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RESEARCH ARTICLE

II. Capsular vaso-mimicry formed by transgenic mammary tumor spheroids implanted ectopically into mouse dorsal skin fold: cellular mechanisms of metastasis [version 1; peer review: 2 approved with reservations]

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V1 First published: 10 Jan 2013, 2:9
<https://doi.org/10.12688/f1000research.2-9.v1>

Latest published: 23 Apr 2013, 2:9
<https://doi.org/10.12688/f1000research.2-9.v2>

Abstract

Most cancer patients die of metastatic disease, not primary tumors, while biological mechanisms leading to metastases remain unclear and effective therapies are missing. Using a mouse dorsal skin chamber model we had observed that tumor growth and vasculature formation could be influenced by the way *in vitro* cultured (avascular) spheroids of N202 breast tumor cells were implanted; co-implantation of lactating breast tissue created stimulating microenvironment, whereas the absence of the graft resulted in temporary tumor dormancy. This report addressed the issue of cellular mechanisms of the vasculogenic switch that ended the dormancy. *In situ* ultrastructural analysis revealed that the tumors survived in ectopic microenvironment until some of host and tumor stem cells evolved independently into cells initiating the vasculogenic switch. The tumor cells that survived and proliferated under hypoxic conditions for three weeks were supported by erythrogenic autophagy of others. However, the host microenvironment first responded as it would to non-immunogenic foreign bodies, i.e., by encapsulating the tumor spheroids with collagen-producing fibroblasts. That led to a form of vaso-mimicry consisting of tumor cells amid tumor-derived erythrocytes (synonym of erythrocytes), megakaryocytes and platelets, and encapsulating them all, the host fibroblasts. Such capsular vaso-mimicry could potentially facilitate metastasis by fusing with morphologically similar lymphatic vessels or veins. Once incorporated into the host circulatory system, tumor cells could be carried away passively by blood flow, regardless of their genetic heterogeneity. The fake vascular segment would have permeability properties different from genuine vascular endothelium. The capsular vaso-mimicry was different from vasculogenic mimicry earlier observed in metastases-associated malignant tumors where channels formed by tumor cells were said to contain circulating blood.

Open Peer Review

Approval Status ? ? ? ✓

	1	2	3	4
version 2 (update) 23 Apr 2013	?		?	✓
	view		view	view
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version 1 10 Jan 2013	?	?		
	view	view		

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Structures similar to the vasculogenic mimicry were seen here as well but contained non-circulating erythrocytes formed between tumor nodules. The host's response to the implantation included coordinated formation of new vessels and peripheral nerves.

Associated Research Article

Witkiewicz H, Oh P and Schnitzer JE » III. Cellular ultrastructures in situ as key to understanding tumor energy metabolism: biological significance of the Warburg effect, F1000Research 2013, 2:10 (<https://doi.org/10.12688/f1000research.2-10.v1>)

Witkiewicz H, Oh P and Schnitzer JE » I. Embryonal vasculature formation recapitulated in transgenic mammary tumor spheroids implanted pseudo-orthotopically into mouse dorsal skin fold: the organoblasts concept, F1000Research 2013, 2:8 (<https://doi.org/10.12688/f1000research.2-8.v2>)

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Competing interests: No relevant competing interests were disclosed.

Grant information: This work was supported by National Institutes of Health Grants (to JES): R01CA119378, PO1CA104898 and R01CA083989.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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How to cite this article: Witkiewicz H, Oh P and Schnitzer JE. **II. Capsular vaso-mimicry formed by transgenic mammary tumor spheroids implanted ectopically into mouse dorsal skin fold: cellular mechanisms of metastasis [version 1; peer review: 2 approved with reservations]** F1000Research 2013, 2:9 <https://doi.org/10.12688/f1000research.2-9.v1>

First published: 10 Jan 2013, 2:9 <https://doi.org/10.12688/f1000research.2-9.v1>

“Discovery consists of seeing what everybody has seen and thinking what nobody has thought”.

– Albert Szent-Gyorgyi

Introduction

Two main problems persisting in oncology are related; they are (1) the incomplete understanding of the mechanism by which tumors spread from primary locations to multiple organs and (2) the lack of selective anti-cancer treatments. A developmental regulatory program involved in embryo implantation, referred to as the “epithelial-mesenchymal transition” (EMT)^{1–4} was adopted to explain how transformed epithelial cells could acquire the ability to metastasize, i.e., to invade surrounding nonmalignant tissues and to disseminate, in a multistep process including entering and leaving the circulatory system^{5,6}. However, no satisfactory mechanism for the spread of non-epithelial tumors to secondary locations was proposed. Therefore, an alternative to EMT regulatory programs playing a role in invasiveness of carcinoma cells should be considered, as pointed out elsewhere⁷.

Attempts made to elucidate the cellular mechanism of metastasis-initiating events have included retrospective extrapolation from the distribution of established metastases, namely the preference of specific tumors to metastasize in certain organs but not in others. One of the earliest studies addressed the issue by injecting fixed and stained tumor cells into the left side of the heart in rabbits and determining their subsequent distribution in tissue sections of several organs. The results supported the hypothesis that the distribution of metastases was determined by the mechanics of circulation and the consequent location of embolic tumor cells but they did not exclude a role of local “soil” factors⁸. Other studies, by making a connection with genetic diversity of tumors, suggested that metastases might have a clonal origin and the dissociated representatives of particular subpopulations could be directed to their related tissues. However, generating biological diversity continued among different metastatic foci⁹. Metastases of particularly aggressive cancers of different types (not only melanoma¹⁰) were associated with patterned vasculogenic mimicry, i.e., a network of periodic acid Schiff-stained (glycoproteins containing¹¹) “loops” that represented blood-containing microvascular “channels”, generated by the aggressive tumor cells without participation of endothelial cells (ECs) and independently of angiogenesis^{12,13}. How these structures facilitated metastasis was not clear^{14,15}. Elevated incidence of metastasis was also correlated with autophagy of internal organelles in tumor cells, although the mechanism behind this was unclear¹⁶. Reports based on a variety of experiments have suggested that, depending on the context, autophagy could either stimulate or prevent cancer¹⁷. Thus, the question regarding the way in which autophagy influences metastasis has remained unanswered¹⁸. Two other intriguing issues were the inefficiency of tumor formation in experimental settings and the targeting of a selected sub-population of tumor cells by an anticancer drug. (1) Theoretically, a single cell could be capable of establishing the tumor but large numbers and a latent period were actually required^{19,20}. (2) A selective uptake of the endoradiotherapeutic 6-[211 At]-astato-2-methyl-1,4-naphthoquinol bis(diphosphate) drug only by those tumor cell nuclei that contained alkaline phosphatase isoenzyme was

demonstrated²¹. Those observations, together with the heterogeneity of tumors known for a long time but not fully understood^{20,22}, suggest the existence of cancer stem cells (CSCs) in spite of the undifferentiated phenotype of the malignant cells. The definitive proof of the CSCs was lacking²³.

