ARTICLE

Leukocyte–Mediated Cell Dissemination and Metastasis: Findings From Multiple Types of Human Tumors

Yan-gao Man,[1]* Jeffrey Mason,[1] Russell Harley,[1] Yeon Ho Kim,[2] Kangmin Zhu,[3] and William A. Gardner[1]

[1]Armed Forces Institute of Pathology and American Registry of Pathology, Washington, District of Columbia 20306
[2]Defense and Veterans Brain Injury Center, Walter Reed Army Medical Center, Washington, District of Columbia 20306
[3]US Military Cancer Institute and Department of Preventive Medicine and Biometrics, Uniformed Service University, Bethesda, Maryland 20850

ABSTRACT

Our previous studies revealed that leukocyte infiltration could trigger human breast and prostate tumor invasion through focal disruptions of the tumor capsule, which selectively favors monoclonal proliferation of tumor progenitors or a biologically more aggressive cell clone overlying the focal disruptions. Our current study, involving multiple types of human tumors, further shows that leukocyte infiltration could also trigger tumor metastasis through the following pathways: [1] more leukocytes migrate to focally disrupted tumor capsules, which forms leukocyte aggregates surrounding newly formed tumor cell clusters, [2] the physical movement of leukocytes into proliferating tumor cells disrupts the intercellular junctions and cell-surface adhesion molecules, causing the disassociation of tumor cells from the tumor core, [3] leukocytes are conjoined with some of these tumor cells through plasma membrane fusion, creating tumor cell–leukocyte chimeras (TLCs), and [4] the leukocyte of TLCs impart migratory capacity to associated tumor cell partners, physically dragging them to different tissue sites. Our findings suggest a novel pathway for tumor cell dissemination from the primary sites and the subsequent journey to new sites. Our findings also provide a unique explanation for the cellular mechanism of leukocytes on tumor invasion and metastasis. If confirmed, our hypothesis and technical approach may significantly facilitate early detection and intervention of tumor invasion and metastasis. J. Cell. Biochem. 112: 1154–1167, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: TUMOR METASTASIS; TUMOR INVASION; TUMOR STEM CELL; LYMPHOCYTES; LEUKOCYTES; INTERCELLULAR JUNCTIONS; SURFACE ADHESION MOLECULES

It has been well documented that the primary cause of cancer mortality arises not from primary tumors, but from disseminated metastatic diseases [Christofori, 2006]. The mechanism(s) of tumor metastasis, however, remains as a subject of debate [Fidler and Kripke, 1977; Talmadge et al., 1982; Li et al., 2007; Polyak and Weinberg, 2009]. Recently, there has been a great deal of interest in the role of immune cells in tumor metastasis, and several models, including tumor-educated macrophages [Pollard, 2004], paracrine loop signaling [Wyckoff et al., 2004, 2007], cancer cell-leukocyte fusion [Pawelek and Chakraborty, 2008], and immune cell-based mediation [DeNarddo et al., 2008], have been proposed to explain how immune cells could facilitate metastasis. Collectively, these models suggest that immune cells facilitate tumor invasion and metastasis through the following mechanisms: [1] macrophages enhance tumor cell migration through secretion of chemotactic and chemokinetic factors, which promote angiogenesis and fibrillogenesis, allowing tumor cells track along collagen fibers to blood vessels [Pollard, 2004; Wyckoff et al., 2004, 2007], [2] T-lymphocytes

indirectly promote invasion and metastasis by directly regulating the phenotype and effector function of tumor associated CD11b(+)Gr1(−)F4/80(+) macrophages [DeNarddo et al., 2008], and [3] macrophages ingest tumor cells, resulting in the fusion of genetic materials of two cell types that creates a hybrid phenotype [Pawelek and Chakraborty, 2008].

Each of these models has its individual strengths and weaknesses [Hunter et al., 2008] and is supported by laboratory findings or clinical data, suggesting that tumor cell metastasis is likely to occur through multiple mechanisms. However, it is not currently possible to discern the mechanistic origin of a given metastatic lesion. In addition, since [1] all epithelium-derived tumors are physically segregated from the stroma and vascular structures by a dense tumor capsule, [2] epithelial cells are held in place by intercellular junctions and cell-surface adhesion molecules, and [3] the stroma normally harbors abundant immunoreactive cells, two critical issues remain to be elucidated: [1] how metastasis-initiating cells are disseminated from their primary sites, and [2] how disseminated tumor cells escape from the immune-surveillance during their journey to new sites. More importantly, the specific molecule and unique morphological feature that define pending or early metastatic lesions remain elusive, making it difficult for early detection and intervention of tumor metastasis.

Our previous studies in human breast and prostate tumors have led to the hypothesis that leukocyte infiltration represents a triggering event for tumor invasion by causing focal disruptions in the tumor capsule. This was shown to selectively favor proliferation of cells overlying these focal disruptions [Man et al., 2003; Man et al., 2005a,b, 2006; Man, 2007, 2010; Hu et al., 2008; Man and Gardner, 2008]. Our current study expands upon these observations to further elucidate the role of leukocytes in tumor cell dissemination and metastasis. Our findings suggest that leukocytes can facilitate dissociation of tumor cells from the primary sites and their subsequent metastasis through the following pathways: [1] leukocytes migrate to focally disrupted tumor capsules in responding to stromal invasion of cells overlying these focal disruptions, which give rise to leukocyte aggregates surrounding newly formed tumor cell clusters, [2] physical movement of leukocytes into the epithelium disrupts the intercellular junctions and cell-surface adhesion molecules, which causes the disassociation of tumor cells from the tumor core, [3] leukocytes are physically conjoined with the plasma membrane of some isolated tumor cells creating what we refer to as tumor cell–leukocyte chimeras (TLCs), and [4] the leukocytes of TLCs impart migratory capacity to associated tumor cell partners by physically dragging them to different sites during migration, thus promoting tumor metastasis. We collectively refer to these pathways as leukocyte-mediated tumor cell dissemination and metastasis. Finally, we show evidence that the formation of TLCs causes altered chromatin organization in tumor cells of the chimera, which may result in genetically distinct daughter cells.

EXPERIMENTAL PROCEDURES

Formalin-fixed, paraffin-embedded tissue blocks from 20 cases of human breast cancer were selected from our previous studies [Man et al., 2003, 2005a,b, 2006]. The predominant lesion of these cases is ductal carcinoma in situ (DCIS) with a high percentage (>30%) of the affected ducts exhibiting focal disruptions in the surrounding tumor capsules. The same number of reduction mammoplasties from individuals with no family history of breast cancer, and no mammographic or histological abnormalities in the breast were used for controls. To further assess the impact of leukocyte infiltration in metastasis, human prostate (N = 5), lung (N = 5), and cervical (N = 5) cancers with extensive leukocyte infiltration were included for the analyses described below. Serial 5–7 μm sections were cut and placed on positively charged slides. The first and last sections from each block were stained with hematoxylin and eosin for morphological classification using our published criteria [Tavassoli and Man, 1995].

Double-immunohistochemistry was utilized to identify tumors with focally disrupted capsules, surrounding leukocyte aggregates, and isolated tumor cells. Antibodies used to identity focal disruptions were a mixture of anti-smooth muscle actin (SMA; clone: 1A4; Sigma, St. Louis, MO) and anti-collagen IV (clone: CIV22) for breast, a mixture of anti-cytokeratin (CK) 34BE12 (clone: M0630) and anti-collagen IV (Dako, Carpinteria, CA) for prostate, and collagen IV alone for lung and cervical tumors. A focal disruption was defined as the presence of a physical gap that is larger than the combined size of at least three epithelial cells in at least three consecutive sections. Antibodies used to identify leukocytes and isolated tumor cells were anti-leukocyte common antigen (LCA, clone: 2B11 + PD7/26), which reacts with all normal hematopoietic cells and their neoplastic transformations, and CK AE1/AE3 (clone: AE1/AE3), which reacts with all epithelium-derived cells.

Double-immunohistochemistry was also used to assess the effect of leukocyte infiltration on intercellular junctions and cell-surface adhesion molecules. Four consecutive sections from each case were first immunostained for LCA, and the antigen–antibody complex was elucidated with a species-specific secondary antibody and a black or red chromogen. Then, the sections were incubated with antibodies to CK AE1/AE3 and three surface adhesion molecules, E-cadherin (clone: 36B5; Lab Vision, Fremont, CA), β-catenin (clone: 17C2), and CD44 (clone: DF-1485; Dako), respectively. The antigen–antibody complexes were elucidated with a species-specific secondary antibody and a different colored chromogen. In addition, consecutive sections from each case were immunostained for epithelial-specific antigen (clone: VU-1D9; Novocastra Laboratories Ltd, Newcastle, UK), D2-40 (clone: D2-40; Signet, Dedham, MA), CD34 (clone: QBEnd/10), p53 (clone: D07), and Ki-67 (clone: MM1; Dako), to further assess the potential impact of leukocyte infiltration on tumor cells and the tumor microenvironment.

Immunostained sections were examined under high magnification to assess whether isolated tumor cells or small tumor cell clusters were exclusively located within leukocyte aggregates adjacent to focally disrupted tumor capsules. Tumor cells or cell clusters were considered to be dissociated from the primary tumor if they were physically separated from the tumor core in at least three consecutive sections. The numbers of isolated tumor cells within leukocyte aggregates adjacent to and distant from disrupted tumor capsules were compared.

