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14. ABSTRACT This DoD Innovation grant was based on the hypothesis that transmissible ER stress (TERS) promotes Epithelial to Mesenchymal Transition (EMT) in differentiated prostate cancer cells, programming cancer towards a different phenotype and greater invasive characteristics. The hypothesis predicted a new and potentially important mechanism in tumorigenesis. Through the work performed during the last year, we have been able to demonstrate a link between prostate tumor ER stress and EMT. The study is not finished yet but we can confidently say that the premises of the original hypothesis have been experimentally validated.					
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Annual Report

DOD Award: W81XWH-12-1-0156
Principal Investigator: Maurizio Zanetti, M.D.

Introduction

How prostate tumor cells cope with tumor microenvironmental stress often dictates successful tumor outgrowth. This may entail the co-opting of a variety of processes, including resistance to programmed cell death or increased vascularization. While these processes enable successful primary tumor growth, they do very little to explain metastasis, the often fatal feature of cancer. One possibility to explain how epithelial tumor cells acquire motile characteristics is through a process known as the epithelial to mesenchymal transition (EMT). This model posits that transformed epithelial cells become polarized and undergo epigenetic changes towards a mesenchymal phenotype, allowing for greater motility and ultimately escape from the primary tumor. While much emphasis has been placed on probing the genetic machinery of this intriguing cellular process, there has been very little consideration for what tumor or tumor microenvironmental cues may initiate EMT.

Recent evidence suggests that tumor cells have an enhanced unfolded protein response (UPR), a programmed cellular response tumor cells employ to cope with endoplasmic reticulum (ER) stress. This is often elicited by *noxae* in the tumor microenvironment. Reports show that the UPR enables tumor cell survival, promotes chemoresistance, and represents a possible link to the initiation of EMT. The induction of UPR in tumor cells also drives the production of Lipocalin 2 (LCN2) and interleukin (IL)-6, proteins that can promote EMT in several cancer types including prostate cancer. Recently, we reported that ER-stressed prostate tumor cells influence tumor-associated myeloid cells in a cell-extrinsic manner, polarizing them into an inflammatory phenotype, an event termed transmissible ER stress (TERS). Provided this novel extrinsic role of the tumor UPR and the potential role the UPR has in EMT, the tumor UPR, and more specifically TERS, may represent the cues tumor cells receive to undergo EMT.

Body

Under the aegis of this DoD grant, we have begun testing the hypothesis that upon as endoplasmic reticulum (ER) stress prostate cancer cells actually direct through cell extrinsic effects EMT changes in otherwise unstressed cancer cells. To this end, we stressed human PC3 prostate cancer cells with a canonical ER stress drug, thapsigargin, or its vehicle control, to generate ER stress or vehicle (Veh) conditioned mediums. Because the former can transmit an ER stress response in receiver cells, its effect has been termed transmissible ER stress (TERS).

We then sought to determine if prostate cancer cells cultured over five days in TERS (which was resupplemented daily) undergo a UPR, as indicated by the fold upregulation of the master UPR gene, *Grp78* (Fig 1a).

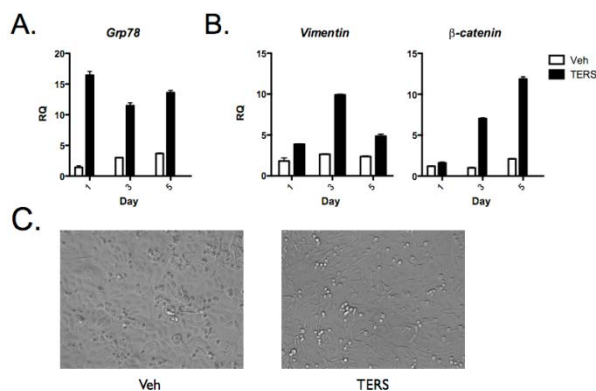


Figure 1: TERS treated prostate cancer cells undergo a UPR and EMT.

PC3 cells were treated to TERS for 5 days and probed for a A. UPR via *Grp78* and B. EMT, as determined by *vimentin* and *β-catenin*, respectively. C. Bright-field visualization of treated cells after 72 hrs.

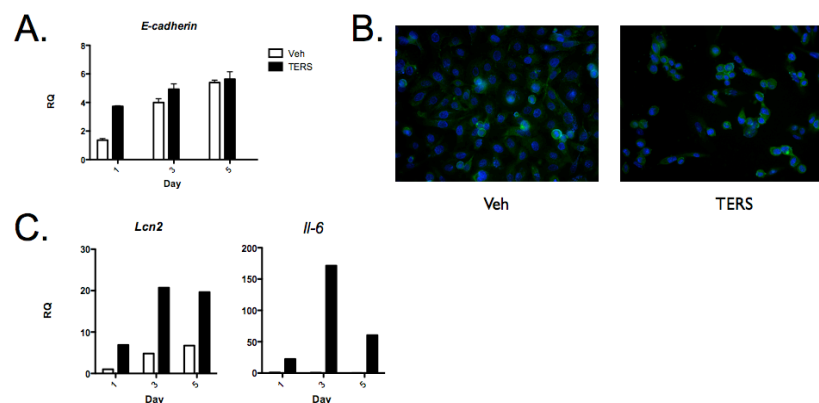
After establishing that the gene is highly upregulated following exposure to TERS, we sought to determine if prostate cancer cells also undergo EMT as determined by microscopy and PCR analysis of EMT-associated genes (*β-catenin* and *Vimentin*) (Fig 1b, c).

