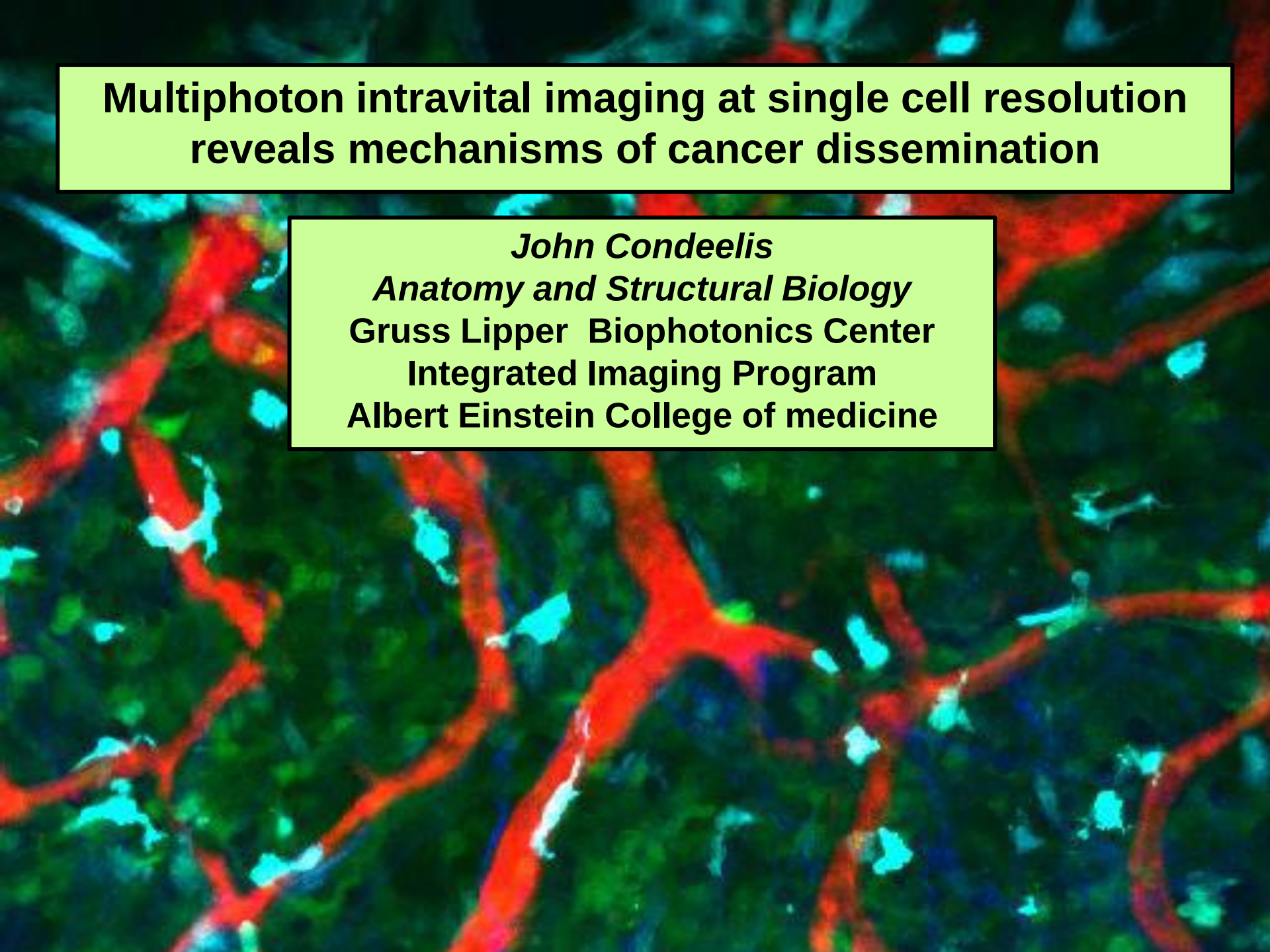
Multiphoton intravital imaging at single cell resolution reveals mechanisms of cancer dissemination



John Condeelis
Anatomy and Structural Biology
Gruss Lipper Biophotonics Center
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Why study metastasis?

Metastasis is what kills the patient.

Barriers to progress:

- **Traditional therapeutic approaches are targeted to growth control which is independent of dissemination.**

- **In addition, single cell resolution in digital pathology is mostly studied in fixed tissue resulting in correlations and inferred mechanisms.**

However, dissemination must be studied at single cell resolution and in real time in vivo to define and test mechanisms of dissemination.

How? Computational Intravital imaging.

Goals of computational intravital imaging:

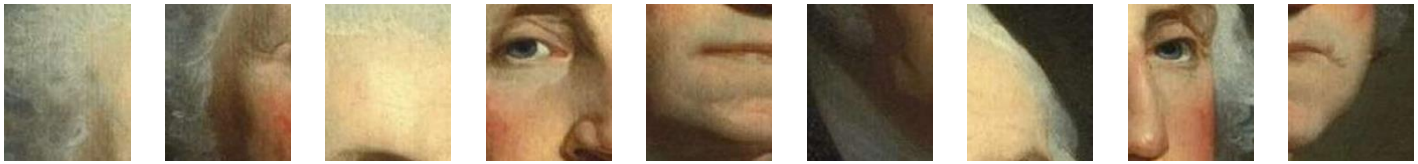
- **Establish single cell phenotypes in vivo in real time in large tumor volumes**
- **Discover microenvironments supporting cause and effect relationships**
- **Define molecular mechanisms driving these phenotypes**

Maja Oktay

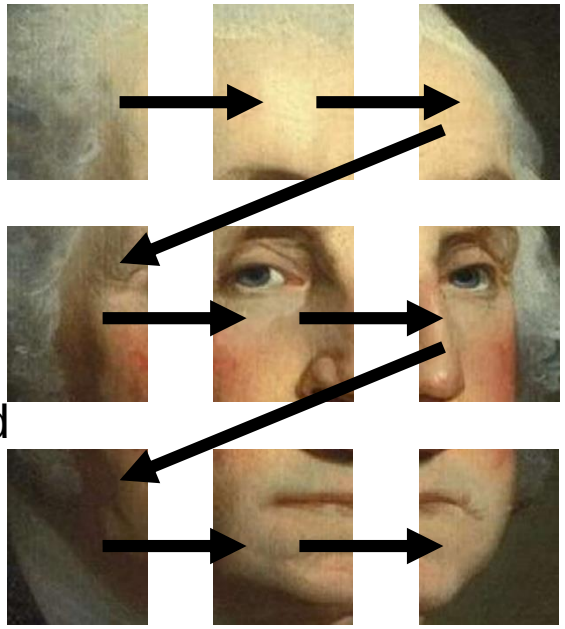
Computational Pathology → Predictive Oncology

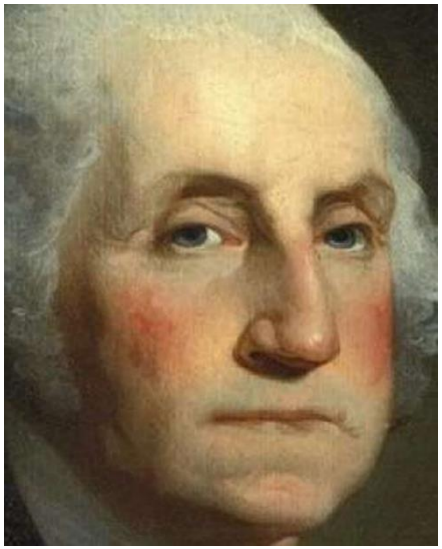
Large volume imaging at high resolution combined with computational/systems analysis

High mag/ high resolution of random fields = No context



High mag/ high resolution ordered fields = context and resolution





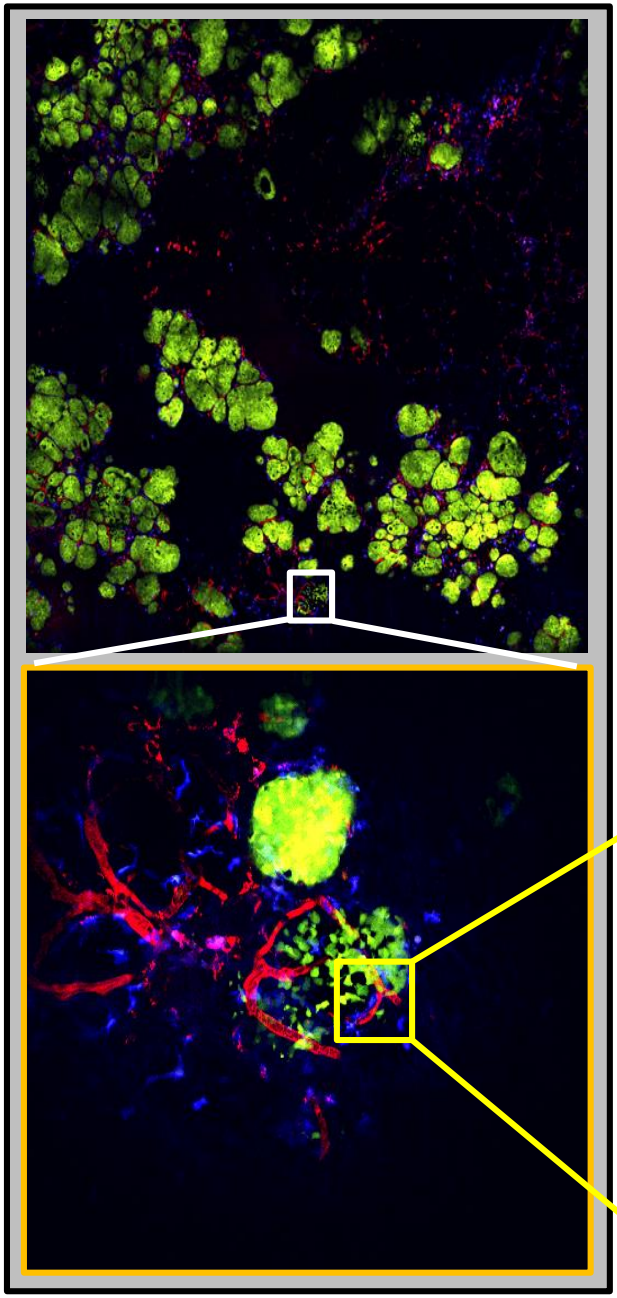
Computational classification leads to new insights into mechanism

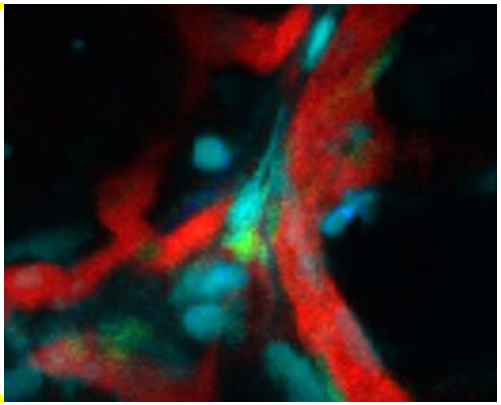
Entenberg et al 2017 LVHR

Large volume high resolution multiphoton imaging of primary mammary tumor

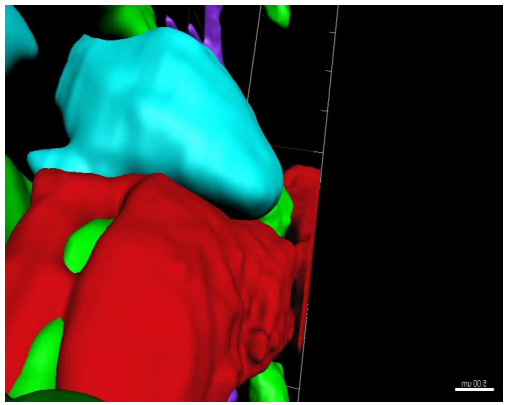
Multiplexed rapid scanning, time lapse and z-series = very large volumes of tumor that can be zoomed to follow single cell phenotypes in real time in live mice over many days.

Large area shown = 4 mm x 4 mm x ~300 um. Resolution = 0.4 um.



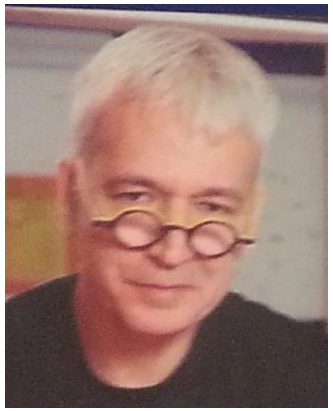


~150 um

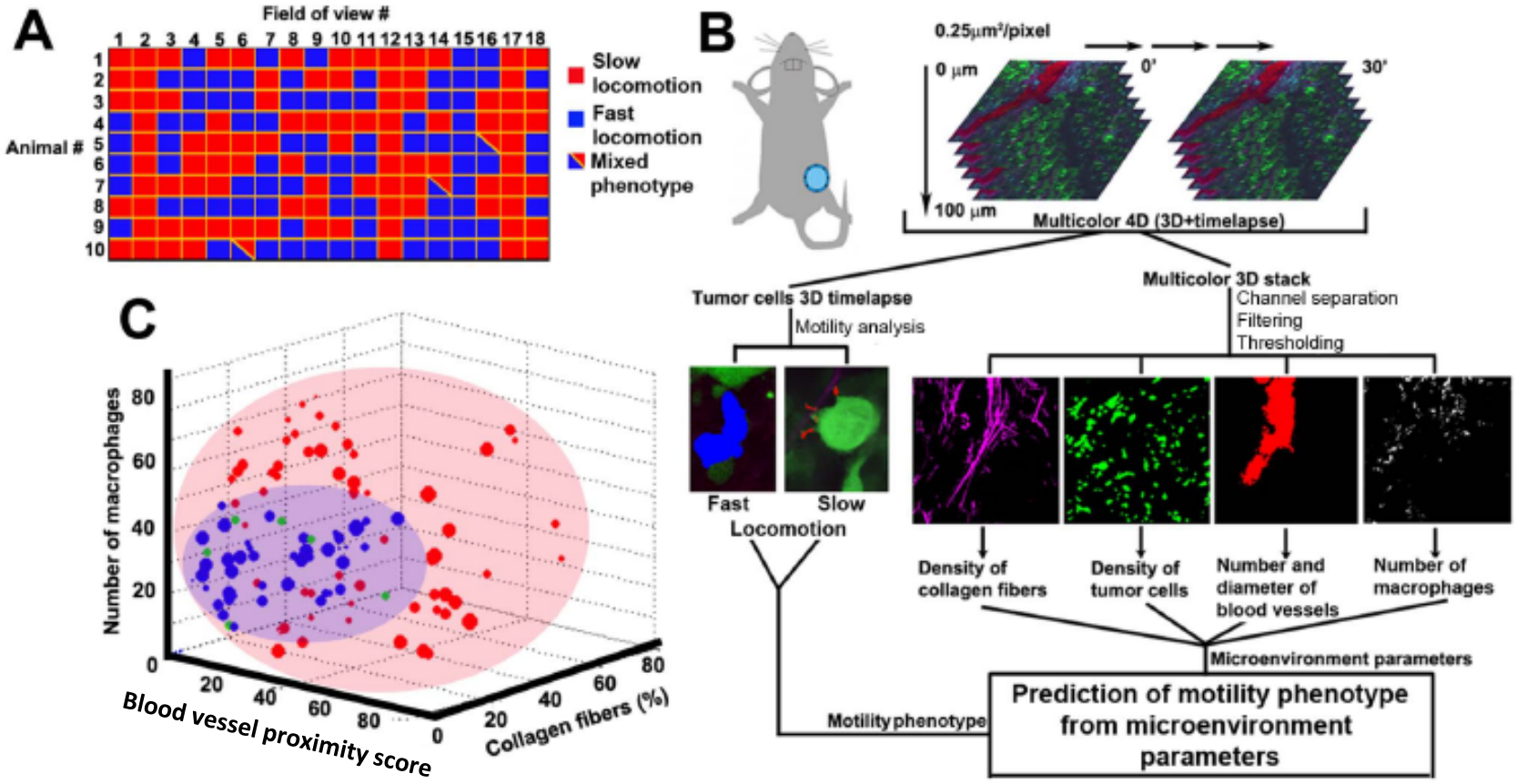


~30 um

Tumor microenvironment complexity requires a non-linear SVM classification of 7 dimensions to objectively define invasive breast tumor cell phenotypes