The intensity and sub-cellular localization of the cell-surface adhesion molecules were arbitrarily defined as normal (++ to +++, cell membrane localization) or aberrant (weaker than ++, or cytoplasmic localization). The expression of these molecules in 300 isolated tumor cells, and the same number of cells within the tumor core were statistically compared using Pearson's chi-squared test. Statistical significance was defined as $P < 0.05$.

The correlation between the presence of leukocyte aggregates and cell dissociation from the tumor core was further studied in multiple consecutive sections of morphologically similar pre-invasive tumors, with and without surrounding leukocyte aggregates, from five selected cases double-immunostained with different biomarkers. The periphery of the tumors was examined to detect the physical signs of cell disassociation.

Double-immunostained sections were examined under high magnification to characterize the physical association between leukocytes and tumor cells. TLCs were defined as tumor-leukocyte pairs that share an extended region over which the two plasma membranes flatten out physically against each other, resulting in increased thickness or changes of the color. To determine if the physical association between leukocytes and tumor cells results from random overlapping or specific membrane fusion, adjacent sections of immunostained slides containing TLCs were double-immunostained for LCA plus CK AE1/AE3 and E-cadherin. The two antigen–antibody complexes were distinguished using secondary antibodies labeled with different fluorophores (DyLight 488 and Dylight 649; KPL, Gaithersburg, MD). The double-immunostained sections were examined with a laser scanning confocal microscope (Fluoview 300; Olympus America, Inc., Center Valley, PA). The two fluorophores were excited simultaneously using Ar-ion (488 nm) and HeNe (632 nm) lasers. The epi-fluorescence signals from the two fluorophores were separated by using a 630 nm dichoric filter and a 660 nm longpass filter. A 515 nm bandpass filter was used to block excitation light. The epi-fluorescence signals were then detected using two photomultiplier tubes, one optimized for each wavelength. The area of interest in the tissue section was optically sectioned in the z-direction, in steps of 0.5 μm, using confocal imaging, resulting in a three-dimensional image profile [Pawley, 2006].

The ability of the leukocytes in the TLCs to facilitate the transport and extravasation of tumor cells into the stromal and vascular structures was examined by identifying the presence of epithelial cells and their physical association with leukocytes within these structures. Finally, individual tumor cells and TLCs were compared to determine if tumor cell–leukocyte pairing affected the size, shape, and morphology of mitotic or proliferating tumors cells, and to detect physical signs suggestive of abnormal cell division in the TLCs.

To identify the specific subtype(s) of leukocytes physically associated with tumor capsule disruptions and tumor cells, consecutive sections from 10 cases with large focal capsule disruptions and leukocyte infiltration (as shown in Fig. 1 below) were double-immunostained for CK AE1/AE3 and a panel of markers, including CD20 (clone L26) for B-lymphocytes, CD3 (clone UCHT1), CD4 (clone MT310), and CD8 (clone C8/144B) for T-lymphocytes, CD14 (clone TUK4) for monocytes, and CD56 (clone

IB6) for natural killer cells. As other immune cells, including mast cells, macrophages, plasma cells, and neutrophils can be defined based on their morphological features or locations, no specific phenotypic markers were devoted to identify and classify them. All antibodies used in this study were purchased from two well-established manufacturers, Dako or Novocastra (Newcastle, UK). After immunostaining, the same areas with focal tumor capsule disruptions and leukocyte infiltration were photographed and enlarged prints were made. The absolute numbers of all these subtypes were counted and statistically compared using the Pearson's chi-squared test. The subtype(s) with the highest frequency of physical association with tumor capsule disruptions and disseminated tumor cells is considered as the most likely leukocyte subtype associated with tumor invasion and metastasis.

Immunostaining was carried out using our published protocol [Man and Burgar, 2003] with monoclonal mouse anti-human antibodies. The secondary antibody, ABC detection kit, and diaminobenzidine (DAB) chromogen kit were obtained from Vector Laboratories (Burlingame, CA). The AP red-chromogen kit was purchased from Zymad Laboratories (South San Francisco, CA). To assess the specificity of the immunostaining, different negative controls were used, including [1] the substitution of the primary antibody with the same isotype or pre-immune serum of the antibody, and [2] omission of the secondary antibody. In addition, the immunostaining procedure was repeated at least twice using the same protocol and under the same conditions. Immunostained sections were independently evaluated by two investigators. A given cell was considered immunoreactive if distinct immunoreactivity was consistently seen in its cytoplasm, membrane, or nucleus, while all negative controls lacked distinct immunostaining.

RESULTS

The pattern and sub-cellular localization of the immunoreactivities seen with each of the biomarkers examined in this study were in accordance with those of manufacturers' descriptions and published data. All negative controls were devoid of distinct immunoreactivity.

A total of 88 hyperplastic and in situ lesions harbored focal disruptions in the surrounding capsule. These focally disrupted tumor capsules were surrounded by or immediately adjacent to leukocyte aggregates, which consisted of about 100 to more than 1,000 individual leukocytes in a given profile. The size of the focal disruption in the tumor capsule correlated with that of the surrounding leukocyte aggregate (not shown). Nearly all hyperplastic or in situ tumors exhibiting a large focal disruption (the absence of 1/5 to 1/3 of the capsule) had a jagged periphery containing variable numbers of irregular-shaped projections of cell masses or isolated individual tumor cells. These epithelial protrusions or isolated tumor cells were immediately surrounded by or intermixed with leukocytes (Fig. 1). Of the 88 leukocyte aggregates, 49 (55.7%) harbored isolated tumor cells. In contrast, the adjacent peripheral regions within the same tumor, but distant from the leukocyte aggregates, completely lacked these projections and isolated cells (Fig. 1). None of the 20 cases of reduction
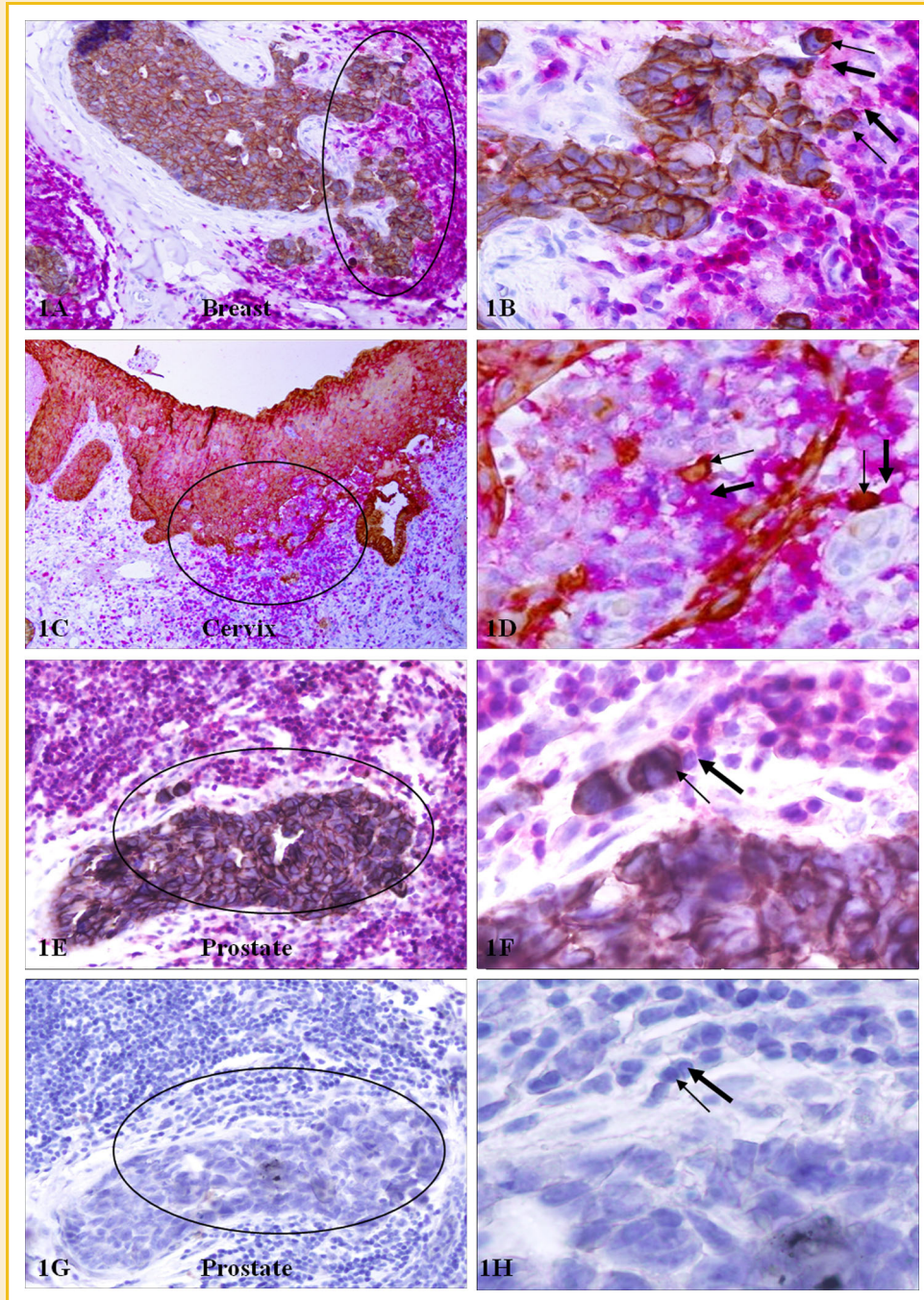
Fig. 1. Leukocyte aggregates with dissociated tumor cells in different tissues. Sections A–F were double-immunostained for CK AE1/AE3 (brown or purplish colored cells/structures) and LCA (red or purplish-pink colored cells). Sections G–H are the adjacent section of E–F, and were double-immunostained with the same antibodies under the same condition except the omission of the secondary antibody. Circles identify cell projections surrounded by or adjacent to leukocyte aggregates. Note that all leukocyte aggregates harbor dissociated tumor cells (thin arrows), which are immediately surrounded by or adjacent to leukocytes (thick arrows). In contrast, adjacent peripheral regions distant from leukocyte aggregates lacked isolated tumor cells. The negative control (G and H) lacks distinct immunostaining and shows only blue color of the counterstaining. A, C, E, and G: 100×. B, D, F, and H: a higher (300×) magnification of A, C, E, and G, respectively.

mammoplasties showed morphologically distinct focal disruptions in the capsules or any surrounding leukocyte aggregate with disseminated tumor cells.

