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14. ABSTRACT
We have successfully devised a 3D tumor invasion assay that integrates 3D tissue engineering and FRET imaging to provide us with a means with which we could directly monitor, in real-time, the endothelial signaling event during tumor intravasation. Using this novel assay, we have demonstrated that breast cancer cells induce endothelial cell myosin light chain kinase (MLCK) activation at the site of tumor invasion, and that this transient MLCK activation correlates with localized myosin II regulatory light chain (RLC) phosphorylation and regional endothelial cell stress fiber shortening. We have also investigated the role of RLC phosphorylation using a high throughput tissue cytometric studies and demonstrated that endothelial RLC phosphorylation played a critical role in determining the routes of tumor intravasation. The majority (~56%) of MDA-MB231 breast cancer intravasation events were transcellular (i.e. through individual endothelial cells). However, when challenged by endothelial cells expressing mutagenized RLC that cannot be phosphorylated by MLCK, cancer cells predominantly (~76%) undergo paracellular invasion, indicating that intraendothelial signaling events can be modulated and play an important role in the tumor intravasation. The development of this novel assay adds significantly to our experimental repertoire to directly study an important yet hitherto under-explored perspective of tumor invasion – that of the underlying endothelium.

15. SUBJECT TERMS
FRET, signal transduction, metastasis, myosin light chain kinase, vasculogenesis, tuor intravasation

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Introduction

We seek to study in this project the signaling cascades in the underlying endothelium triggered by metastatic breast cancer cells during diapedesis. We proposed to examine these transient and localized signaling events using a three dimensional (3D) assay with superior spatio-temporal resolution. Specifically, we planned to develop and fully integrate 3D fluorescence resonant energy transfer (FRET) studies into 3D tissue engineering to create a vasculature network capable of dynamic read-out of signaling events. This system will allow us to monitor the regional interaction between cancer cells and the endothelial layer. We will use this system to (i) decipher how the metastatic tumor cells increase vascular permeability by controlling the signals that converge on the contractile machinery, (ii) to explore the potential anti-metastatic effects of obliterating these signaling components along the vascular permeability pathway, thus potentially offering new rationale to use the inhibitors as anti-cancer agents.

We have spent the three years of the funding period, as planned, working on the goals outlined in the modified Statement of Work. We have made remarkable progress simultaneously on several aspects, including (1) refining the method with which we can introduce the breast cancer cells into the 3D matrix with the engineered vasculature network, (2) performing 3D FRET imaging to study the dynamic activity of myosin light chain kinase (MLCK) during active tumor transendothelial migration of breast cancer cells, (3) standardizing protocol to fix and permeabilize the endothelial vasculature in the 3D matrix in order to perform immunofluorescence, (4) investigating the role of endothelial myosin II regulatory light chian (RLC) phosphorylation in determining the route of tumor cell transendothelial migration.

We have made an unexpected discovery, in the second year of the funding period, of a novel mechanism with which cancer cells invade the vasculature. Using our novel assay, we demonstrated that tumor cells could indeed undergo transcellular (i.e. through individual endothelial cells) intravasation, in addition to the conventional known mechanism of paracellular (i.e. through cell-cell junctions) invasion. This newly observed phenomenon was vitally important for us to understand how cancer cells penetrate the endothelial barrier, and more importantly might significantly change the paradigm of how we think about tumor invasion. We had likewise requested a modification to the Statement of Work to continue to pursue this critical information.

Development planned in Statement of Work

First year of the funding period

We successfully integrated 3D *in vitro* vascular engineering with real time FRET imaging, laying down the foundation needed to bring this project to fruition. We confirmed that endothelial cells in the 3D matrix were capable of (a) sensing mechanical forces in the form of isometric tension; (b) undergoing lumen formation, anastomosis, and braching; and (c) establishing basal-apical polarity in the correct orientation. We also characterized the fluid exchange rate within the 3D matrix to ensure that endothelial cells embedded in the collagen gel are not cultured under hypoxia condition. Using Cell Tracer Green dye (Invitrogen), we confirmed that the fluid exchange rate within the gel was rapid enough to not cause any experimental artifact.

