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The epithelial-mesenchymal transition (EMT) has been found by us to induce normal mammary epithelial cells (MECs) to acquire mesenchymal traits and, in addition, many of the characteristics of mammary epithelial stem cells. At the same time, induction of an EMT in breast cancer cells causes them to acquire to many of the attributes of cancer stem cells (CSCs). However, none of these observations demonstrate that the products of EMT are actually stem cells. To demonstrate this, we developed a protocol for the efficient expression of EMT-inducing transcription factors in vector-infected cells. We have now demonstrated in initial experiments that induction of an EMT in normal mouse MECs results in a ~100-fold increase in their ability to generate mammary ductal trees. Hence, an EMT can indeed increase the formation of normal mammary epithelial stem cells. Attempts to produce the parallel result with human breast cancer cells have not advanced as far. Since we can now produce large numbers of cells with properties of human breast cancer stem cells, we have used these to screen for drugs that preferentially kill human CSCs and have found two agents that preferentially eliminate CSCs.

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**Generation of Breast Cancer Stem Cells by the EMT**
**Robert A. Weinberg, Ph.D.**

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**Introduction** The progression of benign human breast cancer cells into high malignant derivatives is accompanied by the acquisition of mesenchymal cell characteristics, motility, and invasiveness. These multifaceted changes in cell phenotypes are involved in the transdifferentiation program termed the epithelial-mesenchymal transition (EMT). Recently, we discovered that in addition to conferring cell-biological traits of high-grade malignancy, the EMT program also coverts more differentiated mammary epithelial cells into derivatives having many of the attributes of epithelial stem cells, a conversion that operates in both immortalized and in transformed, tumorigenic mammary epithelial cells. However, it remained unclear whether such EMT products are actually stem cells or only cells having many of the properties of stem cells. 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. In addition, we have exploited our ability to produce large numbers of human CSCs to screen for pharmacologic agents that can preferentially kill breast CSCs relative to the non-CSCs in the tumor. Agents of this sort are highly desirable, as diverse observations indicate that CSCs are generally much more resistant to conventional chemotherapeutic treatments, enabling the CSCs surviving initial chemotherapy to regenerate the tumor after cessation of treatment.

**Body**

**1. EMT and the founding of macroscopic metastases** As described previously, a central supposition of the epithelial-mesenchymal (EMT) model is that this change of cell differentiation empowers carcinoma cells to become invasive and metastatic. While this is widely assumed, the evidence providing direct support for this remains to be produced. Accordingly, we were interested in exploring the question of whether the induction of the EMT program in cancer cells that are already disseminated within various sites throughout the body, will allow these cells to colonize the tissues and form macroscopic metastases. Implicit in this model is the notion that the activation of this program is sufficient on its own to impart this trait of high-grade malignancy. More specifically, we have found previously that activation of the EMT program imparts stem-cell traits to normal or neoplastic epithelial cells. Among these traits is the self-renewal potential that we believe is required in order colonize sites of metastatic dissemination.

Our initial experimental plan was to exploit fusion proteins between the estrogen receptor (ER) and two of the most intensively studied EMT-inducing transcription, Twist and Snail; in each case, addition of tamoxifen, a ligand of the ER, to cells expressing such a fusion protein is expected to liberate the fusion protein from cytoplasmic sequestration by heat shock proteins, allowing these proteins to move into the nucleus and function as active transcription factors (TFs). We then expressed each of these fusion protein TF constructs in breast cancer cells and introduced these, in an “experimental metastasis model” into the venous circulation of mice, allowing these cells to lodge in the lung microvasculature and to extravasate into the lung parenchyma. Thereafter, we activated the TFs by systemic administration of tamoxifen. This experiment was ultimately stopped, because even in the absence of tamoxifen administration, there was already a basal level of Twist and of Snail function.