The irregular-shaped projections of tumor cell masses or disseminated individual tumor cells associated with leukocyte aggregates consistently showed aberrant expression of all cell-surface adhesion molecules. Of 300 disseminated tumor cells examined within leukocyte aggregates, 269 (89.7%) had either a substantially reduced expression or cytoplasmic localization of these adhesion molecules (Fig. 2). In sharp contrast, only 22 (7.3%)
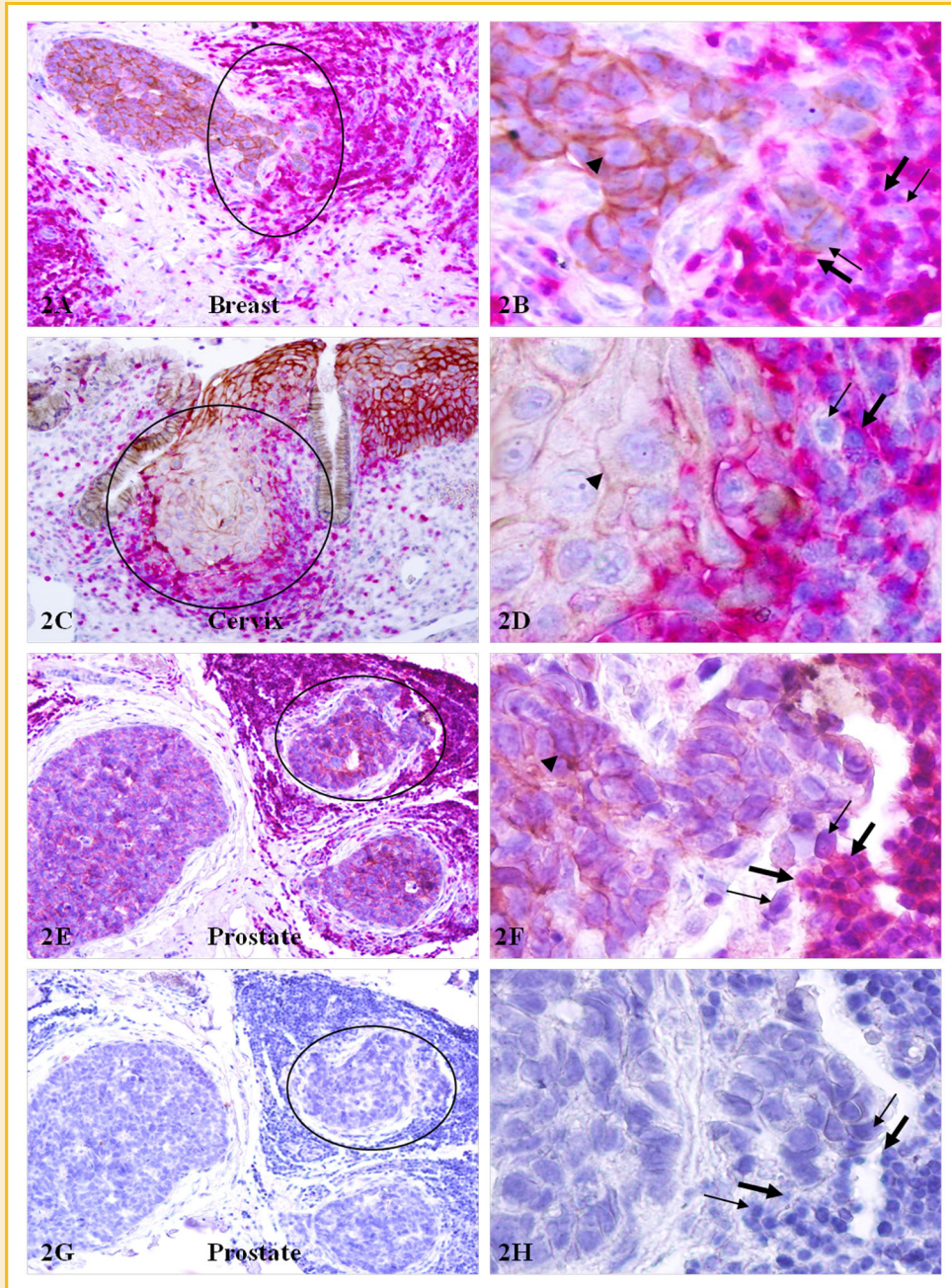
Fig. 2. Reduced cell-surface adhesion molecule in tumor cells within leukocyte aggregates. Sections A–F were double-immunostained for LCA (red or purplish-pink colored cells) and E-cadherin (brown or reddish orange colored cells/structures). Sections G and H are the adjacent section of E and F, and were double-immunostained with the same isotype of anti-LCA and E-cadherin antibodies under the same condition. Circles identify cell projections surrounded by or adjacent to leukocyte aggregates. Thick arrows identify leukocytes. Thin arrows identify tumor cells. Note that nearly all dissociated tumor cells show reduced or no E-cadherin expression, but cells within the tumor core show normal membrane localization of E-cadherin (arrowheads). The negative control (G and H) lacks distinct immunostaining and shows only blue color of the counterstaining. A, C, E, and G: 100×. B, D, F, and H: a higher (300×) magnification of A, C, E, and G, respectively.

of the 300 cells examined within the tumor core showed aberrant expression of cell-surface adhesion molecules. This difference is statistically significantly ($P < 0.001$).

Examination of consecutive sections of most irregular-shaped cell mass projections consistently detected variable numbers of isolated individual or clusters of tumor cells, which appear to be disseminated from the tumor core. In addition, some disseminated tumor cells were seen within the leukocyte aggregates at a distance from the tumor core. Figure 3 shows an example of such changes in consecutive sections of DCIS immunostained for different biomarkers. One end of this tube-like DCIS is surrounded by a leukocyte aggregate, whereas the other end is largely devoid of leukocytes. Of these consecutive sections, the end with no apparent leukocyte infiltration showed little change in its morphological and
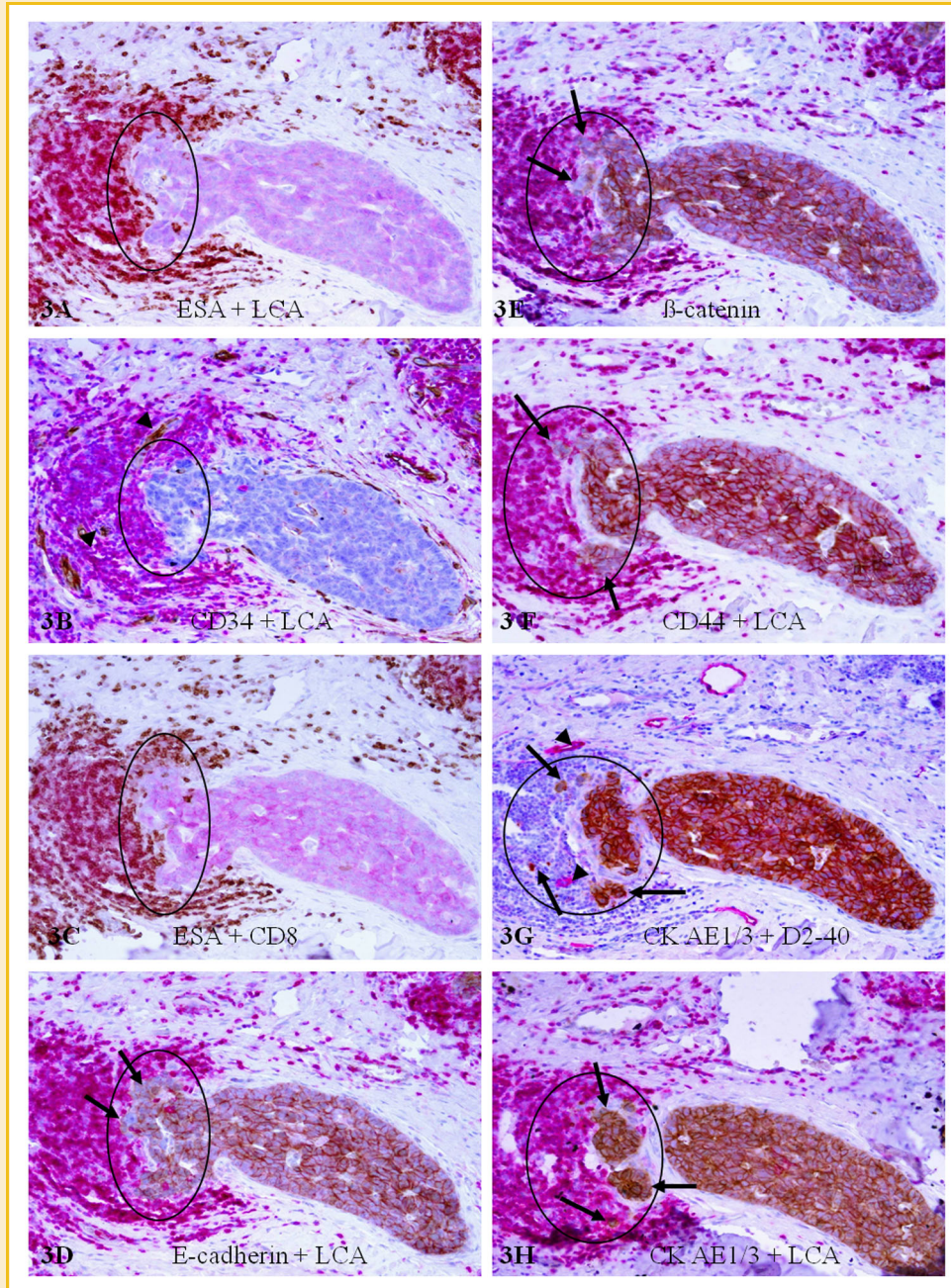
Fig. 3. Disassociation of isolated or large clusters of tumor cells from the tumor core. Consecutive sections from a ductal carcinoma in situ (DCIS) were immunostained for different biomarkers. One end of this tube-like DCIS is surrounded by a leukocyte aggregate (circles), while the other end is largely devoid of leukocytes. Compared to its counterpart, the end surrounded by leukocytes (circles) has the following unique features: [1] a rough and irregular edge, [2] dissociation of isolated or large clusters of tumor cells (arrows) from the tumor core, and [3] a substantially increased vascular structures (arrowheads). Note that the end without surrounding leukocytes shows little change. 200×.

