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Sheep, Wolf, or Werewolf: Cancer Stem Cells and the Epithelial-to-Mesenchymal Transition

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Abstract

Multiple cancers contain subpopulations that exhibit characteristics of cancer stem cells (CSCs), the ability to self-renew and seed heterogeneous tumors. Recent evidence suggests two potentially overlapping models for these phenotypes: one where stem cells arise from multipotent progenitor cells, and another where they are created via an epithelial to mesenchymal transition. Unraveling this issue is critical, as it underlies phenomena such as metastasis and therapeutic resistance. Therefore, there is intense interest in understanding these two types of CSSs, how they differ from differentiated cancer cells, the mechanisms that drive their phenotypes, and how that knowledge can be incorporated into therapeutics.

Keywords

EMT; Cancer Stem Cells; Epithelial-Mesenchymal Transition; Cellular Plasticity

1. Introduction

It was first proposed in the mid-1800's that neoplasias are comprised of two subpopulations--stem cells and differentiated cells (reviewed in [1]). Over a hundred years later, evidence of these distinct populations were seen in leukemia. Cancer stem cells (CSCs) were isolated by selecting for cell-surface markers and then showing that these cells could establish disease by transplanting them into new hosts in limiting dilutions [2; 3]. These CSCs, or alternatively, tumor initiating cells, exhibit the capacity to self-renew and seed new tumors with the same cellular heterogeneity in immunocompromised mice [4; 5]. Using similar approaches, stem cells were subsequently isolated from a range of solid

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Conflict of Interest

JTC and SAM have no conflicts of interest.

tumors, including breast [6], brain [7; 8], melanoma [9], prostate [10; 11], colon [12; 13], pancreas [14; 15], and lung [16].

To identify the subpopulation of cells that exhibit the CSC phenotype, characteristic cell surface markers can be used. A common one is CD44, a glycoprotein [17] that was originally identified as a cell surface protein in NIH-3T3 cells [18]. Subsequently, it was discovered that CD44⁺ cells (in conjunction with secondary markers such as CD24) have an increased ability to seed new tumors in breast [6], prostate [10], pancreatic [14], ovarian [19], and colon [20] cancers. However, its accuracy remains controversial, and it does not appear to be universal as a CSC marker [21]. Cell surface markers for CSCs have also been selected from those expressed during the normal hierarchical development of the tissue. As one example, acute myeloid leukemia cells expressing CD34⁺/CD38⁻, characteristic of early hematopoietic precursors, have a capacity to seed new tumors [2; 22]. Similarly, melanoma-initiating cells express CD271, a marker for development of the neural crest, the precursor cells of melanocytes [23].

In several different kinds of cancer, CSCs have been proposed to originate from stem cells through transformation (reviewed in [1]). However, work over the last decade has revealed unexpectedly that differentiated epithelial cancer cells can also serve as a source of CSCs by reactivation of a latent developmental process called an epithelial-mesenchymal transition (EMT) (reviewed in [24]). In this review, we will summarize the current knowledge on the connections between the EMT and stem cells, in particular the plasticity observed in cancer cells during disease progression and cover implications for clinical treatment.

2. The developmental EMT program can be reactivated in cancer

EMT is a reversible developmental process that induces cells to convert from the epithelial to mesenchymal state, engendering a profound change in cellular and molecular properties ([25] and other reviews). During the development of multicellular organisms, EMT is coupled sequentially with the reverse process, called the mesenchymal-epithelial transition (MET), to form complex structures and organs including the kidney, lung, and skin [26; 27]. In short, the EMT process demonstrates that the cells found in embryos possess an enormous plasticity that is driven by reversible molecular programs.

Although the EMT program is normally activated during development, it can also be aberrantly activated in adult cancer tissues [28]. The developmental and cancer programs share many similarities such as the signals that can initiate an EMT and the phenotypic changes that result [29; 30]. However, the extent of the differences between the two programs is not yet fully clear.