In response to this, we have switched to an alternative strategy of inducing the function of these EMT-inducing TFs. Thus, we now have tetracycline-inducible constructs that encode these TF, and indeed, upon addition of doxycycline to cells expressing these two constructs, there is a 1000-fold induction of the function of these TFs and an induction of the EMT *in vitro*. These cells are currently (10/09) being introduced into mice in order to gauge the biological responses, specifically those enumerated above.

**2. EMT and the formation of normal mammary gland stem cells and tumor-initiating breast cancer stem cells** One question of great interest to us is the nature of the normal cell-of-origin that undergoes transformation to yield the self-renewing cancer stem cells (CSCs) within mammary tumors. One way to examine these mechanisms, we reasoned, was to induce normal mammary epithelial cells (MECs) to pass through an EMT and generate normal mammary stem cells (SCs). Related to this is the antecedent

question of whether we could create normal mammary SCs that could then serve as the targets of transformation by introduced oncogenes.

Accordingly, we initially began to pose the question of whether forced passage through an EMT can indeed form normal mammary gland SCs. We first spent a number of months attempting to optimize the conditions of infection of normal mouse MECs by lentivirus vectors. Indeed, we developed conditions that now allow a ~90% infection efficiency, which is more than 10-fold greater infection rate than was previously possible.

In additional preliminary experiments, we undertook to determine the identities of the various EMT-inducing TFs that might function specifically in the context of the mammary gland to create or at least be associated with normal mammary gland SCs.

The operational test for this involved the implantation of various MEC populations into cleared mouse mammary stromal fat pads, with the hope that the proportion of mammary gland-generating cells would be greatly increased by prior exposure of cells to an EMT-inducing TF.

To address this issue, we undertook to separate the normal mammary gland SCs, the transit-amplifying/progenitor cells, and the differentiated MECs from mouse mammary glands on the basis of their CD49f/CD61 cell-surface antigen display. The goal was to determine which of the half dozen or so EMT-TFs was specifically associated with mammary gland SCs (Figure 1).

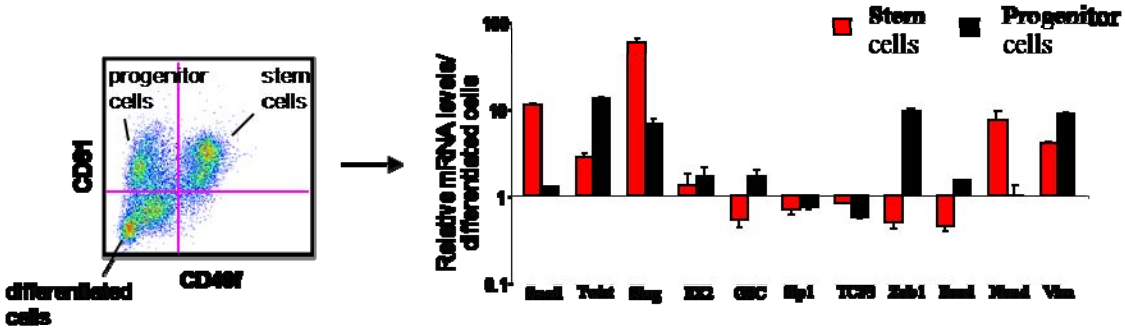


Figure 1. (left panel) Use of fluorescence-activated cell sorting (FACS) to fractionate normal mouse MECs into differentiated, progenitor and stem cell subpopulations. ((right panel) use of RT-PCR to quantify the relative levels of various EMT-inducing TFs expressed in either the SC or progenitor population relative to the differentiated cell population.

As we found, relative to the differentiated mammary MECs, the normal mammary gland SCs expressed very high levels of Slug (~90x) and Snail (~15x) relative to differentiated mammary epithelial cells. This persuaded us that Snail and Slug were likely to be excellent candidates for the regulators of the mammary SC state.

