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14. ABSTRACT

The epithelial-mesenchymal transition (EMT) has been found by us and others to induce normal and neoplastic mammary epithelial cells (MECs) to acquire mesenchymal traits and, in addition, many of the characteristics of stem cells. However, none of these observations showed that the products of EMT are actually *bona fide* stem cells. To demonstrate this, I had optimized efficient EMT induction in several different cell types, tested new ways of separating stem cell and non-stem cell populations of normal and breast cancer cells and identified EMT transcription factors most likely involved in stem cell biology. Preliminary results directly demonstrate that transient induction of EMT increases the number of mammary epithelial stem cells capable of generating an entire mammary ductal tree. With these observations and optimizations I can now move closer to uncovering the connection between EMT and entrance into a stem-cell state. The outcome of these experiments holds important implications for the mechanisms controlling the formation of normal mammary epithelial stem cells as well as the formation of breast cancer stem cells.

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The Role of Epithelial-Mesenchymal Transition in the Formation of Normal and Neoplastic Mammary Epithelial Stem Cells

Zuzana Keckesova, Ph.D.

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INTRODUCTION

Metastatic invasion, the most feared feature of cancer, requires neoplastic epithelial cells to loose cell-cell attachment and to gain motility. The molecular process known to be responsible for this malignant change is called epithelial-mesenchymal transition (EMT). Recently, our laboratory and others uncovered an unanticipated, indirect link between the EMT and stem cells (Mani et al., 2008; Morel et al., 2008). Stem cells have the unique ability to self-renew almost infinitely and to give rise to all cell types in the body. Tumor tissues also contain such self-renewing, stem-like cells, which are called cancer stem cells (CSCs) and which carry the source of a tumor's ability to grow and regenerate. It was shown that the cells that underwent EMT acquired some of the properties of stem cells; mainly the expression of stem cell surface markers and an increased ability to form mammospheres. My project aims to identify whether EMT is involved in *in vivo* formation and maintenance of normal and cancer stem cells.

The first part of my study focuses on the role of EMT in the formation of normal stem cells. I am testing whether induction of an EMT in murine mammary epithelial cells (MECs) is able to convert them into *bona fide* stem cells capable of forming the entire mammary gland *in vivo*. The first year of my research involved the optimization of *in vitro* culture conditions for primary murine epithelial cells, improving the lentiviral transduction efficiency of these cells and examining the suitability of individual EMT transcription factors for induction of EMT in primary murine epithelial cells. In addition, I have also performed a preliminary study of the effect of EMTed cells on mammary gland reconstitution.

In the next part of my project I am examining the role of EMT in the formation of breast cancer stem cells and studying whether EMT-inducing transcription factors also play a role in the maintenance of CSCs within tumors. The first year of my research was directed towards cloning of inducible human versions of EMT-transcription factors, optimization of conditions for efficient EMT induction and preliminary examination of tumor-initiating abilities of EMTed cells in xenograft breast tumor model.

Solving and setting up these conditions allows me to move closer towards elucidating the role of EMT in normal and cancer stem cell processes. Better understanding of mammary stem cell biology will ultimately lead us to a better understanding of breast cancer stem cells, which is crucial for the future development of anti-cancer therapies. By finding an easy way to generate large numbers of cancer stem cells at will, we will gain the opportunity to study these in great detail, to uncover their maintenance mechanisms, and to screen for therapeutic agents to specifically eliminate them. This would be a highly effective approach to finding cures, since it would strike right at the "heart" of a tumor, halting the eventual regrowth of the tumor mass and thus preventing clinical relapses.

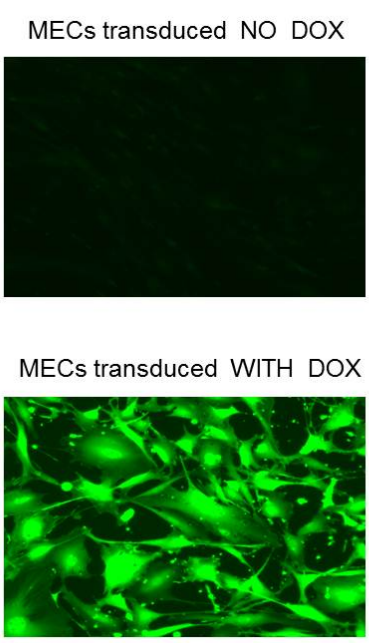
BODY

1. The Role of EMT in the Formation of Mammary Epithelial Stem Cells

I am investigating whether induction of an EMT in primary mouse MECs can significantly and efficiently increase their mammary gland-reconstituting activity, thereby converting them into *bona fide* stem cells. For this purpose, murine mammary epithelial cells have to be isolated from mouse mammary gland, incubated *in vitro* and transduced with lentiviral vectors carrying doxycycline inducible EMT-transcription factors (EMT-TFs). These cells will then be induced to undergo EMT and will be injected into cleared mammary fat pads, followed by the examination of their gland-reconstituting activity.