immunohistochemical profiles. In sharp contrast, the end with surrounding leukocytes displays several unique features: [1] an irregular shaped edge (Fig. 3A–H), [2] protrusion of isolated tumor cells or cell clusters into the leukocyte aggregate, which show significantly reduced expression of surface adhesion molecules (Fig. 3D–F), [3] increased vascular structures with dilated lumens (Fig. 3B,G), and [4] dissociation of tumor cell clusters from the tumor core (Fig. 3G,H).

A significant number of these disseminated tumor cells located within leukocyte aggregates appear to be conjoined with leukocytes to create TLCs through the fusion of their plasma membranes, which is suggested by the thickening of the cell membrane or change of the color at the junctions. Examples of TLCs are shown in Figure 4. Leukocytes associated with TLCs were uniform in size with large round, densely stained nuclei and limited cytoplasm, typical features of lymphocytes. Further immune- and statistical
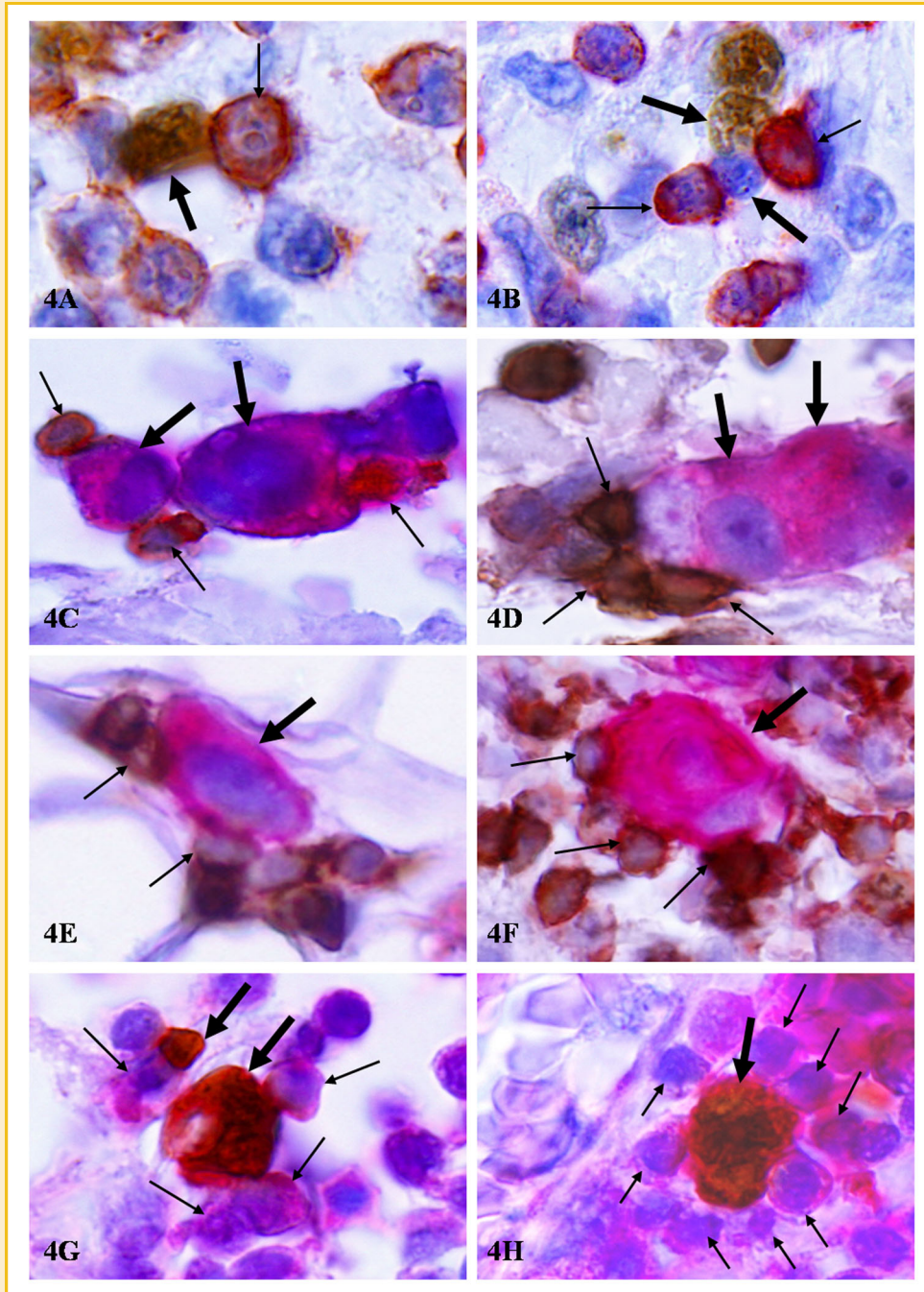
Fig. 4. Plasma membrane conjunction between tumor cells and leukocytes in different sites. Sections from different tissues were immunostained for different markers. Thick arrows identify leukocytes. Thin arrows identify tumor cells. Note that the plasma membrane of two p53 positive and one p53 negative breast tumor cells (A,B) and three prostate tumor cells (C,D) within leukocyte aggregates appears to be conjoined with that of leukocytes to form tumor cell–leukocyte chimeras (TLCs), suggested by the increased thickness of the cell membrane at the membrane junctions. Similar TLCs are also seen in the stromal tissues distant from leukocyte aggregates (E,F). Most tumor cells conjoined with leukocytes show no sign of apoptosis or degeneration. As shown in two immediate adjacent sections (G,H), a large breast tumor cell surrounded by several leukocytes appears to be in the process of division. 1,500×.

analyses have consistently shown that the predominant leukocyte subtypes physically associated with focal tumor capsule disruptions and disseminated tumor cells are CD4 and CD8 positive lymphocytes. The number of leukocytes physically associated with a given tumor cell varied substantially. For some large tumor cells, leukocytes formed ring-like structures that partially or completely surrounded tumor cells (Fig. 4D). TLCs were not only seen within leukocyte aggregates (Fig. 4A–D) but also in the stromal tissues distant from leukocyte aggregates (Fig. 4E,F). Most tumor cells conjoined with leukocytes displayed no sign of degeneration or apoptosis. Instead, many of these tumor cells are in the process of division (Fig. 4G,H).

The formation of TLCs was found to greatly influence the morphology of condensed chromosomes of the mitotic tumor cells. The majority of mitotic tumor cells of the TLCs displayed unique morphological alterations, including disorganized arrangement of condensed chromosomal bands (Fig. 5A–D), and variably shaped small particles or rod-like structures separated from the main chromatin structures (Fig. 5E–H). In contrast, no abnormal nuclear morphology was observed in the leukocytes of the TLCs.