The problem with nonspecific side-effects causing activities of anti-cancer drugs was to be circumvented by targeted drug delivery. That hope was based on the observation that endothelial surfaces had variable, organ-specific properties^{24–29}. However, how molecules cross the endothelial barrier and successfully reach the intended vascular destination has turned out to be another problem. The targeted destruction of established tumor vessels themselves resulted only in reduction of the tumor growth³⁰ (as expected by J. Folkman³¹) because tumors could regenerate their vasculature. The approach did appear effective in some non-neoplastic diseases^{32,33}. The issue of the tumor vessels’ permeability is rather perplexing. On the one hand, the vessels prevent anticancer drugs from penetrating the tumors, while on the other hand, they are known to be abnormally leaky^{34,35}.

We had observed earlier that in our model, formation of the tumor vasculature (vessels and blood) could be accelerated by availability of homologous tissue stem cells (TSCs) from a co-implanted graft³⁶. Without them, the process was relatively slow and the growth of implanted avascular tumor spheroids was limited, yet eventually the vasculogenic switch did happen. That raised the following questions: (1) how did the tumor survive the lag period (about three weeks) without vasculature and (2) how was the problem solved eventually? In addition to providing answers to those questions, the results shown below suggest a new cellular mechanism for initiating metastasis. We use the term TSCs according to the 2002 functional definition by M. Loeffler and I. Roeder^{37,38}. It refers to the stemness as a capability of a system rather than individual cell lineages.

We present a new type of vaso-mimicry in the murine model of breast tumor that was morphologically different from the vasculogenic mimicry previously described and postulate its role in facilitating the passive transport of tumor cell clusters to secondary locations and in determining the increased permeability of such fake vessels. Understanding the process is critical from the clinical point of view. If correct, it would bring the focus of future studies to the energy metabolism-related initial steps (as discussed elsewhere³⁶) and could result in the identification of new methods of inhibiting some of these steps before the angiogenic switch has had a chance to evolve, therefore, potentially preventing the metastases.

Materials and methods

The study was performed according to protocols approved by Sidney Kimmel Cancer Center’s OLAW-approved Institutional Animal Care and Use Committee (Assurance No A4128-01). The protocol numbers were: 03-16A and 05-11 for Grants CA104898 and CA119378, respectively. No human specimens were involved in any of the experiments outlined here.

Two recipient mice of the 5 used in the two accompanying articles were assessed in this report. The same numbering system was used in all three articles. The experimental design is summarized in [Table 1](#).

Table 1. Experimental design.

Figure numbers	Host mouse no	Tumor cell line	Graft tissue	Incubation time (days)
1 (A & C–I), 2 (B–F), 3 (A–E), 4 (A–D)	4	N202.1A+H2B-GFP	None	22
1 (B), 2 (A)	1	N202.1A+H2B-GFP	None	21

Animals, tumor spheroids, chambers and antibodies

The host mice were 8–9 weeks old athymic nude females purchased from Harlan. The mice were housed in the SKCC animal care facility. For surgery, they were anesthetized (7.3 mg ketamine hydrochloride and 2.3 mg xylazine/100 g body weight, inoculated i.p.) and placed on a heating pad. Immediately before tissue harvesting the tumor hosting mice as well as the graft donors were euthanized with pentobarbital overdose (100 mg/kg i.p.).

The N202.1A+H2B-GFP cell line (generated by stable transfection of the parental murine breast cancer cell line, N202.1A³⁹ to express GFP under histone H2B promoter⁴⁰) was obtained from Drs. J. Lustgarten and P. Borgstrom and used to form tumor spheroids by culturing 2×10^5 cells per well for 2–3 days prior to implantation. A week after establishment of mouse dorsal skin chambers, the tumor spheroids were implanted directly on skin (ectopically)⁴¹. The tumors were incubated in the chambers for three weeks (Table 1). Their final size was about 1–3 mm in diameter. The GFP-specific rabbit polyclonal IgG (ab290) was from Abcam; and non-reactive goat polyclonal IgG (sc-34284) were from Santa Cruz.

Tissue processing

The tumors with some surrounding tissues were dissected out and cut in halves perpendicular to the host skin surface while immersed in cold fixative (4% paraformaldehyde in 0.1 M Na cacodylate pH 7.4). The skin region served later as a reference to distinguish between edges of the tumor facing the skin and those facing the glass window of the chamber. The halves were then separated and processed independently for TEM and immunocytochemistry.

TEM

The tissues were transferred into a stronger fixative (4% paraformaldehyde/2.0% glutaraldehyde in 0.1 M Na cacodylate pH 7.4) to better preserve the ultrastructures before further cutting. They were cut into 1 mm thick slices in planes perpendicular to the plain of the first cut and to the skin surface, finally, into $\sim 1 \text{ mm}^3$ blocks, transferred into a fresh portion of the fixative in which they were cut and incubated for 2 hrs at 4°C. The fixed tissue blocks were washed with 0.1 M Na cacodylate – HCl buffer pH 7.4 ($3 \times 15 \text{ min}$) and post fixed in 1% OsO_4 in 0.1 M Na cacodylate buffer, pH 7.0 for 60 min. on ice, washed with water and stained with 1% uranyl acetate at RT for one hour. The blocks were embedded in EMbed-12 (EM Sciences, Cat No. 14120). The resin-embedded tissues were cut into 60 nm sections, on a Leica Ultracut UCT ultramicrotome and stained with lead citrate⁴² or viewed without further contrasting.

