Defining the function of epithelial to mesenchymal transition during tumor progression in breast cancer

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Abstract

Metastasis is the process where cancer cells escape the primary tumor and establish themselves in different locations around the body. It is estimated that metastasis is responsible for about 90% of cancer deaths. Epithelial to mesenchymal transition (EMT) is a crucial step in the process of metastasis and results in mesenchymal and invasive cells. EMT is essential during embryonic development, organogenesis, wound healing, and disease progression. These newly gained characteristics include increased mobility, increased invasive ability, and altered growth factor receptor signaling, which can result in metastasis. Mesenchymal cells are disorganized, with a loss of cell to cell attachments and loss of polarity genes expression. We have previously shown there is a heterogeneous expression of EMT markers as well as various invasive phenotypes, such as collective and single cell invasion. These different invasion mechanisms may be explained by the heterogeneous activation of programs known to control master regulators of EMT. Here, we show signaling pathway interactions that augment invasion within C3T mouse model primary tumors. Dysregulation of these pathways can induce EMT at inappropriate times, resulting in the induction of metastasis and disease progression.

Background

Triple negative breast cancer (TNBC) lacks ER, PR, and HER2 on its cells and makes up 15% of all diagnosed cases. It also has the worst prognosis of the three subtypes and has a high proliferative rate as well as an aggressive nature. TNBC also exhibits more basal-like tumors (71%) than the other subtypes (29%) and can appear phenotypically different. TNBC has been shown to have heterogeneous expression of EMT markers, such as vimentin and EMT master regulators such as Zeb, Slug, Snail, and Twist. The induction of EMT results in loss of cell polarity and a cadherin switch which can augment disease progression through increased invasion and metastasis. the development of TNBC is more of a mystery, as it cannot be traced to the overexpression of receptors, however, there are trends in de novo mutations in certain pathways, such as AKT/PI3K, JAK/STAT, and ERK. Basal-like breast cancers have an activated Ras-ERK gene signature relative to other breast cancer subtypes. Using human cell line-based models, we have previously shown that ERK activation promotes tumor cell migration and invasion. By understanding the mechanisms behind different levels of invasiveness and EMT induction, this could reveal molecular targets that can reduce invasive behaviors of cells and hinder metastasis.

Conclusions

1. Phosphorylated ERK and its downstream targets may contribute to the level of invasive capabilities of cells within the primary tumor
2. TGF-β enhances invasive capabilities cells, which may lead to a subpopulation of cells with a higher metastatic potential
3. EMT induction may be regulated by the interaction of these two pathways through the regulation of Zeb1/2 transcription.

Results/Discussion

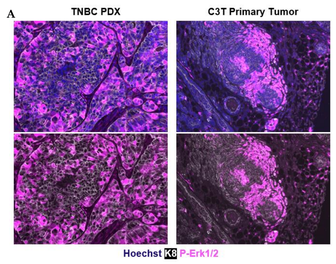

Figure 1. Erk1/2 is activated in TNBC patient tumors and C3T tumors. Immunohistochemistry staining reveals ERK signaling pathway is heterogeneously activated in our C3(1)SV40 T Antigen Mouse model primary tumors and triple negative breast cancer patient derived xenograft tumors.

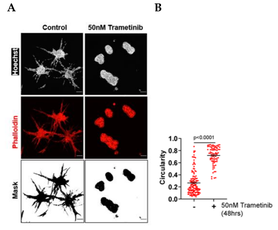

Figure 2. Inhibition of Erk1/2 phosphorylation suppresses invasion of C3T organoids. A. Immunofluorescence staining of organoids generated from primary C3T, treated with Trametinib. **B.** Treated organoids were less invasive than control. To explore the function of active ERK signaling is contributing to invasion , we used Trametinib which is a MEK inhibitor. In the control, you can see our C3T organoids have an intrinsic invasive ability. This invasive phenotype is suppressed when MEK is inhibited. Organoids invasive behaviors were measured using circularity calculations. The closer the number is to 1, the less invasive it is and the more circular it is. If the organoid is more invasive, it will have a circularity score closer to 0. Comparing control to MEK inhibitor, we see a higher circularity value which is indicated by to a less invasive behavior, thus ERK could be regulating these phenotypes.

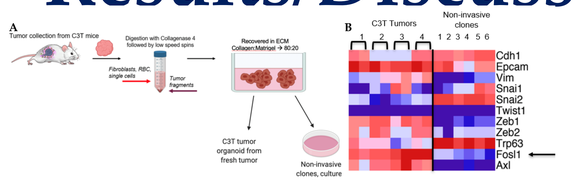

Figure 3. Fosl1, a direct target of ERK exhibits increased expression in C3T tumors. A. Primary tumors were collected from C3T mice, digested and then plated in Collagen: Matrigel (80:20). **B.** RNAseq of non-invasive clones vs. C3T tumors. To identify potential downstream targets that could be contributing to invasion, we Used RNAseq to look at differentially expressed genes between invasive C3T tumors, each having their own replicate, and non- invasive clones which were cultured in vitro. Fosl1, which is a direct target of ERK , is increased in more invasive C3T Tumors , which means it could be regulating these invasive phenotypes we are seeing in our organoids