At the time this project was launched, a commercial software was not available that can perform 3D ratiometric imaging analysis – critical for this study. We collaborated with Improvision, Inc., to develop the first 3D FRET software module that is now available in Volocity software version 4.0 and higher.

Second year of the funding period

As reported in our 2008 (2^{nd} year) progress report, we successfully generated an adenovirus expression vector to ensure that we could achieve ~100% transfection efficiency for the endothelial cells to be used in our 3D vascular network. We also standardize the protocol needed to introduce the MDA-MB231 breast cancer cells into the 3D collagen matrix to facilitate tumor invasion *in vitro*.

Using this fully developed, novel 3D tumor invasion assay, we characterized the real-time tumor-endothelial cell interaction. We presented the first preliminary data showing that breast cancer cells are capable of undergoing both paracellular (i.e. through cell-cell junctions) and transcellular (i.e. through individual endothelial cells) migration during intravasation. More importantly, tumor cell undergoing transcellular migration triggered a transient and localized endothelial MLCK activation at the site of tumor invasion, as reported by our MLCK FRET-based biosensor.

Third (Final) year of the funding period

For the final year progress, we will list the accomplishment according to the modified statement of work. *Note that we have requested a modification to our Statement of Work in 2008.*

A. Confirming transcellular migration by electron and light microscopy [months 25-26]

During the 3rd year of the grant, we have developed methods to directly document the ability of tumor cells to undergo transcellular migration during intravasation. As shown in Figure 1, we have demonstrated conclusively, using both transmission electron microscopy and confocal microscopy, that tumor cells indeed can undergo transcellular invasion into endothelial cells.

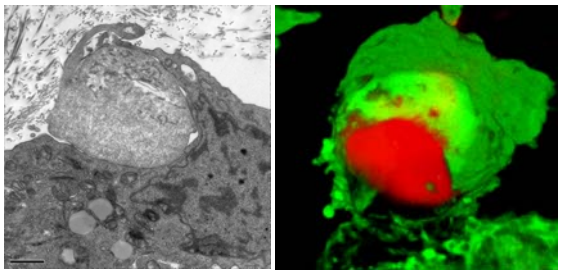


Figure 1 MDA-MB231 breast cancer cells undergo transcellular migration during intravasation. (A) Electron micrograph demonstrating the endothelial cytoplasmic deformation as an MDA-MB231 cell invades into the cytoplasm. Note that the tumor cell entered an area right next to the endothelial cell nucleus that is devoid of endothelial cell-cell-junction. Bar = 1µm. (B) 3D reconstruction of confocal microscopy showing the engulfment of an MDA-MB231 cell (red) by the endothelial cell (green).

B1. Determining the pattern of endothelial RLC phosphorylation at tumor invasion site. (months 33-36)

MLCK is a highly specific kinase with only one known physiological substrate: the 20kDa RLC that regulates the actin-activated ATPase of myosin II. Unlike Rho kinase (ROCK), which mono-phosphorylates RLC at residue Ser-19, MLCK is capable of diphosphorylating RLC at Ser-19 and Thr-18. Phosphorylation of the RLC at Ser-19 potentiates myosin ATPase 10-100 fold, while diphosphorylation of RLC at Ser-19 and Thr-18 maximally activates myosin II. The marked *in situ* activation of MLCK suggests that the endothelial myosin II RLC may be locally targeted for maximal phosphorylation. To test this hypothesis, we performed immunofluorescence using antibodies specific for the diphosphorylated form of RLC (Fig. 2A) and total myosin II (Fig. 2B). The relative level of RLC phosphorylation was obtained by normalizing the level of phosphorylated RLC to total myosin II local concentration (Fig. 2D). It is important to point out that immunofluorescence would inevitably stain myosin II and RLC in both endothelial and cancer cells. Note the local enrichment of phosphorylated endothelial RLC surrounding the invasion site (Fig.2D). While the invading tumor cell contained high concentration of myosin II, it displayed low ratio of diphosphorylated RLC relative to myosin.