The migratory ability of leukocytes to transport tumor cells of TLCs to different tissue sites was further suggested by the presence of TLCs in the lumens of vascular structures within the cancerous tissue. Of 68 tumor cells detected within these vascular structures, 48 (70.6%) were presented as TLCs (Fig. 6). The morphology and
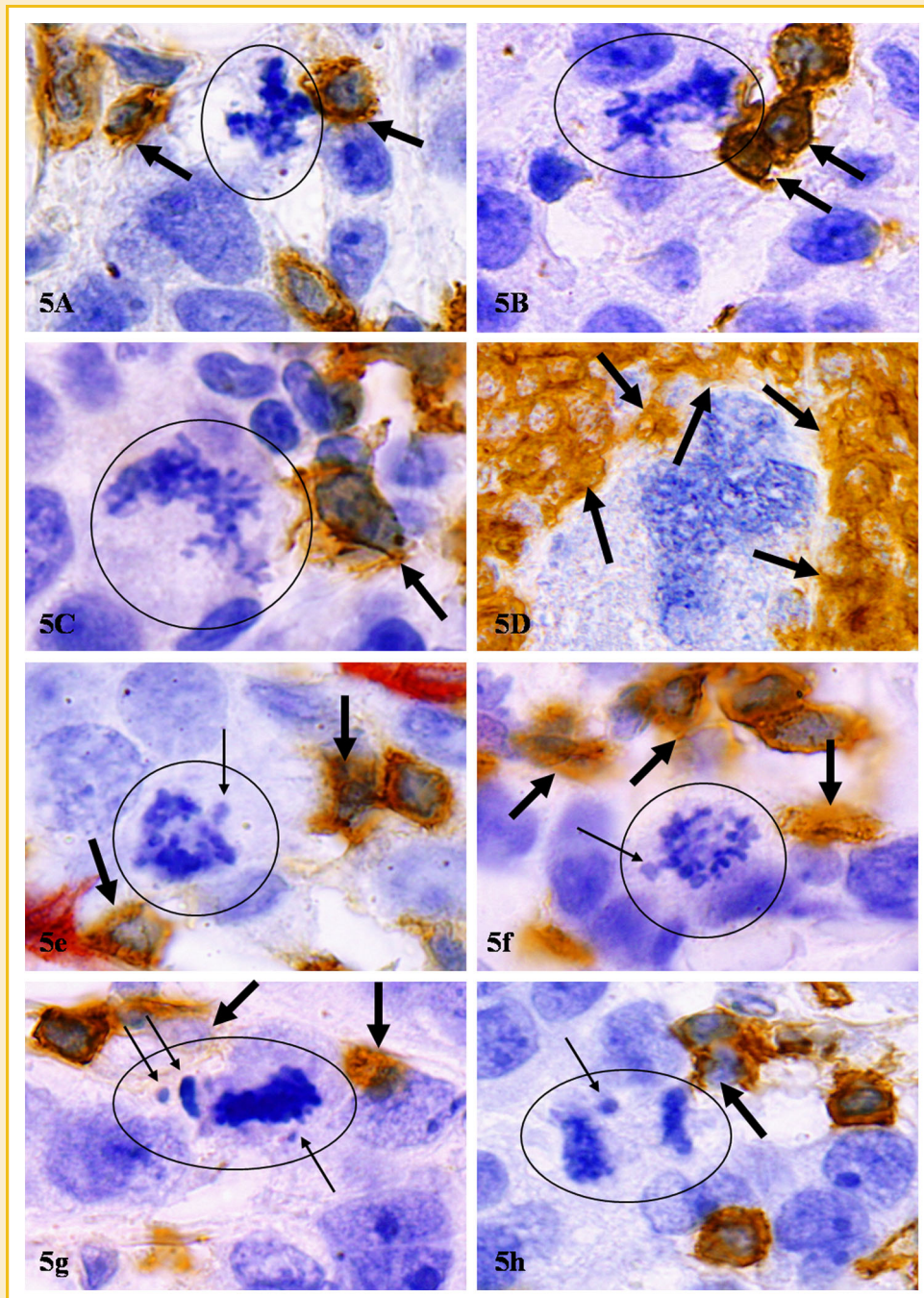

Fig. 5.   Plasma membrane conjunction of leukocytes with mitotic tumor cells. Sections from different tumors were immunostained for LCA (brown). Note that the condensed chromosomes (circles) of some mitotic cells are physically associated with the plasma membrane of leukocytes (thick arrows). Mitotic tumor cells physically associated with leukocytes often show disorganized arrangement of condensed chromosomal bands (A–D), or variably shaped small particles and rod-like structures separated from the main chromatin structures (E–H; thin arrows). 1,500×.
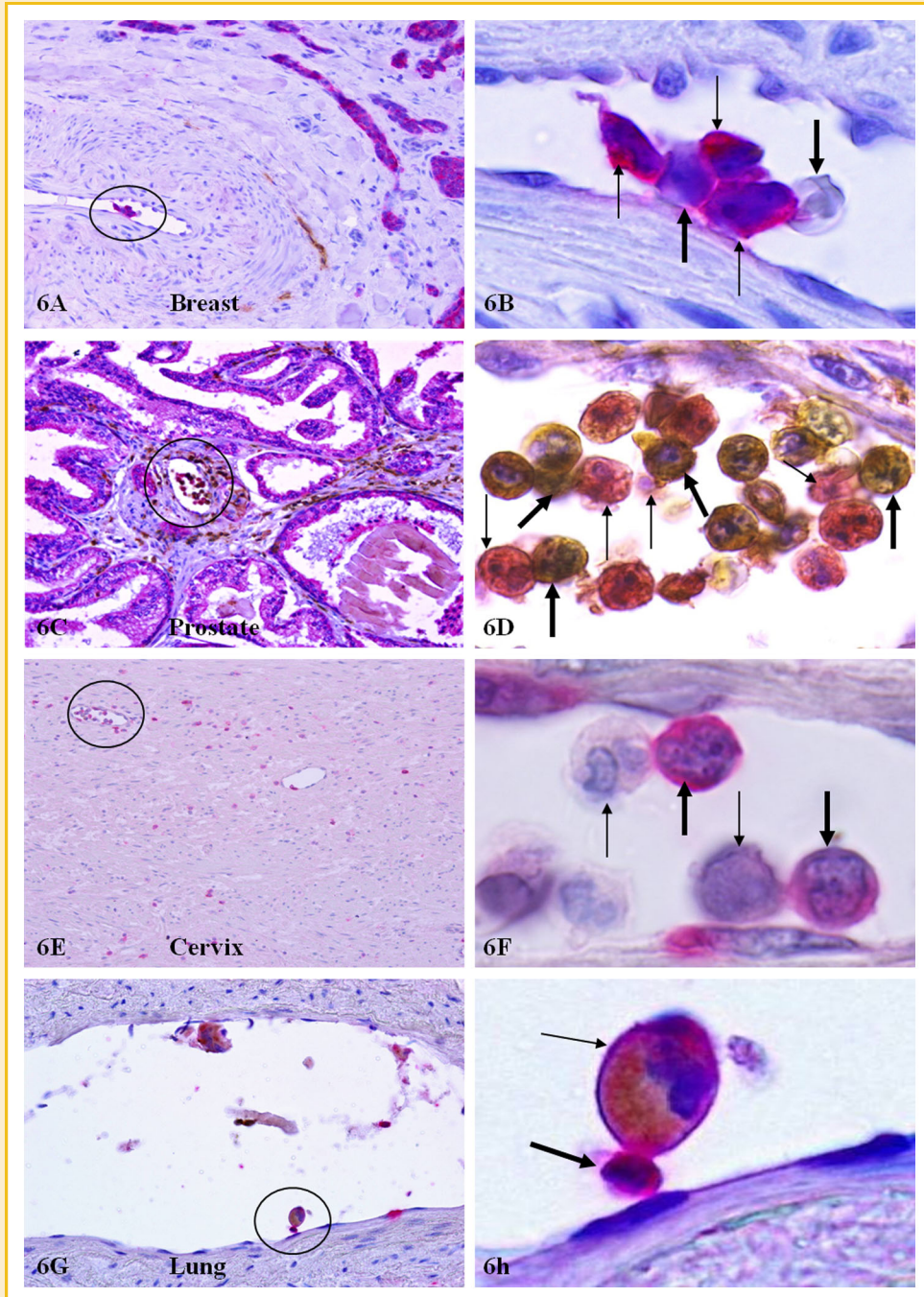
Fig. 6. Plasma membrane conjunction between tumor cells and leukocytes within vascular structures. Sections A and B were immunostained for CK AE1/3 (red), sections C,D and G,H for CK AE1/3 (brown) and LCA (red), and sections E,F for LCA (red). Circles identify vascular structures with tumor cells. Thick arrows identify leukocytes. Thin arrows identify tumor cells. Note that a vast majority of tumor cells within vascular structures are conjoined with leukocytes to form TLCs. A, C, E, and G: 100×. B, D, F, and H: a higher (1,500×) magnification of A, C, E, and G, respectively.

properties of the leukocyte-tumor cell couplings seen within vascular structures were very similar to the TLCs seen within leukocyte aggregates. The leukocytes associated with the TLCs within the vascular structures showed typical morphological features of lymphocytes. The percentages of TLCs within the capillary, vein, small artery, and lymphatic duct appeared to be similar. The vast majority of the tumor cells of the TLCs displayed no sign of degeneration or apoptosis.

