AWARD NUMBER: W81XWH-14-1-0296

TITLE: Microenvironments and Signaling Pathways Regulating Early Dissemination, Dormancy, and Metastasis

PRINCIPAL INVESTIGATOR: John Condeelis

CONTRACTING ORGANIZATION: Albert Einstein College of Medicine
Bronx, NY 10461-1931

REPORT DATE: September 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
September 2016	Annual	09/01/2015 - 8/31/2016

4. TITLE AND SUBTITLE

Microenvironments and Signaling Pathways Regulating Early Dissemination, Dormancy, and Metastasis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0296

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
John Condeelis, Julio Aguirre-Ghiso

E-Mail:john.condeelis@einstein.yu.edu

5d. PROJECT NUMBER
0010533520

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Albert Einstein College of Medicine
1300 Morris Park Ave LBBY
Bronx, NY 10461-1931

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

N/A

14. ABSTRACT

We devoted effort to both aims, to optimize methods and publish the first phase of efforts. **SA1** is to understand the intrinsic mechanisms of dissemination by early-progressed cancer cells and how the microenvironment in these primary sites named P-TMEM (**P**rimary **T**umor **M**icro**e**nvironment of **M**etastases) contribute to early dissemination. We now have new data suggesting that a signature of early dissemination markers consisting on $HER2^{HI}$/p-$ATF2^{LO}$/E-$cadh^{LO}$ could identify motile early-progressed tumor cells and predict for dissemination. We further show that mammary tissue macrophages and $HER2^{HI}$/p-$ATF2^{LO}$/E-$cadh^{LO}$ tumor cells cooperate to disseminate early. This further supports our hypothesis that even in early cancer lesions macrophages and tumor cells assemble with endothelial cells a TMEM structure. We have also made progress in generating a standardized triple staining that captures all these cell types in all tissues and we have used intravital imaging to document intravasation in early cancer lesions (see also partnering PI progress report). Our work revealed that the presence of intraepithelial macrophages in DCIS samples in mouse models or humans could accurately predict dissemination in 70% of the cases of patients with bone marrow DTCs (**D**isseminated **T**umor **C**ells). **SA2** focuses on elucidate how S-TMEM (**S**econdary **T**umor **M**icro**e**nvironment of **M**etastases) contributes to the dormancy phase of early DTCs. We found that early DTCs appear to undergo a p38 independent but TWIST-dependent dormancy in secondary organs revealing a new pathways for dormancy. Our new data also show that depletion of macrophages during early dissemination steps significantly reduces metastasis that develop into the late stages of progression, providing functional support for macrophages in P- and S-TMEM structures in metastasis development. This work is now part of two papers, one accepted for publication in the journal ***Nature*** and the other under review in ***Nature Communications***. Progress has also been made in the sorting protocol to isolate CFP+/HER2+ DTCs from lungs and CTCs in the MMTV-Neu-CFP model and we have also performed single cell RNA-seq which will allow us in the next year to identify the early and late gene signatures in DTCs we proposed in our grant.

15. SUBJECT TERMS

dormancy, early dissemination, EMT, macrophages, TMEM

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
"U"	"U"	"U"	"UU"	6	

Table of Contents

Page

PROGRESS REPORT- DoD grant Number: W81XWH-14-1-0296 (Partnering PI John Condeelis)

Title: Microenvironments and Signaling Pathways Regulating Early Dissemination, Dormancy, and Metastasis.

Original Specific Aim 1- To test how ErbB2high/P-p38low Mammary epithelial cells (MECs) assemble primary Tumor Microenvironment of Metastasis structures (P-TMEM) during early dissemination.

SA1.1. Objective: Use intravital imaging and MMTV-ErbB2-CFP, MMTV-PyMT-EGFP-c-fms-CFP transgenic mice and modulation of ErbB2 and p38 signaling to detect P-TMEM function during intravasation of "early" and "late" ErbB2+ MECs.

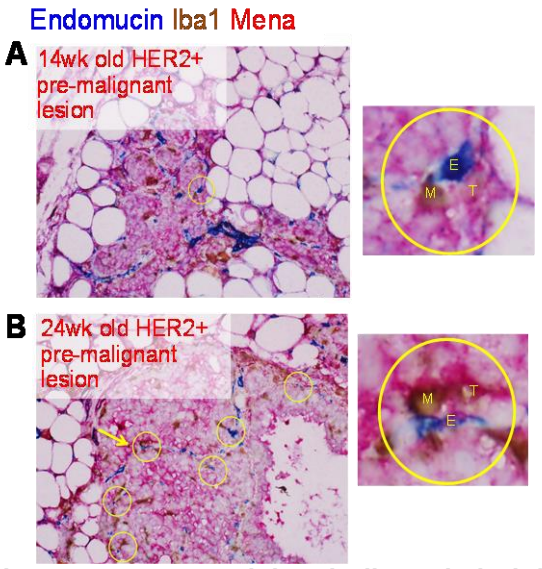

Figure 1. TMEM staining (yellow circles) in early HER2+ lesions. Early cancer cells (T), macrophage (M) and endothelial cell (E) comprise P-TMEM.