Aviv Bergman
Chair
Computational /systems

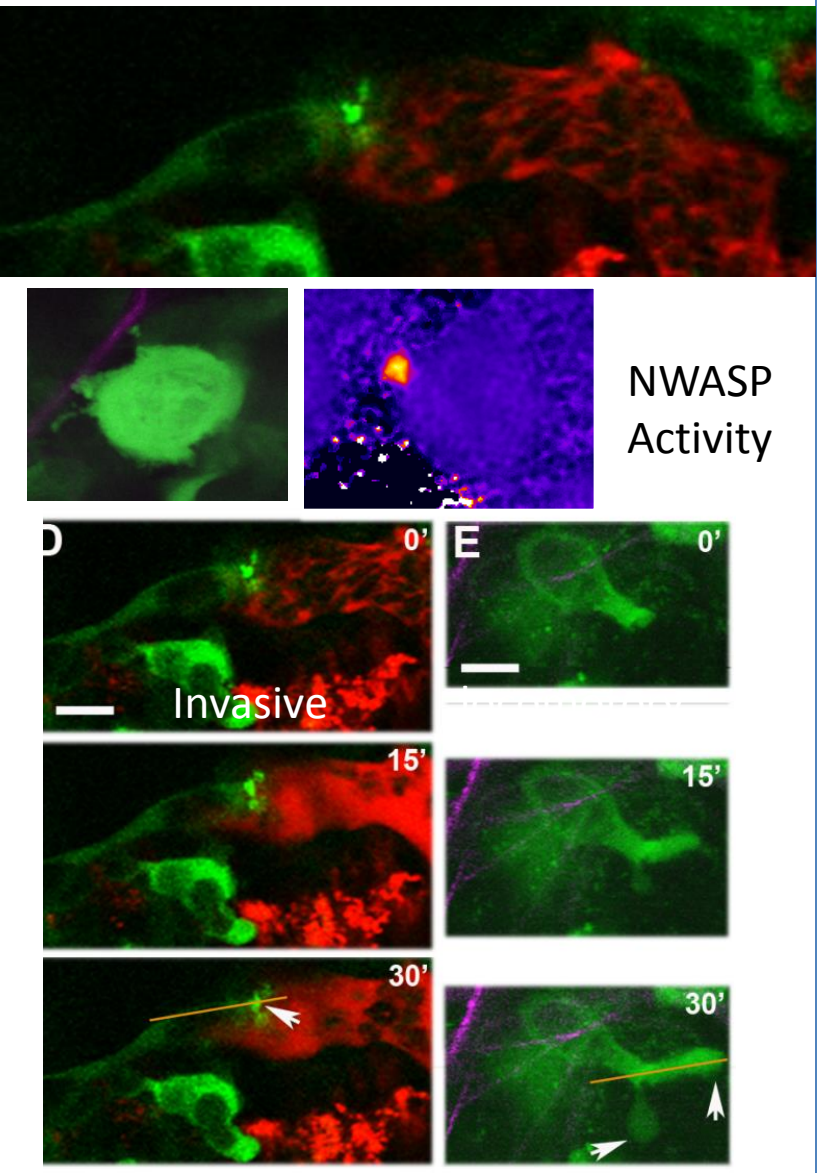


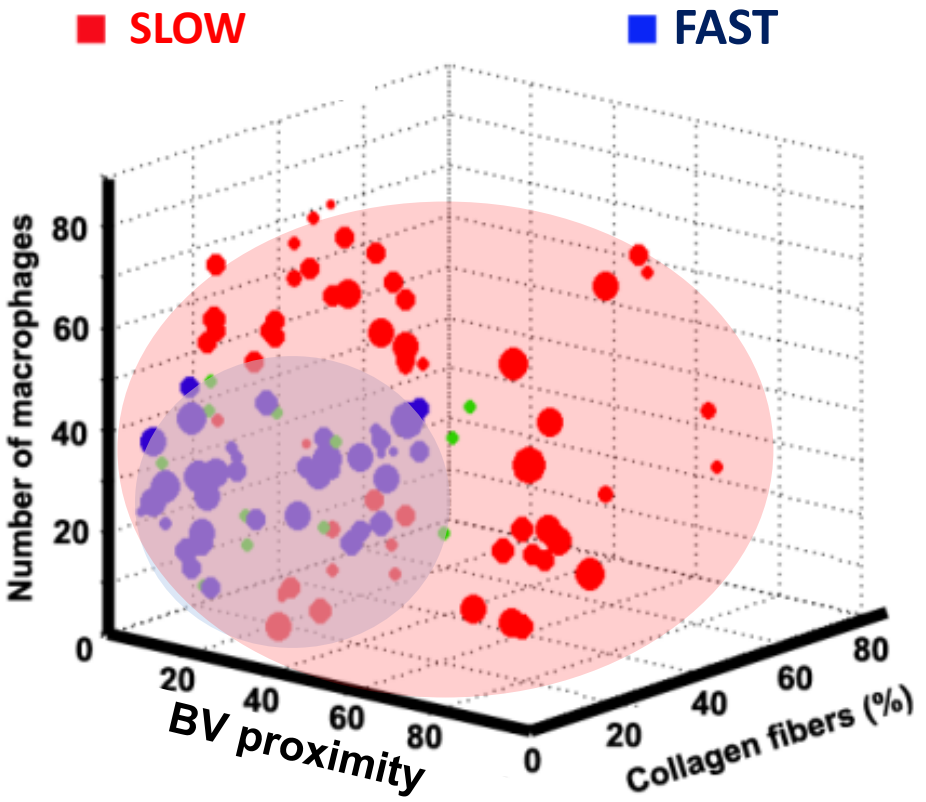
$$S = \{(x_i, c_i) | x_i \in \mathbb{R}^n, c_i \in \{-1, +1\}\}, i = 1 \ldots m$$

Bojana Gligorijevic et al 2014 Plos Biol

Systems analysis recognizes microenvironments associated with two tumor cell phenotypes:

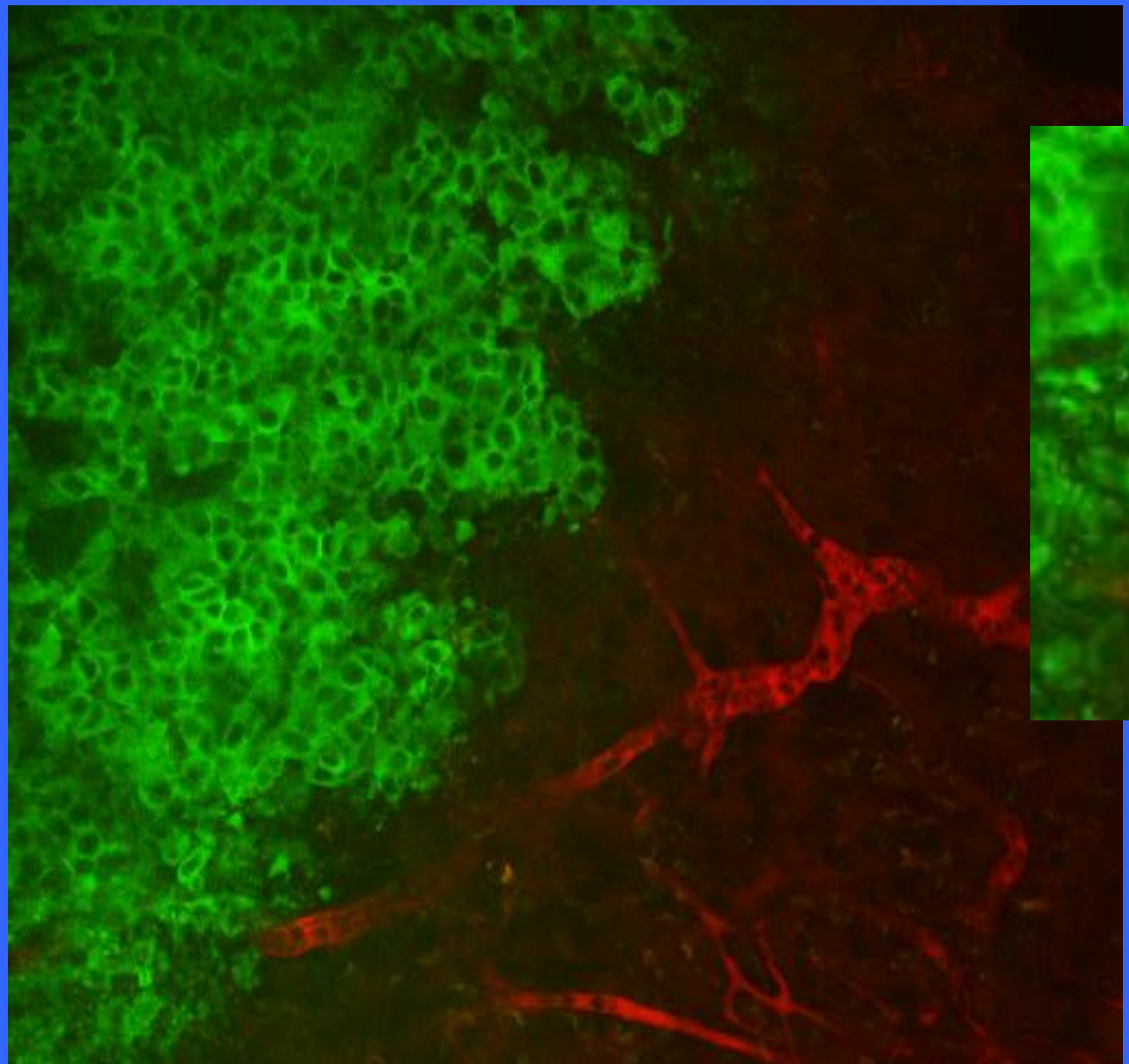
1. Fast (Locomotory)
2. Slow (Invasive)

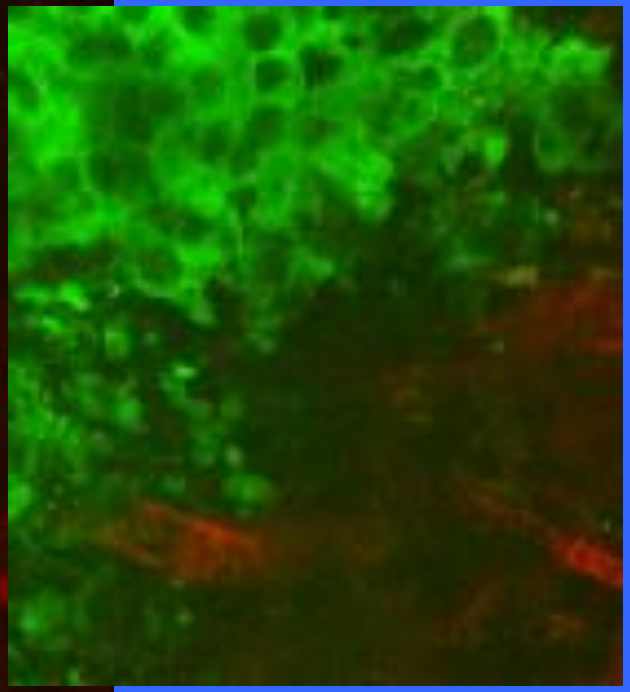




Bojana Gligorijevic et al 2014 Plos Biol

FAST phenotype = tumor cells (Green) migrate by streaming toward blood vessels (red)