Immunocytochemistry

During cutting into $\sim 1 \text{ mm}^3$ blocks as described above, the tissues were kept in the mild fixative to protect the antigenic epitops (4% paraformaldehyde in 0.1 M Na cacodylate pH 7.4). The tissue blocks

were vitrified by infiltrating the pieces with 50% PVP (polyvinylpyrrolidone) containing 2.3 M sucrose in 0.1 M Na-cacodylate buffer, pH 7.4, for 2 hrs or overnight, mounted on metal pins and frozen in liquid nitrogen, as described by Tokuyasu⁴³. Frozen tissues were cut into 70 nm sections, on a Leica Ultracut UCT ultramicrotome with the cryo-attachment. The sections were picked from the knife with 2.3 M sucrose and floated on 1% ovalbumin (Sigma, Cat No. A5378) in 0.1 M Na-cacodylate buffer for at least one hour before incubation with specific or non-reactive antibody (50 $\mu\text{g/ml}$), at RT for one hour. Sections were then rinsed eight times with 0.1% ovalbumin in the same buffer and incubated for one hour with 10 nm Au coupled to protein A (from Dr G. Posthuma; Cell Microscopy Center, university Medical Center Utrecht, The Netherlands). The eight rinsing steps were repeated before fixation of the immune complexes with 1% glutaraldehyde. After rinsing three times with water, the immunostained cryosections were contrasted with a mixture of uranyl acetate and methyl cellulose (25 centipoises, Sigma M-6385) in water, at final concentration of 1.3% each, for 10 min at RT. Excess liquid was removed and the sections were dried at RT.

Viewing

All sections were viewed and the images captured at 100 kV using a Morgagni 268 electron microscope equipped with a MegaView III digital camera. Images were transmitted from the microscope camera to an iTEM imaging platform from Olympus Soft Imaging Solutions and archived in a designated database. We used the graphics editing program, Adobe PhotoShop, to add cell type-specific color-coding shown in the twin set of images included in the Supplement.

Results

Three weeks after the ectopic implantation of tumor spheroids, the vasculature formation, i.e., formation of tumor-supporting blood and vessels was evidently retarded in comparison to pseudo-orthotopically implanted tumors described elsewhere³⁶. However, the host response to the surgical injury was well advanced (Figure 1 & Figure S1). A multi-cellular layer of connective tissue was growing between the tumor and the glass wall of the chamber, therefore, it was also generating its own vasculature ([A] in Figure 1 & Figure S1). Here, the term “vasculature” includes vessels and blood and “erythrocyte” is used as a synonym for the “erythrocyte”, because the latter is not a cell³⁶. Acellular areas of collagen matrix contained erythrocytes that were vessel-free, although not extravasated. Those areas were not necrotic. Occasionally some blood cells were in close contact with supporting nucleated cells ([B & C] in Figure 1 & Figure S1). Outside the tumor capsule, a primitive forming vessel morphologically resembled some of those seen around pseudo-orthotopically implanted tumors after only five days ([D] in Figure 1 & Figure S1). Fibroblasts also encapsulated small tumor nodules. The population of tumor cells inside the encapsulated nodules was heterogeneous ([E–G] in Figure 1 & Figure S1). Evidently the tumor cells were also capable of

converting into erythrocytes and by doing so in a non-synchronized fashion, they could enable survival of other tumor cells. However, the tumor's ability to generate the genuine vessels was limited at that stage; therefore, the tumor could not grow. Some tumor cells (CSCs?) did however begin regenerating their vasculogenic potential that had been dormant during the years of *in vitro* culturing ([F & G] in Figure 1 & Figure S1). Thus the vasculogenic switch did occur in the absence of the homologous tissue stem cells (TSCs) from the graft but only after a considerable delay (about two weeks). Until that time, some tumor cells survived at the expense of others.

Most tumor cells displayed ultrastructural features characteristic of hypoxia, i.e., mitochondrial changes and dilated endoplasmic reticulum (ER) cisternae without ribosomes. In some locations,

hypoxic tumor nodules were breaking apart via prominent anoikis (loss of attachment between cells⁴⁴) with abundant nano-tentacles ([A–C] in Figure 2 & Figure S2). Commonly, cells located next to each other had mitochondria changing in opposing ways. They were either losing their internal cristae without shrinking and thus generating electron-lucent vacuoles (seemingly empty or containing whorled membranes that might be intermediate stages of the internal membranes degradation) or becoming smaller and electron dense. The first type of the morphological changes of mitochondria had been shown to occur as a result of genetically simulated hypoxia followed by necrosis⁴⁵. The second type at first resembled the appearance of mitochondria during mitosis and later, they were indistinguishable from the dark granules in erythroblasts ([D–F] in Figure 2 & Figure S2) and consistent with published images of

Figure 1. Delay in tumor vasculature development and capsular vaso-mimicry in the ectopic environment. Three weeks after implantation, the tumor environment consisted of single migrating cells that were secreting ECM components, mainly collagen and others converting into erythrocytes [A]. The erythroblast [B] and the eosinophil [C] were each in contact with a nucleated supporting cell on one side. A small primitive vessel located next to the tumor [D] resembled those early ones seen in the pseudo-orthotopic environment five days after implantation (Figure 2 in³⁶). Tumor cells were encapsulated by fibroblasts and the cell population inside the capsules appeared heterogeneous. There were erythrocytes [E], and the nuclei of some cells had sinuses [F & G] similar to those in ECs of a forming artery (Figure 11 in³⁶). Featured in [H] is a part of a larger elongated capsule containing a cluster of tumor cells in the center and multiple erythrocytes around it, all covered with typical flat, collagen-producing fibroblasts, whereas a similar structure in [I] contained mainly erythrocytes and platelets. Both were remarkably similar to a vein.

peroxisomes^{46–49}. Such opposing changes occurring simultaneously in cells sharing the same microenvironment suggested different fates for them. The one with initiated necrosis could potentially recover when the other had completed its conversion into erythrocyte(s). That is because erythrocytes are capable of secreting anaerobically generated ATP⁵⁰. Oxygen is not critical for erythrocytes themselves because they do not have mitochondria to use it. Initially, electron-dense regions of the tumor cell nucleus contained chromatin in both cases. However, that changed with the progression of the erythrocytic conversion when detecting histone H2B simultaneously exposed electron dense regions of the nucleus that did not contain chromatin ([E] in Figure 2 & Figure S2). Iron accumulation could be a good alternative reason for such increased electron density not attributable to chromatin condensation. Chromatin degradation products contained all the elements needed to make hemoglobin except iron. Therefore, a cellular conversion into erythrocytes during the neo-hematopoiesis would require soliciting iron from outside the tumor. Indeed, in tumor bearing mice the accumulation of iron was reported to shift from the spleen and liver to the tumor site⁵¹. The fibroblasts that encapsulated the tumor were of host origin, similar to the host connective tissue “membrane” encapsulating orthopedic implants⁵². The GFP-labeled mitotic chromosomes identified the tumor cell whereas the unlabeled fibroblast, on the other side of collagen layer separating the two, must have been of host origin ([F] in Figure 2 & Figure S2). Together,

the host fibroblasts and the encapsulated tumor-derived blood elements created the capsular vaso-mimicry that morphologically resembled veins ([G–I] in Figure 1 & Figure S1). The tumor cell population was clearly heterogeneous; therefore, it could survive by some cells feeding on others, initially via lactic acid secretion⁵³ and then via erythrocytic autophagy³⁶. In this way, the metabolic requirements of the encapsulated tumor nodules appeared to be responsible for initiating the capsular vaso-mimicry.

