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

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

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

Bojana Gligorijevic et al 2014 Plos Biol
Systems analysis recognizes microenvironments associated with two tumor cell phenotypes:
1. Fast (Locomotory)
2. Slow (Invasive)
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

Primary Tumor

Invasion and streaming

EGF

CSF1
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
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

Green = HGF
Red = CD31 Blood vessel
Blue = DAPI

\[ y = 1212.2e^{-0.005x} \]
\[ R^2 = 0.8078 \]
Endothelium directed streaming in vivo is dominated by HGF/c-Met which is potentiated by the MenaCalc expression pattern.
TC2

EGF/CSF1

MenaClassichi
MenaINVhi
Mena11alo

TC1

HGF

Blood vessel

MenaCalc

Fast

Blood vessel diameter (μm)

Collagen fibers (%)

Number of macrophages
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.
TMEM function

TMEM

VEGF

TIE2

Integrin

Transendothelial migration

Local permeability (Bursting)
Frequency of macrophage – tumor cell collisions increase with vascular proximity.

Leung et al 2016 ONC
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.

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

Max Weidmann et al 2016
Jeanine Pignatelli et al 2016
Mena\textsuperscript{INV} expression drives invadopodium formation leading to transendothelial migration by tumor cells at sites of vascular leakiness induced by the TMEM macrophage

Max Weidmann et al 2016
Jeanine Pignatelli et al 2014; 2016
Digital Pathology/Predictive Oncology studies

**summary: TMEM, Mena\(^{\text{Calc}}\) as prognostic markers in the clinic**

**Breast cancer: Successful prediction of risk of distant metastasis**

<table>
<thead>
<tr>
<th><strong>TMEM</strong></th>
<th>Robinson et al 2009 Clinical Can. Res.</th>
<th>30 case-control pairs (60 cases)</th>
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<tr>
<td></td>
<td>Rohan et al 2014 JNCI</td>
<td>259 case-control pairs (518 cases)</td>
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<tr>
<td></td>
<td>Sparano et al 2017 Nature-JBC In Press</td>
<td>600 cases (E2197 cohort)</td>
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<th><strong>Mena(^{\text{Calc}})</strong></th>
<th>Agarwal et al 2012 Breast Cancer Res.</th>
<th>797 cases</th>
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<td></td>
<td>Forse et al 2015 BMC Cancer</td>
<td>406 cases</td>
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**Machine vision scored TMEM and Mena\(^{\text{Calc}}\) markers** predict distant recurrence (dissemination) but not tumor growth (Karagiannis et al 2016).
Digital Pathology/Predictive Oncology summary: TMEM, Mena$^{\text{Calc}}$ as prognostic markers in the clinic

Breast cancer: Successful prediction of risk of distant metastasis

**TMEM**
- Rohan et al 2014 JNCI
- Sparano et al 2017 Nature-JBC In Press

**Mena$^{\text{Calc}}$**
- Agarwal et al 2012 Breast Cancer Res.
- Forse et al 2015 BMC Cancer
How can we inhibit TMEM function?
The TIE2 receptor that regulates TMEM macrophage function has multiple inputs.

Local permeability (Bursting)

TC *invadopodium* required for transendothelial migration
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
- 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)

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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.
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 only sites on blood vessels where tumor cells intravasate.

4. TMEM has two functional steps: 1) macrophage-mediated induction of MenaINV 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.


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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