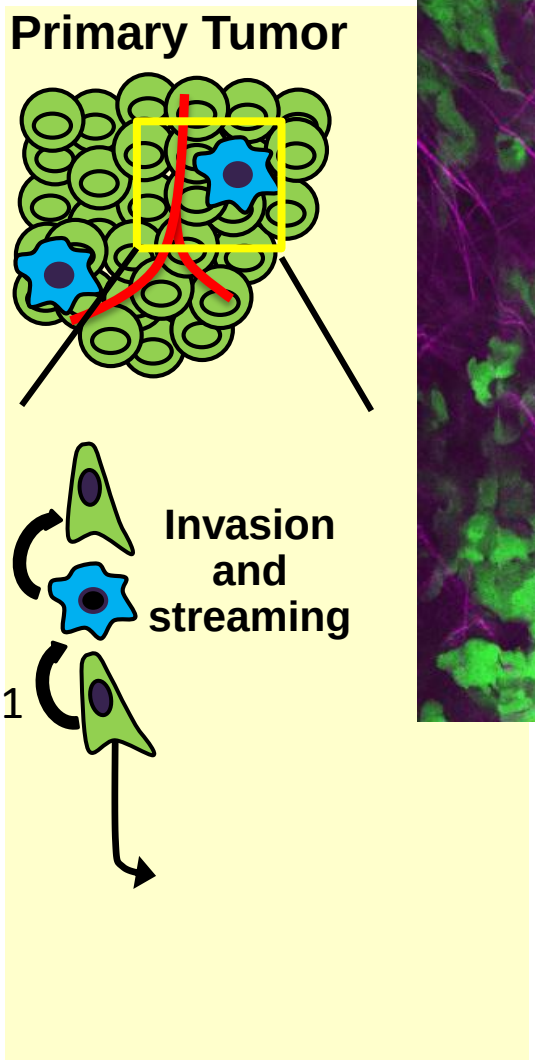


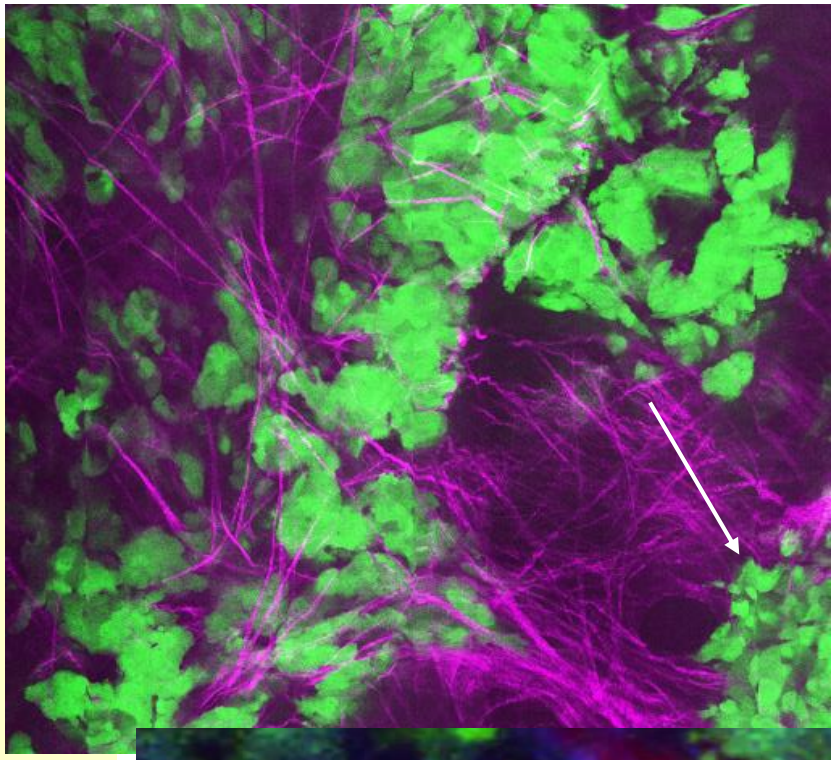


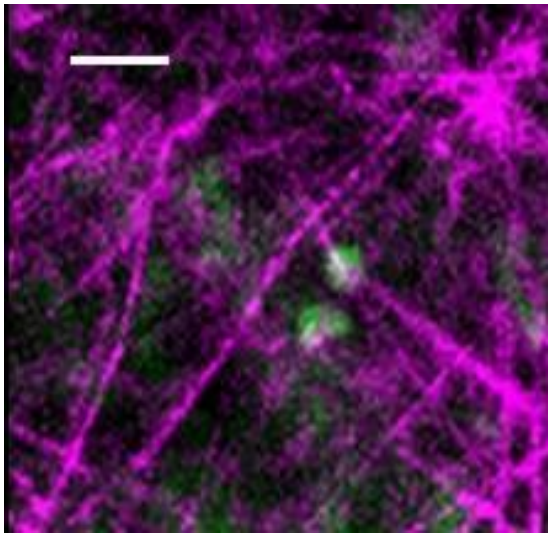
Roussos et al 2011
Nature Rev Cancer

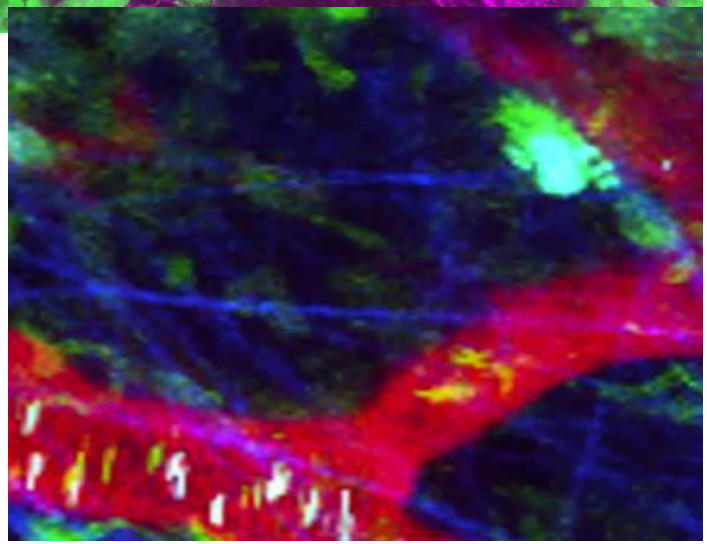
Patsialou et al 2013

Tumor cell – macrophage Streaming









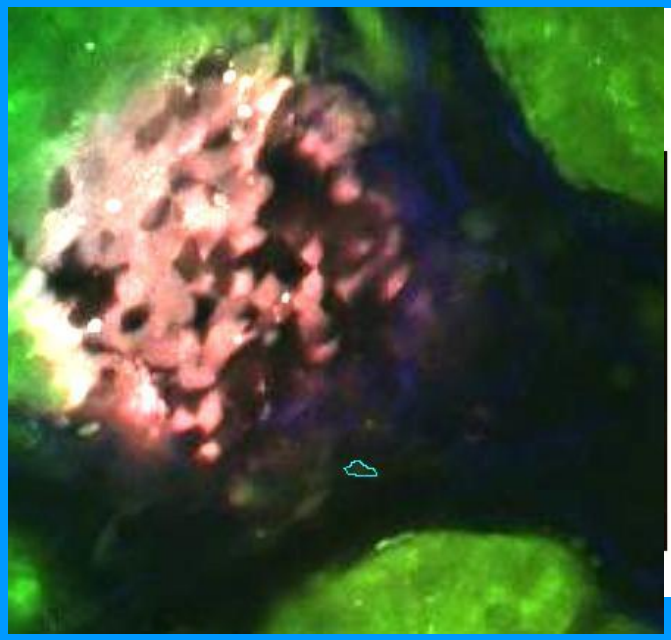
Found

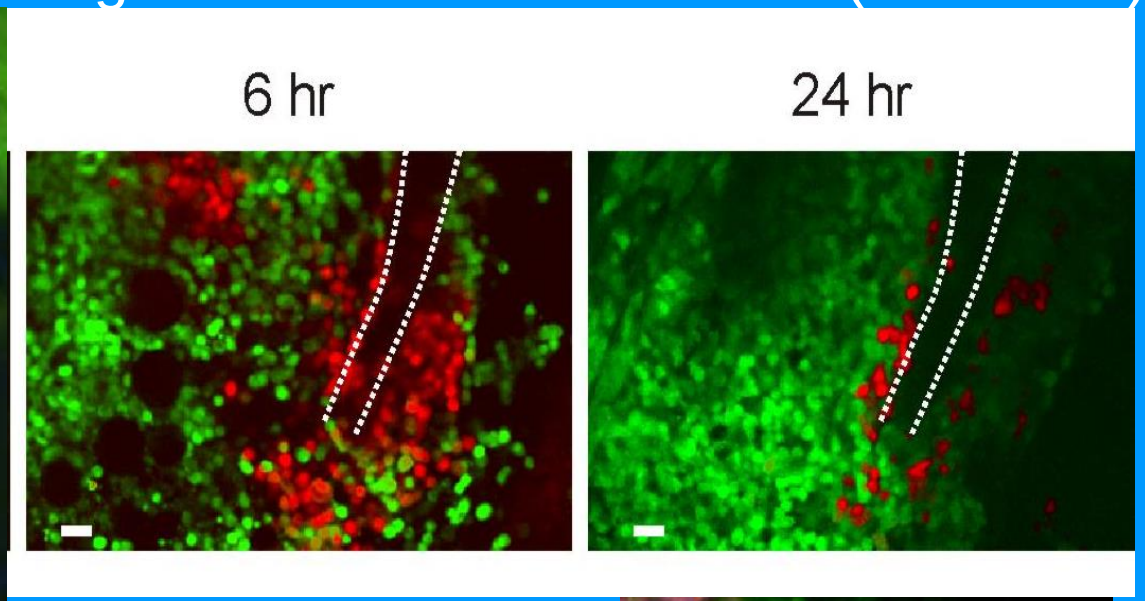
1. FAST phenotype involves pairing and streaming* of tumor cells with macrophages on linear paths directed by 2 um diameter fibers toward blood vessels.

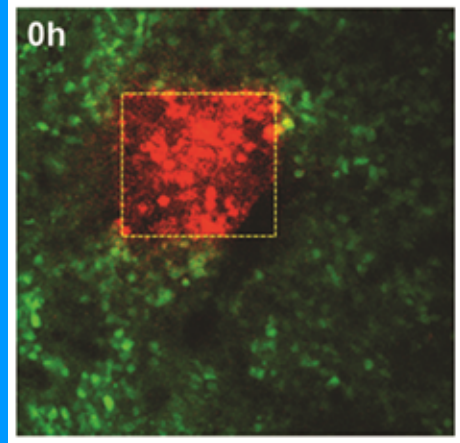
- Streaming = cells not touching but exhibiting unidirectional migration following of a linear path in single file.

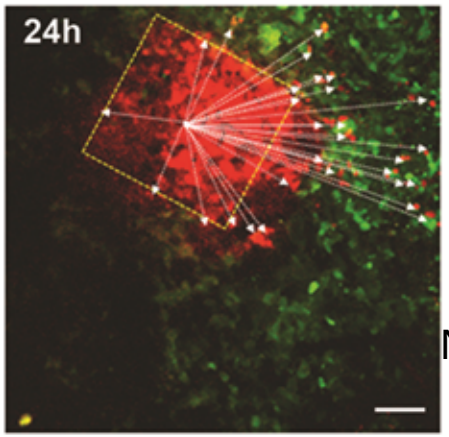
Question: What is the molecular mechanism driving blood vessel directed streaming?

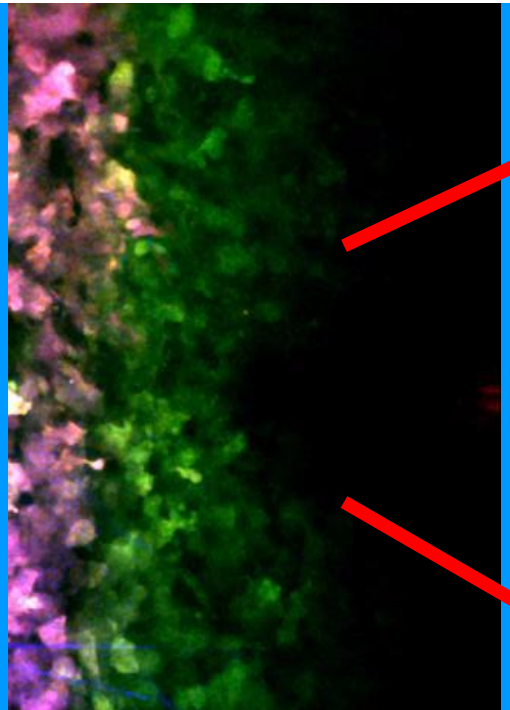
Blood vessel signals responsible for attraction of fast tumor cells were discovered using an artificial blood vessel (NANIVID)









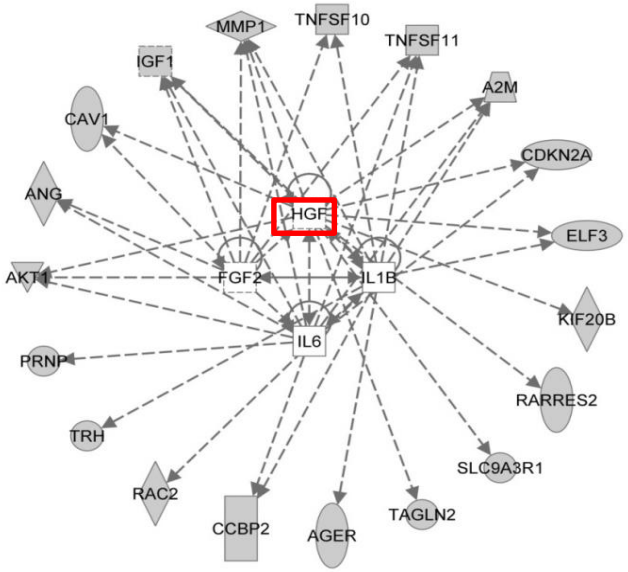


Wyckoff et al 2004, 2007 Cancer Research
Patsialou et al 2009; 2012; 2013
Raja et al 2010 Integrative Biol

Human Invasion Signature

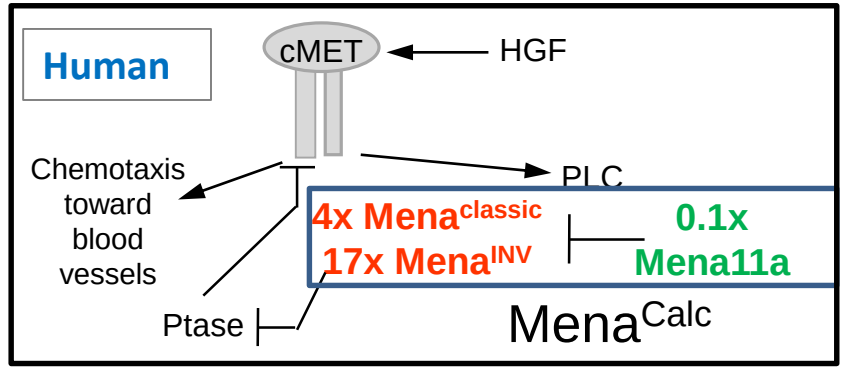
GENE SET	ES	NES	NOM p-val	FDR q-val
Ramalho_Stemness_Up.grp	0.448	1.629	0.000	0.023
Wong_Embryonic_Stem_Cell_Core.grp	0.468	1.643	0.000	0.032
Bhattacharya_Emryonic_Stem_Cell.grp	0.483	1.436	0.039	0.082
BenPorath_ES_1.grp	0.302	1.220	0.162	0.186

•Gene Set Enrichment Analysis of the Human Invasion Signature (HIS) found significant enrichment of the HIS for gene signatures of embryonic stem cells.