These steps involve a number of technical obstacles. One of the hurdles is the known difficulty to transduce primary murine epithelial cells efficiently. Even though I am planning to use powerful lentiviral systems for the delivery of EMT-TFs into the cells, it has been my experience and that of others that one round of infection will give around 8% of transduction efficiency. By concentrating the virus the efficiency might increase to 30%. My experiments require better delivery efficiency and since primary MECs are very sensitive to cell sorting techniques, it is difficult to separate the transduced population. Also, primary murine

epithelial cells grow in culture only up to 12-15 da ys before undergoing senescen ce and/or cell death. This limitation prevents me from using drug selection as one of the possibilities to elim inate cells that were not transduced. Therefore, I attempted to optimize the conditions of infection of prim ary mouse MECs by lentivirus vectors. Indeed, by optimizing transfection and transduction protocols I developed conditions that now allow ~90% infection efficiency, which is m ore than 10-fold greater infection rate than was previously possible (Figure 1).



Transfection: 293T cells are incubated in DME+15% heat inactivated FCS (no antibiotics). They are split every 2 days with the ratio of 1:4 (10cm plates). Transfection is performed the next day after the split.

The transfection mixture for one 10cm plate contains:

-1µg pMDG (encoding the pantropic VSV-G envelope)

-1µg of the packaging vector (Gag-Pol expression plasmid)

-1.5 µg of the marker-encoding transfer vector

Mix together and add PBS to 15 µl.

Mix 200 µl Opti-MEM with 18 µl FuGENE (let stand the mix for 5 minutes). Then add the DNA mixture to the Fugene mixture and incubate at room temperature for 15-20 minutes before adding dropwise to the almost confluent 293-T cells in 10 cm petri dish with 8 ml of media. Transfected 293-T cells are incubated overnight at 37oC and with 5-10% CO2. Media is changed after 24 hrs, and supernatant is harvested at 48, 72 and 96 hours post-transfection and passed through a 0.45 µm filters. The supernatant mixed with polybrene (50µg/ml) and media (ratio 2:3) is then used directly to transduce the primary murine cells for 8 hours.

Figure 1: Primary murine cells transduced with doxycycline (DOX) inducible lentiviral GFP vector with and without doxycycline addition. Included is the optimized transfection and transduction protocol.

Next, I had to establish which of the m any EMT-transcription factors is most suitable for my experiments; seeking the EMT factor that is ca pable of inducing potent EMT and that is specifically associated w ith mammary gland stem cells. Even though Twist was show n to be a powerful EMT inducer in hum an cells these effects are tissue and species-specific and m ight not be the sam e in the context of murine cells. Therefore, I separated, by cell sorting, three distin ct subpopulations of murine MECs pr epared from mammary glands of ~3 month-old m ice using cell-surface antigenic markers developed by others (Asselin-Labat et al., 2007; Stingl et al., 2006). More specifically, the stem cell-enriched $CD49f^{high}/CD61^{+}$ basal population was separated from $CD49f^{low}CD61^{+}$ luminal progenitor cells and from $CD49f^{low}CD61^{-}$ fully differentiated luminal cells (Figure 2). I then used quantitative RT-PCR to examine the expression profile of eight major EMT-TFs in these subpopulations. As co mpared to the differentiated m ammary MECs, the normal mammary gland stem cells e xpressed high levels of Slug (~90x) and Sna il (~15x) and a m ildly elevated levels of Twist (~4x) (Figu re 2). This persuaded m e that Slug, Snail and/or Twist were likely to have some function in the mammary stem cell state. Consistent with previous reports (Lim et al., 2010; Mani et al., 2008), the cells in stem cell-enriched basal population also expr essed relatively high levels of mesenchymal markers, such as N-cadherin, vim entin, and low levels of epithelial m arkers, specifically E-cadherin (Figure 2). The analysis of published m icroarray data of various hum an mammary epithelial cell subpopulations confirmed that S lug is also the EMT-TF that is m ost highly expressed by hum an stem cell-enriched cell population (Lim et al., 2010).

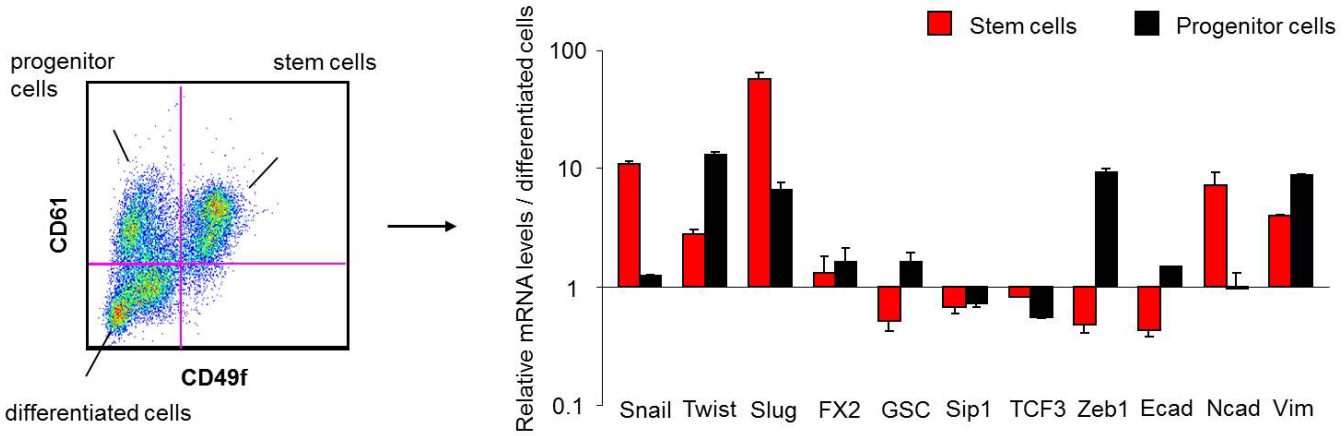


Figure 2: Primary murine epithelial cells were separated by FACS into 3 distinct sub-populations and analyzed by RT-PCR for the expression levels of EMT-transcription factors and mesenchymal and epithelial markers.

