercellular bridges (Figs. 3d,e) and individual filopodia (Fig. 3f) indicative of microtubule bundles greater than a micron in diameter.

The involvement of filopodia in cell locomotion is well established. This study suggests that filopodia have additional roles in culture. Liquid-overlay culture generates spheroids of cancer cells used for drug testing [14]. The cells preferentially attached to each other rather than to the attachment-limiting agar substrate. In this environment, filopodia contacted neighboring cells (fig. 2a,b), and intercellular bridge formation was evident during cell aggregation to form nascent spheroids (Fig. 1f). Figure 4 shows the involvement of intercellular bridges in cell communication. Video microscopy suggests that the bridges can form microtunnels connecting adjacent cells. In the sequence of time-lapse images, membrane vesicles with 1 to 3 µm diameters traveled through these intercellular tunnels; some vesicles appeared to fuse in the process (see video at http://www.jcmm.ro/jcmm/download/jcmm008.003.11.avi).

Additional research on intercellular communication was achieved by mixing two groups of DU 145 cells labeled with two different dyes, DiI and DiO. Eight hours after mixing the cells together, fluorescence microscopy detected exchange of membrane material between the two groups: vesicles of one color were present in the cytoplasm of cells with the contrasting color (Figs. 5a-f). After 12 h, vesicles of mixed color are prevalent (Figs. 5g-i), probably due to fusion of native endomembranes and imported vesicles and to lipid diffusion in membranes.

Discussion

DU 145 cells exhibited the remarkable capacity to physically interact in culture over several cell diameters through contiguous filopodial extensions and direct communication was established between adjacent cells across intercellular bridges. The following discusses the functional meaning, interrelationship, and composition of these cellular structures, as well as the significance of these findings for other culture systems.

Insight into the functional meaning of filopodial dynamics comes from the epithelial origin of DU 145 and other adenocarcinoma cells. It is likely that filopodia interactions among DU 145 cells are sim-