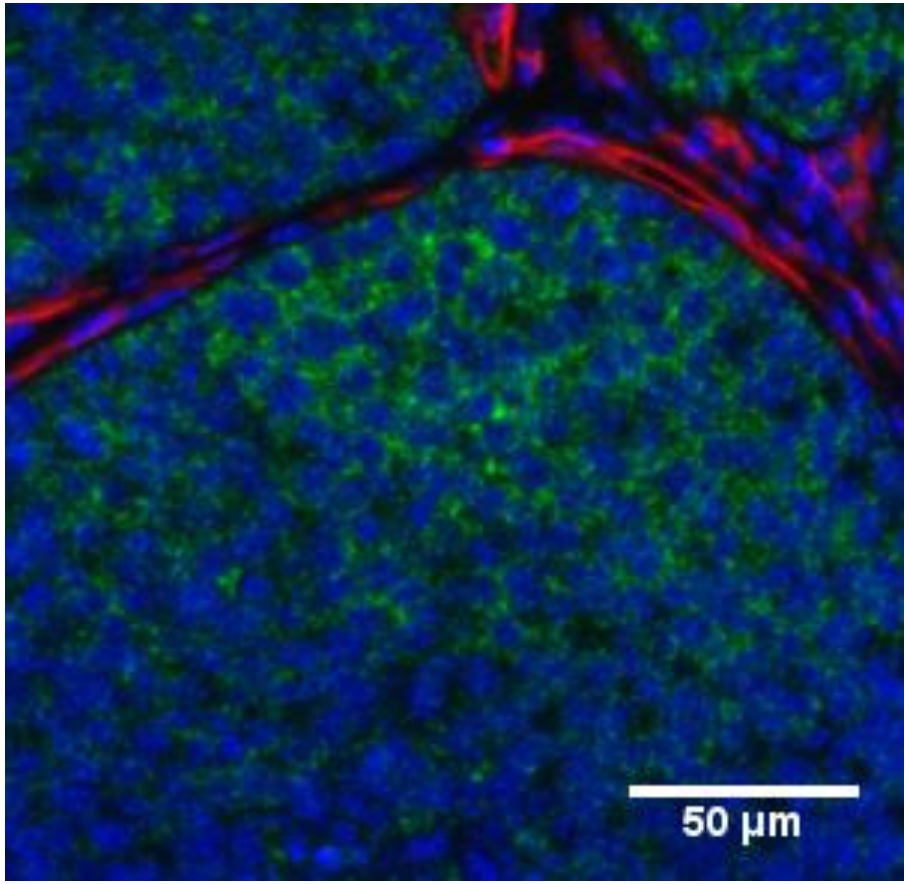
HGF signaling is the dominant signaling network in the FAST phenotype. HGF is synthesized and secreted by endothelial cells.

Tumor cells exhibiting the HIS express the Mena isoform pattern MenaCalc which increases sensitivity of RTK signaling and chemotaxis.

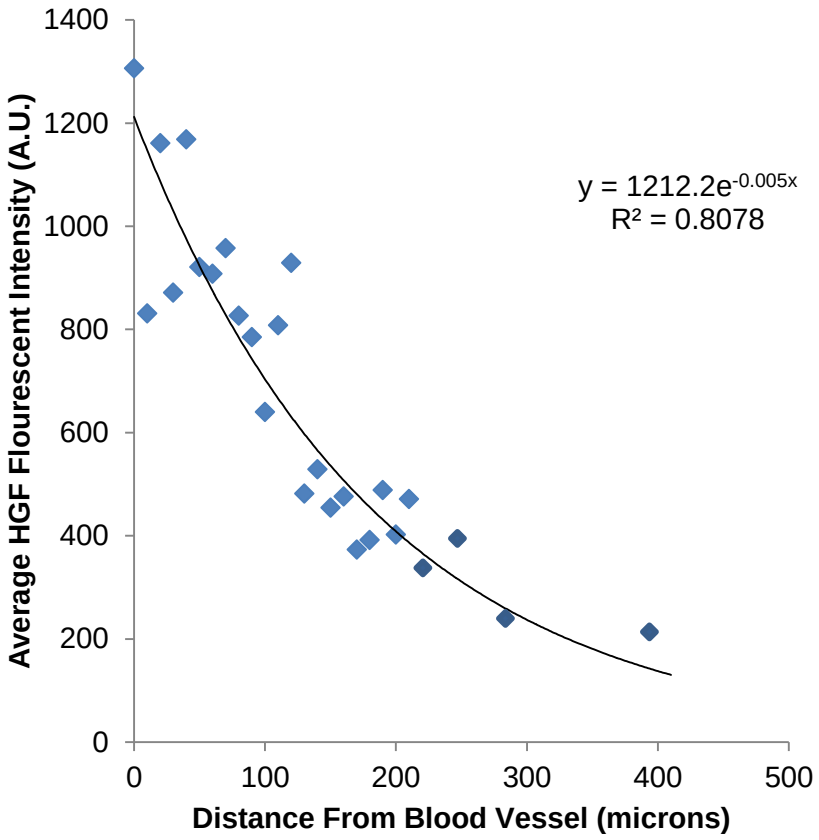


HGF gradient extends out from blood vessels for hundreds of microns

A)



B)

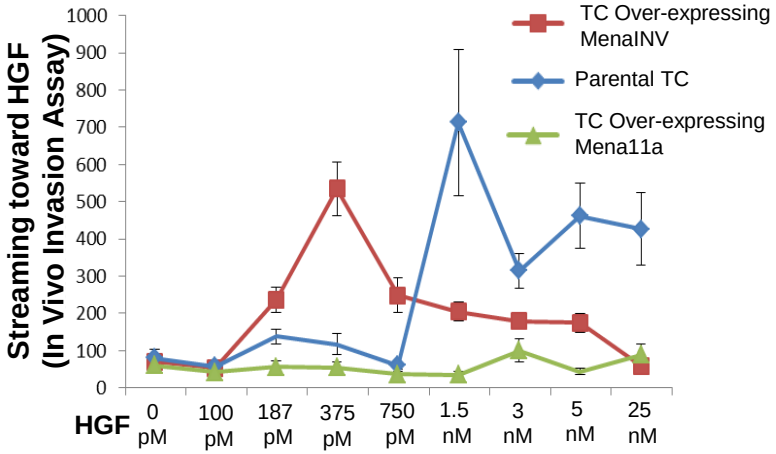


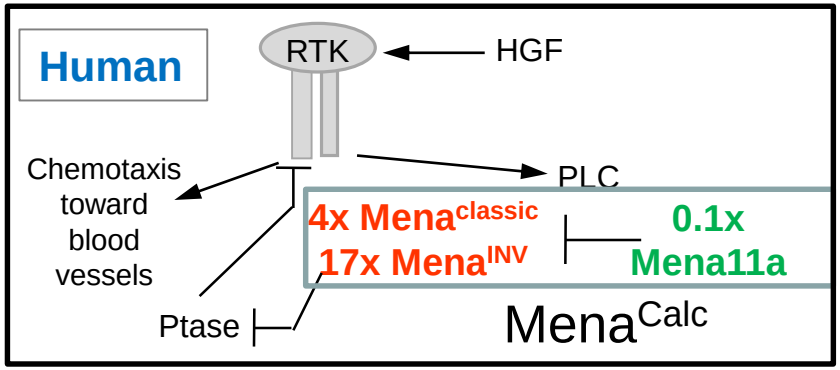
Green =HGF

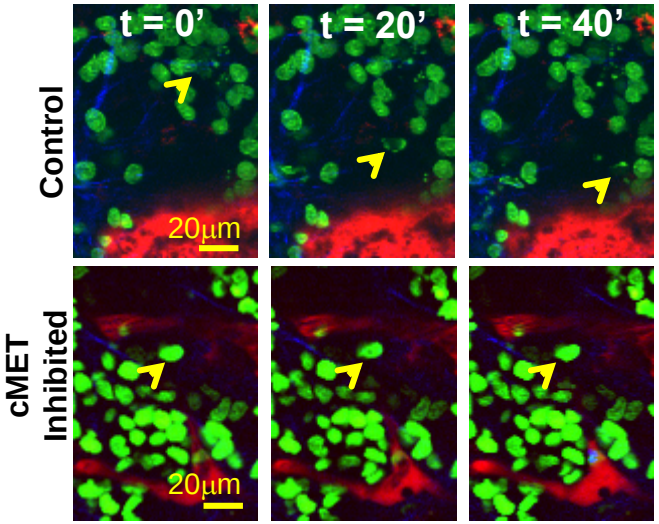
Red = CD31 Blood vessel

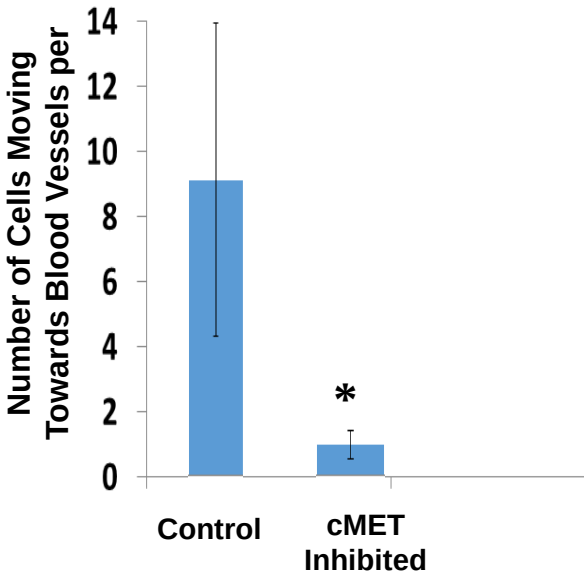
Blue = DAPI

Endothelium directed streaming in vivo is dominated by HGF/c-Met which is potentiated by the MenaCalc expression pattern.