The formation of TLCs appears to directly impact the division process in proliferating tumor cells of the pair. These proliferating cells display several unique features: [1] two nuclei with a common plasma membrane (Fig. 7A,B), [2] Ki-67 immunostaining in both of
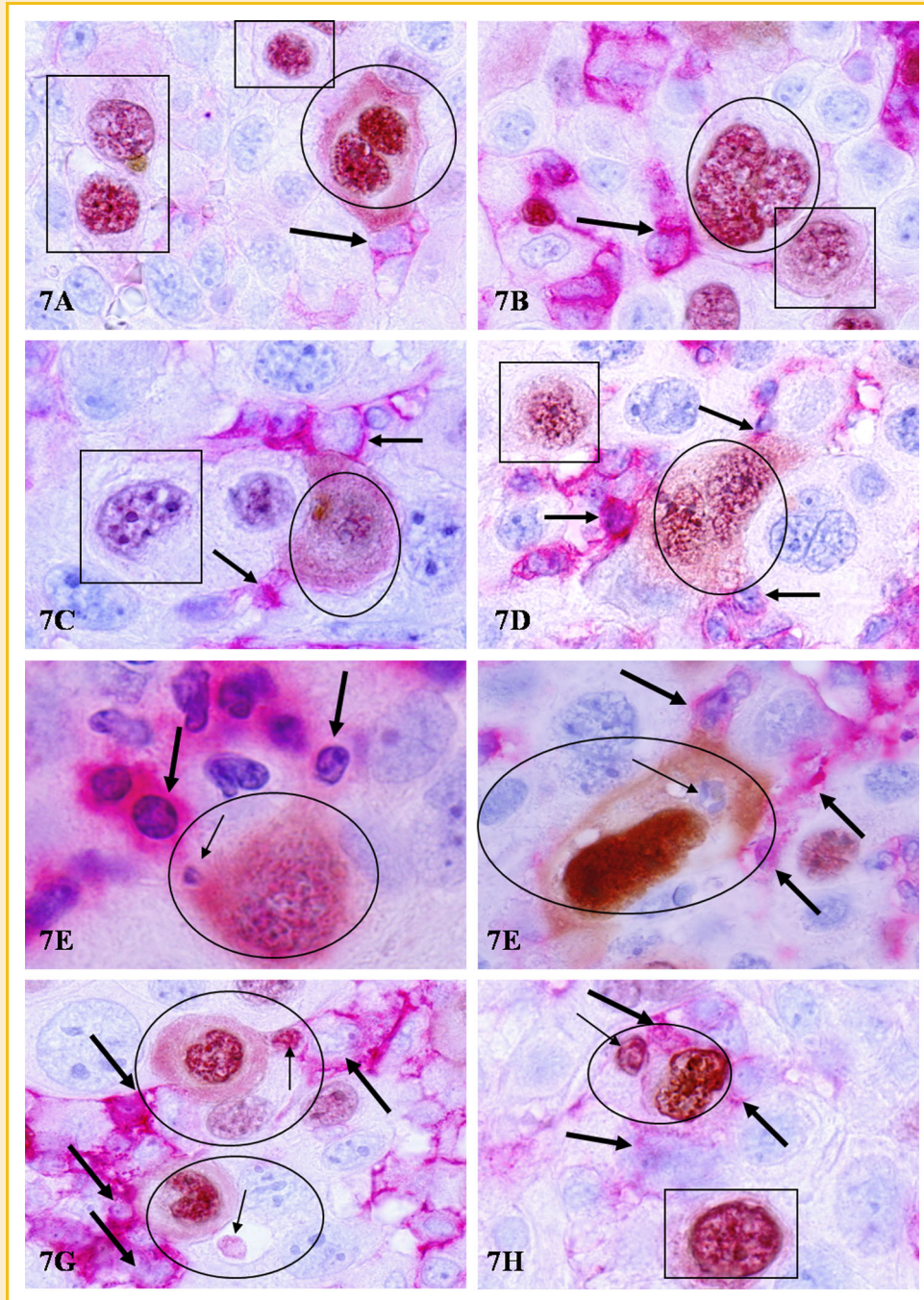
Fig. 7. Impact of leukocyte infiltration in proliferating cells. Sections from breast (A–D) and lung (E–H) tumor tissues were double-immunostained for Ki-67 (brown or purplish colored cells) and LCA (red or purplish-pink colored cells). Circles identify proliferating cells with Ki-67 immunostaining. Thick arrows identify leukocytes. Thin arrows identify small particles within the proliferating cells. Note that the nuclei of proliferating cells distant from leukocytes were well-defined with only nuclear Ki-67 positivity (squares), whereas proliferating cells physically associated with leukocytes display several unique alterations, including two nuclei within a common plasma membrane (A,B), Ki-67 immunostaining in both cytoplasm and nucleus (C,D), small particles adjacent to the nuclei (E,F), and un-even size of the daughter cells (G,H). 1,500×.

the cytoplasm and nucleus (Fig. 7C,D), [3] small particles adjacent to the nuclei (Fig. 7E,F), and [4] an un-even size of daughter cells (Fig. 7G,H). Most proliferating tumor cells distant from leukocytes produced daughter cells with similar size, shape, Ki-67 immunostaining, and a well-defined plasma membrane.

Examination of the immunostained sections under a confocal microscope indicated that the physical association between leukocytes and tumor cells results from the plasma membrane conjunction of these two cell types. As shown in Figure 8, several leukocytes are immediately adjacent to tumor cells and
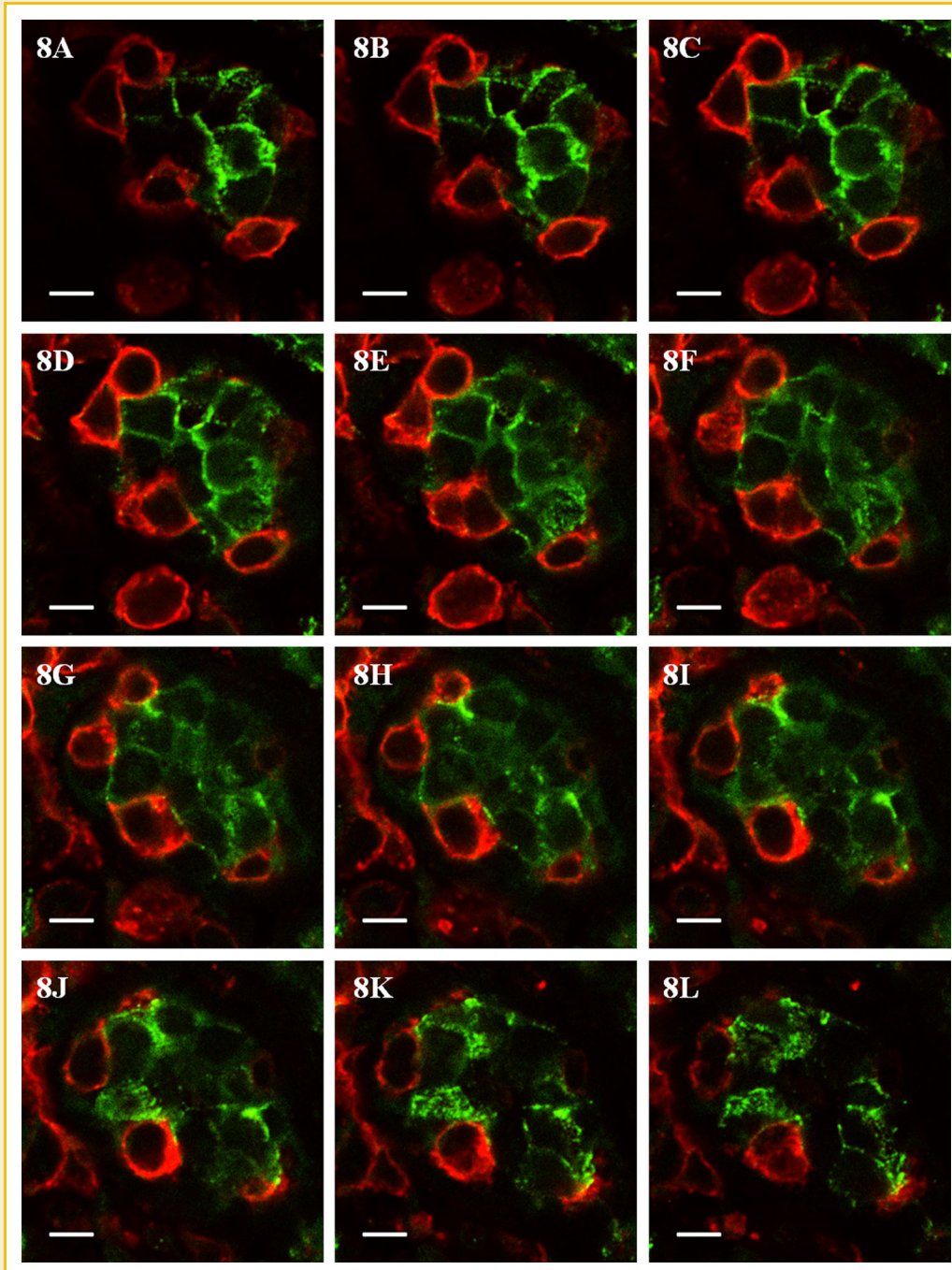
Fig. 8. Confirmation of tumor cell–leukocyte chimeras using confocal microscopy. A 6 μm thick lung tissue section was double-immunostained for CK AE1/AE3 (green) and LCA (red). Circles identify conjoined tumor cells and leukocytes. The scale bars are 5 μm. The panels were obtained in steps of 0.5 μm from A (bottom) to L (top) in the Z-direction, which is perpendicular to the plane of the panels. Note that the conjoined plasma membranes of the leukocytes and the tumor cell are evident at all the 12 panels. 400×.

appear to form TLCs. The intimate association of the plasma membranes of these leukocytes and their associated tumor cell is visible in all the twelve 0.5 μm thick z-scans. In all of these scans, the mirror-image morphology of the two membranes is maintained in both lung and breast tissues examined. These observations argue strongly that the plasma membranes of the

TLCs form tight junctions and are not simply touching or overlapping.