Not all tumor nodules were successfully encapsulated at the time of tissue harvest. In some regions, the fibroblasts appeared trapped between possibly faster-growing tumor nodules and, commonly, such fibroblasts were undergoing the erythrocytic autophagy as well. Typically, their cytoplasmic remnants were still present between the erythrocytes and tumor cells. The elongated cells like the one shown between the tumor nodules (Figure 3 & Figure S3) had large nuclei undergoing conversion into erythrocytes and a sparse, metabolically active cytoplasm generating energy and synthesizing protein. What appeared in the two-dimensional image as a single file of erythrocytes between the tumor nodules was not a rouleau of circulating erythrocytes.

The non-malignant tissue repair included formation of new blood vessels and peripheral nerves (Figure 4 & Figure S4). Developing vessels and nerves arranged in heterotypic pairs suggested a coordinated regulation of their morphogenesis (Figure 4 & Figure S4).

Figure 2. Hypoxia in tumor cells and involvement of host cells in forming the capsular vaso-mimicry. Hypoxia in tumor cells was manifested by structural changes of mitochondria resulting in their condensation into smaller and more electron dense (darker) forms or in degradation of cristae (left and right, respectively) [A], occasionally leading to autophagic vacuoles with whorls of membranes (arrows in [B & C]) or to peroxisomes (arrows in [D]). Partial separation of the tumor cells from one another (anoikis⁴⁴) with emergence of meandering nanotentacles (stars in [A & C]) created intercellular passages and greatly increased the cell surface. Immunocytochemical detection of GFP-labeled histone H2B in tumor cells is shown in [E & F]. The tumor cell in [E] had a nucleus with electron-dense regions that did not contain H2B, except for a small upper region, demonstrating incomplete chromatin degradation (arrow). The upper left corner of [F] corresponds to the boxed area of the insert and showed H2B-specific label in a mitotic chromosome of the tumor cell. A layer of collagen fibers (cf) separated that cell from the elongated fibroblast with unlabeled nucleus (upper right corner) of [F]. The arrow in the insert points to a condensed mitochondrion.

In some regions, ECs converting into erythrocytes (hemogenic endothelium) were also seen (Figure 8 in⁵⁴).

Discussion

Surviving without vasculature

Survival of the ectopically implanted breast tumor cells for three weeks without the support of a host circulatory system was possible due to the erythrocytic autophagy³⁶. The ability of cells to undergo such nucleocytoplasmic conversion was not tumor-specific. The initial host response to tumor nodules by encapsulating them with fibroblasts was simultaneous with the response to surgical injury caused by the implantation. During the repair process, as the layer of connective tissue grew thicker beneath the glass wall of the chamber, it too was forming new vasculature. That finding showed similarity between the cellular mechanisms of vasculature morphogenesis in growing malignant and non-malignant tissues. Hypoxia, the common metabolic denominator, could force erythropoiesis upon different types of cells, including differentiated ones for example ECs⁵⁴, if the condition persisted.

The tumor ecological niche resembled a *perpetuum mobile* in its ability to survive without blood vessels. There was a turnover of tumor cells; they kept proliferating and succumbing to erythrocytic autophagy. The system was not really isolated because it used metabolites from the

microenvironment, but depending solely on diffusion for that purpose, the tumor could not increase its size. Non-vasculogenic tumors do not grow over several weeks although the tumor cells keep proliferating at a rate similar to that of vasculogenic tumors; “They have no or non-functional vessels”⁵⁵. Those “non-functional” vessels could have been non-circulating erythrocytes, most likely derived from the tumor cells. That was, in fact, an experimental result demonstrating that some tumor cells could survive at the expense of others. Such postnatal extramedullary erythropoiesis at a location other than the bone marrow (*medulla ossea*) was not unprecedented; spleen⁵⁶⁻⁵⁹ and adipocytic tissues⁶⁰ have that potential as well.

The relevance of the variable metabolism within a single tumor nodule was that tumor-derived erythrocytes might indeed extend viability of adjacent tumor cells by supplying them with vital energy in the absence of vasculature. Hemoglobin has evolved to bind oxygen cooperatively, i.e., most efficiently when it is abundant (in lungs where the oxygen concentration is about 100 torr) and gradually less and less efficiently as erythrocytes move through arteries and veins (in peripheral tissues, the oxygen concentration is about 20 torr)⁶¹. In tumors experiencing hypoxia, one would expect the binding of oxygen to hemoglobin to be least efficient, so the erythrocytes would not compete for oxygen with the tumor cells.

Figure 3. Erythrocytic autophagy in elongated cell between tumor nodules; resemblance to the vasculogenic mimicry. Consecutive splitting of an erythrocyte (red-splitting-body) into erythrocytes (erythrocytes); one fragment completely separated and the rest in the process of splitting, all surrounded by leftover cytoplasm of the cell producing them [A]. Four enlarged regions of that cell [B–E]. A distinct region of the erythrocyte indicated the location of the next, already initiated, split (arrows) [A & C]. Vesicles on both sides of the mitochondrion contained collagen fibers (arrows) [E].

Figure 4. Coordinated morphogenesis of blood vessels (V) and peripheral nerves (N). Two neuro-vascular pairs. The upper pair were less mature; the boxed area of the nerve in [A] (enlarged in [B]) showed incomplete myelination of the neuron (arrows). The pair located next to the muscle cells (MC) [C] appeared more mature; the myelination of one neuron was completed (arrow). Morphology of the erythrocytes surrounded by endothelium was also more mature in [C]. The boxed area, enlarged in [D], featured abundant caveolae (arrows) of the multilayered neurothelium and a difference in thickness of the collagen fibers (cf) between those in endoneurium (thinner) and in surrounding ECM (thicker) [D].