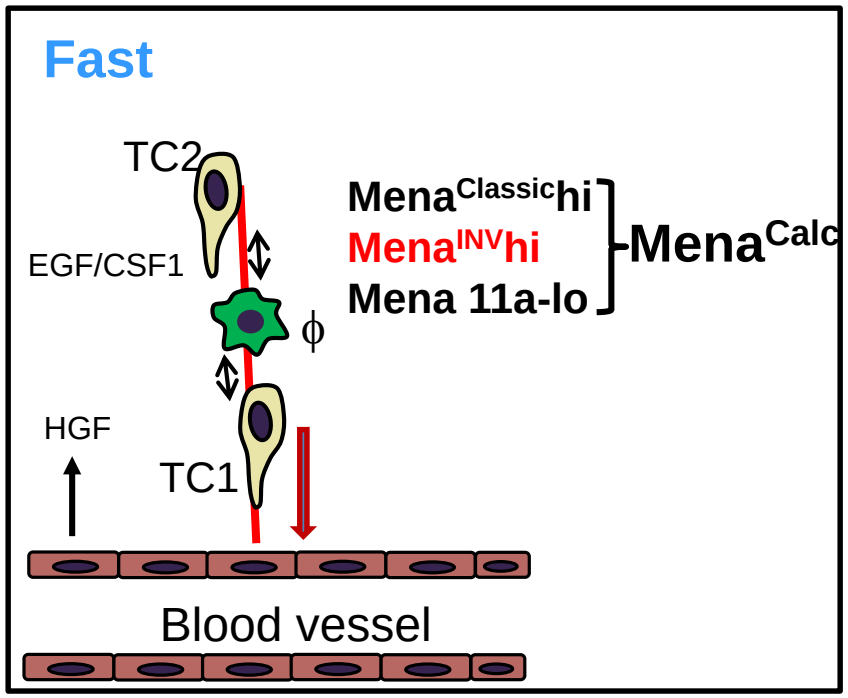


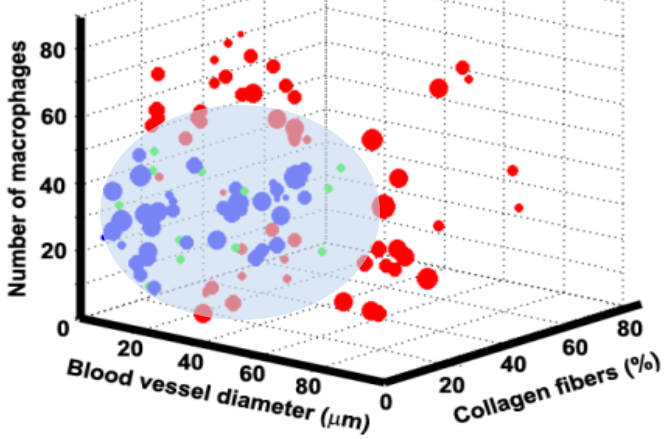




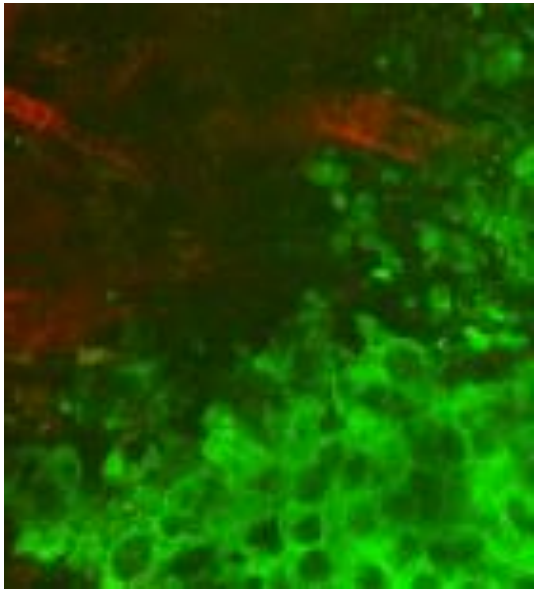


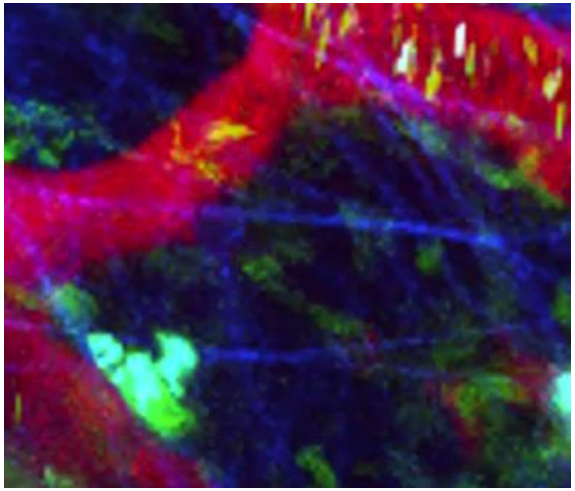
Hughes et al 2015
Leung et al 2016

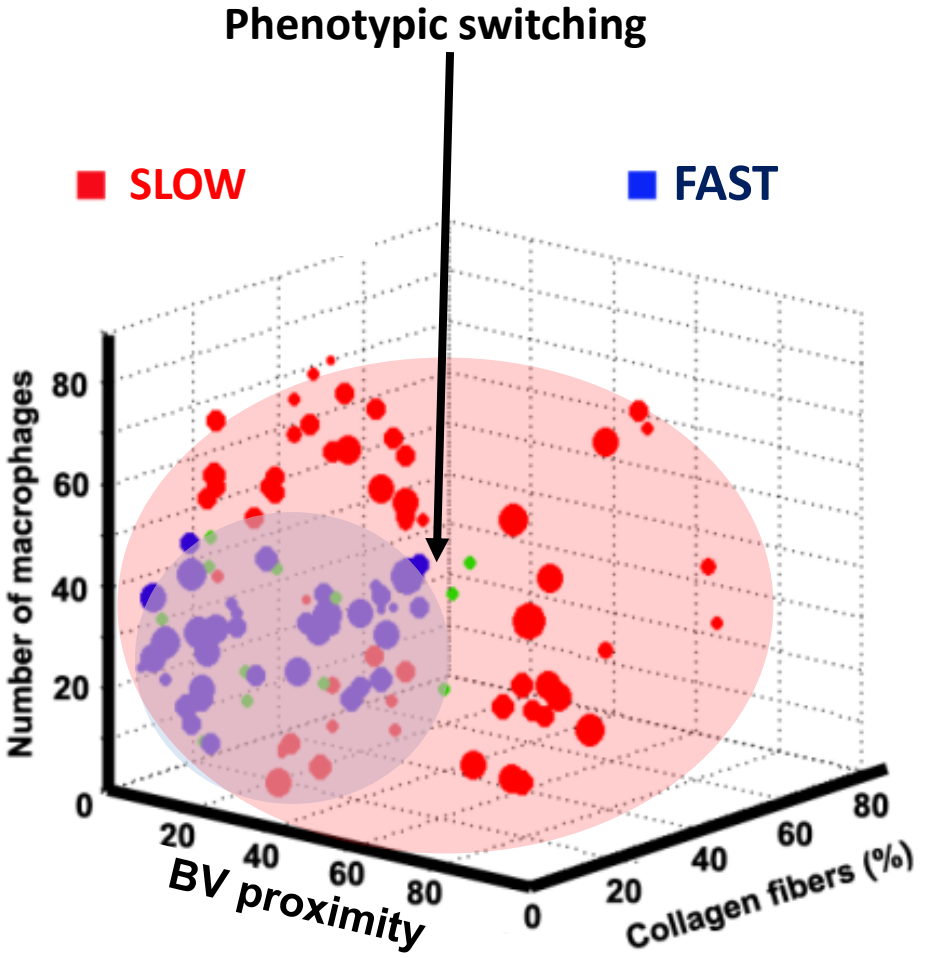

Fast
TC2
EGF/CSF1
ϕ
HGF
TC1
Blood vessel
Mena^Classic hi
Mena^INV hi
Mena 11a-lo
Mena^Calc


Number of macrophages
0
20
40
60
80
Blood vessel diameter (μm)
Collagen fibers (%)

High resolution multiphoton imaging identifies the location and kinetics of phenotypic switching between the fast and slow phenotypes



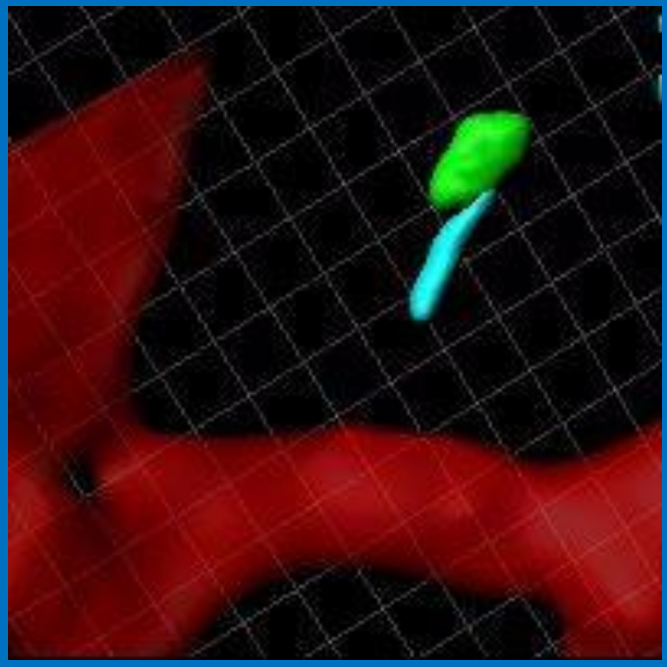


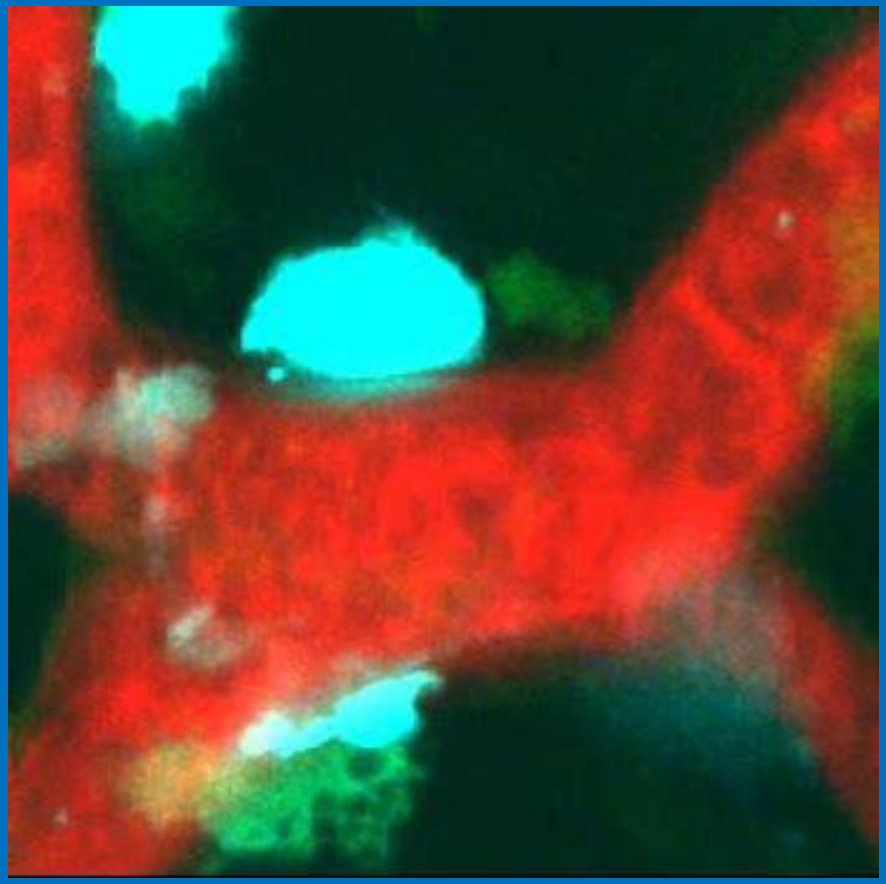


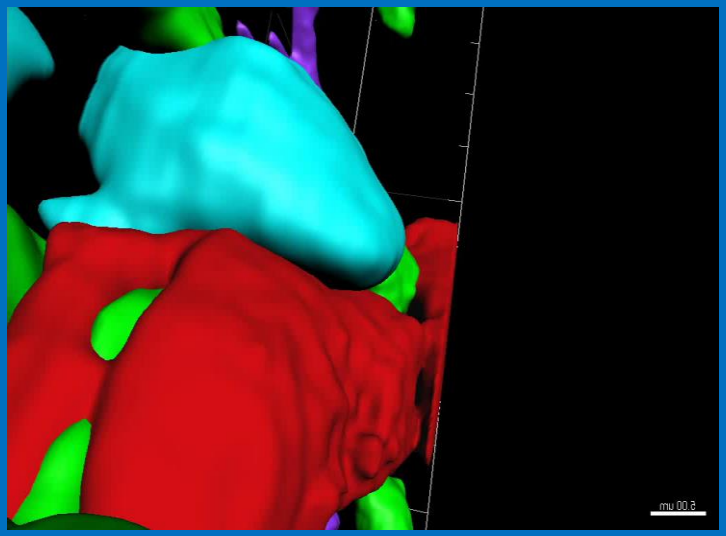
Bojana Gligorijevic et al 2014 Plos Biol

When macrophage and tumor cell pairs arrive at the blood vessel the phenotype switches from fast to slow. This leads to the assembly of TMEM, the “tumor microenvironment of metastasis.”

TMEM is the doorway for tumor cell intravasation.



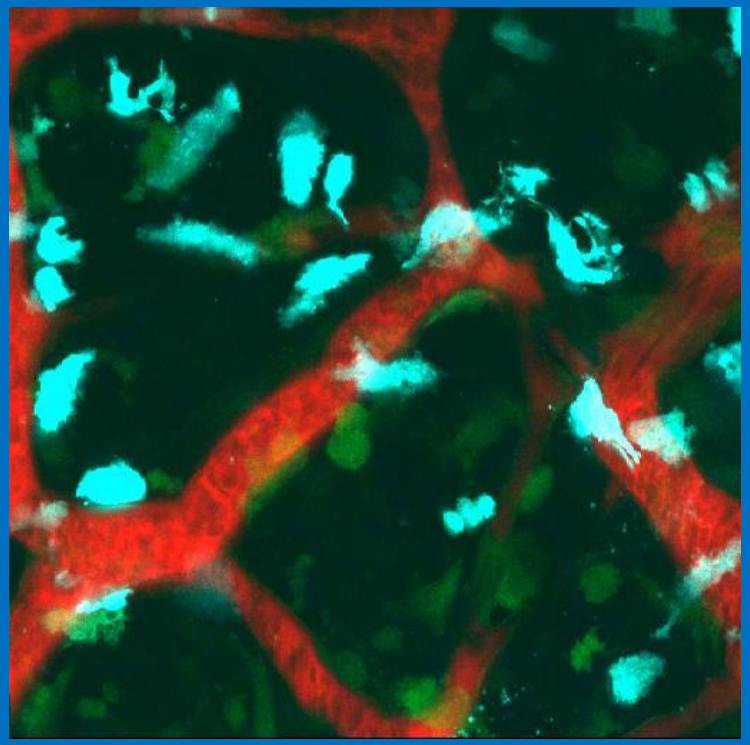


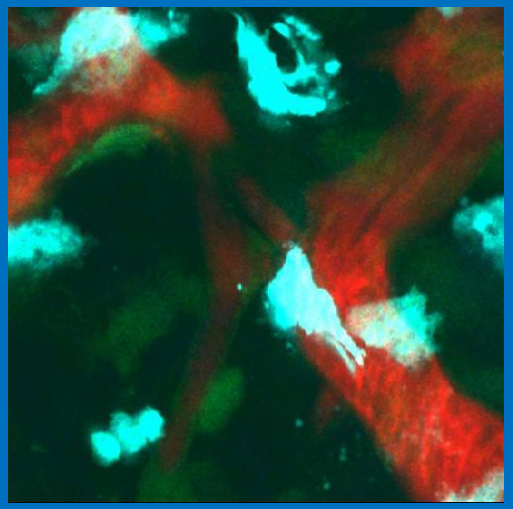


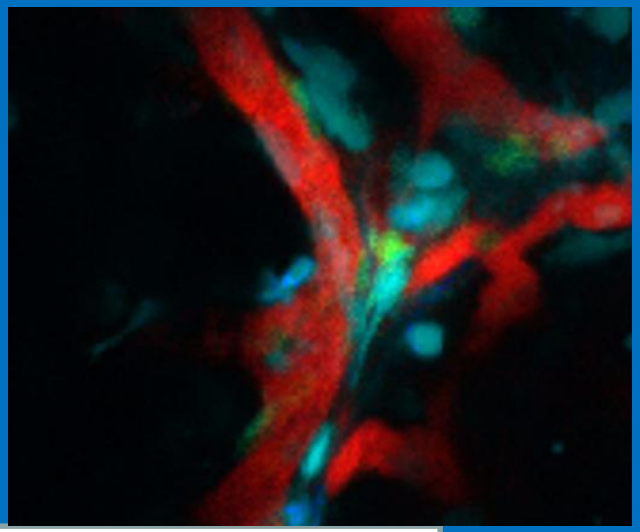
TMEM is composed of a MenaCalc tumor cell, Tie2+ macrophage and endothelial cell in direct, stable contact.

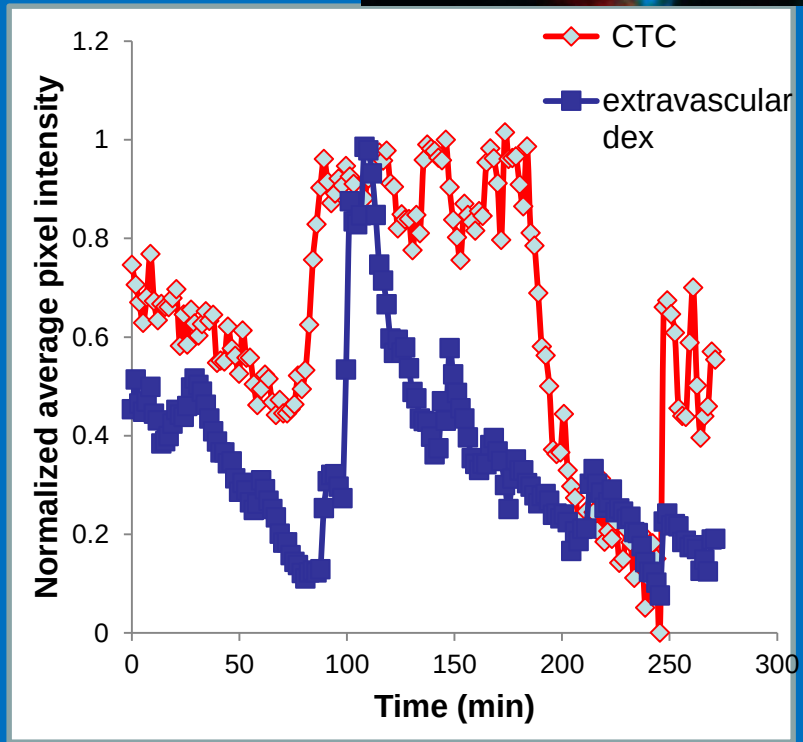
How does TMEM work?