Accordingly, we used tetracycline-inducible expression constructs encoding either the Slug or Snail EMT-inducing TFs as well as green fluorescent protein (GFP). We introduced these constructs, via lentivirus vectors, into normal mouse MECs *in vitro*, activated the expression constructs by exposure to tetracycline for 10 to 12 days, mixed with red fluorescent protein (RFP)-labeled control MECs, and then implanted these mixed cell populations into cleared mammary stromal fat pads (Figure 2).

We then monitored the behavior of these cells, implanted at various dilutions, at various time points after implantation. (Figure 2, above) At one week after implantation, we noticed no difference in the numbers of cells that had or had not been induced to undergo an EMT. However, when monitored at 6 weeks post-implantation, the abilities of these cells to regenerate mammary ductal trees, when compared with control cells, was increased by 100-fold.

This experiments, because it was internally well-controlled, already allowed some solid conclusions; nonetheless, it requires reproduction. Most importantly, we can now conclude that not only does an EMT create cells with properties of epithelial stem cells, but beyond this, by the most rigorous criterion, it creates cells that are indeed mammary epithelial stem cells. We are, as a consequence, now poised to determine whether the SC products of the EMT are indeed the preferred targets of transformation that generate mammary CSCs and thus mammary tumors, as described above. These experiments are currently underway.

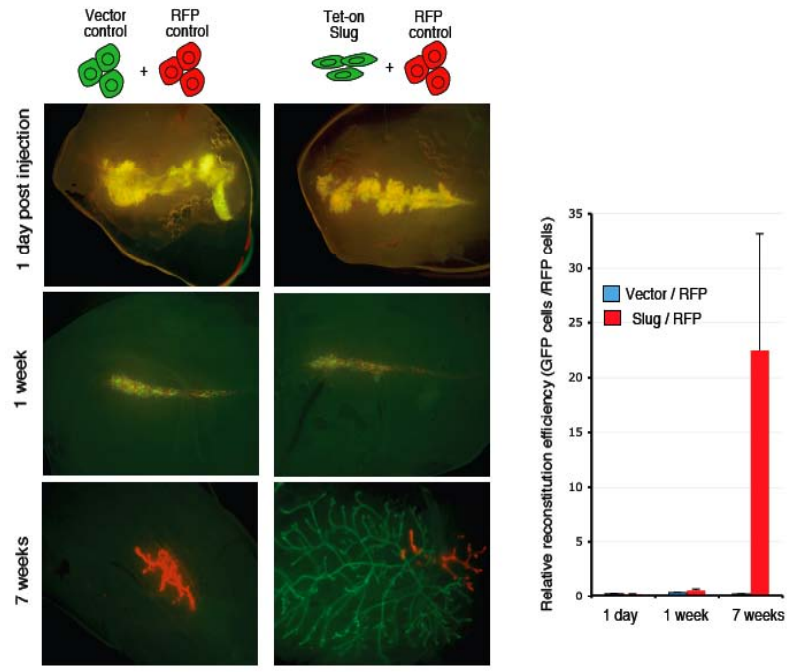


Figure 2. Left panels: images of mammary fat pads injected with the indicated cell types at different time points. Right graph: quantification of the ratio of contribution by GFP vs RFP cells.

**3. The EMT and its effects on CSCs and metastasis** In these experiments, we intended to induce human breast cancer cells to undergo an EMT either *in vitro* or *in vivo* in order to observe EMT-related biological effects. To begin, we used MCF7ras human breast cancer cells that were induced to undergo an EMT through the actions of the Twist or Snail transcription factor, expressed in each case as a tamoxifen-activatable ER fusion protein. Upon tamoxifen treatment, neither the Twist-ER nor the Snail-ER fusion constructs induced significant EMTs after 10 days of tamoxifen treatment. However, the joint expression of both TF s led to clearly visible morphological conversion to a mesenchymal state with some induction of vimentin by 5 days, while E-cadherin was still not repressed after 10 days of treatment. Nonetheless, in spite of the incomplete induction of an EMT, we ascertained whether these cells had higher percentages of mammary CSCs. In fact, as seen below, there was a clear increase in the percentage of CSCs in Snail-ER or Snail+Twist ER; however, in both cases, the extent of induced increase of the CSC population was several-fold and therefore only marginal.