The ability to convert into erythrocytes was not limited to erythropoietic lineage derived from myeloid precursors. Understandably so, because the inducing factor, hypoxia, affected the most fundamental function of all living cells, i.e., the respiration, generating vital energy aerobically. Under hypoxia, they all had only one alternative to extend their existence, namely, anaerobic generation of energy indispensable to sustain life. That metabolic pathway being shared by all cells experiencing hypoxia imposed the formation of similar ultrastructural features on all of them (convergence). Therefore, knowing what the cells do and how they do it regardless of cell lineages is important to control tumor growth. Unfortunately anaerobic metabolism is not unique to tumor cells; others are neutrophils and muscle cells, precursors of erythrocytes in bone marrow, and any dividing cells at mitosis⁵⁴.

Vasculogenic switch

The microenvironment determined the fate of tumor cells in a way similar to controlling the fate of other cells. The interactions were

mutual and ever changing. The unrelated cells could become similar enough to act as “relatives”. In other words, the heterologous environment did not kill the transplanted tumor but gradually the exogenous tissue acquired the ability to engage in the paracrine dialog with local TSCs (perhaps by acquiring proper cell adhesion molecules⁶²), or the tumor activated its own SCs (CSCs). Trans-differentiation of tumor SCs into ECs was also reported earlier in glioblastoma^{63–66}. When that happened, the dormant tumor underwent a vasculogenic switch⁶⁷. If the process was slow enough, it might not be completed within the life span of the host and such tumors would be unnoticed due to their small size, limited by 100–200 μm oxygen diffusion range⁶⁸. Reported dormant tumors had < 1 mm diameter, possibly including fibroblastic coats and necrotic centers⁶⁷.

Capsular vaso-mimicry as a cellular mechanism of metastasis

Two cellular mechanisms normally beneficial to the organism when acting independently, one involved in tissue nourishment and the other

in healing, i.e., erythropoiesis and scar formation (or foreign body encapsulation) respectively, became deleterious by creating the capsular vaso-mimicry when they coincided in the ectopically implanted tumor. The newly emerged vessel-imitating structures contained cells of tumor and host origin. They did not contain circulating blood initially, but could potentially fuse with the morphologically similar host lymphatic vessels or veins. If the conversion of the tumor cell population into erythrocytes were incomplete at the time of the merger, the fusion would facilitate metastasis. The anastomosis with lymphatic vessels might be more likely than with blood vessels (particularly arteries) because the former are comprised of a single endothelial cell layer, have no pericytes and only incomplete basement membrane. That would be consistent with the observation that metastases of most cancers occurred initially through the lymphatics⁶⁹. The dissociation of tumor cells from one another, i.e., anoikis⁷⁰, commonly seen in necrotic regions, might be due to hypoxic stress and starvation. That way each cell would gain direct access to interstitial fluid and the cell surface would greatly increase through multiple, meandering nano-tentacles that appeared to be an early sign of stress. At the same time, the loss of attachment to other cells could facilitate their dissemination by breaking the tumor tissue into small cell clusters or single cells that could be carried away by blood flow. Whereas converting a fraction of the growing tumor population into erythrocytes solved the immediate energy metabolism problem for the rest of the population temporarily, the capsular vaso-mimicry could assure a future steady supply of new energy resources in the end.

Concerning the prospect of controlling initiation of metastasis, the cellular mechanism presented here appeared more manageable because it did not depend on great biological diversity of primary tumors. The initiation of capsular vaso-mimicry was governed by metabolic requirements rather than the genetic repertoire of the tumor. Clusters of primary tumor cells could be passively carried to different tissues by blood flow and become immobilized when they reached vessels narrower than their own dimensions, in a tissue non-specific manner. However, the fate of such randomly dispersed metastatic tumor “seeds” would depend on their phenotypic compatibility with the local “soil”⁷¹. Thus, the vasculogenic switch could occur in the secondary locations either immediately or after a variable period of latency depending on the initial degree of relevant similarities. Liver being formed relatively early during embryogenesis and later maintaining primitive vasculature might be most compatible with tumors for that reason and therefore most prone to metastases, as observed clinically. If the metastasized tumors did not adjust their properties as needed to establish paracrine dialog with local TSCs, they would stay dormant. The dormancy would not necessarily be permanent because surviving via erythropoietic autophagy was accompanied by proliferation ([F] in Figure 2 & Figure S2) and therefore equipped the tumor with a source of the biological diversity. The concept of the distant niche anticipating an invader and getting ready for it^{72,73} could be adopted as follows. Dissemination of tumor cells through circulating blood could occur due to capsular vaso-mimicry targeting all organs but a successful metastasis would only develop in tissues somewhat similar to the one where the primary tumor developed. This would be consistent with the seed and soil theory^{71,74,75}. On the other hand, if the tumor was large enough to produce meaningful levels of cytokines and growth factors in circulating blood, the effect on un-invaded homologous tissues should be comparable to that caused by a smaller number of tumor cells that have metastasized. That is how anatomically distant but phenotypically compatible tissue could become activated by the tumor before metastasizing cells got there.

Vasculogenic mimicry

The structures shown in Figure 3 & Figure S3 and the earlier reported vasculogenic mimicry¹⁰ could be of the same nature. The remnants of cells that produced erythrocytes could be responsible for PAS staining due to their glyco-lipid components and, more importantly, for fusion with capillaries of the main circulatory system, at stages later than analyzed here. Our tumors were significantly smaller (diameter of < 1 mm) than those described in the literature (1 cm or more). A lack of hierarchy in the network pattern of the aggressive tumors suggested a lack of blood circulation. However, small molecules used to study intra-tumoral microcirculation by injecting a dye into a vessel located close to it could rapidly diffuse through such spaces^{12,13}. If blood were circulating through the vascular mimicry, there should be no problem with drug delivery to such tumors. Therefore, that kind of mimicry probably is a form of fibroblastic autophagy associated with the presence of metastatic tumors. The metastases could have been initiated via capsular vaso-mimicry earlier, when the primary tumor nodules were small.