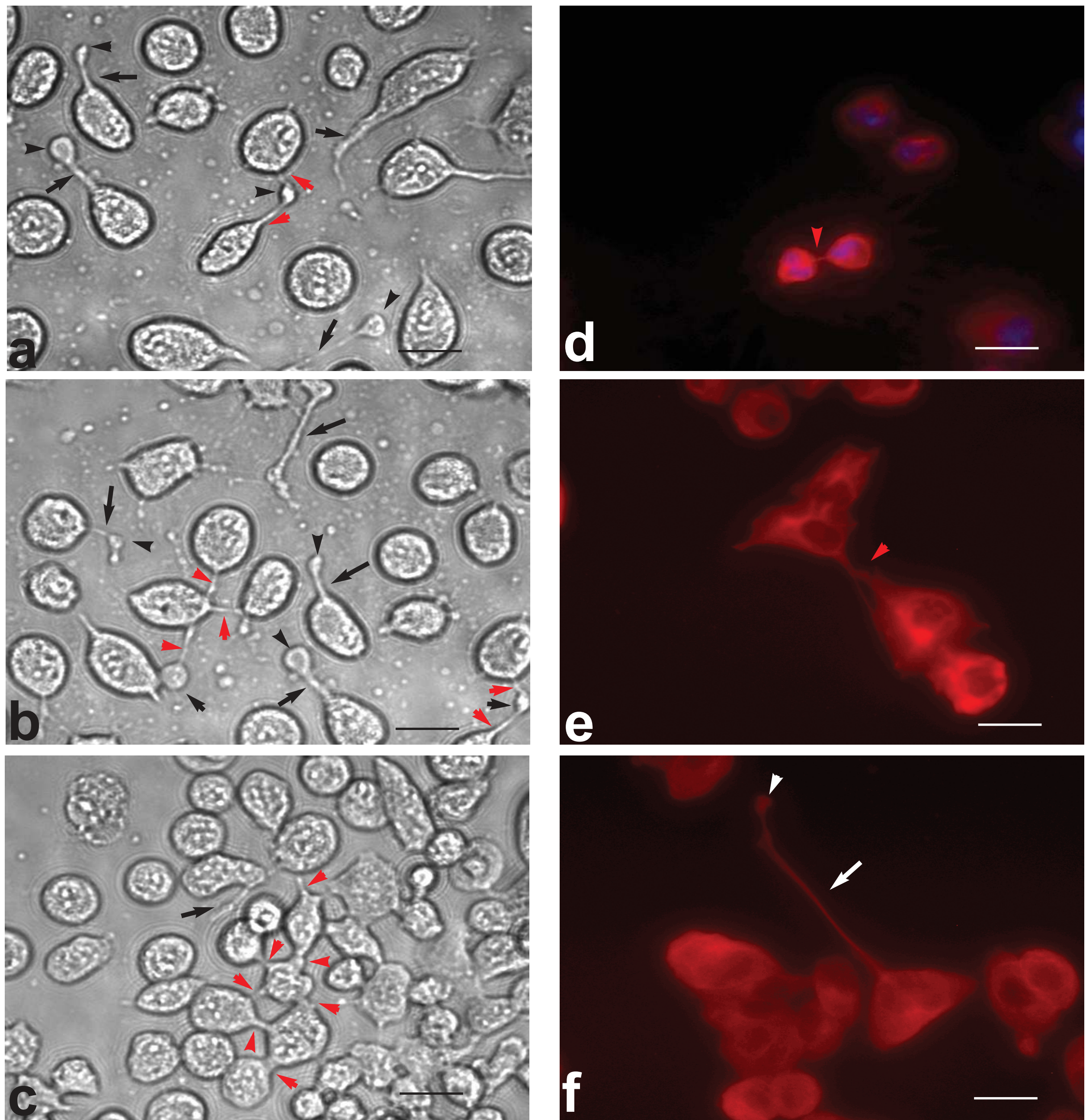

Fig. 3 Membrane extensions in DU 145 monolayer culture. Phase-contrast microscopy: (a - c) lipid bulges and adhesion pads (black arrowheads) on filopodia (black arrows) and intercellular bridges (red arrowheads). Immunofluorescence with anti-alpha tubulin antibodies: (d - f) filopodia (white arrows) and intercellular bridges (red arrowheads). Scale bar: 25 µm.

ilar to epithelial sheet sealing during embryogenesis. The latter is a biphasic process of transient followed by stable interactions that involves penetration of actin-rich filopodia into opposing cells, actin depolymerization until the two epithelial fronts meet and evolution of these weak interactions into stable adhesion mediated by transmembrane dimerization of cadherins [15,16]. As in the present study, filopodia involved in epithelial sheet sealing are upward of 70 µm in length, can be less than a micron in diameter, and extend/retract at a rate of ~10 µm/min. The filopodia extending from DU 145 cells may aid in directing locomotion towards another cell and in subsequent cell aggregation.