These attempts at increasing the number of tumor-initiating cells gave results that were far less than we anticipated, which caused us to reconfigure this experimental protocol. Thus, we made six major changes in the way this experimental protocol is designed. First, we substituted a human Twist reading frame for the mouse Twist that was used in the initial experiments. Second, we introduced the reading frames of Snail and Twist into a retroviral vector in which each was expressed together with a GFP marker, the two reading frames being separated by an IRES segment; this will allow us to monitor more accurately the

| | GFP | TwER | SnER | Sn+TwER |
|---|---|---|---|---|
| 10^5 | 12/12 | 12/12 | 12/12 | 12/12 |
| 10^4 | 0/6 | 2/6 | 4/4 | 4/6 |

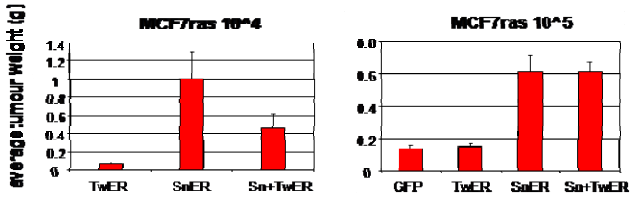


Figure 3 (top, bottom) Limiting dilution experiments indicate a marginal effect of expressing either Twist-ER (Tw-ER) or Snail-ER (Sn-ER) in increasing the representation of CSCs; however co-expression of both led to a larger, albeit still-modest increase in the repesentaiton of CSCs

efficiency with which vector infections proceed. Third, we replaced the ER fusion constructs with a tetracycline-inducible transcriptional promoters, which allowed us lower, uninduced basal levels of expression and a greater relative increase of expression/function upon induction. Fourth, we used as a control vector one that encodes the Bcl-XL protein in order to gauge the contribution, if any, of the anti-apoptotic effects of the EMT-inducing proteins specified by the experimental vectors.

A fifth change involves a shift in the type of human breast cancer cells. We began to use experimentally immortalized HMLE cells that were then transformed through expression of high levels of the HER2 protein; this protein is now expressed at levels comparable to those expressed by the BT474 human breast cancer cells, as validated by FACS analyses (Figure 4). A detailed study of the CSC fraction of the resulting HMLE-Neu cells using RT-PCR measurements revealed that while Snail and Twist are overexpressed 5-6 fold relative to the non-SCs, the Zeb1 EMT-inducing TF is overexpressed by a factor of ~50 relative to the non-SCs (Figure 4). Hence, Zeb1 is now being included as a third EMT-inducing TF in experiments currently underway designed to prove definitively that transient induction of an EMT by one or another EMT-inducing TF can result in a great increasing in the numbers of tumor-initiating CSCs. These experiments are designed to deliver definitive tests of the hypothesis that passage through an EMT has a profound effect on the number of CSCs within a population of breast carcinoma cells.