I cloned the mouse versions of Slug, Snail, Twist and Luciferase control into doxycycline inducible lentiviral vectors (also expressing green fluorescent protein-GFP). Freshly isolated murine epithelial cells were then transduced with these vectors according to my protocol and the EMT factors were induced with doxycycline for 12 days. I have examined the EMT status of these cells by morphology and Western Blot analysis. Expression of Slug and Snail in primary MECs induced a robust EMT as judged by morphological changes observed in monolayer culture, specifically a conversion from a cobblestone epithelial morphology to one of dispersed, mesenchymal-like cells (Figure 3). This change was more rapid in cells over-expressing Slug (5 days) than in cells over-expressing Snail (12 days). Slug and Snail also induced higher expression of mesenchymal markers in primary MECs (Figure 3). Surprisingly, even though cells over-expressing Snail and Slug manifested almost complete mesenchymal morphology, the protein levels of E-cadherin were not decreased. Similar observation was noted by others in human MCF7 cell line. When this cell line was manipulated to undergo EMT its E-cadherin protein levels remained the same, instead it was the E-cadherin subcellular localization that had changed from a membrane to a cytosolic position (Micalizzi et al., 2009). Immunofluorescence analysis will need to be performed to clarify these issues in the context of primary murine epithelial cells. Expression of Twist had very toxic effect on primary murine epithelial cells and I have, therefore, excluded it from further analysis (Figure 3).

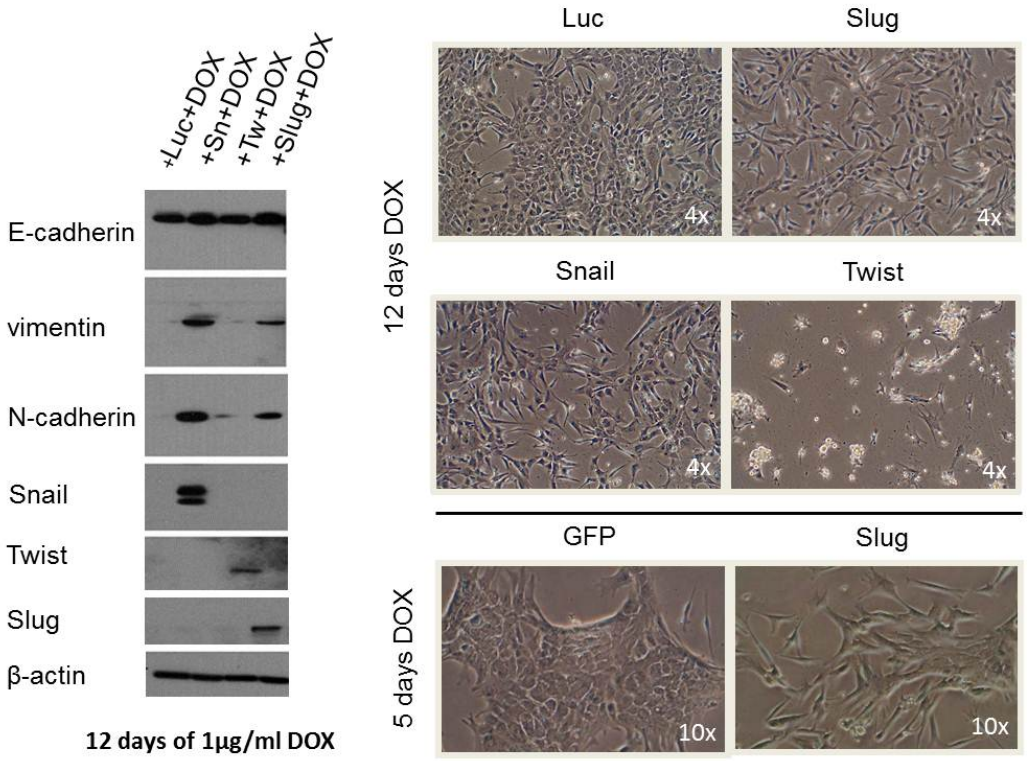


Figure 3: The EMT status of primary murine epithelial cells transduced with Luciferase control, Snail, Slug and Twist is examined by morphology and Western Blot analysis. Included are mesenchymal (vimentin, N-cadherin) and epithelial (E-cadherin) markers.

Next, I have used the *in vivo* cleared mammary fat pad reconstitution assay to measure whether Slug- and Snail-induced EMTed primary murine cells contain more mammary stem cells than the non-EMTed cells. This mouse *in vivo* regeneration assay was developed by Smith and Medina (1988), and is aimed at isolation and characterization of mammary epithelial stem cells. In this assay, 3-week old mice have the portion of their stromal compartment of the mammary gland (called mammary fat pad), containing the mammary epithelial tree, cleared away. Cells of interest are injected into such cleared fat pads and if the injected cells contain mammary epithelial stem cells, an entire mammary ductal tree can be regenerated. Otherwise, the cleared fat pad remains devoid of mammary epithelium. The preliminary test I have performed involved the implantation of EMTed and non-EMTed cells into cleared mouse mammary stromal fat pads, with the hope that the proportion of mammary gland-generating stem cells would be greatly increased by prior exposure of cells to an EMT-inducing transcription factors. This experiment was done in collaboration with my colleague Dr. Wenjun Guo, who is studying the master regulators of mammary gland morphogenesis and who has an extensive knowledge of the fat pad reconstitution essay.