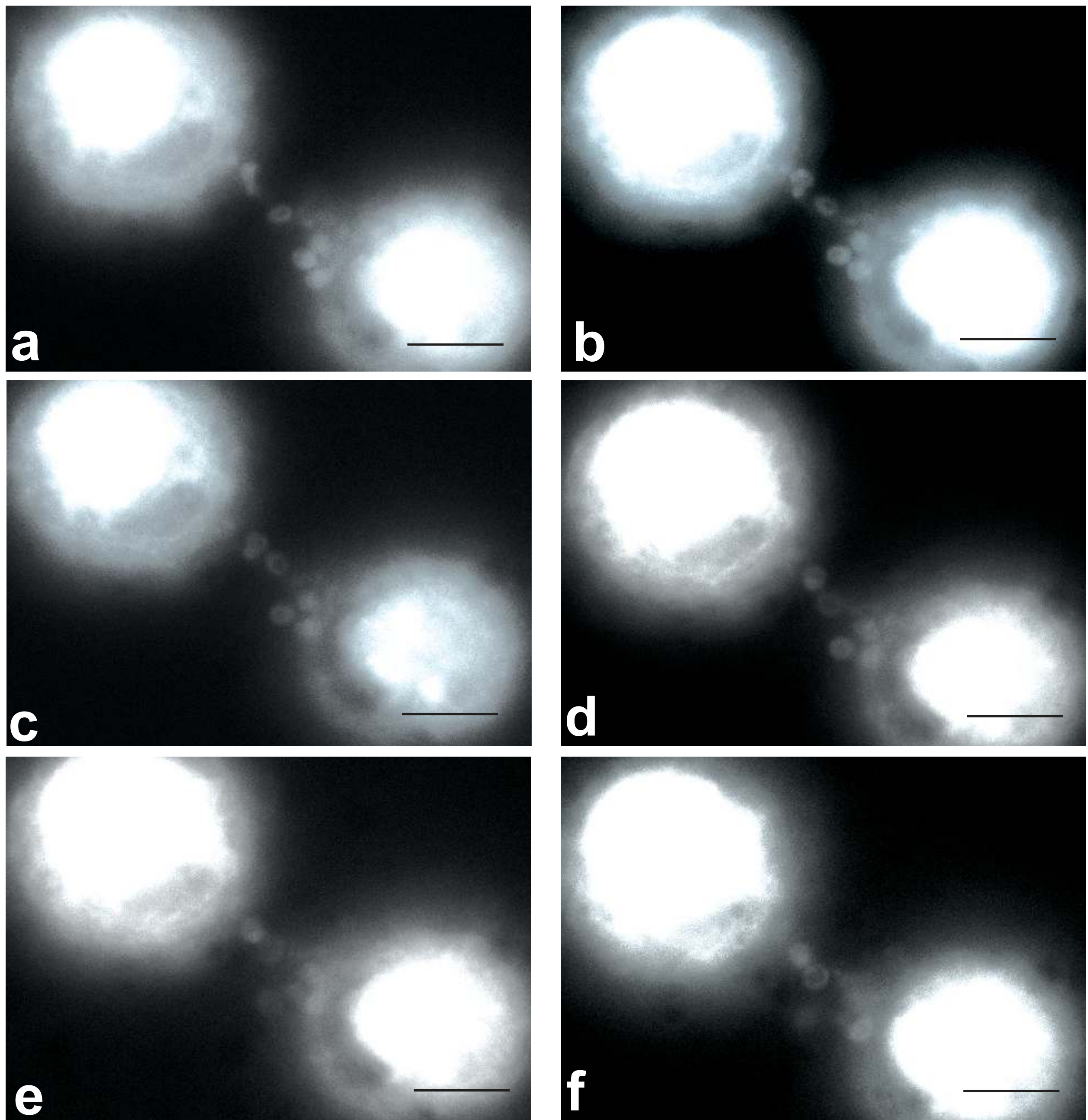

Fig. 4 Vesicle traffic between two DU145 cells stained with DiI in liquid-overlay culture. Vesicle diameter is ~1 to 3 mm. Time-lapse frames were acquired at 30 s intervals. Scale bar: 10μm.

Do filopodia form intercellular bridges? The recognized mechanism of intercellular bridge formation is incomplete separation at the end of cytokinesis [17,18]. Previous research has proposed that bridges between transformed cells result from the fusion of filopodia-like protrusions [2], but incomplete separation at the end of mitosis cannot be excluded. The data presented in this study demonstrates that DU145 cells are inherently forming filopodia and intercellular bridges in both monolayer and liquid-overlay culture. Filopodia architecture and movement suggest that they may be used to build intercellular bridges. This mechanism is consistent with epithelial sheet sealing described above.

This study demonstrates that the bridges between DU 145 cells contain alpha-tubulin. These