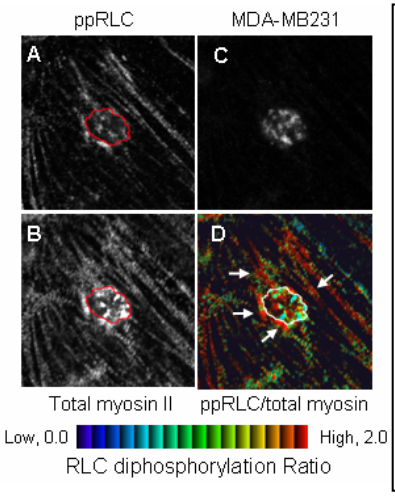


Figure 2 Enrichment of diphosphorylated RLC and localized myosin II contraction at invasion site in endothelial cell. (A) Immunofluorescence for RLC diphosphorylated at Thr-18 and Ser-19, ppRLC. (B) Immunofluorescence of total myosin II. (C) CellTracker Red-labeled MDA-MB231 cell; this picture was used for the cancer cell outlines used in the other three panels. (D) The distribution of diphosphorylated RLC was assessed by determining the ratio of phosphorylated RLC (from panel A) to total myosin II (panel B), and displayed according to the ratio bar. Myosin with very high phosphorylated RLC is indicated in red (white arrows).

B. Determining the myosin contractile activity in the 3D vascular system at the site of tumor invasion. [months 27-30]

We previously reported that invading tumor cells triggered a transient and localized endothelial MLCK activation at the site of tumor intravasation. MLCK is a specific kinase with only one known physiological substrate – myosin II regulatory light chain (RLC). MLCK, when activated, diphosphorylates RLC at Ser-19 and Thr-18. Diphosphorylation of RLC maximally activates myosin II activity. We therefore hypothesize that the localized activation of MLCK would lead to active myosin II contractile function, measurable by stress fiber shortening. We report here we have successfully shown, in real-time in the 3D assay, localized stress fiber shortening at the tumor invasion site. This result indicates that during transcellular intravasation, invasive tumor causes the activation of endothelial MLCK that directly activates myosin II contractile function *in situ* at the invasion site.

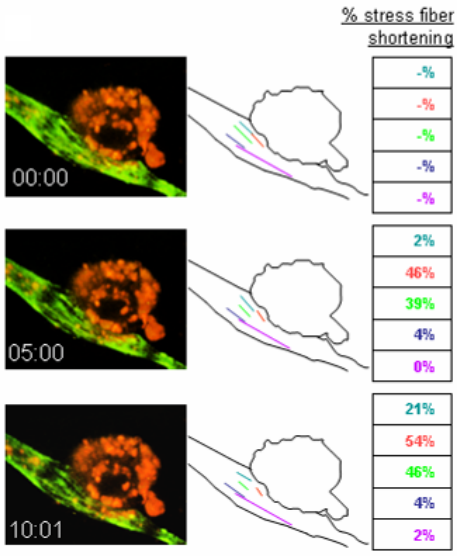
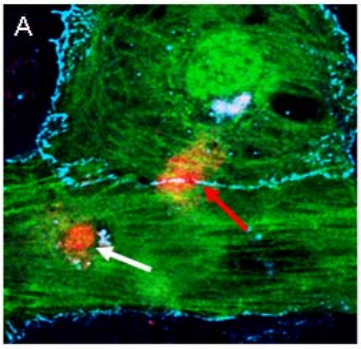
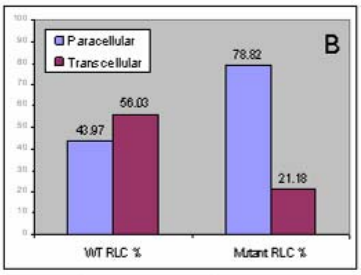


Figure 3 Localized endothelial myosin II contraction at the site of tumor invasion. (left panels) Confocal micrograph of MDA-MB231 (red) transcellular invasion into an endothelial cell expressing GFP-RLC. (middle panels) Outline of the cells and five prominent endothelial stress fibers are presented. (right panels) Myosin II contraction is expressed as a percentage stress fiber shortening as compared to the initial lengths, with the color of the % matches the color of the stress fiber outline. (Representative results from N of 4 invasion events). Indicated time points are in minutes:seconds.