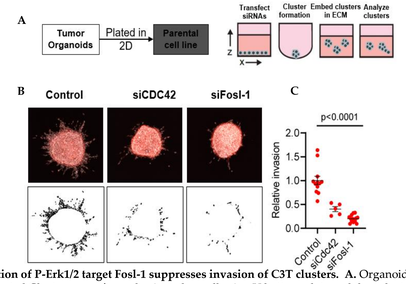

Figure 4. Depletion of P-Erk1/2 target Fosl-1 suppresses invasion of C3T clusters. A. Organoids were plated in 2D and transfected Clusters were formed using a low adhesion U bottom plate and then clusters were embedded in ECM. **B.** Knockdown of Fosl1 reduced invasiveness of clusters. **C.** Relative invasion decreased in both Cdc42 and Fosl1 knockdown clusters. To explore FOSL1's role as a downstream effector of ERK activation we used organoids to established cell lines in 2D. Then we knocked down FOSL1 expression using siRNAs , so first we transfect the cells using a pool of siRNAs , and then we form clusters in a low adhesion round bottom dish, then we embed the clusters in ECM and analyze their invasiveness CDC42 implicated in protrusion formation and is a positive control for suppression of invasion . In order to quantify Invasion, we removed the central cell mass and measured the area of the nuclei in the cells invading outward. Compared to control, the knock down of Fosl1 suppressed invasion, thus it has a lower relative invasion score. This suggests that Fosl 1 is contributing to invasion and is regulated by Phosphorylated ERK

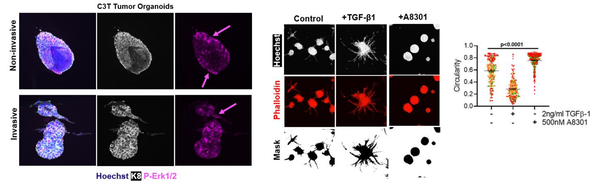

Figure 5. TGF-β1 treatment induces an enhanced invasion in C3T organoids and may explain heterogeneous invasive behaviors within primary tumors. A. IHC of C3T organoids reveals that ERK signaling is not sufficient for invasion. **B.** Images of organoids treated with TGF-β1 or A8301. **C.** Circularity measures reveal that TGF-β1 increases invasiveness while A301 decreases the amount of protrusions. we have shown ERK and Fosl1 contribute to this invasive phenotype however since we know its not sufficient for invasion. One possible signaling pathway that could be inducing invasion is TGF-β. To see if TGF beta is contributing to this invasive phenotype, we treated organoids with exogenous TGF-β1 or A8301, which is a TGF-β receptor inhibitor. There is an intrinsic invasive ability in our C3T organoids, and this is enhanced when they are treated with TGF-β . This autocrine signaling and invasion is suppressed when an organoids are treated with a TGF beta receptor inhibitor . TGF beta treated organoids also had Lower average circularity score, which is reversed when treated with A8301. This shows that TGF beta induces invasion in our organoids.

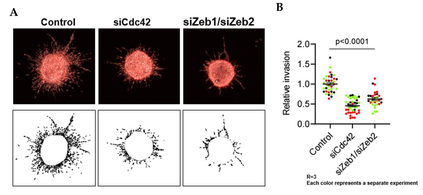

Figure 6. Depletion of Zeb1/2 restricts TGF-β1 induced invasion. A. Images of C3T organoids that have been treated with exogenous TGF-β1 transfected with siCdc42 or siZeb1/2. **B.** Relative invasion was decreased when treated organoids were transfected with siCDC42 and siZeb1/2. Zeb seems to play an important role in invasion and may be the downstream target of TGF beta signaling. It is also upregulated within our C3T tumor cells when compared to non-invasive clones . To see if this might be the downstream target of TGFB signaling we knocked down expression of Zeb1 and 2 in the presence of TGF-β1 and saw a clear suppression of invasion. Additionally, Relative invasion in cells depleted of Zeb was also lower than the control. These results tell us that Zeb could be the downstream target of TGF-β1 and be involved in this enhanced invasion phenotype .

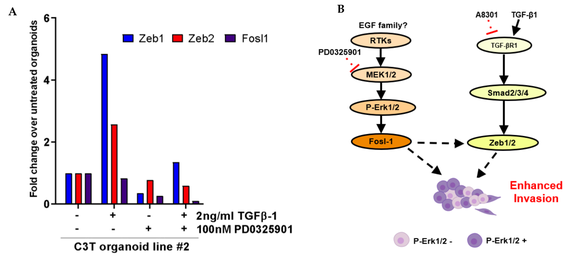

Figure 7. Inactivation of the ERK1/2 pathway reduces Fosl-1 as well as TGF-β1 dependent upregulation of Zeb1. A. RT-qPCR of organoids treated TGF-β1 with or with a PD0325901. **B.** Model for invasion regulation within C3T tumors. To see if FOSL1 or Zeb respond to TGF beta signaling, we either treated organoids with TGF beta or with a MEK inhibitor. Zeb expression increased with the Exogenous TGF beta,. When the MEK inhibitor is given, we see a reduction in FOSL1 as well as a reduction in Zeb. We also seen that when TGF beta and the MEK inhibitor is given together, you see reduction in upregulation in Zeb that we saw earlier when TGF beta is given alone, and there is also a continued reduction of Fosil1 , suggesting that the two may be interacting to regulate this enhanced invasive behavior in the primary tumor.

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