A- Results – detection of P-TMEM in the MMTV-Her2 model during early stages of cancer: In FY2 we aimed to identify P-TMEM characterized by the interaction of early cancer cells, macrophages and the vascular endothelial cells where TMEM are identified using antibody staining against the TMEM-tumor cell using anti-Mena, the TMEM-endothelial cell using anti-CD31 and the TMEM-macrophage using anti-CD68. TMEM are identified as a structure when all three stained cells are in direct contact. We therefore optimized this triple staining to detect TMEM structures by IHC in collaboration with the Aguirre-Ghiso lab (**Fig.1**). We found that all 3 TMEM cell types could be detected as a triple cell complex in which the cells are in direct contact with each other thereby identifying TMEM in these early lesions. Optimization of this staining will be required to ensure quantitative scoring. Additional staining of epithelial cells and their cell-cell junctions in adjacent sections might help solve the optimization in the future.

We found that P-TMEM structures could be detected as pre-invasive lesions in both non-proliferative 14wk old and proliferative 24wk old MMTV-HER2 mice but were more frequent in proliferative lesions (**Fig.1B**). Non-proliferative structures often contained intra-epithelial macrophages (**Fig.2A**). Interestingly, during those early stages, dissemination rates are low but can be stimulated through the inhibition of p38 signaling **(Fig. 2B)** which results in the activation of an EMT program and enhanced recruitment of macrophges and vascularization (unpublished observation Aguirre-Ghiso lab). In contrast, in more progressed but still pre-invasive stages (**Fig.1B**; 24wk old MMTV-HER2 glands), P-TMEM structures are frequently found and this is correlated with enhanced early dissemination (Linde et al., under review *Nature Communications* and also see progress report from Partnering PI Aguirre-Ghiso, JA). **Conclusion**: We found that TMEM staining works in the MMTV-HER2 mouse model and that the TMEM structure is present in these early lesions.

B- Imaging early dissemination and P-TMEM in premalignant stages of Her2+ mammary cancer: In the last progress report we showed how we optimized a mammary gland imaging window to perform intravital imaging and detect P-TMEM function during early stages of progression in the MMTV-HER2 model. This work has progressed significantly and we were able to not only document local invasion as in last year's progress report but also document the interaction of early disseminating cancer cells with the vasculature and entry into the vasculature that was corroborated by the detection of circulating cancer cells at this stage and disseminated cancer cells in target organs such as lung and bone marrow. Briefly, in **Fig 2** we show that we have been able to monitor early dissemination using high-resolution intra-vital imaging[14] of MMTV-Her2-CFP transgenic mice (**Fig2**). Using a new mammary imaging window and 2-photon imaging in vehicle treated Her2-CFP mice[14] we found that while at 10 weeks (normal ductal structure) no invasion was detected (**Fig2A, left)**, at 15 and 18 weeks local invasive CFP+ cells were detected (**Fig2A**) invading into the stroma (**Fig2A,**). CFP reports faithfully for Her2 overexpressing cells as confirmed using double Her2 and CFP IF (>90% Her2+ cells were CFP+; not shown). p38α/β inhibition with SB203580 stimulated (**Fig2A-D**) invasion and now CFP+ cells were found intravasating (**Fig2B, C and D**). 3D reconstruction of the videos showed unambiguously how individual cells from the ductal pre-malignant lesions (**Fig2B'-C**) enter the lumen of blood vessels (**Fig2C-D**).

The intravasation documented in the movies led to successful dissemination as we found Her2+/CK8/18+ early disseminated cancer cells (eDCCs) detected in the blood, BM and Her2+ eDCCs in lungs of 100% of 14-18 weeks old mice (**Fig2E-G**). Also, systemic inhibition of p38 for 2 weeks significantly increased numbers of early circulating cancer cells (eCCCs) (**Fig2E**), eDCCs in BM (**Fig2F**), and eDCCs in lungs (**Fig2G**). (14. This work is part of a manuscript now

accepted for publication in ***Nature***: *Harper KL, Soledad MS, Entenberg D, Cheung J, Nobre R, Valderas AA, Nagi C, Hosseini H, Girnius N, Davis RJ, Farias EF,* ***Condeelis J****, Klein C,* ***Aguirre-Ghiso JA****. Mechanism of early dissemination and metastasis in HER2+ mammary cancer. Accepted, Nature 2016.* Please see progress report from the partnering PI – Attachment 2).