Vascular permeability (bursting) and tumor cell intravasation occur at TMEM

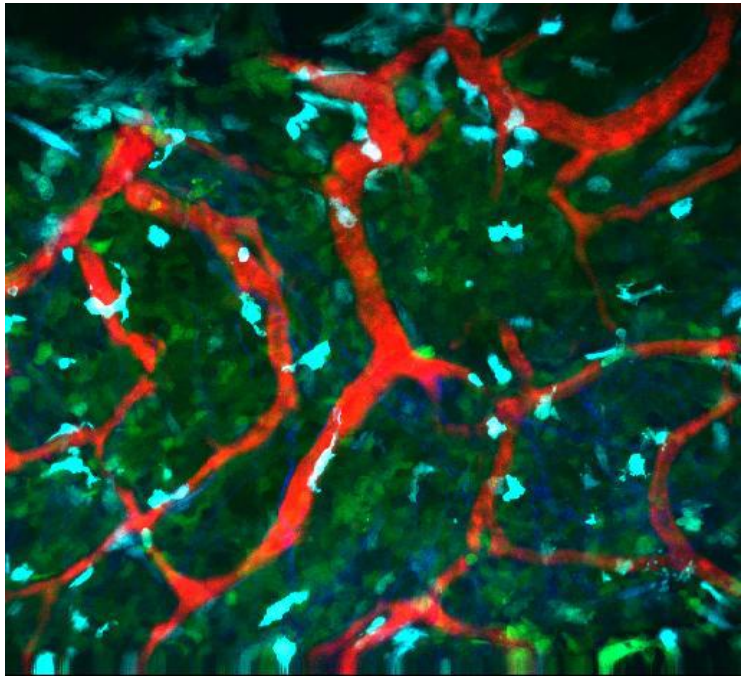


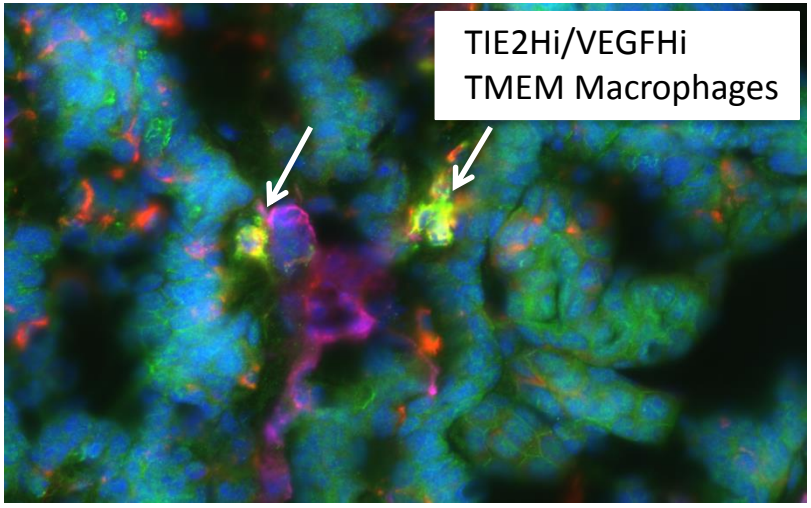


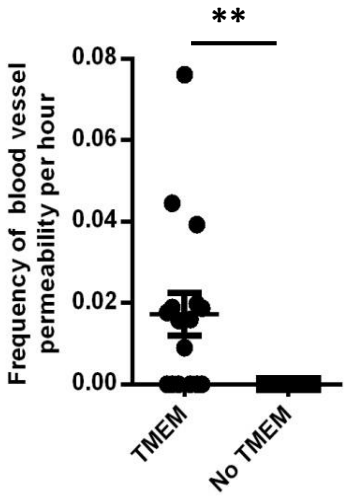


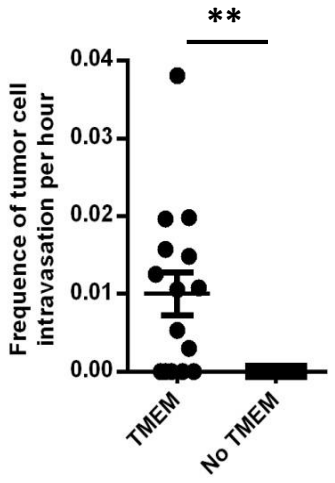


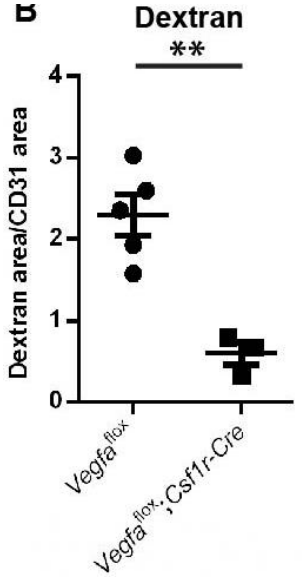
Tumor vascular leakiness and tumor cell intravasation occur only at TMEM and require TMEM macrophage VEGF

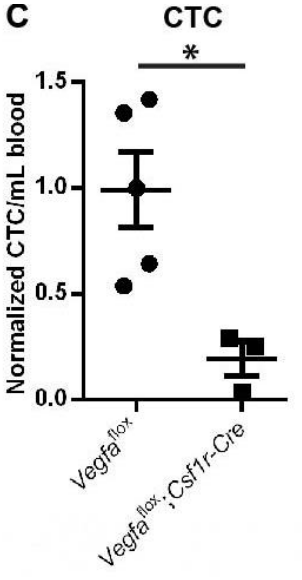






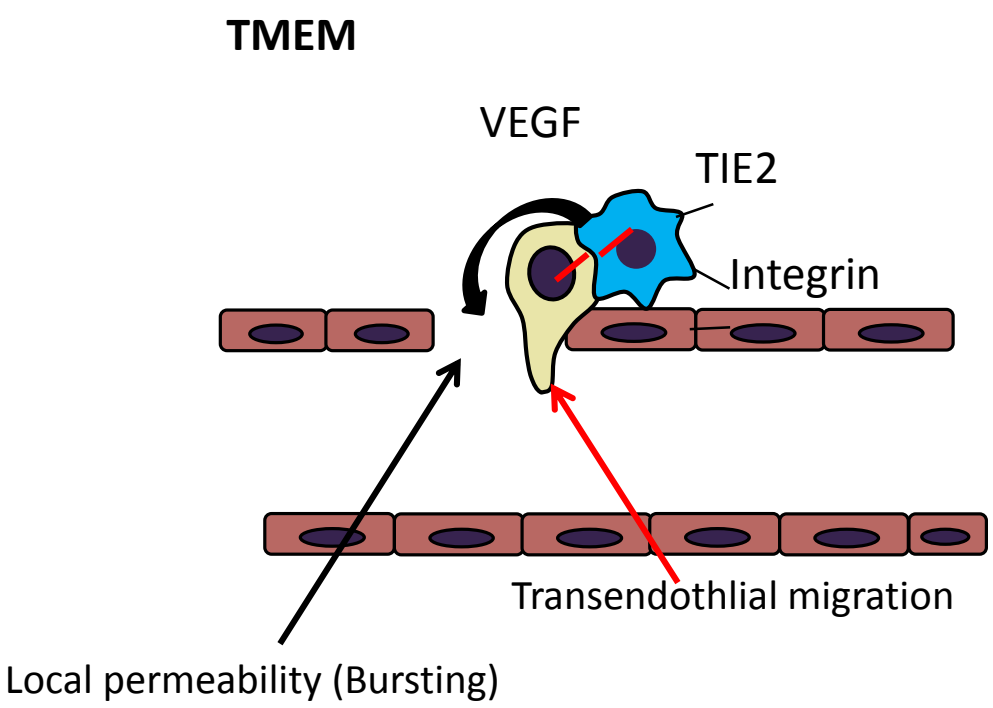




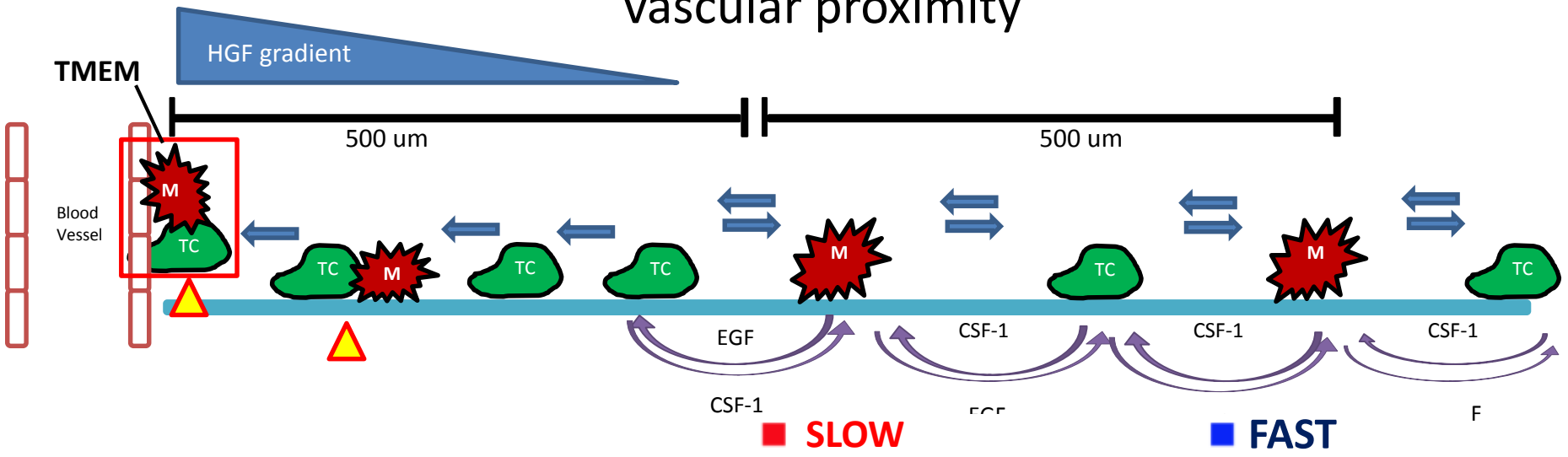


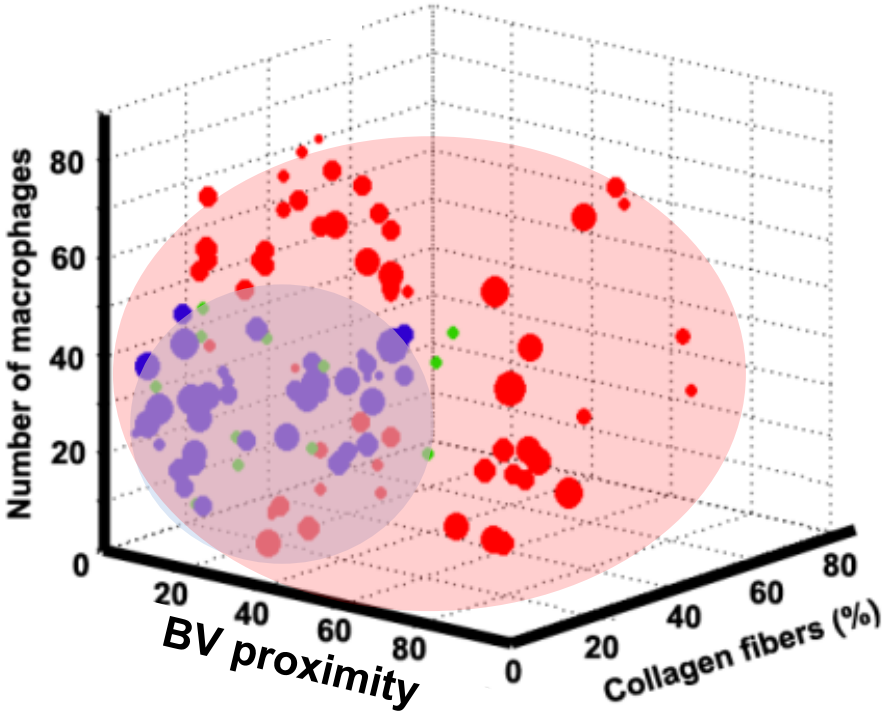
Harney et al 2015
Cancer Discovery

TMEM function



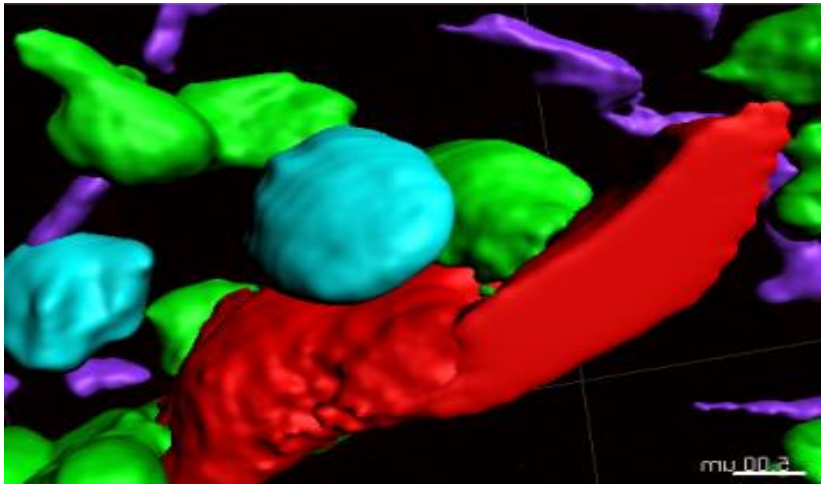
Frequency of macrophage – tumor cell collisions increase with vascular proximity