C. Determine the importance of regulatory light chain phosphorylation in mediating the tumor transcellular entrance into the vascular system (Months 30-32)

Results obtained thus far indicate that endothelial cell contractile activity is potentiated in response to tumor transcellular migration, through the *in situ* activation of MLCK. To determine the role of endothelial regulatory light chain diphosphorylation in tumor invasion route, we challenge the invasive breast cancer cells MDA-MB231 with endothelial cells expressing either wild type RLC or mutagenized RLC that cannot be phosphorylated (Alanine replacement of Ser-19 and Thr-18, hereafter referred to as 18A19A RLC). To obtain high invasion events for statistical analysis, we have performed this assay in 2D, in stead of 3D condition. We stained the endothelial cell-cell border with VE-cadherin in order to help differentiate transcellular from paracellular invasion events. Using a novel high throughput cytometric imaging system (TissueGnostics, Vienna, Austria), we have devised a sequential filtering method to score for cancer cells undergoing either transcellular or paracellular invasion into the underlying 2D endothelium. As shown in Figure 4, MDA-MB231 breast cancer cells, when challenged with endothelial cells expressing wild type RLC, undergo both transcellular (56%) and paracellular (44%) invasion. However, faced with endothelial expressing non-phosphorylatable 18A19A RLC, MDA-MB231 undergo predominantly paracellular (79%) invasion. This result indicates that endothelial contractile function plays significant role in determining the route of breast cancer intravasation. More importantly, it highlights the hitherto neglected intraendothelial signals as potential determinant in tumor invasion route that cannot be overlooked.





	WT RLC		Mutant RLC	
	Cell Count	%	Cell count	%
Paracellular	62	43.97	67	78.82
Transcellular	79	56.03	18	21.18

Figure 4. **MDA-MB231 transcellular invasion is dependent on endothelial myosin II RLC phosphorylation.** (A) Confocal micrograph showing examples of how paracellular (red arrow) and transcellular (white arrow) invasions are scored. (B) Bar graphs and table showing the actual cell count and the percentage of MDA-MB231 invasion via para- vs. transcellular route when challenged by endothelial cells expressing wild type or mutant RLC.

Key Research Accomplishments (Cumulative)

- Generation of viral vectors to deliver the FRET sensor constructs to 100% of the endothelial cells used to generate the 3D vasculature
- Standardization of the condition for endothelial cells to reproducibly establish vasculature with lumen in 3D matrix
- Standardization of the condition for endothelial cells expressing the FRET biosensors to establish lumenized 3D vasculature
- Characterization of the fluid exchange rate of the 3D matrix for subsequent drug treatment experiments
- Development of 3D FRET ratio-imaging software module with Improvision, Inc.
- Performing the first 3D FRET confocal microscopy
- Confirmation of the polarization of endothelial cells in the engineered vasculature
- Standardization of cell injection protocol and co-culture condition to introduce the metastatic breast cancer cells into the 3D matrix system.
- Utilizing 3D FRET imaging to dynamically characterize the *in situ* and transient activation of MLCK activity during tumor transendothelial migration.
- Development of an *in vivo* 3D vasculature system expressing FRET sensors which will allow us to examine the signaling events involved in active tumor transcellular entry.
- Demonstrate that breast cancer cells can undergo transcellular as well as paracellular intravasation.
- Demonstrate, using our novel assay, that endothelial MLCK is transiently and locally activated at the site of tumor invasion.

- Demonstrate, using ratiometric imaging, that endothelial MLCK activation correlates with localized increased in RLC diphosphorylation and regional myosin II contraction.
- Establish the role of endothelial myosin II RLC phosphorylation in determining the route of breast cancer cell intravasation; endothelial RLC diphosphorylation at Ser-19 and Thr-18 is required for tumor cells to undergo transcellular invasion.