As mentioned earlier, primary murine epithelial cells grow in culture only up to 12-15 days before undergoing senescence and/or cell death. Experimenting with different *in vitro* culturing media, as well as growing these cells under a hypoxic (~3% oxygen) conditions that more closely correspond to intravital physiologic conditions, did not extend their life span (data not shown). This limitation prevented me from inducing full EMT in vitro. We were, therefore, inducing Snail, Slug and Luciferase cells with doxycycline for 5 days in vitro, followed by injection of these cells into female NOD/SCID mice (2-3 mice per group) at limiting dilutions (2000 cells and 400 cells) and continued inducing the factors for one more week in vivo. We then analyzed the mammary glands 8 weeks after implantation. The ability of EMTed cells to regenerate mammary ductal trees was increased several fold when compared with control cells (Figure 4). This preliminary experiment will require reproduction on a larger number of mice. It is possible that, in order to increase the efficiency of the conversion of EMTed cells into stem cells, I will need to over-express a

combination of EMT-inducing transcription factors or that EMT factors might need a cofactor. These experiments and optimizations are currently underway.

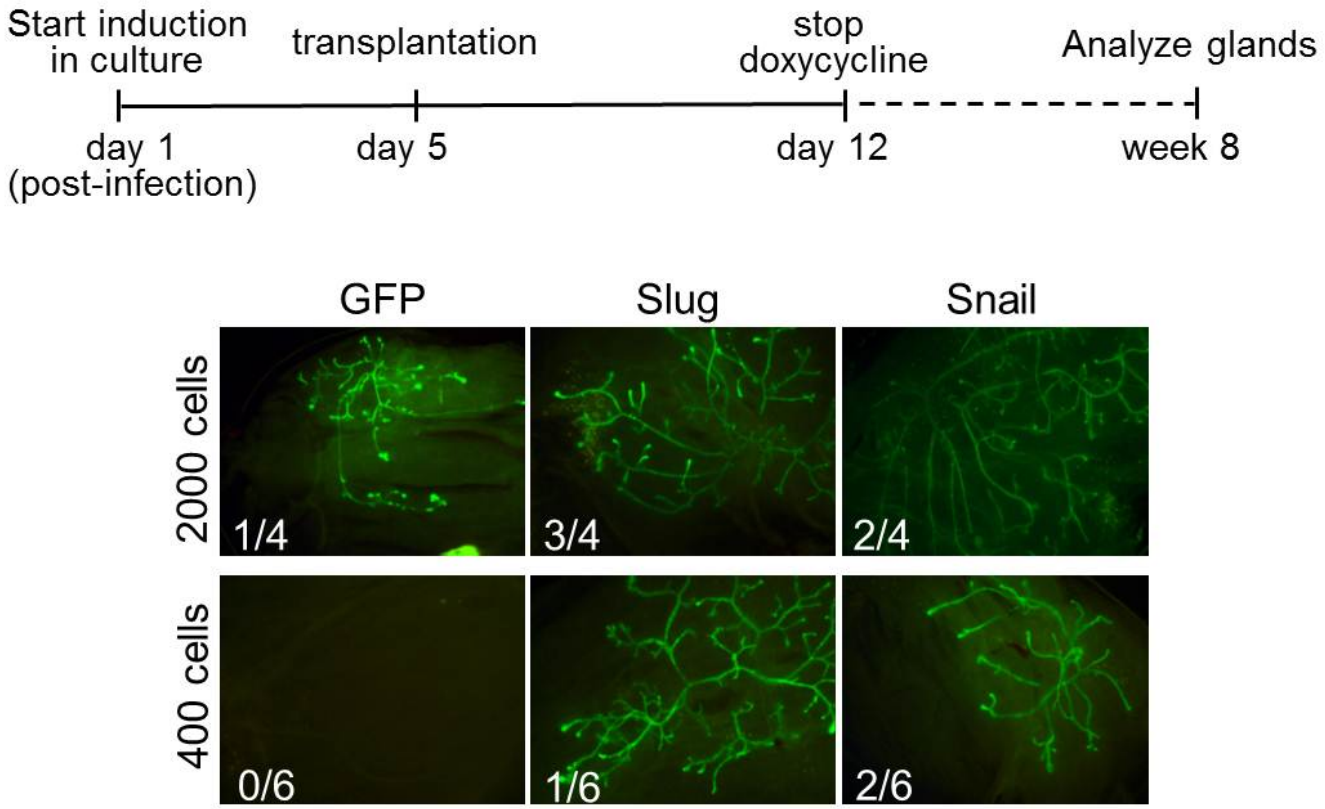


Figure 4: Schematic diagram of mammary fat pad reconstitution experiment. Shown is the incidence of the regeneration of mammary ductal trees (green) at different dilutions in control primary murine cells and in cells over-expressing Slug and Snail.