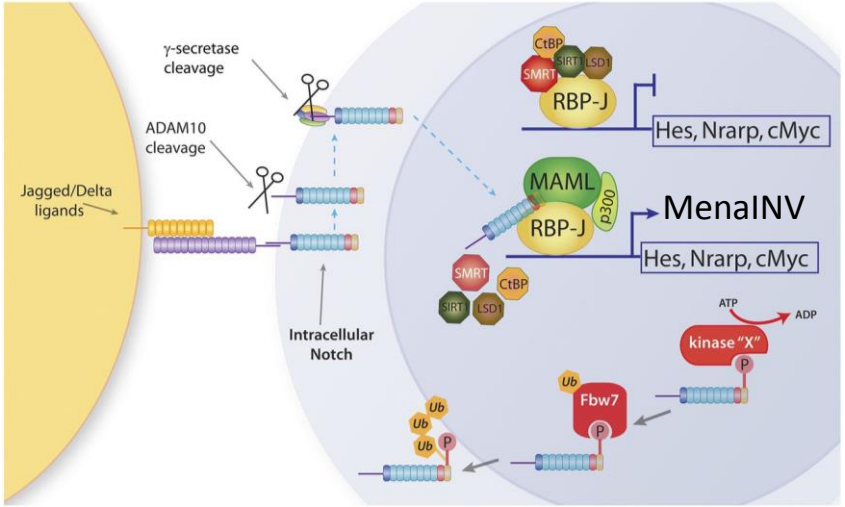


Leung et al 2016 ONC

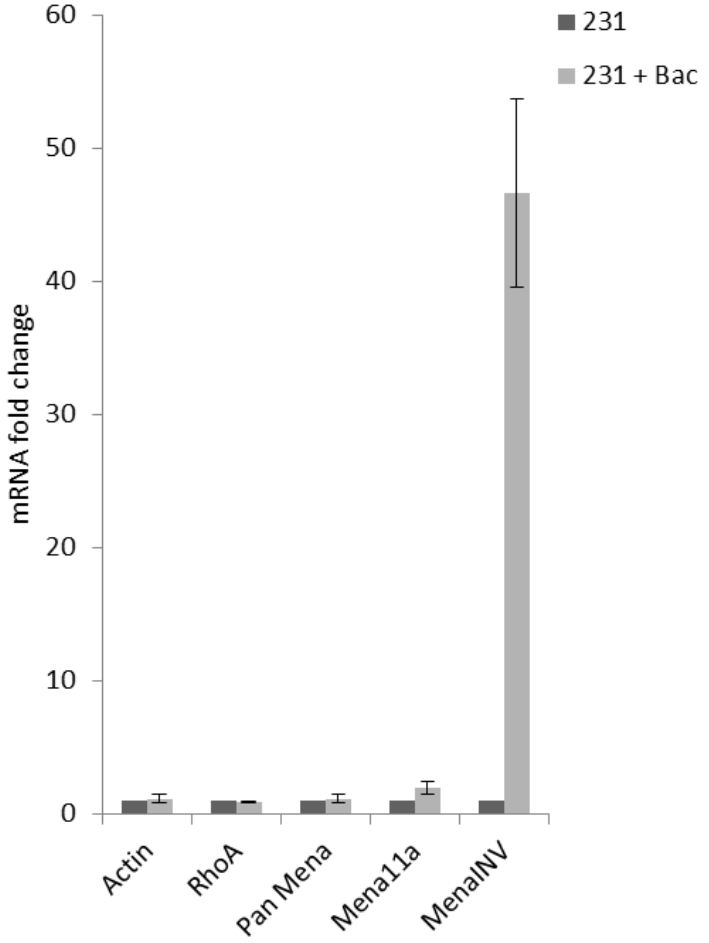
Macrophage- tumor cell contact induces MenaINV expression in response to Notch signaling



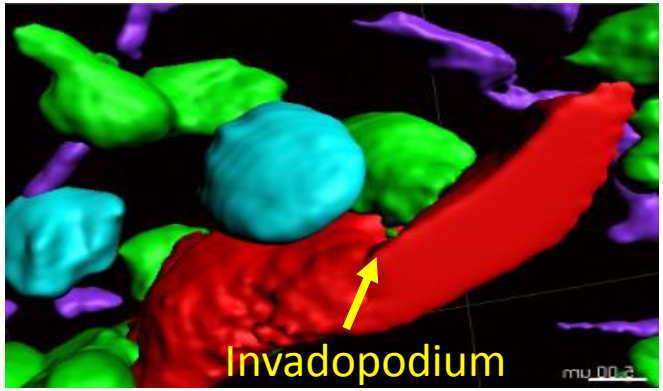
During streaming and at TMEM macrophages and tumor cells come in direct contact.



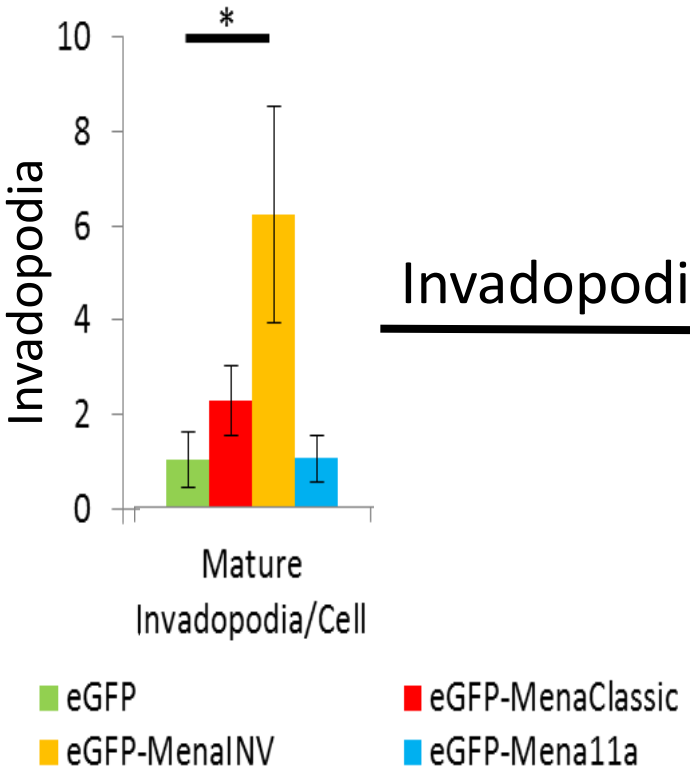
Macrophages express Jagged/Delta ligands and tumor cells express their Notch1 receptor.



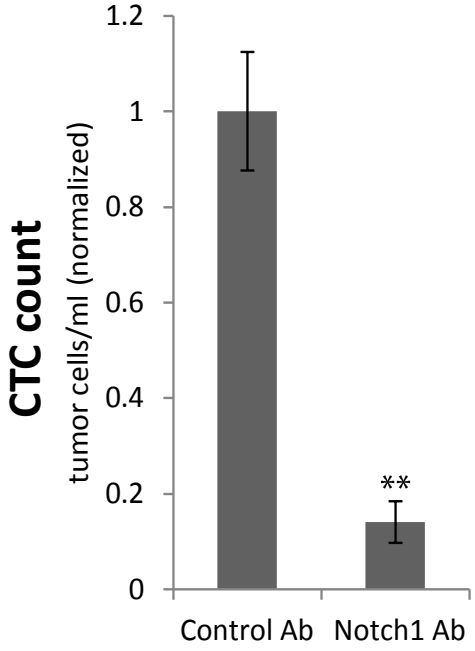
Max Weidmann et al 2016
Jeanine Pignatelli et al 2016

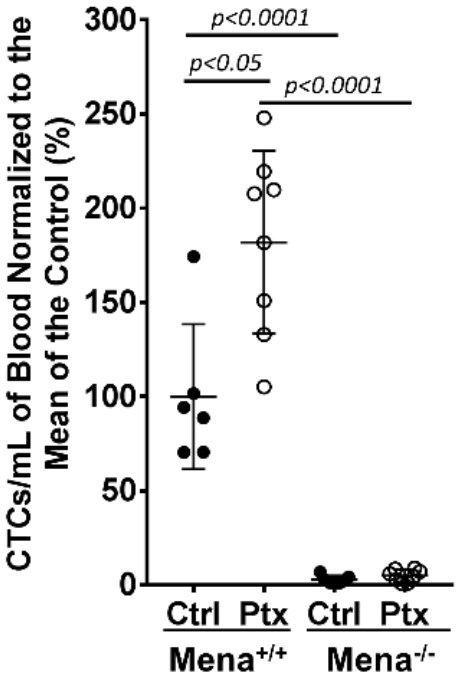


Mena^{INV} expression drives invadopodium formation leading to transendothelial migration by tumor cells at sites of vascular leakiness induced by the TMEM macrophage

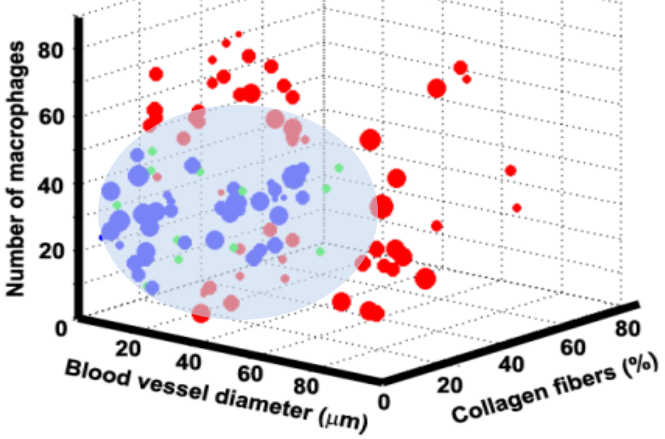


Invadopodia





Max Weidmann et al 2016
Jeanine Pignatelli et al 2014; 2016



Fast

TC2

EGF/CSF1

ϕ

HGF

TC1

Blood vessel

Mena^{Classic}hi
Mena^{INV}hi
Mena 11a-lo
} Mena^{Calc}

Slow

TMEM Φ

VEGF

Tie2

Ang2

TMEM TC

TMEM EC

TMEM associated
vessel permeability

Combined

TC3

EGF/CSF1

ϕ

EGF/CSF1

HGF

TC2

VEGF

Tie2

Ang2

TC1

TMEM associated
vessel permeability

Digital Pathology/Predictive Oncology studies summary: TMEM, Mena^{Calc} as prognostic markers in the clinic

Breast cancer: Successful prediction of risk of distant metastasis

TMEM

Robinson et al 2009 Clinical Can. Res.	30 case-control pairs (60 cases)
Rohan et al 2014 JNCI	259 case-control pairs (518 cases)
Sparano et al 2017 Nature-JBC In Press	600 cases (E2197 cohort)

Mena^{Calc}

Agarwal et al 2012 Breast Cancer Res.	797 cases
Forse et al 2015 BMC Cancer	406 cases

Machine vision scored TMEM and Mena^{Calc} markers predict distant recurrence (dissemination) but not tumor growth (Karagiannis et al 2016).

Digital Pathology/Predictive Oncology summary: TMEM, Mena^{Calc} as prognostic markers in the clinic