Reportable Outcomes

- 3D FRET and ratio-imaging modules now incorporated in Volocity software (Improvision) version 4.0.1 and onward.
- *Abstract*: "Hijacking the Endothelial Cell Contractile Machinery during Transcellular Invasion by Breast Cancer Cells: a 3D FRET study." American Society for Cell Biology 2008 Meeting, San Francisco
- *Presentation*: "Endothelial myosin II regulation and function during tumor invasion" Ludwig Boltzman Cancer Research Institute, Vienna, Austria
- *Manuscript in review*: "Transcellular Intravasation of Breast Cancer Cells Requires Myosin Light Chain Kinase Signal in the Underlying Endothelial Cells: A 3D FRET Study."

Conclusion

We have successfully accomplished all the tasks in the approved statement of work for the 3-year funding period. These accomplishments, taken together, demonstrate that we have standardized the 3D assay system with which we can now dynamically monitor, with extremely high spatiotemporal resolution, the transient and *in situ* signaling events within the vasculature network triggered by invading breast cancer cells.

In the second year of funding, we have accomplished the first specific aim of the proposed project. Using the vasculature network engineered in the 3D matrix, combined with our newly developed time-lapsed 3D FRET imaging techniques, we have studied the process of transendothelial migration of tumor cells with vastly improved spatio-temporal resolution. Our data showed that the interaction with cancer cells caused a general wave of elevation of MLCK activity within the endothelial cells. This is likely due to the calcium release triggered by the engagement of various adhesion molecules on the surface of the endothelial cell. We have indeed reciprocally confirmed the general elevation of calcium signaling in the underlying endothelial cell being invaded using Cameleon Ca^{2+} FRET sensor. The actual process of diapedesis of the tumor cell triggered a marked regional activation of MLCK at the site of invasive entry – implicating a tumor-mediated biphasic Ca^{2+}/calmodulin response in the endothelial cell.

In the third year of funding, we have followed the modified (documented in previous year's progress report) statement of work and focused our effort in deciphering the downstream effect of endothelial MLCK activation on tumor invasion. We documented the regional enrichment of diphosphorylated myosin II RLC at the site of invasion and that this pattern of RLC phosphorylation correlates well with endothelial myosin II localized contractile function, as measured by stress fiber shortening. More importantly, we have directly probed the role of endothelial RLC phosphorylation in tumor intravasation. When challenged with endothelial cells expressing mutagenized RLC that cannot be phosphorylated, MDA-MB231 drastically changed the route of invasion to predominantly paracellular.

Another critical advancement we have made is the development of the methodology and software module to perform three-dimensional FRET. This microscopy technique will prove to be a powerful improvement not only for those working in breast cancer, but for any investigators who need to expand their FRET imaging repertoire to study dynamic signaling, protein processing, protein-protein interactions as well as protein conformational changes in three dimension either in single cells or in thicker tissue specimens. It also marks the successful combination of advance microscopy technique with *in vitro* tissue engineering.

This study provides new insight of how the endothelial signaling cascades may be modulated by the invading tumor, and highlights the importance of examining the process of tumor invasion from an important yet hitherto under-explored perspective – that of the underlying endothelium. The dependence of tumor invasion route on endothelial contractility provides an important reminder that tumor intravasation is a host-disease interaction, and that the endothelium may serve as key determinant in tumor invasive potential. Successful tumor invasion is the sum result of many factors that constitute the tumor microenvironment (Condeelis and Pollard, 2006; Kedrin et al., 2008; Li et al., 2007; Lunt et al., 2008; Witz,

2006; Witz, 2008), which is experimentally difficult to scrutinize. Our system offers a powerful way to reconstitute the cellular, chemical and physical environment, one element at a time if necessary, to dissect the tumor invasion mechanisms. The engineered vascular network as a tumor invasion platform underscores our ability to control the genotype of the cellular players in the tumor microenvironment at-will, allowing us to introduce cell type-specific gene manipulation without having to resort to drug treatment which invariably affects all cells in the tumor milieu.

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APPENDICES

Statement of work

Molecular Mechanisms in Compromised Endothelial Barrier During Breast Cancer Metatasis

We anticipate that the first specific aim will take longer than the second aim because most the technical development and integration have to be first standardized in the first aim. We expect to accomplish the first aim in the first two years.