2. The Role of EMT in the Formation of Breast Cancer Stem Cell

In this part of the project I am studying the role of EMT in the formation of breast cancer stem cells and examining whether EMT- inducing transcription factors also play a role in the maintenance of CSCs within tumors. To begin with, I wanted to induce EMT in human breast cancer MCF7ras cells through inducible-EMT transcription factors *in vitro*, followed by injection of these EMTed cells into fat pads and later analysis of resulting tumor formation. I wanted to test whether the tumor initiating ability, one of the major characteristics of CSC, of EMTed cells increases as compared to non-EMTed cells.

Since my previous studies and those of my colleagues have shown the leakiness of the tamoxifen inducible system (there was a significant expression of the cloned genes even in the absence of tamoxifen) I have decided to use doxycycline system instead. Human versions of various EMT transcription factors (Snail, Twist1, Slug, Zeb1) and control molecules (Luciferase, BclXL) were cloned into doxycycline Tet-ON vector with or without IRES-GFP sequence (Figure 5). I have chosen to use the IRES-GFP sequence for easier control of the expression status of cloned EMT factors. One of the explanations of getting a higher tumor-initiating ability with EMTed cells might be the anti-apoptotic effect of EMT transcription factors (Maestro et al., 1999; Cheng et al., 2007; Onder et al., 2008). Therefore, I have included anti-apoptotic molecule BclXL as a control. As an initial cell line I have chosen human breast cancer MCF7ras cells which is an epithelial, non-metastatic, widely characterized cell line. I have transduced MCF7ras cell line with the cloned vectors and induced the expression of EMT factors and control factors with doxycycline for 5 days *in vitro*.

Their efficient, non-leaky expression was confirmed by fluorescent microscopy, Western Blot and RT-PCR (Figure 5).

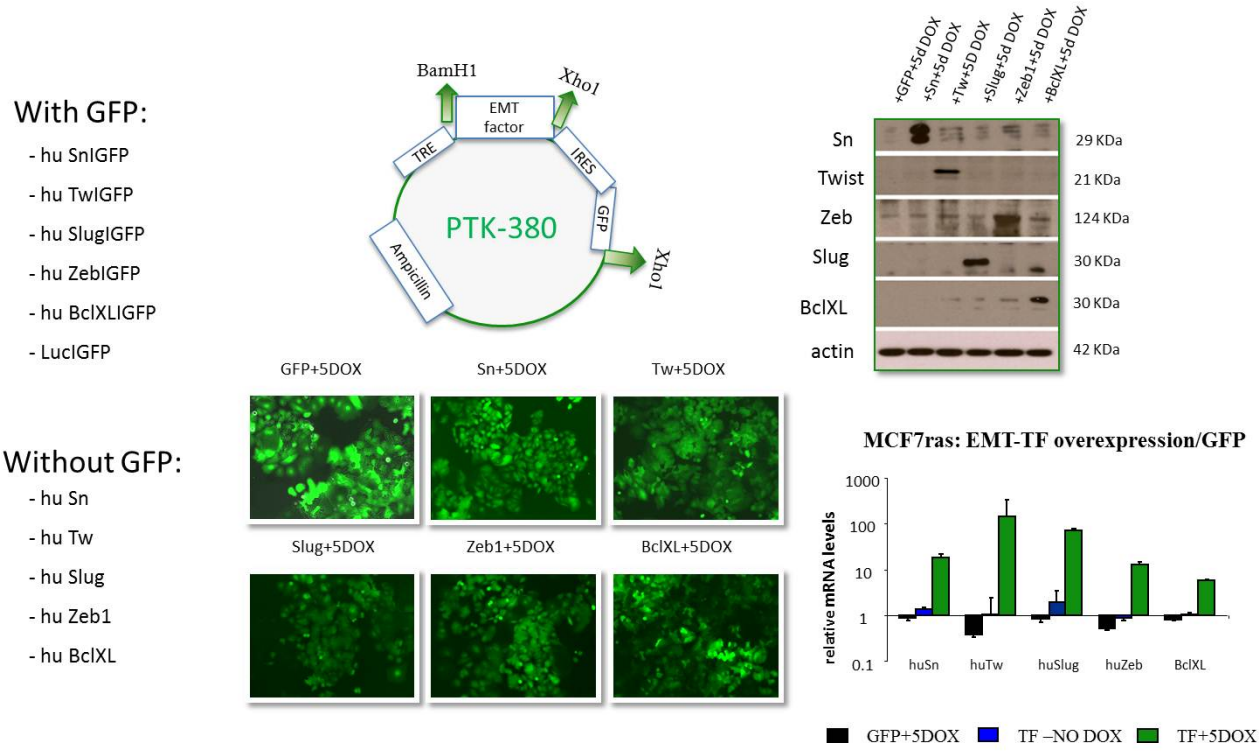


Figure 5: Cloning of doxycycline-inducible vectors. The expression of the cloned genes was verified by fluorescent microscopy, Western Blot and RT-PCR.

The correct activity of the cloned BclXL molecule was examined by incubating MCF7ras-BclXL cells (with or without 5 day pre-treatment with doxycycline) with 10μM apoptotic reagent Doxorubicin. Western Blot analysis was performed to visualize the levels of cleaved PARP molecule (Figure 6).