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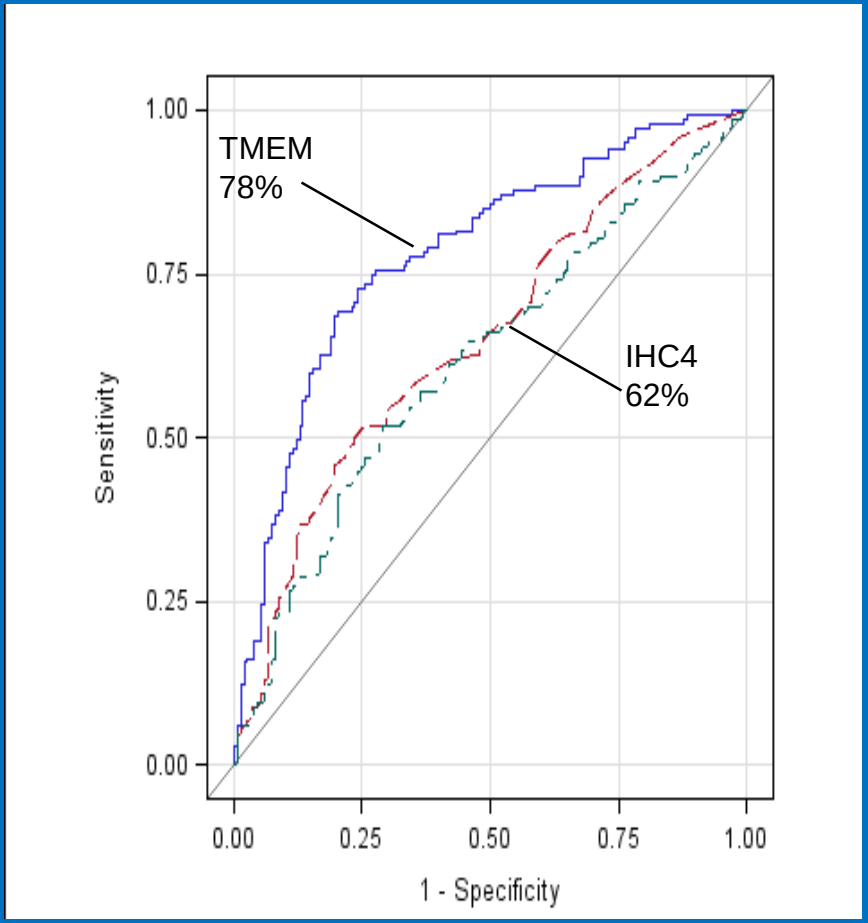
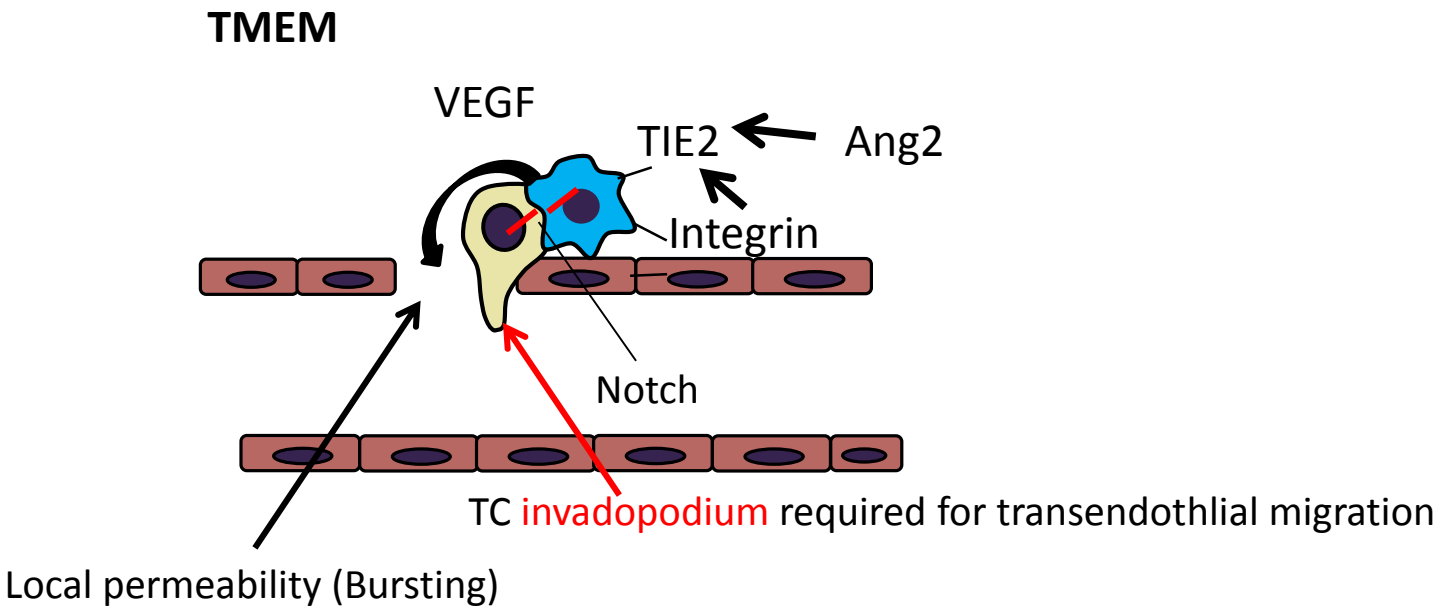
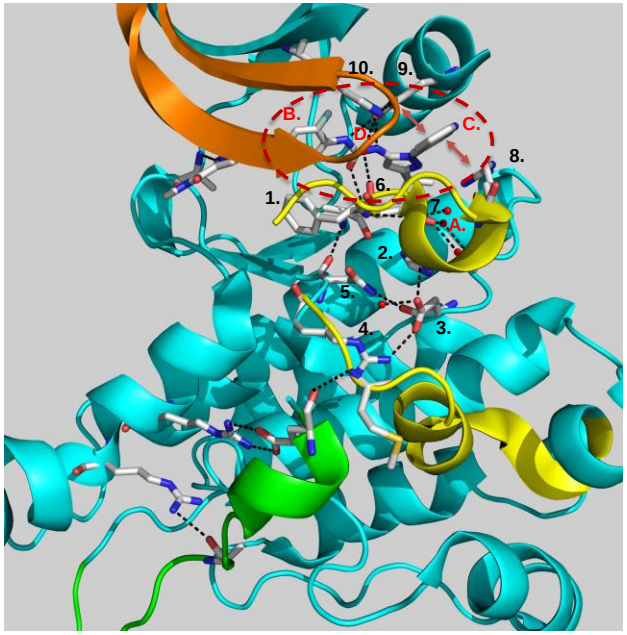


How can we inhibit TMEM function?

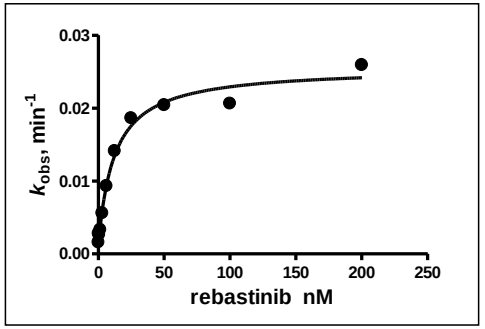
The TIE2 receptor that regulates TMEM macrophage function has multiple inputs.



Rebastinib is a TIE2 specific inhibitor developed in collaboration with Deciphera Pharmaceuticals



- Rebastinib occupancy of Switch Pocket (dashed red oval)
- Induced DFG-out conformation (inactive conformation of switch (yellow)
  1. **Phe983** **A. t-Butyl** **B. F-Phenyl**
- H-Bond collapse of catalytic residues
  2. His962 **3. Asp964** **4. Arg968**
  5. Asn969 **6. Asp982** **7. Gly984**
- Key region of electrostatic stacking
  8. Arg987 ⟷ **C. Quinoline** ⟷ **9. Glu872**
- H-bond to conserved switch salt bridge
  D. urea **9. Glu872** **10. Lys855**
- Network of 30 Hydrogen Bonds
- Retention of C-terminal substrate binding inhibitory motif (green)
- 6 Regions of Hydrophobic Collapse (Figure S1)

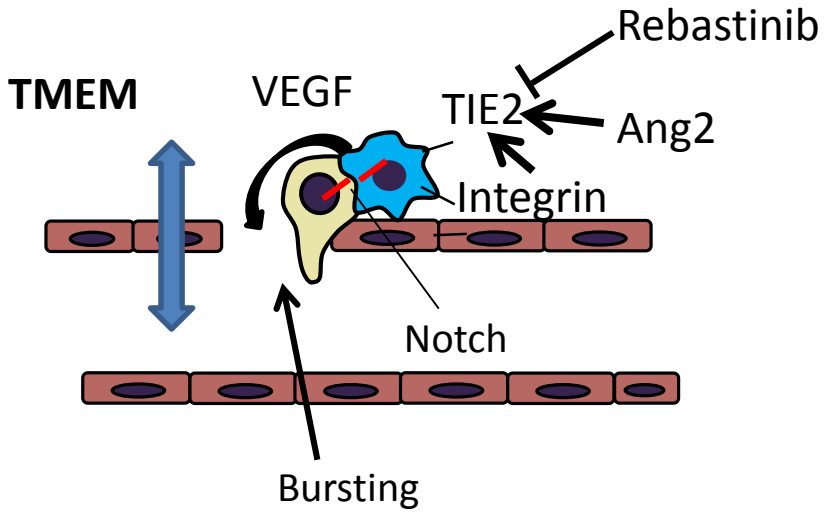


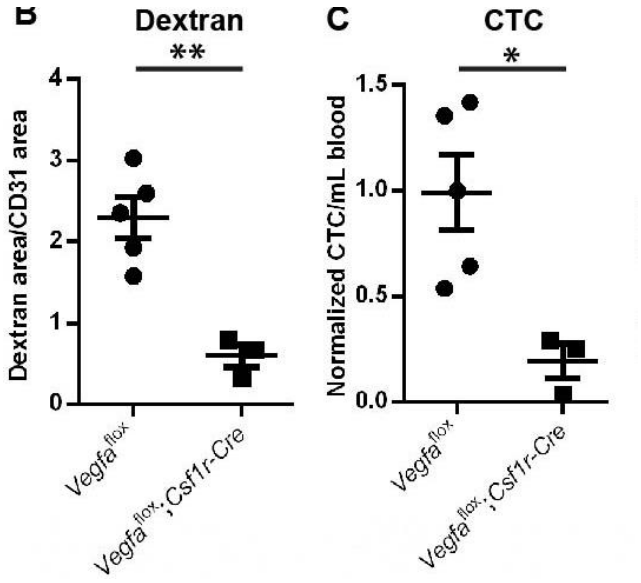
$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} E{\cdot}I \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E{\cdot}I^*$$

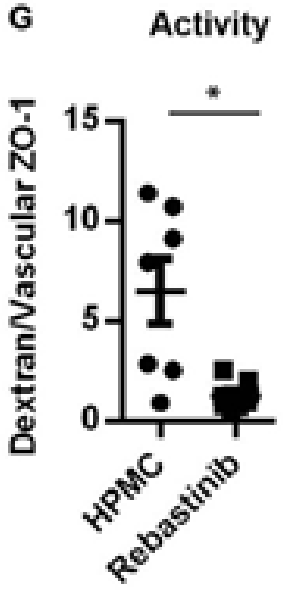
rebastinib/Tie2	min^{-1}	$t_{1/2}$, min
$K_4 = k_{off}$ (Off rate)	0.0012	577
$K_3 = k_{on}$	0.024	28
K_I	12 nM	
K_d*	0.57 nM	

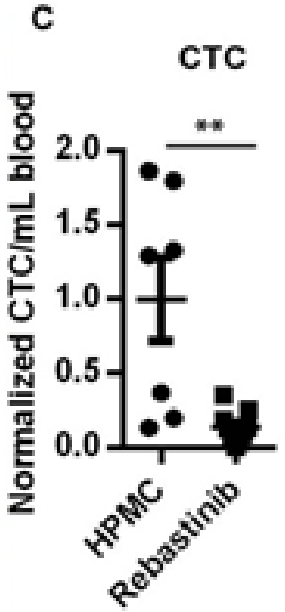
Harney, Karagiannis et al 2017
Karagiannis et al 2017

Rebastinib inhibits TMEM function and metastasis to levels seen when TMEM macrophage VEGF is knocked out

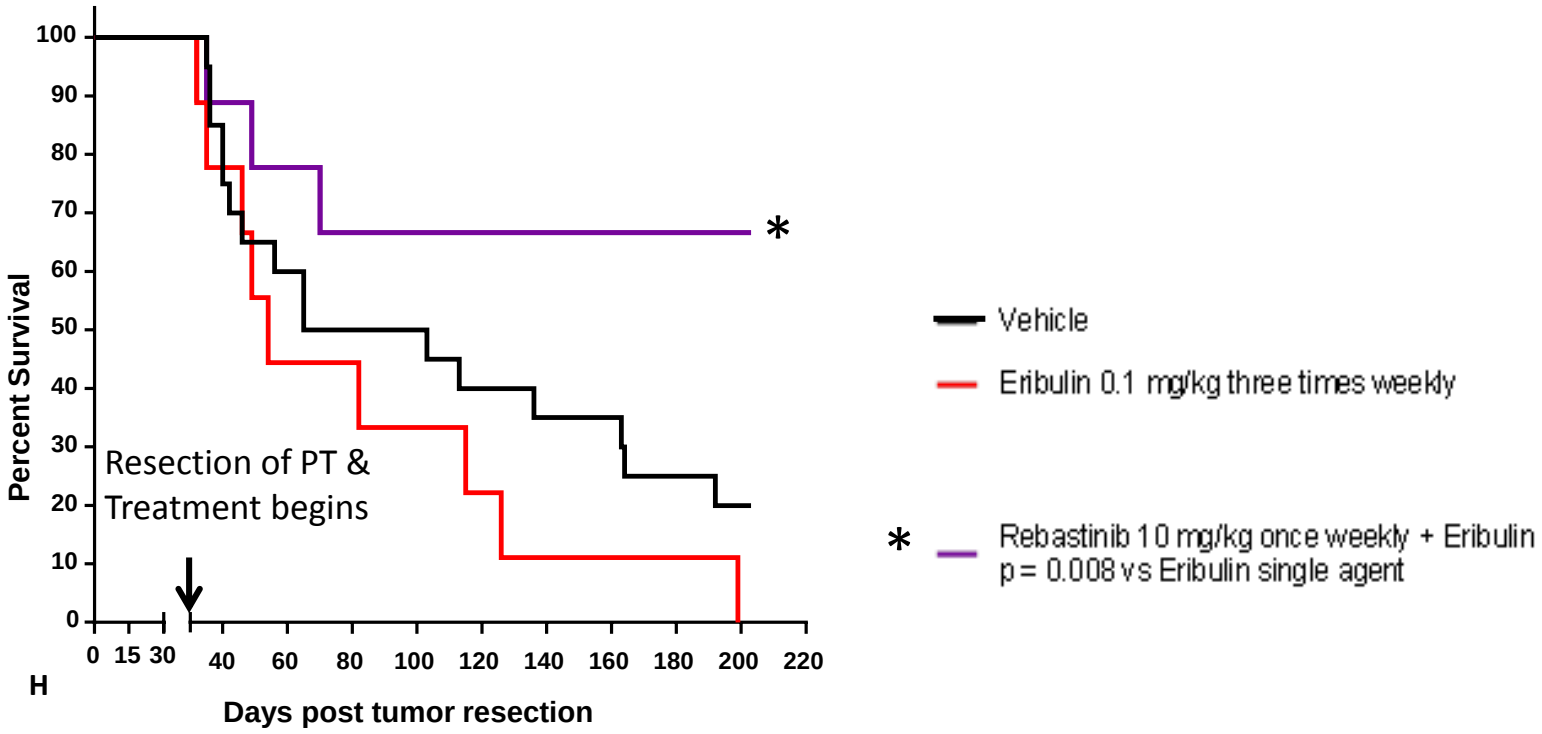








MOUSE: Rebastinib can be used in combined cytotoxic chemotherapy to increase survival in mice with very aggressive PyMT mammary tumors.



Found

1. **Tumor cells are attracted to blood vessels in response to HGF.**

2. **TMEM are sites where fast to slow phenotypic switching occurs.**

3. **TMEM are the <u>only</u> sites on blood vessels where tumor cells intravasate.**

4. **TMEM has two functional steps: 1) macrophage-mediated induction of $Mena^{INV}$ expression in tumor cells endowing transendothelial migration activity; 2) local release of macrophage VEGF causing local vascular leakiness allowing trans-endothelial migration.**

5. **Inhibition of Tie2 on TMEM macrophages with rebastinib inhibits TMEM function.**

6. **Rebastinib inhibits TMEM-dependent tumor cell dissemination in mice.**

Conclusions

1. Chemotherapy induces macrophage recruitment and tumor cell dissemination in a TMEM and Mena^{INV}-dependent manner.

2. Inhibition of Tie2 on TMEM macrophages with rebastinib inhibits intravasation at TMEM.

3. Rebastinib inhibits tumor cell dissemination in mice and human breast cancer patients causing inhibition of metastasis in mice and potentially in human patients.

4. **The combination of classical chemotherapy with inhibition of microenvironmental contributors to dissemination (e.g. TMEM sites) will be most effective approach for improving long term outcome.**

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