Spectral *in vivo* oxygenation

The new understanding of the cellular mechanisms involved in the tumor neovasculature formation provokes some additional retrospective thoughts on earlier published results regarding vasculature-related issues that were also based on the model of breast tumors grown in the mouse skin fold chamber. For example, abnormal microvascular oxygen transport demonstrated by spectral imaging of hemoglobin saturation^{76,77}. Anastomoses between vessels with significantly different oxygenations could be explained by the fusion of hypoxic capsular vaso-mimicry with a vein containing circulating blood; the direction of the flow initiated that way would be expected to be the same as in the vein involved in the fusion, as observed (Figure 8 in⁷⁷). The resulting larger hybrid vessel would initially have a flattened profile, as seen in malignant neurilemmoma grown in a hamster cheek pouch chamber⁷⁸. What looked like “acute local stoppage of blood flow” could correspond to the lack of the flow in the vascular vaso-mimicry before the anastomosis⁷⁷. Shunting of inspired oxygen into tumor venules, presumed to occur due to arterio-venous anastomoses (Figure 10 in⁷⁶) could alternatively be explained by stable saturation of hemoglobin located in non-circulating erythrocytes within the tumor capsule, as well as within the regions mimicking vessels (Figure 1 & Figure S1). The oxygen could have remained bound to hemoglobin if the surrounding cells did not have structurally sound (functional) mitochondria. We have shown that mitochondria of tumor cells in the microenvironment of such stagnant erythrocytes were structurally impaired (converted into peroxisomes or necrotized) and accompanied by calmyrites. Such ultrastructural features are consistent with anaerobic metabolism. In the absence of functional mitochondria, the tumor cells would have no use for the abundant oxygen. Consequently, it shouldn't be surprising that increased oxygenation of breast adenocarcinoma by treatment with, for example, darbepoetin alpha, had no desirable effect on the tumor's responsiveness to radiotherapy⁷⁹. Oxygenation was probably increased in the erythrocytic component of the tumor, not in the tumor cells (a distinction impossible to make by clinical radiology).

Leakiness of tumor vessels

The distance between capillaries in tissue sections suggested that, within the 100–200 μm zones, cell membranes did not present a barrier for diffusion of nutrients or oxygen. On the other hand, toxic metabolic products can be sequestered into intracellular

vesicles to protect the cytoplasm. Although indistinguishable by TEM, the bi-layer lipid membranes vary with regard to their molecular composition. The leaky outer cell membranes permit passage of small molecules whereas the more selective inner vacuolar membranes provide a mechanism for intracellular compartmentalization. Considering what we now know about cytoevolution leading to ECs differentiation³⁶, one could make a premise that the luminal surface of the polarized endothelial cell was a functional equivalent of the inner membrane. Therefore, it could present a barrier preventing uncontrolled diffusion of some molecules. Caveolae would serve as a compensation for such an indiscriminate barrier and would provide a structural basis for selective (controlled) transport across the membrane. That might be why ECs have them in great numbers and the absence of caveolae in brain endothelium correlates with the functional blood-brain barrier⁸⁰. Vascular lumen in that context would be a functional equivalent of an intracellular vesicle. One could conclude that host fibroblasts encapsulating the tumor and creating the capsular vaso-mimicry by positioning themselves around erythrocytes should not present a barrier for the diffusion process because they did not go through the process of cytoevolution resulting in polarization of the outer cell membrane into luminal and abluminal. Morphologically, the tumor capsular vaso-mimicry resembled lymphatic vessel or vein, however it was neither. It had fibroblasts in place of ECs, therefore diffusion across the walls of the capsular vaso-mimicry (and further) would not be restricted. That could be a new explanation for the leakiness of the tumor pseudo-vessels, whereas in genuine tumor-supporting vessels, control of permeability would remain tight.

Conclusions

The results demonstrated that a balance between tumor growth and formation of its own vasculature could shift reversibly as dictated by a changing microenvironment. *In vitro*, where proper atmosphere and nutrients were available, tumor cells did not need vasculature and none formed. That changed when they found themselves back in the live mice but not connected to the host vasculature. Hypoxia forced some tumor cells to change their energy metabolism to an anaerobic pathway. That way, they could salvage the remaining tumor cells: by secreting lactic acid⁵³ or ATP⁵⁰ (similar to muscle cells and erythrocytes, respectively), and by initiating the vaso-mimicry. Time gained by the metabolic switch allowed for triggering the genuine vasculogenic switch and exposed self-organizing potential of the malignant tissue (limited to formation of its own vasculature). Establishing the metastatic tumors by creating the capsular vaso-mimicry required sufficient numbers of cancer cells in the initiating nodule. While some tumor cells were evolving into erythroblasts and megakaryocytes and inducing differentiation of ECs, others kept proliferating. Such activation of differentiation in some cancer cells was consistent with the organoblasts concept³⁶. By definition, they could be referred to as cancer stem cells (CSCs). However, alkaline phosphatase cannot be used as a marker specific for CSCs because it was detected in erythroblasts⁸¹. If the enzyme plays a role in degradation of chromatin during

the erythrogenic conversion of erythroblasts it could be associated with growth of any tissue, not only malignant tissue.

Eventually, the heterologous host TSCs also engaged in paracrine dialog with the tumor (via cytokines and growth factors⁷²). The distinction between the two sources of SCs was based here on the location where early stages of vasculature formation were seen - within the tumor capsule or next to it (Figure 1 & Figure S1). Whereas the existence of somewhat controversial CSCs^{23,82} was exposed in the ectopic environment, it was masked by availability of homologous TSCs in the pseudo-orthotopic one³⁶. Such interpretation regarding the role of homologous TSCs in neo-vasculature morphogenesis was consistent with earlier reports stating that it was not bone marrow-derived EC precursors^{83,84} but rather TSCs from the tumor microenvironment differentiated into vasculature that supported the tumor growth⁸⁵. They were also shown to support vascular nonmalignant engraftment⁸⁶.

On the other hand, after pseudo-orthotopic implantation, TSCs from grafted breast tissue formed vasculature for the breast tumor because malignant tissues maintained some characteristics of their tissue of origin. The two related cell types cooperated in executing the tissue self-organizing potential³⁶. The source of SCs that generated tumor vasculature under different circumstances (tumor, host or grafted homologous tissue) mattered with regard to how soon the vasculature formation could begin. However, in each case, hematopoiesis supporting the growing tissues was extramedullary. That observation was new and suggested a physiological role for the aerobic glycolysis (the Warburg phenomenon) in tissue morphogenesis, as addressed elsewhere⁵⁴.

Author contributions

JES conceived the study and participated in the interpretation of results; PO conducted the tissue culture and animal surgery; HW did the electron microscopy and wrote the manuscript. All authors participated in the design of the study and approved the final version of the manuscript.

Competing interests

No relevant competing interests were disclosed.

Grant information

This work was supported by National Institutes of Health Grants (to JES): R01CA119378, PO1CA104898 and R01CA083989.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

The authors thank Mrs. Carol Casarjian for proofreading the manuscript, Mr. Fred Long for his expert technical Figure preparation assistance and Mr. Zbigniew Malas who contributed the color versions of the figures included in the Supplement.