Task 1 To characterize the signal transduction in endothelial cell in 3D matrix during tumor invasion (months 1-24)

- Obtain FRET-based biosensors for MLCK, Rho, and Rac **[accomplished]**
- Standardizing simultaneous CFP/YFP FRET imaging techniques **[accomplished]**
- Generate adenoviral transfection system for FRET-based MLCK biosensors **[accomplished]**
- Generate adeno- or retroviral vector for FRET-based Rho and Rac biosensors **[accomplished]**
- Design and set up apparatus for casting 3D collagen matrix system in cell culture incubator. **[accomplished]**
- Adapt endothelial cells to 3D matrix for long term growth **[accomplished]**
- Standardizing the condition for endothelial cells to reproducibly establish vasculature with lumen in 3D matrix **[accomplished]**
- Standardizing the condition for endothelial cells expressing the various FRET biosensors to establish vasculature with lumen in 3D matrix **[accomplished]**
- Standardizing the microinjection conditions to introduce MDA-MB 231 and MCF-7 breast cancer cells into the 3D matrix **[accomplished]**
- Characterization of the fluid exchange rate of the 3D matrix, for introducing various inhibitors of ROCK (Rho kinase), MLCK as well as to introduce simvastatin **[accomplished]**
- Performing 3D FRET imaging to study the dynamic activity of MLCK during active tumor transendothelial migration of breast cancer cells. Compare the endothelial response to MDA-MB-231 and MCF-7 **[accomplished]**
- Standardizing protocol to fix and permeabilize the endothelial vasculature in the 3D matrix in order to perform immunofluorescence staining. **[accomplished]**

NOTE: The entire task 1 is now accomplished as planned within the first 24 months of funding.

Original Statement of Work for the 3rd year of Funding Period

Task 2 To delineate the anti-metastatic mechanisms of statins, Rho kinase inhibitor and MLCK inhibitor (months 24-36)

- Standardize the "uncaging" condition of activating CMNB-caged fluorescein in the 3D co-culture matrix. This task will subsequently allow us to measure the permeability of the 3D vasculature. **[months 25-27]**
- Performing fluorescence uncaging experiment to determine if (i) simvastatin, (ii) Y27632 (ROCK inhibitor), (iii) ML-7 (MLCK inhibitor) are capable of preventing VEGF-induced endothelial hyperpermeability **[month 28-29]**
- Determining the inhibitory effects of simvastatin on the dynamic translocation of VEGF-activated Rho and Rac to plasma membrane. **[months 30-31]**
- Performing ratio immunofluorescence experiment to assess the inhibitory effect of (i) simvastatin, (ii) Y27632 (ROCK inhibitor), (iii) ML-7 (MLCK inhibitor) on VEGF-induced myosin phosphorylation. **[months 32]**
- Performing *in situ* cytometric quantification to determine if (i) simvastatin, (ii) Y27632 (ROCK inhibitor), (iii) ML-7 (MLCK inhibitor) can directly attenuate MDA-MB-231 transendothelial migration in the 3D matrix **[months 33-36]**

Amendment to Statement of Work for the 3rd Year of Funding Period

Task 2 To delineate the role of myosin II regulatory light chain phosphorylation in tumorigenic transcellular invasion in to the vascular system (months 24-36)

- Confirm transcellular migration event by electron microscopy and live cell imaging with endothelial cells expressing GFP-cadherin. **[months 25-26]**
- Determine the myosin contractile activity in the 3D vascular system at the site of tumor invasion. This can be easily achieved by GFP-tagged myosin regulatory light chain which we have generated years ago. **[months 27-30]**
- Determine the importance of regulatory light chain phosphorylation in mediating the tumor transcellular entrance into the vascular system, using GFP-tagged phosphorylation site mutants of myosin II regulatory light chain. **[months 30-32]**
- Determine the myosin regulatory light chain phosphorylation response to MDA-MB-231 to probe if the ability to induce myosin activation in endothelial cells can be established as one of the metastatic factors for tumor cells. **[months 33-36]**