We found that TERS treated cells greatly upregulate both genes after 3 days and continue stable upregulation for another two days of TERS culture, suggesting that TERS elicits EMT in prostate cancer cells.

Having established that TERS drives EMT polarization, we probed other known facilitators of EMT. To our surprise, we found no significant decrease in the gap junction protein E-cadherin at both the genetic and protein levels (Fig 2a,b). In contrast, two other EMT facilitators, IL-6 and lipocalin-2 (*Lcn2*), are greatly upregulated (Fig 2c).

Figure 2: TERS evoked EMT does not affect E-cadherin but activates EMT cytokines.

A. E-cadherin expression of TERS treated PC3 for five days. B. Immunostain for E-cadherin (green) and nuclear (blue) after three days of TERS stress. C. Genes expression for known EMT cytokines lipocalin 2 (*Lcn2*) and IL-6



Collectively, these results suggest that TERS treated prostate cancer cells undergo incomplete, or partial, EMT polarization after short time treatment.

We further interrogated a new EMT facilitator, Twist, and asked the question of whether its induction can be mediated by TERS. Two new reports suggest Twist is necessarily upregulated for EMT and the extravasation of cancer cells from a primary tumor to occur. Further, Twist is then downregulated once cancer cells have colonized distal metastasis.

We treated PC3 cancer cells with TERS for three days followed by a refractory period of five days (Fig 3a). Over this period, cells were harvested daily and probed for their expression of *Twist* (Fig 3b).

Our findings reflect that TERS is a potent propagator of Twist and once removed from culture conditions, prostate cancer cells can be restored to their original epithelial state.

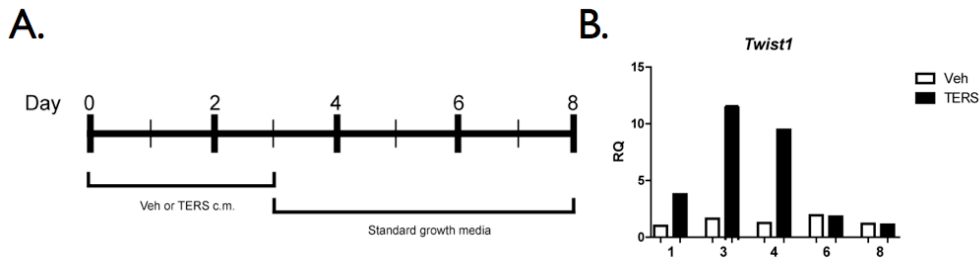


Figure 3: Twist expression is dependent of TERS.

A. Dosing regimen of TERS over PC3 over eight days.

B. *Twist* gene expression over treatment periods

We are currently pursuing the functional implications of our studies as well duplicating them in another human prostate cancer cell line, LNCaP.

Emerging data suggest that TERS treated PC3 cells do not have enhanced motility, as determined by transwell and wound healing assays. However, these results are provisional and need to be repeated.

In lieu of activated β -catenin, we have begun to pursue if Wnt signaling occurs during TERS driven EMT. Given that this signaling process has tumor implicated roles in aggressive phenotype, these data could further establish that TERS has marked and deleterious effects on unstressed tumor cells.

Finally, a new report suggests that Hsp27, another ER stress response gene, regulates EMT and can be propagated through exposure to IL-6. While we continue to elucidate this potential axis of TERS evoked EMT, we now extend these studies to TERS primed macrophages and whether the cytokines they release, including IL-6, can also advance EMT machinery.

Key Research Accomplishments

- Prostate cancer cell lines, when undergoing a UPR through transmissible ER Stress (TERS), exhibit many key characteristics of an epithelial-to-mesenchymal transition.
- TERS-treated prostate cancer cells also undergo changes that are not typical of an EMT such as an upregulation in E-cadherin
- TERS-treated cells undergo a change in morphology similar to those seen undergoing EMT
- Various EMT cytokine genes, including IL-6 and Lipocalin 2, are elevated in TERS treated prostate cancer cells
- TERS-treated cells exhibit an activation of the Wnt-signaling pathway
- Twist expression is directly linked with the presence of TERS

Reportable Outcomes

We have verified the basic tenet of the DoD application that transmissible ER stress is causative of changes in unstressed prostate cancer cells that are consistent with EMT except for a down-regulation of E-cadherin. We are provisionally working under the hypothesis that the changes imparted by TERS are causative of partial or incomplete EMT.

Conclusion

The work to date allows us to say that transmissible ER stress is causative of changes in unstressed prostate cancer cells that are consistent with EMT except for a down-regulation of E-cadherin. Experiments that will be performed in future months through a NCE will bring to conclusion this study and we will begin writing a manuscript for publication based on the new findings discussed herein.

References

No paper has been submitted to publication yet.