Supplementary figures

Figure S1. Delay in tumor vasculature development and capsular vaso-mimicry in the ectopic environment. Three weeks after implantation, the tumor environment consisted of single migrating cells that were secreting ECM components, mainly collagen (yellow) and others converting into erythrocytes (red) [A]. The erythroblast [B] and the eosinophil [C] were each in contact with a nucleated supporting cell on one side (green). A small primitive vessel, constituted mainly by erythrocytes (red) held together with megakaryocytes and platelets (purple), was located close to the tumor (brown) [D]. It resembled those early ones seen in the pseudo-orthotopic environment five days after implantation (Figure 2 in³⁶). Tumor cells were encapsulated by fibroblasts (yellow) and the cell population inside the capsules appeared heterogeneous [E]. There were erythrocytes (red) and the nuclei of some cells had sinuses (green) [F & G] similar to those in ECs of a forming artery (Figure 11 in³⁶). Featured in [H] is a part of a larger elongated capsule containing a cluster of tumor cells (brown) in the center and multiple erythrocytes around it, all covered with typical flat, collagen-producing fibroblasts, whereas a similar structure in [I] contained mainly erythrocytes and platelets (purple). Both were remarkably similar to a vein.

Figure S2. Hypoxia in tumor cells and involvement of host cells in forming the capsular vaso-mimicry. Hypoxia in tumor cells was manifested by structural changes of mitochondria resulting in their condensation into smaller and more electron dense (darker) forms or in degradation of cristae (left and right from the red line, respectively) [A], occasionally leading to autophagic vacuoles with whorls of membranes (arrows in [B & C]) or to peroxisomes (arrows in [D]). Partial separation of the tumor cells from one another (anoikis⁴⁴) with emergence of meandering nanotentacles (stars in [A & C], no color added) created intercellular passages and greatly increased the cell surface. Immunocytochemical detection of GFP-labeled histone H2B in tumor cells is shown in [E & F]. The tumor cell in [E, light red] had a nucleus with electron-dense regions that did not contain H2B, except for a small upper region, demonstrating incomplete chromatin degradation (arrow). The upper left corner of [F] corresponds to the boxed area of the insert and showed H2B-specific label in a mitotic chromosome of the tumor cell (brown). A layer of collagen fibers (cf) separated that cell from the elongated fibroblast (yellow) with unlabeled nucleus (upper right corner) of [F]. The arrow in the insert points to a condensed mitochondrion.

Figure S3. Erythrocytic autophagy in elongated cell between tumor nodules; resemblance to the vasculogenic mimicry. Consecutive splitting of an erythrocyte into erythrocytes (red); one completely separated and the rest in the process of splitting, all surrounded by leftover cytoplasm of the cell producing them (yellow) [A]. Four enlarged regions of that cell [B-E]. A distinct region of the erythrocyte indicated the location of the next, already initiated, split (arrows) [A & C]. Vesicles on both sides of the mitochondrion (orange) contained collagen fibers (arrows) [E].

Figure S4. Coordinated morphogenesis of blood vessels (V) and peripheral nerves (N). Two neuro-vascular pairs. The upper pair were less mature; the boxed area of the nerve (blue) in [A] (enlarged in [B]) showed incomplete myelination of the neuron (arrows). The pair located next to the muscle cells (MC) [C] appeared more mature; the myelination of one neuron was completed (arrow). Morphology of the erythrocytes (red) surrounded by endothelium (green) was also more mature in [C]. The boxed area, enlarged in [D], featured abundant caveolae (arrows) of the multilayered neurothelium (emerald) and a difference in thickness of the collagen fibers (cf) between those in endoneurium (thinner) and in surrounding ECM (thicker) [D].

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Reviewer Report 24 April 2013

<https://doi.org/10.5256/f1000research.359.r762>

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Maria Vinci

The Institute of Cancer Research, Belmont, Sutton, Surrey, UK

The authors describe an interesting model that involves the ectopic implantation of tumour spheroids (N202 breast tumour cell line) into the mouse skin fold chamber. By using high quality ultra structure images, the authors describe tumour survival to hypoxia and formation of capsular vaso-mimicry as the event that determine the vasculogenic switch, 3 weeks after implantation and that facilitate metastasis.

Although the work is valuable for the novelty of the process described, on the other side, it also presents some issues, for which I have reservations. The work is based only on one tumour model, the number of animals used for some of the observations is too low, the analysis is performed only by *in situ* ultrastructure and no quantitative data are provided as well as no information on the number of fields of view/slide analysed.

Also the images shown are all in very high power magnification. It would be useful to include images at lower power magnification to have a better overview of the surrounding tissue. Also, the authors do not provide any evidence of tumour metastasis, or tumour dissemination occurring from the original implantation, but imply that the observed vaso-mimicry process could facilitate metastasis. The authors should have looked at the process at different time points: earlier and later than the 3 weeks time point selected for the study.

Minor comments: the article is in general too wordy with the results often containing discussion points. Typos: in the title "ectopicly" should be "ectopically" and on page 9, "phenotipicly" should be "phenotipically".

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 23 Apr 2013

Halina Witkiewicz, Proteogenomics Research Institute for Systems Medicine, San Diego, CA, USA

Magnification

The article has been amended to include examples of images taken at lower power magnification. Figure 5 shows how the overall topography of each section was examined before focusing on the most interesting regions.

Implications for tumor metastasis

In the title of the amended article we have added the word “implications” to reflect the distinction between the new results and their implications more precisely. In other parts of the article that distinction is clear. The original findings (including the blood elements and tumor cells inside the vessel-resembling structure, i.e., the capsular vaso-mimicry) are described in the Results section whereas the implications derived from those data and their significance are placed in the Discussion section. For the reader’s work such implications may be most useful when they represent new ideas and envision new directions for the future research. Without hinting in the second part of the title on significance of the new findings some readers could miss the article despite its relevance to their studies.

***In situ* ultrastructure only**

The issues addressed in our study are central in tumor biology and have been approached in many ways. However, without the type of validation possible by the *in situ* analysis of a particular living system at high resolution, the published work left many questions unanswered and created multiple controversies. Yet, our in depth analysis of the TEM images would not have been possible without the other approaches. We drew freely from the abundant experimental data accumulated in the literature regardless of the method used to generate them. The visual information from our work inspired logical connections between various kinds of earlier published data, often derived from various sources. Therefore, our conclusions and hypotheses do not rely solely on TEM. Our approach fills up the essential void; it “connects the dots”. The approach and the model we used are the strength of our study (as discussed in [article III](#)).