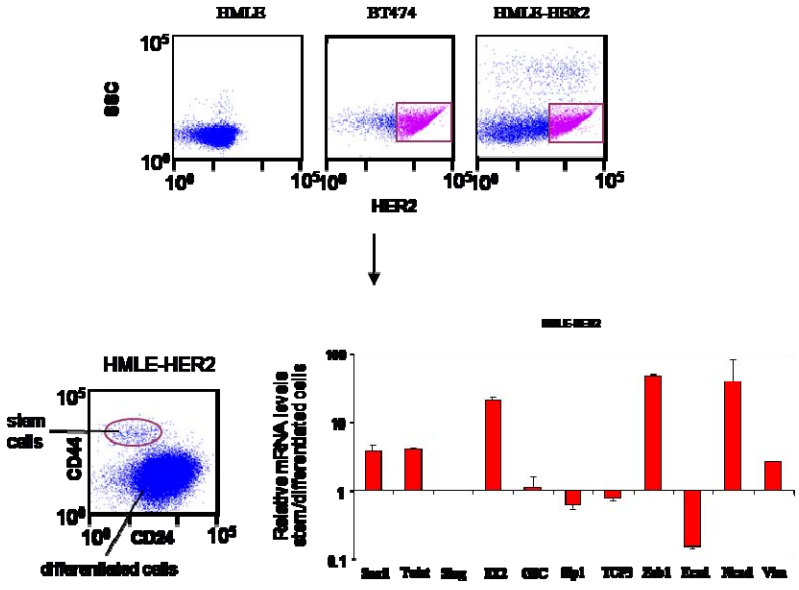


Figure 4 (above) FACS analyses used to verify the levels of expression of the HER2 protein in HMLER-HER2 cells relative to cells of the human BT-474 breast cancer cell line; (below) use of FACS (left panel) to fractionate CSCs from non-CSCs in these transformed cell population, which were then (right panel) subjected to RT-PCR analyses in order to determine the relative expression levels of various EMT-inducing TFs.

4. **Screen for anti-CSC therapeutics** As described previously, we intended to screen a library of more than 16 thousand compounds for those agents that preferentially kill cells with cancer stem-cell (CSC) properties versus bulk cancer cells that do not have these properties. This project has now advanced to a very successful conclusion. To begin,

we generated large numbers of cells with CSC properties by taking experimentally transformed HMLER human breast cancer cells and forcing them through an epithelial-mesenchymal transition (EMT) by downregulating E-cadherin. We found that such cell populations, having passed through an EMT, exhibited a ten-fold increase in cells with the characteristic $CD44^{high}/CD24^{low}$ cell-surface antigenic phenotype, as revealed by fluorescence-activated cell sorting (FACS), This was in consonance with our previously published work that indicated that normal and neoplastic human mammary epithelial cells passing through an EMT acquire many of the attributes of normal and neoplastic stem cells, respectively. Hence, cells that have passed through an EMT acquire mesenchymal markers and, at the same time, exhibit a phenotypes that closely resemble those of stem cells.

Indeed, when we took transformed human mammary epithelial cells and forced them through an EMT via downregulation of E-cadherin, such cells acquired a ~100-fold increase in tumor-initiating ability, in consonance with the notion that they contained greatly increased numbers of tumor-initiating cells. Interestingly, such cells, as we have found in other contexts, proliferate more slowly than non-stem cells in culture. Moreover, we discovered, as we had predicted, that such CSCs exhibited a significant increase in resistance to the cytotoxic effects of commonly used chemotherapeutic drugs. For example, they were 20-fold more resistant to paclitaxel and 5-fold more resistant to doxorubicin. This echoes the work of others demonstrating that CSCs within breast tumors exhibit increased resistance to some of the commonly deployed chemotherapeutic agents. The mechanistic reasons for this increased resistance remain elusive. Of additional interest, the normal, untransformed precursors of the breast cancer cells used in this experiment – the immortalized HMLE cells – also exhibited an increase resistance to drug killing after having passed through an EMT. This provide indication, to our knowledge for the first time, that such acquired resistance is intrinsic to passage through an EMT rather than passage from an untransformed to a transformed state.