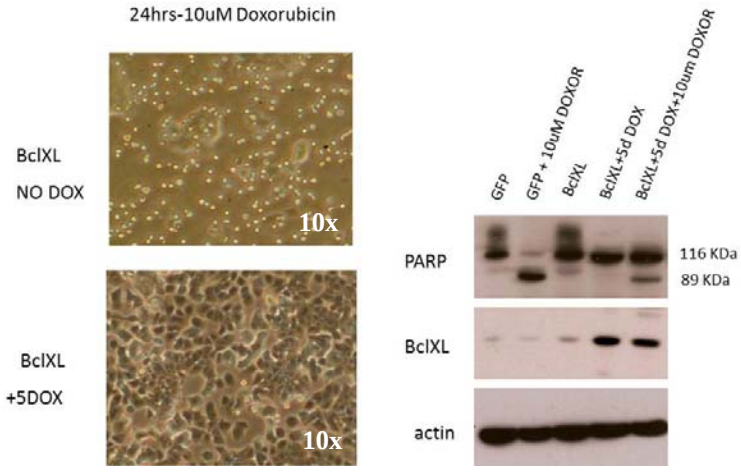


Figure 6: Activity of BclXL molecule in MCF7ras-BclXL cells in the presence or absence of doxorubicin was verified by light microscopy and Western Blot analysis.

However, I was unable to induce EMT with these factors *in vitro* as gauged by absent mesenchymal morphology phenotype and negative Western Blot for changes in the levels of epithelial and mesenchymal markers (data not shown). I tested higher doses (up to 10 µg/ml) and longer exposures of doxycycline (up to 28 days), plating cells on collagen (to test whether more suitable matrix will help), detaching cells from plate through adding trypsin (hoping that temporary break-down of cell-cell adhesions might help induce EMT), and combination of EMT transcription factors without success. Next, I wanted to test whether *in vivo* environment coupled with the expression of EMT factors might be more appropriate to induce EMT. Some authors have reported that *in vivo* environment might help to induce EMT in breast cancer cells (Santisteban *et al* 2009). Mesenchymal stem cells were also shown to increase the *in vivo* growth kinetics and metastatic potential of MCF7ras cells (Karnoub et al 2007) and it might be possible that this effect occurs through the induction of EMT *in vivo*. Therefore, I injected MCF7ras cells which were transduced with EMT factors and control factors into NOD-SCID mice at 10^5 cells/fat pad. I induced EMT-factors and GFP control cells with doxycycline for 14 days *in vivo* (through the doxycycline-containing water). Cells were then extracted from mice, separated by cell sorting using GFP as a marker and analyzed by RT-PCR for the expression of epithelial and mesenchymal markers (Figure 7). The RT-PCR results had shown EMT induction in Snail, Slug and Zeb-1 over-expressing MCF7 ras cells as opposed to GFP control (I didn't retrieve enough Twist-containing cells from mice to perform RT-PCR).

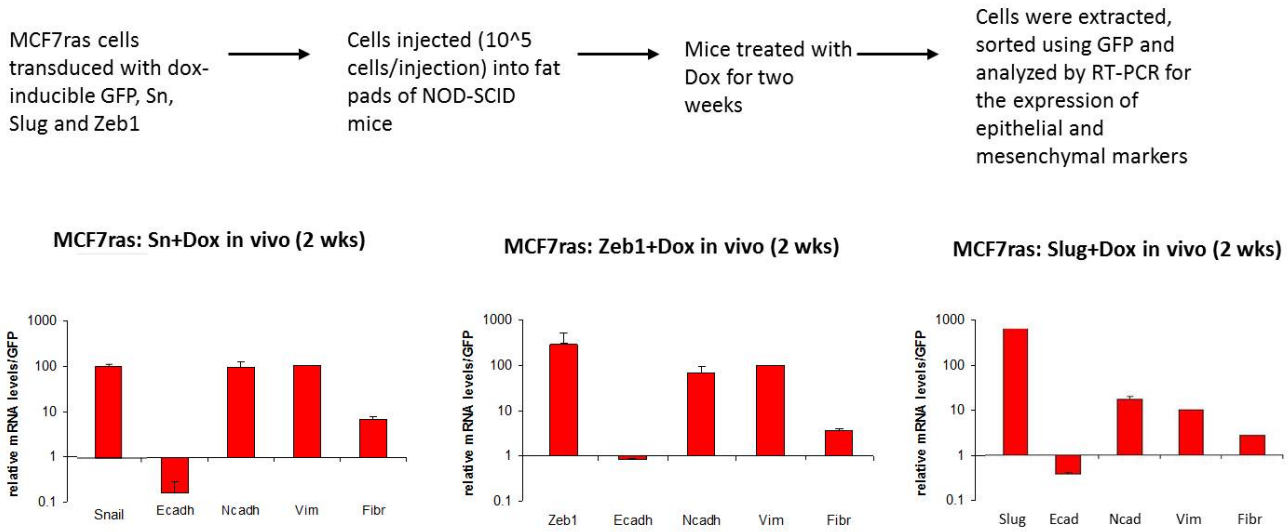


Figure 7: RT-PCR analysis of induction of EMT in MCF7ras cells over-expressing EMT factors *in vivo*.

Since the EMT induction *in vivo* proved successful I then injected 10^4, 10^3 and 10^2 of MCF7ras cells transduced with GFP, BclXL, Snail, Twist1 and Zeb1 cells into NOD-SCID mice (fad pad injections, 4 mice per group). At the day of injection I started treating injected mice for 2 weeks with doxycycline. The number and size of resulting tumors was analyzed 2.5 months post-injection (Figure 8). No increase either in tumor initiation or in tumor size was seen as compared with control GFP cells (except the tumor size of Twist in 10^2 group).