The overall pattern of the above changes was very similar among breast, prostate, lung, and cervical tissues. However, lung tumors were more vascular with a substantially higher number of tumor cells present within the vascular structures.

DISCUSSION

In this report, we propose a novel hypothesis, whereby immune cells can facilitate dissemination of tumor cells from the primary site and journey to new sites, based upon histopathological observations. In this hypothesis, metastasis is facilitated by the infiltration of leukocytes that causes tumor cell disassociation from the tumor core through disruption of the intercellular junctions and surface adhesions molecules, and by adhesion of leukocytes to the plasma membrane of isolated tumor cells to form TLCs. The tumor cells are then shuttled to blood vessels or lymph ducts by the natural migratory behavior of the immune cells. This leads to the dissemination of the tumor cells and their introduction into distant organs, which is again facilitated by the natural migratory properties of the leukocytes. We refer to this mechanism as leukocyte-facilitated tumor dissemination and metastasis as depicted in Figure 9. One important consequence of our hypothesis is that it provides a unique explanation for the vexing problem of unknown-primary metastatic disease [Pentheroudakis et al., 2007], where patients present with disseminated disease in the absence of a detectable primary tumor. In our hypothesis, tumor cell dissemination is correlated with the inflammatory response induced by the tumor rather than its physical size. It is conceivable that a small primary lesion with a strong immune response could induce the formation of a large number of TLCs leading to wide-spread dissemination even if the primary tumor failed to increase in size or ultimately regressed.

Our hypothesis proposes that leukocytes can promote tumor cell dissociation from the primary tumor and subsequent metastasis through three interrelated mechanisms: [1] leukocytes disrupt the intercellular junctions and cell-surface adhesion molecules of cells overlying focally disrupted tumor capsules, [2] leukocytes physically fuse with the plasma membrane of tumor cells to form TLCs, and [3] the leukocytes of the TLCs facilitate migration and intravasation/extravasation of the tumor cells of the TLCs by way of their natural migratory behavior and ability to infiltrate cellular barriers. In addition, the formation of TLCs in mitotic cells may interfere with the distribution of genetic materials in daughter cells, resulting in genetically distinct daughter tumor cells. Based on our hypothesis, leukocytes can alter the tumor microenvironment and behavior of tumor cells, but without the requirement for fusion of their nuclear contents with that of the tumor cells [Pawelek and Chakraborty, 2008]. Our hypothesis is consistent with a number of previous observations: [1] increased leukocyte infiltration was associated with substantially elevated tumor cell proliferation in prostate tumors [Smith and Gardner, 1987], [2] increased leukocyte infiltration correlated with progression of oral epithelium from hyperkeratosis to dysplasia and carcinoma [Gannot et al., 2002], [3] pre-invasive prostate tumors with chronic inflammation had a significantly higher rate of subsequent invasive tumors than their morphologically similar counterparts without chronic inflammation[MacLennan et al., 2006],and [4] pregnancy associated and inflammatory breast cancers, which have extensive leukocyte infiltration, have the most aggressive clinical course and worst
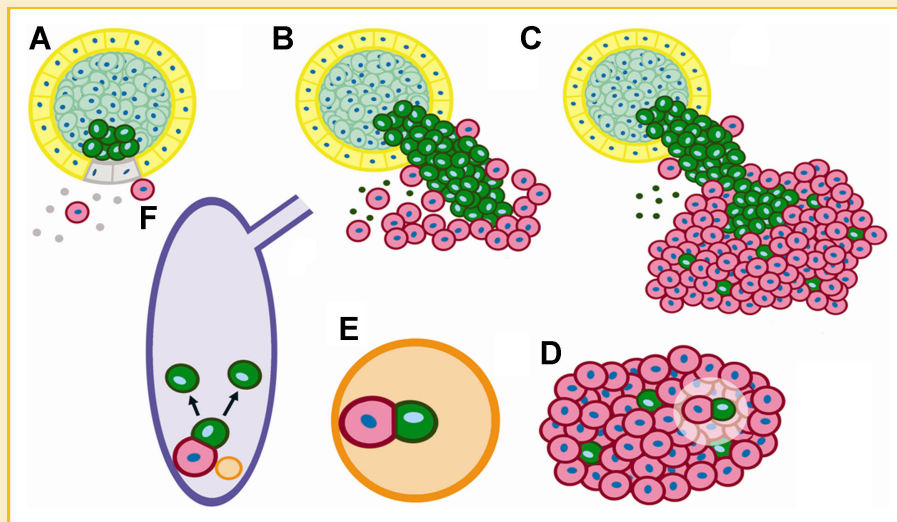

Fig. 9. Hypothesized contributions of leukocytes in tumor metastasis. A: Ductal carcinoma in situ with myoepithelial layer (yellow) and intra-luminal tumor cells (light green). A cluster of cells representing an aggressive progenitor tumor clone (dark green) is shown located over a region of degeneration or chronic inflammation (light gray) in the myoepithelial layer. The degenerating myoepithelial cells release cellular breakdown products (gray dots) that attract leukocytes (red). B: Leukocytes clear the degenerated myoepithelial cells leaving a focal disruption (gap) in the myoepithelial layer. The aggressive progenitor cells overlying this focal disruption then invade into the stroma forming a finger-like projection. These progenitor cells may release microvesicles from their plasma membranes (dark green dots) that contain proteins that act as self-epitopes to attract additional leukocytes; C: Leukocytes penetrate into the distal end of the tumor cell projection, resulting in disruption of the intercellular junctions and cell-surface adhesion molecules. This causes dissociation of tumor cell clusters and individual tumor cells from the tumor core. D: Within the leukocyte aggregate, some of the tumor cells attach to leukocytes to form tight junctions creating tumor cell–leukocyte chimeras (TLCs). One such TLC is shown highlighted by the translucent white circle, (E) the ability of leukocytes to migrate and cross cell barriers facilitates the extravasation of the TLCs into blood vessels and lymph ducts (orange circle), (F) the circulating TLCs can then reach distant organs, such as the lung (purple), where the TLCs can intravasate into the tissue and form metastatic lesions. Our previous studies established the pathways shown in A and B, and the current study has identified the pathways shown in (C–E), and the ability of the tumor cells of the TLCs to undergo mitosis as shown in (F).

prognosis among breast malignancies [Mathelin et al., 2008; Rodriguez et al., 2008].

It has been well documented that macrophages can fuse with tumor cells or themselves to differentiate into giant cells or multinucleate osteoclasts that play a central role in chronic inflammatory diseases and osteoporosis, respectively [Vignery, 2006]. It has also been well documented that lymphocytes can fuse with normal host cells in vitro or in vivo to generate an invasive and metastatic phenotype [De Baetselier et al., 1984]. To our knowledge, a focal membrane fusion between leukocytes and tumor cells that allow leukocytes to alter the mitotic process of the tumor cell or to physically shuttle tumor cells within tissues, however, has not been previously reported. The mechanism responsible for the formation of the tumor cell–leukocyte junction is not known, but may involve the formation of microvesicles by the tumor cells. Microvesicles released from the membranes of tumor cells contain proteins that may act as self-epitopes [Bari et al., 2009; Lima et al., 2009; Wysoczynski and Ratajczak, 2009], which stimulate production of corresponding auto-antibodies, or activate a subset of leukocytes. Prior to their release, these microvesicles are small particles embedded within the tumor cell plasma membrane, and could potentially function as receptors for the leukocytes, leading to the formation of the TLCs. Another possibility is that, as these tumor cells of the TLCs are associated with the leading edge of the invasive protrusions from focally disrupted tumor capsules, they may represent a population of tumor progenitors or a biologically more aggressive sub-clone with enhanced metastatic potential [Man et al., 2003, 2005a; Nguyen and Massagué, 2007]. Thus, it is possible that these cells may have elevated aberrant expression of cell plasma membrane-related molecules, including RunX2 [Pratap et al., 2008, 2009], CD44, aldehyde dehydrogenase 1 (ALDH1), and erythropoietin receptor (EpoR) [Phillips et al., 2007; Nakshatri et al., 2009], which may function as receptors for the leukocytes, leading to the formation of the TLCs. Our speculations are supported by findings from our previous studies, which have demonstrated that in both breast and prostate tumors the vast majority of the leukocytes infiltrates are located at or near focally disrupted tumor capsules [Man et al., 2003; Yousefi et al., 2005; Man, 2007; Man and Gardner, 2008]. Our recent in vitro study has also revealed that protease-degraded collagen I fragments could function as a specific mediator to attract macrophage infiltration in pregnancy-associated breast cancer [O'Brien et al., 2010].