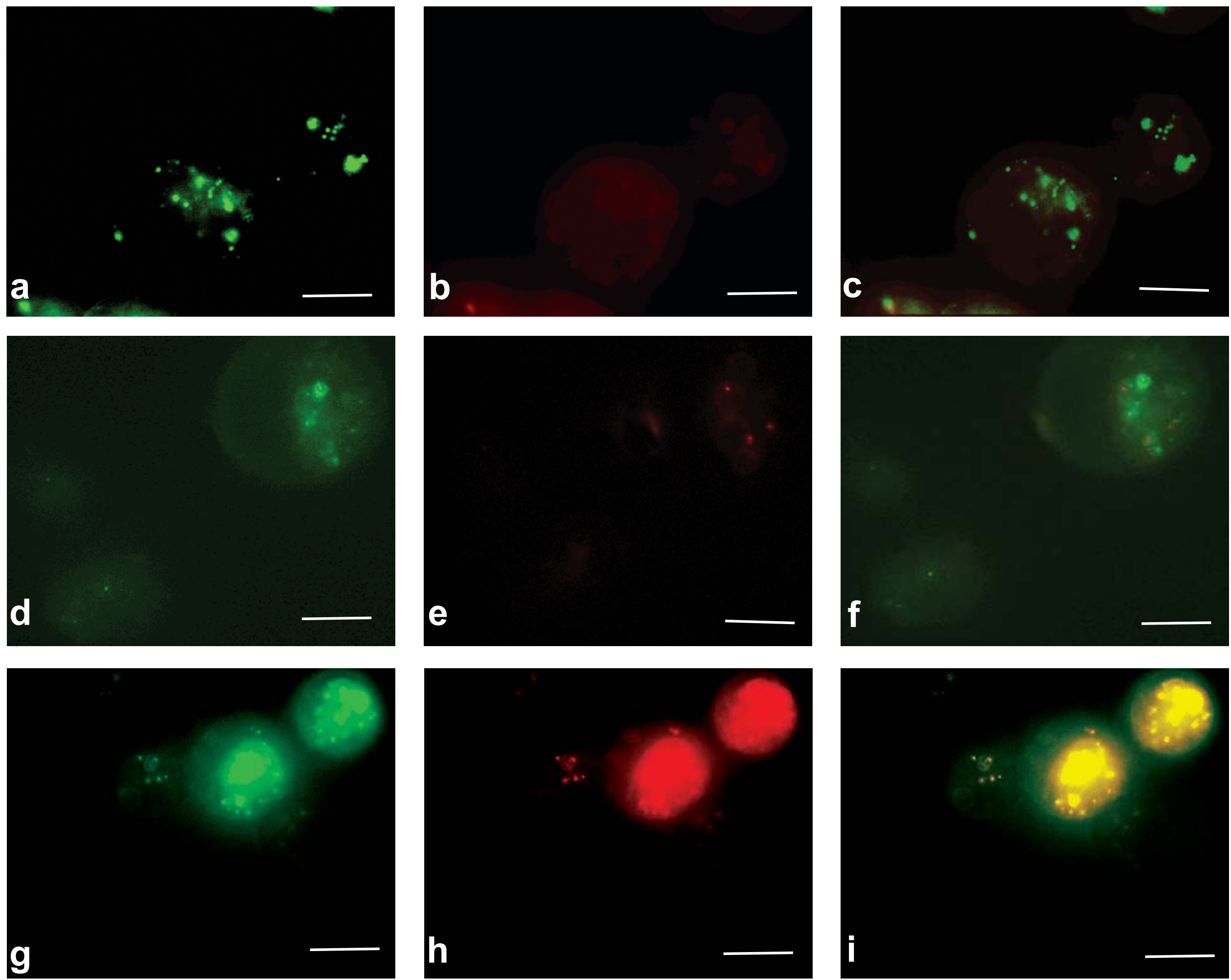

Fig. 5 Exchange of membrane material between cultured DU 145 cells in liquid-overlay culture. Two populations of cells have been stained with DiI and DiO, respectively, and then mixed. Images of green (a, d, g) and red (b, e, h) fluorescence have been merged (c, f, i). After 8 h, vesicles of one color are found in the cytoplasm of cells with the contrasting color: a – c) green vesicles in red cells; d – e) red vesicles in green cells. After 12 h vesicles have mixed red and green fluorescence that appear yellow (g – i). Scale bar – 10 µm.

data are consistent with previous studies on the composition of these membrane extensions [18-20]. Microtubule inhibitors prevent organelle movement through intercellular bridges [18]. In addition, delta-tubulin forms intercellular bridges between spermatogenic sister-cells [20]. If actin-rich filopodia form intercellular bridges, then the actin filaments could serve as a template for microtubule assembly. In future research, the presence or absence of actin in intercellular bridges will help resolve the mechanism of intercellular bridge formation.

Once assembled, intercellular bridges can serve as a conduit for exchange of cellular material. In contrast to gap junctions that permit direct passage of small molecules up to 1 kDa between cells [21], intercellular bridges provide for the exchange of larger material. In the present study, membrane vesicles, 1 to 3 µm in diameter, are transported through these structures. Rustom et al. described very thin intercellular bridges (50 to 200 nm wide) between pheochromocytoma cells as nanotubular highways facilitating organelle transport [2]. In a direct demonstration of organelle traffic through cytoplasmic bridges in mammalian spermatogenesis, Ventelä et al. described a multitude of small (ca. 0.5 µm) granules that move continuously over the bridges, but only 28% of those entering the bridge were actually transported into the other cell [18].

For DU 145 and many other cancer cells that are deficient in gap junctional intercellular communication, flow of cellular material across intercellular bridges may partially compensate for the impeded/arrested transport through defective/inoperative gap junctions.

Intercellular bridges may have an important role in communication between stem cells. As in many cancer cells, gap junctional intercellular communication is non-functional in stem cells and becomes operative upon their differentiation [8]. Direct transfer of cytoplasmic molecules and organelles across intercellular bridges may help synchronize cell differentiation by enabling transfer of large molecules or even organelles [18]. For example, germ cell progeny may need to transfer material as a way to counteract heterogeneity due to stochastic gene expression. This process may require the exchange of protoplasm via wide intercellular bridges to achieve similar levels of mRNAs, proteins and organelles in sister cells. This direct exchange of cellular material may also serve to synchronize populations of germ cells [3]. Testis-brain-RNA-binding protein, which is associated with microtubules and mRNA transport, is involved in the sharing of mRNAs between germ cells by movement through intercellular bridges [22]. Molecules involved in death-inducing mechanisms may spread through intercellular bridges, leading to apoptosis of chains of interconnected spermatogonia [17]. In certain cases, vesicle movement may be related to the construction and dissolution of the intercellular bridge itself or associated structures such as the bridge partitioning complex in germ cells [19].

Research on the structure, mechanism of formation and role of intercellular bridges as a possible common structural feature of cancer cells and stem cells may help elucidate the potential involvement of stem cells in carcinogenesis, and the relationship between differentiation and intercellular communication.

Acknowledgements

This work was supported by NASA grant NAG 9-1351. Dr. Cristina Vidulescu was supported by a Fulbright grant and by NASA grant NAG 9-1351. The authors are grateful to Dr. Cesar Fermin for helpful discussions. We thank Li Xu for technical assistance.

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