-fat pad injections (10^4, 10^3 and 10^2 cells/injection), Nod/Scid,
-2 weeks in vivo treatment with DOX, 2 months NO DOX

	GFP	Sn	Tw	Zeb	BclXl
10^4	8/8 (0.26)	8/ 8 (0.26)	8/8 (0.2)	7/8 (0.28)	6/8 (0.26)
10^3	4/8 (0.2)	4/8 (0.16)	4/8 (0.25)	5/8 (0.22)	5/8 (0.28)
10^2	1/8 (0.05)	1/6 (0.03)	2/8 (0.28)	1/6 (0.04)	2/8 (0.05)

Incidence of tumors and average weight of tumors (in brackets (g))

Figure 8: Tumor size and incidence of MCF7ras cells over-expressing GFP, Snail, Twist, Zeb1 or BclXL factors.

I could not include all the known EMT-factors in my study so I have chosen to work with Snail, Twist1 and Zeb1 since they rep resent members of different EMT-transcription factor families and they have been extensively characterized in our la b. However, the f ailure of these th ree factors to incr ease the tum or-initiating properties of EMT-ed cells m ade me wonder whether I have used the appropriate EMT - transcription factors. While it is relatively easy to find out which EMT-factors are over-expressed in the stem cell portion of som e breast cancer cell lines (thr ough known CD24/44 m arkers) it is not easy in MCF7ras cells where CD24/44 markers does not seem to work (Figure 9). In order to separate the stem-cell enriched MCF7ras population I have used the m ethod described in the Pece et al, 2010, where the stem cells are separated on the basis of their ability to retain the lipophilic dye PKH26 as a conseque nce of their quiescent nature. I labeled MCF7ras cells with red fluorescent dye and plated them in the mammosphere media. After 12-15 days of m ammosphere culture m ajority of cells within the f ormed mammospheres lost red fluorescence and only small percentage of cells had retained it. These cells were separated by cell sorting and their EMT-expression profiles and mammosphere forming ability were analyzed as opposed to the cells that did not re tain the dye (Figure 9). Mammosphere forming-ability of label-retaining cells was 8 f old higher than that of non-retaining cells confirming that the lab el-retaining cells are enriched for stem cells. RT-PCR profile showed that Slug is greatl y over-expressed (over 600 fold) in th e stem cell-enriched population of MCF7ras cells (Figure 9). Therefore, I have decided to start using Sl ug in m y future exp eriments with MCF7ras cells and the tum or-initiating studies usi ng Slug are currently underw ay. This presented an interesting convergence with the first part of m y project where I have also characterized Slug as being the EMT-factor most likely to be involved in mammary stem cell biology.

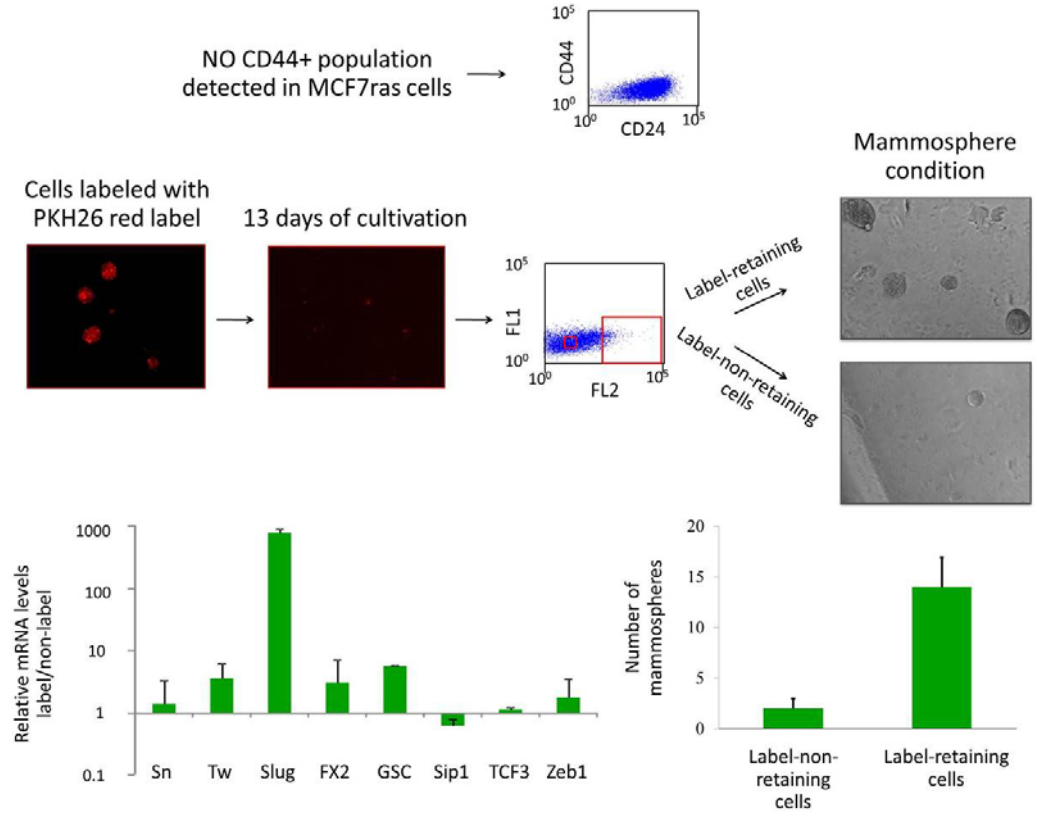


Figure 9: Slug is over-expressed in the MCF7ras cancer stem cell – enriched subpopulation.