No definitive conclusions can be drawn from the current study, as our sample size was small, and clinical follow-up data were not available. In addition, the morphological and immunohistochemical methods used in our study do not permit the identification of the underlying cellular and molecular alterations associated with our observations. Furthermore, as this hypothesis is based exclusively on morphological and immunohistochemical observations, and no established models are available to validate our results, the speculations derived from our observations might not adequately reflect the intrinsic events of tumor metastasis. The authors full understand that the demonstration of transmigration of TLCs into vessels and to new sites is the single most important requirement to validate their hypothesis. The authors also understand that the confirmation of their hypothesis needs not only to show the positive correlation between the absolute number of TLCs and extent of metastatic potential among different tumors, but also to demonstrate the physical and dynamic association between tumor cells and leukocytes at different tissue sites during metastasis, and also to identify the specific molecules involved in the formation of TLCs. On the other hand, as it is not known how metastasis-initiating cells are disseminated from their primary sites, and the precursors of metastatic lesions have not been defined, our findings and hypothesis may have provided a novel cellular mechanism, and also a unique target for early detection and intervention of tumor metastasis. In addition, as morphologically similar TLCs are seen in all types of human tumors we examined in this study, our hypothesis is likely to be applicable to all epithelium-derived tumors. However, as the samples sent to our institute are from around the world for second-opinion diagnosis, it is very difficult, if not impossible, to procure follow-up specimens that would allow for longitudinal and different mechanistic studies. Thus, the intent of our publication is to release our findings to stimulate collaboration and interest in this field.

ACKNOWLEDGMENTS

REFERENCES

Bari R, Zhang YH, Zhang F, Wang NX, Stipp CS, Zheng JJ, Zhang XA. 2009. Transmembrane interactions are needed for KAI1/CD82-mediated suppression of cancer invasion and metastasis. Am J Pathol 174:647–660.

Christofori G. 2006. New signals from the invasive front. Nature 441(7092): 444–450.

De Baetselier P, Roos E, Brys L, Remets L, Feldman M. 1984. Generation of invasive and metastatic variants of a non-metastatic T-cell lymphoma by in vivo fusion with normal host cells. Int J Cancer 34(5):731–738.

DeNarddo DG, Johansson M, Coussens LM. 2008. Immune cells as mediators of solid tumor metastasis. Cancer Metastasis Rev 27(1):11–18.

Fidler IJ, Kripke M. 1977. Metastasis results from preexisting variant cells within a malignant tumor. Science 197:893–895.

Gannot G, Gannot I, Vered H, Buchner A, Keisaris Y. 2002. Increase in immune cell infiltration with progression of oral epithelium from hyperkeratosis to dysplasia and carcinoma. Br J Cancer 86:1444–1448.

Hu M, Yao J, Caroll DK, Weremowicz S, Chen H, Carrasco D, Richardson A, Violette S, Nikolskaya T, Nikolsky Y, Bauerlein EL, Hahn WC, Gelman RS, Allred C, Bissell MJ, Schnitt S, Polyak K. 2008. Regulation of in situ to invasive breast carcinoma transition. Cancer Cell 13:394–406.

Hunter KW, Crawford NP, Alsarraj J. 2008. Mechanisms of metastasis. Breast Cancer Res 10(Suppl 1):S2.

Li F, Tiede B, Massague J, Kang Y. 2007. Beyond tumorigenesis: Cancer stem cells in metastasis. Cell Res 17:3–14.

Lima LG, Chammas R, Monteiro RQ, Moreira ME, Barcinski MA. 2009. Tumor-derived microvesicles modulate the establishment of metastatic melanoma in a phosphatidylserine-dependent manner. Cancer Lett 283(2): 168–175.

MacLennan GT, Eisenberg R, Fleshman RL, Taylor JM, Fu P, Resnick MI, Gupta S. 2006. The influence of chronic inflammation in prostatic carcinogenesis: A 5-year follow-up study. J Urol 176:1012–1016.

Man YG. 2007. Focal degeneration of aged or injured myoepithelial cells and the resultant auto-immunoreactions are trigger factors for breast tumor invasion. Med Hypotheses 69(6):1340–1357.

Man YG. 2010. Aberrant leukocyte infiltration: A direct trigger for breast tumor invasion and metastasis. Int J Biol Sci 6(2):129–132.

Man YG, Burgar A. 2003. An antigen unmasking protocol that satisfies both immunohistochemical and subsequent molecular biological assessments. Pathol Res Pract 199:815–825.

Man YG, Gardner WA. 2008. Focal degeneration of basal cells and the resultant auto-immunoreactions: A novel mechanism for prostate tumor progression and invasion. Med Hypotheses 70:387–408.

Man YG, Tai L, Barner R, Vang R, Saenger JS, Shekitka KM, Bratthauer GL, Wheeler DT, Liang CL, Vinh TN, Strauss BL. 2003. Cell clusters overlying focally disrupted mammary myoepithelial cell layers and adjacent cells within the same duct display different immuno-histochemical and genetic features: Implications for tumor progression and invasion. Breast Cancer Res 5:R231–R241.

Man YG, Zhang Y, Shen T, Vinh TN, Zeng X, Tauler J, Mulshine JL, Strauss BL. 2005. cDNA expression profiling identifies elevated expressions of tumor progression and invasion related genes in cell clusters of in situ breast tumors. Breast Cancer Res Treat 89:199–208.

Man YG, Shen T, Zhao YG, Sang QX. 2005. Focal prostate basal cell layer disruptions and leukocyte infiltration are correlated events: A potential mechanism for basal cell layer disruptions and tumor invasion. Cancer Detect Prev 29:161–169.

Man YG, Zhao CQ, Wang J. 2006. Breast tumor cell clusters and their budding derivatives show different immunohistochemical profiles during stromal invasion: Implications for hormonal and drug therapies. Cancer Ther 4: 193–204.

Mathelin C, Annane K, Treisser A, Chenard MP, Tomasetto C, Bellocq JP, Rio MC. 2008. Pregnancy and post-partum breast cancer: A prospective study. Anticancer Res 28(4C):2447–2452.

Nakshatri H, Srour EF, Badve S. 2009. Breast cancer stem cells and intrinsic subtypes: Controversies rage on. Curr Stem Cell Res Ther 4(1):50–60.

Nguyen DX, Massagué J. 2007. Genetic determination of cancer metastasis. Nat Rev Cancer 8:341–352.

O'Brien JO, Lyons T, Bell KP, Monks J, Lucia S, Wilson RS, Hines L, Man YG, Borges V, Schedin P. 2010. Alternatively activated macrophages and collagen I remodeling characterize the post partum involuting mammary gland across species. Am J Pathol 176(3):1241–1255.

Pawelek JM, Chakraborty AK. 2008. The cancer cell–leukocyte fusion theory of metastasis. Adv Cancer Res 101:397–444.

Pawley JB. 2006. Foundations of confocal scanned imaging in light microscopy. In: Fawley JB, editor. Handbook of biological confocal microscopy. Shinya Inoue, Berlin: Springer. pp 1–19.

Pentheroudakis G, Briasoulis E, Pavlidis N. 2007. Cancer of unknown primary site: Missing primary or missing biology? Oncologist 12(4):418–425.

Phillips TM, Kim K, Vlashi E, McBride WH, Pajonk F. 2007. Effects of recombinant erythropoietin on breast cancer-initiating cells. Neoplasia 9(12):1122–1129.

Pollard JW. 2004. Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 4(1):71–78.

Polyak K, Weinberg RA. 2009. Transitions between epithelial and mesenchymal states: Acquisition of malignant and stem cell traits. Nat Rev Cancer 9(4):265–273.

Pratap J, Wixted JJ, Gaur T, Zaidi SK, Dobson J, Gokul KD, Hussain S, van Wijnen AJ, Stein JL, Stein GS, Lian JB. 2008. Runx2 transcriptional activation of Indian Hedgehog and a downstream bone metastatic pathway in breast cancer cells. Cancer Res 68(19):7795–7802.

Pratap J, Imbalzano KM, Underwood JM, Cohet N, Gokul K, Akech J, van Wijnen AJ, Stein JL, Imbalzano AN, Nickerson JA, Lian JB, Stein GS. 2009. Ectopic runx2 expression in mammary epithelial cells disrupts formation of normal acini structure: Implications for breast cancer progression. Cancer Res 69(17):6807–6814.

Rodriguez AO, Chew H, Cress R, Cress R, Xing G, McElvy S, Danielsen B, Smith L. 2008. Evidence of poorer survival in pregnancy-associated breast cancer. Obstet Gynecol 112(1):71–78.

Smith CJ, Gardner WA, Jr. 1987. Inflammation–proliferation: Possible relationships in the prostate. Prog Clin Biol Res 239:317–325.

Talmadge JE, Wolman SR, Fidler IJ. 1982. Evidence for the clonal origin of spontaneous metastases. Science 217:361–363.

Tavassoli FA, Man YG. 1995. Morphofunctional features of intraductal hyperplasia, atypical hyperplasia, and various grades of intraductal carcinoma. Breast J 1(3):155–162.

Vignery A. 2006. Macrophage fusion: Are somatic and cancer cells possible partners? Trends Cell Biol 15(4):188–193.

Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, Graf T, Pollard JW, Segall J, Condeelis J. 2004. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. Cancer Res 64(19):7022–7029.

Wyckoff JB, Wang Y, Lin EY, Li JF, Goswami S, Stanley ER, Segall JE, Pollard JW, Condeelis J. 2007. Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. Cancer Res 67(6):2649–2656.

Wysoczynski M, Ratajczak MZ. 2009. Lung cancer secreted microvesicles: Underappreciated modulators of microenvironment in expanding tumors. Int J Cancer 125(7):1595–1603.

Yousefi M, Mattu R, Gao C, Mans YG. 2005. Mammary ducts with and without focal myoepithelial cell layer disruptions show a different frequency of white blood cell infiltration and growth pattern: Implications for tumor progression and invasion. Appl Immunohistochem Mol Morphol 13:30–37.