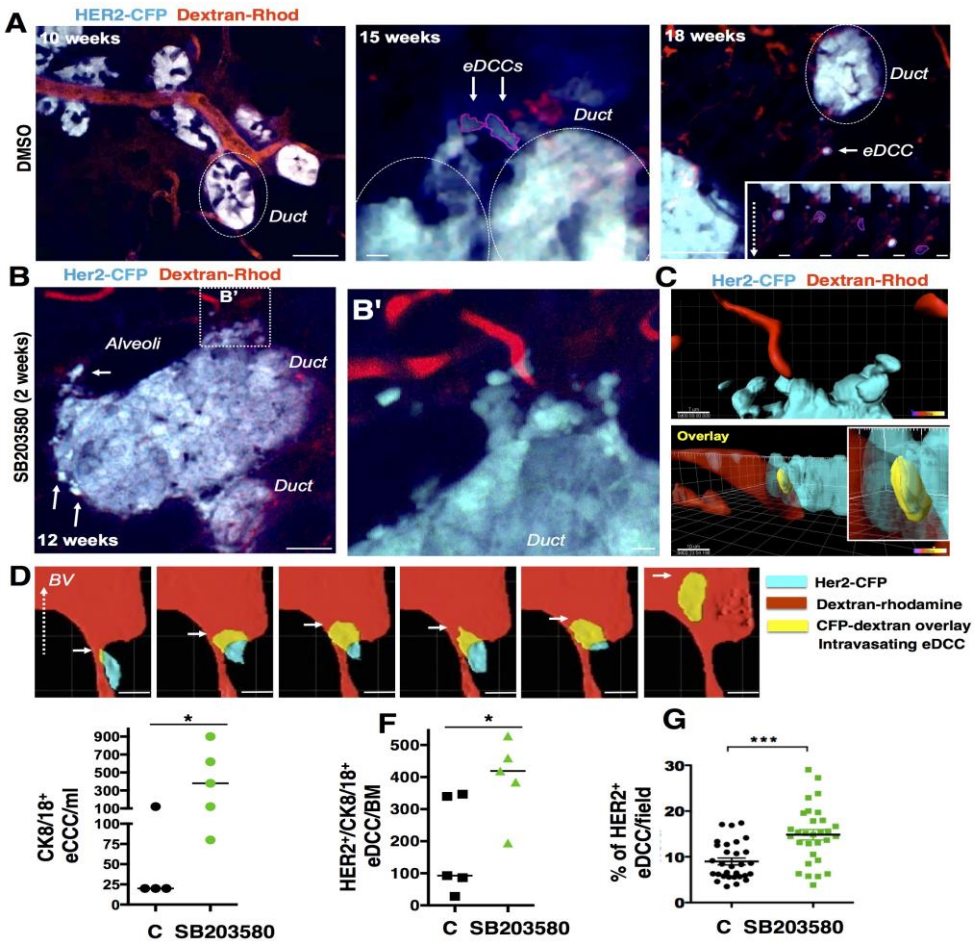

Figure 2. Intra-vital imaging of intravasation and systemic detection of Her2^{hi}/Pp38^{lo} eDCCs. (A) Intra-vital images of 10 (left), 15 (middle) and 18 (right) weeks old early lesions (EL) in MMTV-Her2-CFP mice, scale bars= 38, 5 and 56 µm, respectively. Blue-white signal = CFP+ cells RED signal= rhodamine-dextran injected vasculature. Dotted ellipses define a duct. Right panel inset: direction of movement of an eDCC at 5 consecutive time points (2 min each panel). Dotted arrow= direction of movement, scale bar= 7.8 µm. **(B)** Images from of an EL in an MMTV-Her2-CFP mouse treated with SB203580 for 2 weeks, scale bar= 25.5 µm. **B'** high power high resolution intra-vital still from a movie of the area boxed in B' showing active breaching of MMTV-Her2-CFP cells from the ductal structure into the stroma and interacting with blood vessels (red) scale bar= 5.2 µm. **(C)** Frames of a 3D computer generated reconstruction of a movie in **B'**; bottom panel is a rotated projection to show invasion (yellow) of early cancer cells (CFP) into blood vessels (red). Scale bar=6.6 µm. **(D)** Image from an EL cell in MMTV-Her2-CFP mice treated with SB203580 for 4 weeks. A single cell (in blue) is followed (arrows) as it intravasates (cell in yellow inside the red blood vessel). **(E)** (left most panel) eCCCs detected as CK8/18+ as in **Fig 2E** in blood of MMTV-Her2 mice (age 14-18wk) treated for two weeks with DMSO (C) or the p38α/β inhibitor SB203580 (n=5 mice/group). **(F)** eDCCs detected as CK8/18+ as in **Fig 2G** in BM of MMTV-Her2 mice treated as in **H** (n=5 mice/group). **(G)** eDCCs detected in the lung of MMTV-Her2 mice carrying early lesions only as in **F** and treated as in **H**. Graph= % of Her2+ DCCs/field is each group (n=30 fields, 3 mice/treatment). *p<0.05, **p<0.01, ***p<0.001.