Even though MCF7ras cell line was the initial cell line for my studies, similar experiments are being performed by me using HMLE cell line. This will allow me to examine whether EMT has a role in the formation of cancer stem cells in a variety of cancer cell lines. HMLE system is well characterized by our lab and is known to undergo a robust EMT when given appropriate signals. In order to make HMLE cells tumorigenic I over-expressed wild type HER2 molecule in these cells to a level comparable to naturally occurring HER2-positive breast cancer cell line BT474 (Figure 10). I separated the stem cell ($CD24^{-}44^{+}$) and non-stem cell ($CD24^{+}44^{-}$) fractions of these cells using the known CD24/44 cells surface markers and defined the EMT-factors that are up-regulated in the stem cell fraction of HMLE-HER2 cells by RT-PCR (Figure 10). These measurements revealed that while Snail and Twist are over-expressed 5-6 fold relative to non-stem cells, Zeb1 EMT-inducing transcription factor is over-expressed by a factor of ~50 relative to non-stem cells. The contribution of these EMT factors to the stem cell state of HMLE-HER2 cells is now being investigated *in vivo*.

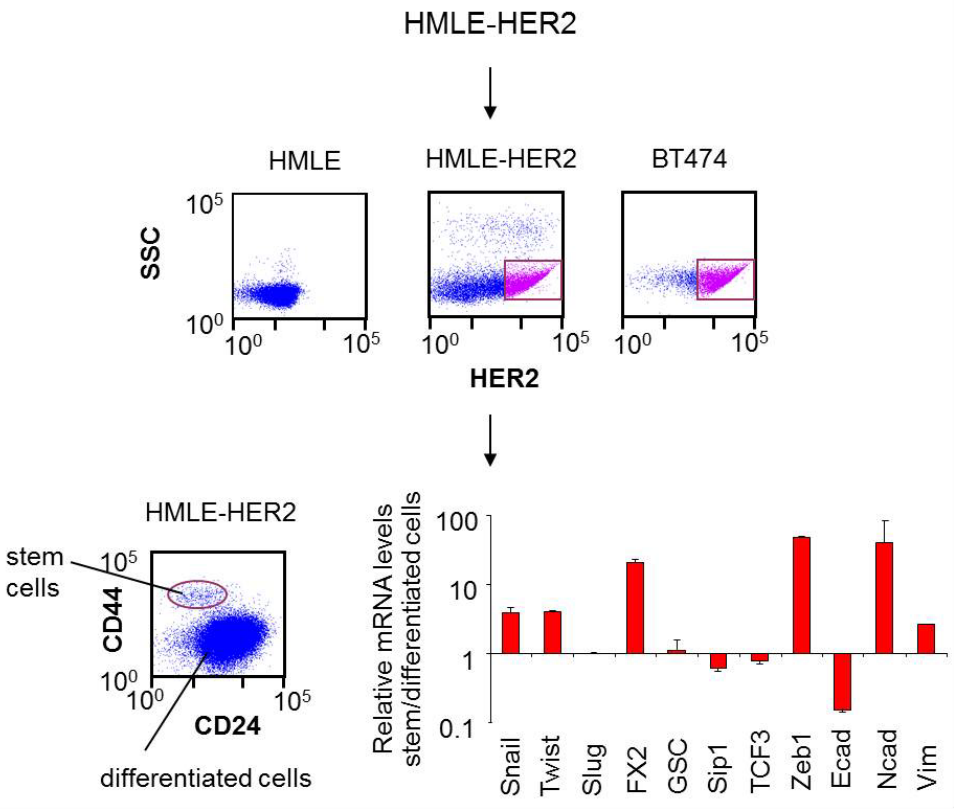


Figure 10: Defining the EMT-factors up-regulated in the stem cell fraction of human breast tumor HMLE-HER2 cells.

KEY RESEARCH ACCOMPLISHMENTS

- I*n vitro* and *in vivo* conditions for effective EMT induction of prim ary murine epithelial cells and hu man breast cancer cells have been optimized.
- EMT-factors most likely to be inv olved in m ammary stem cell and cancer stem cell biology have been identified.
- Preliminary mammary gland reconstitution experiments showed the ability of epithelial-mesenchymal transition to increase the number of mammary epithelial stem cells *in vivo*.

REPORTABLE OUTCOMES

This research project is in its first year of funding and has not led to any manuscript yet. This project was presented in a poster format at the Era of Hope Conference, Department of Defense, Orlando, 2011.

CONCLUSION

The first year of my research project was successful in r eaching the milestones outlined in my work of statement. I had optim ized various steps in m y study design for efficient EMT induc tion in several different cell types, tested new ways of separating stem cell and non-stem cell populations of normal and cancer cells

and identified EMT transcription factors most likely involved in stem cell biology. Major, albeit preliminary, results directly demonstrated that transient induction of EMT increases the number of mammary epithelial stem cells capable of generating an entire mammary ductal tree. With these observations and optimizations I can now move closer to uncovering the connection between EMT and entrance into a stem-cell state. The outcome of these experiments holds important implications for the mechanisms controlling the formation of normal mammary epithelial stem cells as well as the formation of breast cancer stem cells.

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