The tissue ultrastructural analysis *in situ* brings together aspects of tumor biology traditionally treated independently: hematopoiesis, vascular biology, tumor metastasis and metabolism. Such exposure of the interdependence among those aspects introduces the tissue level into the general conversation on tumor biology at cellular & molecular levels. What a single experimental approach is depends on the definition. One can base the criteria on instruments used or on targets analyzed. Bridging the tissue and molecular levels, we used TEM to characterize tissues, cells, organelles and specific proteins *in situ* (immunocytochemistry). We used a genetically engineered, GFP-labeled cell line and the graft-donor mice. Intra-vital light and fluorescent microscopy helped to monitor the rate of tumor growth *in vivo*. The future belongs to this sort of combination of methods, i.e., the use of ultrastructural analyses *in situ* to validate other methods.

One tumor model

Even though our initial observations have been limited to the breast cancer model, there

are some indications that the capsular vaso-mimicry is not unique to that tumor type. As discussed in the article, the structure related to the capsular vasomimicry (Figure 3) was of the same nature as the vascular mimicry first seen in melanoma [PM:10487832]. Such mimicry was subsequently found in tumors of ovary [PM:11290546; PM:11839501], prostate [PM:11813211; PM:12359755] and in glioblastoma [PM:20375132]. Reporting studies based on one tumor type in one animal model is not unusual. The phenomenon of encapsulating tumors with fibroblasts is common among various types of tumors, although not every encapsulation forms the capsular vasomimicry. The prominent fibrous capsule associated with non-invasive insulinoma in the mouse model of pancreatic islet tumorigenesis did not coincide with the invasive tumor type either [PM:12086849]. Unlike the monolayer, a thick layer of fibroblasts encapsulating the tumor did not resemble a vascular wall. Figure 5 in the amended version of the article shows examples of both.

Quantitative data & time points

Our goal was accomplished by generating unprecedented photographic documentation of spontaneous intercellular relations leading to formation of the capsular vaso-mimicry. The nature of the study was exploratory and observational therefore the results are qualitative. The images represented raw data, i.e. a direct demonstration of the phenomena that had actually occurred *in vivo*. The animals were not subjected to any kind of treatment except implantation of the tumor. The conventional thinking in terms of comparing treated and control groups over a period of time to measure an anticipated effect and to evaluate its statistical significance was not applicable here. The time progression was not critical either, as opposed to the embryonal growth and development, because the tumor never matures. *In vivo* the cells were responding to locally variable microenvironment therefore they were not functionally synchronized. Consequently a range of different stages of the observed processes could be found simultaneously within single sections.

To illustrate our conclusions we selected representative images after careful analysis of many (several hundreds per mouse at various magnifications). One should keep in mind that the tumors were about 1 mm in “diameter” when viewed through a dissecting microscope. The photographed fields were selected, not randomly picked; therefore stating how many they were would be meaningless. We stopped the analysis when the findings became redundant and we understood the observed phenomena. In the literature the number of studied animals tends to be inversely proportional to complexity of assays. For example, one study had animal groups of variable size: n=18 for flow cytometry and n=6 for histology (PM:15883207). For electron microscopy studies it is common to use samples from small number of individuals (PM:20439620). In all five mice that we used (in this and the accompanying articles) the fundamental cellular mechanism of initiating the vasculature formation turned out to be the same. Thus, the number of animals was adequate for the goal of this particular study. Increasing the number of sacrificed animals above the necessary minimum, just for the number sake, would be superfluous and against the animal welfare rules.

Minor comments

Dealing with images as raw data makes it difficult to find the right balance between describing what they show (and what the readers can see) and what they mean. We have tried our best to justify the interpretation by indicating what criteria were used to identify

the presented structures. That often involved including literature references. Some improvements have been made in the amended version of the article. Both forms of the adverbs "ectopically" & "ectopically" as well as "phenotypically" & "phenotypically" are used by different sources. Another example of such inconsistency is the pair of words "tumor" & "tumour". We leave the choices to the editor.

Competing Interests: No competing interests were disclosed.

Reviewer Report 21 January 2013

<https://doi.org/10.5256/f1000research.359.r713>

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Ygal Haupt

Tumour Suppression Laboratory, Peter MacCallum Cancer Centre, East Melbourne, VIC, Australia

The authors report here on how tumour cells survive in an ectopic environment by implantation of spheroids of N202 breast cancer cells into a mouse dorsal skin chamber. Their major finding is that the implanted tumour spheroids survived for 3 weeks without any support of the host circulatory system.

They propose that vasculogenic switch and erythrocytic autophagy of some tumour cells are the key process driving the survival and growth of the tumour cells. They find that tumour cells secrete ECM component and can generate their vasculature by converting into erythrocytes thereby supporting other tumour cells. The authors propose that this process is governed by anaerobic metabolism. The limitations of this study are: 1) the study is based on *in situ* ultra structural analysis of tumour growth and survival. There was no metastatic response or circulating tumours in their model, hence no cellular mechanism of metastasis as the title implies. Further, the study is based on one mouse model and one breast tumour cell line. While the study highlights the importance of anaerobic metabolism in metastasis, this concept has been discussed in the literature.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 23 Apr 2013

Halina Witkiewicz, Proteogenomics Research Institute for Systems Medicine, San Diego, CA, USA

***In situ* ultrastructural analysis**

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No metastatic response

In the title of the amended article we have added the word “implications” to reflect the distinction between the new results and their implications more precisely. In other parts of the article that distinction is clear. The original findings (including the blood elements and tumor cells inside the vessel-resembling structure, i.e., the capsular vaso-mimicry) are described in the Results section whereas the implications derived from those data and their significance are placed in the Discussion section. For the reader’s work such implications may be most useful when they represent new ideas and envision new directions for the future research. Without hinting in the second part of the title on significance of the new findings some readers could miss the article despite its relevance to their studies.

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coincide with the invasive tumor type either [PM:12086849]. Unlike the monolayer, a thick layer of fibroblasts encapsulating tumor did not resemble a vascular wall. Figure 5 in the amended version of the article shows examples of both.

The concept

The capsular vaso-mimicry has not been discussed in the literature and the issue of leakiness of the tumor vessels remains unclear. No mechanism was proposed to make logical connections between the molecular events and the intercellular relations leading to the metastasis via the erythrocytic autophagy. Previously reported correlations between anaerobic metabolism and metastasis were used to predict clinical outcome.

Competing Interests: No competing interests were disclosed.

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