In consonance with these observations were the substantial increases in $CD44^{high}/CD24^{low}$cells, as gauged by FACS analysis, after treatment with either paclitaxel or doxorubicin, confirming the selective killing by these conventional chemotherapeutic agents of non-CSCs within tumor stem cell population. Thus, the percentage of $CD44^{high}/CD24^{low}$cells increased from 7.9% to 90.3% following conventional drug treatment. Among the untransformed, immortalized HMLE precursors of HMLER tumor cells, the percentage of SCs increased from 1.7% to 47.2% following drug treatment. In both cases, these results validated the notion that chemotherapeutics in current use actually result in a substantial increase in the number of CSCs or immortalized SCs, rather than eliminating these cells. Of course, in the case of breast cancer, this leaves the residual surviving CSCs able to regenerate the tumor mass following cessation of therapy.

Of the ~16,000 compounds that were screened for their killing effects in a high-throughput screen, 32 showed significant preferential killing of CSCs over non-CSCs. Of these, four were evaluated for further characterization, because of their availability, and one was ultimately characterized in great detail because of its 8- to 10-fold increased toxicity for CSCs versus non-CSCs. Strikingly, we found that salinomycin treatment reduced the representation of CSCs down to 0.2%, more than 300-fold decrease in the relative representation of these cells in the cell population. In addition, we noted that salinomycin halted tumor growth in vivo and caused extensive necrosis within tumors.

This paper was published in *Cell* (Gupta, P. et al, 2009, 138:1-15) and has attracted much favorable attention. It establishes a proof-of-principle that one can develop high throughput screens for drugs that preferentially target breast CSCs. Importantly, this screening was undertaken with a relatively small library of compounds, and the hits that were obtained indicated that there are likely to be many dozens of compounds present in existing chemical compound libraries that are likely to have comparable effects in the preferential killing of breast CSCs.

**Key Research Accomplishments**

- The induction of an EMT has now been demonstrated to convert a normal murine mammary epithelial cells into mammary epithelial stem cells that can, following orthotopic implantation, generate an entire mammary ductal tree. This observation offers us for the first time a definitive proof of the connection between EMT and entrance into a stem-cell state.
- Screening of a compound library of 16,000 pharmacologic agents has yielded a number of compounds that preferentially kill breast CSCs relative to non-CSCs. This represents the first proof of principle that it is possible to identify compounds that deprive a tumor of its subpopulation of CSCs. Extension of this strategy to larger scale screens should yield a significant number of chemical agents that will exhibit potent effects on the treatment of solid tumors.

**Reportable Outcomes**

This research has led to the publication of a research report on the successful identification of compounds that selectively kill cancer stem cells:
Gupta, P.B., Onder, T.T., Jiang, G., Tao, K., Kuperwasser, C., Weinberg, R.A. and Lander, E.S. (2009) Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell, 138:1-15.
This grant also supported the writing of a review article:
Gupta, P.B., Chaffer, C.L. and Weinberg, R.A. (2009) Cancer stem cells:mirage or reality? (2009) *Nature Medicine* 15:1010-1012.

**Conclusion** One major implication of the reported work derives from experiments reported herein in which we have succeeded in greatly increasing the proportion of normal mammary epithelial stem cells in a population of differentiated mammary epithelial cells, doing so by expressing EMT-inducing transcription factors in these cells transiently. Since the EMT can otherwise be induced by contextual signals that have been and are being identified, this suggests that a protocol can be developed involving exposure of differentiated epithelial cells to a mixture to EMT-inducing signals that can substantially increase the proportion of normal epithelial stem cells in these cultures. This holds, in turn, important implications for future modalities of regenerative therapy designed to restore damaged epithelial tissues through implantation of normal, syngeneic epithelial stem cells.

A second major outcome of this work is the demonstration that one can produce large numbers of breast cancer stem cells (via forced passage through an EMT) that can then be used in high-throughput screens to identify compounds that preferentially kill breast cancer stem cells relative to more differentiated cancer cells. In an initial proof-of-principle screen, we discovered two compounds – salinomycin and abamectin – that were characterized in detail. These showed enhanced killing of breast CSCs created by forced passage through an EMT, and as such, represents a model for how millions of compounds can be screened by others who are intent on discovering drugs that preferentially kill breast cancer stem cells.