The first evidence that cancer cells can exhibit characteristics of the EMT program came from *in vitro* studies [31; 32]. Cells lines from a range of distinct tissues exhibit different phenotypes including epithelial or mesenchymal properties. Although there is a wealth of evidence that EMT can occur *in vitro*, there remains some controversy on its relevance in the clinic [33; 34; 35]. However, there is now direct *in vivo* evidence in a PyV-mT mouse model that support its importance in metastasis. Carcinoma cells that migrate away from the primary tumors have been shown to contain an activated mesenchymal-specific expression marker [36]. When the FSP1 marker gene was deleted in a mouse model, the number of metastases was reduced, showing that the mesenchymal transcriptional program is an important requirement for metastasis.

EMT was first proposed as a contributor to cancer due to a remarkable concordance between the mesenchymal phenotype and the characteristics required for cells to metastasize. Specifically, they need the ability to detach from other cells and the extracellular matrix,

migrate through the lymphatic or circulatory system, and colonize a distant site [37]. Phenotypically, conversion of an epithelial cell to a mesenchymal phenotype is marked by a dramatic loss of adhesion to adjoining cells and the extracellular matrix, increased capacity for migration and invasion [38], as well as increased resistance to apoptosis [39]. Thus, while an epithelial cell lacks the characteristics required for metastasis, an EMT can provide these traits [30; 40].

Cellularly, the key distinction between the epithelial and mesenchymal states is the loss of cell-cell adhesions. In epithelial cells, those adhesions are mediated by E-cadherin at adherens junctions. Loss of E-cadherin leads to their disruption and is considered a hallmark of EMT [41]. This is accompanied by a range of profound changes in cellular phenotypes described above. Furthermore, it has been found to be a critical step in the development of invasive pancreatic carcinoma [42] and has been reported in a range of cancers (reviewed in [43]).

Although genetic mechanisms can directly lead to the loss of E-cadherin [44; 45; 46; 47; 48], it is believed that more often it is functionally silenced by external signals (for a summary figure, see [49]). From developmental models, there is much known about the molecular pathways that induce and maintain an EMT. The major accepted signals are from the TGF- β superfamily [50; 51] and Wnt pathways [52], although many other pathways are involved, such as Notch, Sonic Hedgehog, EGF, and IGF [53]. High expression of TGF- β is a marker for progression in breast cancer [54]. Activation of TGF- β can lead to EMT in carcinoma cells, and has also been linked to increased invasive and metastatic ability in mouse models and as markers in human cancers [55; 56; 57]. In many epithelial cells, TGF- β activation leads to loss of E-cadherin, at least in part, due to a Smad-dependent pathway. The Smad transcription factors, along with Snail co-factors, can repress E-cadherin expression [58; 59] via E-box elements in the E-cadherin promoter [58; 60]. The E-box elements are required for repression of E-cadherin via transcription factors [61], and ectopic expression of embryonic transcription factors can induce EMT [62; 63]. Other E-box binding repressors include the Snail relative Slug [64; 65], Zeb1 [66], and Zeb2 [67]. Indirect repressors of E-cadherin include Twist [68; 69] and others [70].

A potential source of EMT-inducing signals is cancer-associated fibroblasts (CAFs). In cell culture and co-culture models, it has been shown that they secrete factors can induce an EMT in a number of different cancer cell types ([71; 72; 73; 74]). In sum, much is known about the signaling pathways that drive the EMT process, but the drivers of the signals are still being sorted out.

Another area that is still under investigation is the relationships among the EMT inducers. To understand their distinctions, the global gene expression profiles of mammary epithelial cells ectopically expressing Twist, Snail, Goosecoid, TGF- β , and an siRNA for E-cadherin were compared [75]. While there are inducer-specific transcriptional programs, a shared signature was identified. This suggests that, regardless of the inducer, a core EMT program is shared across physiological conditions [76].

3. Epithelial cancer cells acquire stem cell properties through an EMT

Although they shared molecular and phenotypic similarities, an understanding of the EMT and stem cell phenotypes was advanced independently until the surprising discovery that epithelial cells induced to undergo an EMT acquire stem cell properties [77; 78]. After EMT, human mammary epithelial cells acquire a mesenchymal phenotype and show the CD44^{high}/CD24^{low} antigenic profile that is characteristic of breast CSCs [77]. They have an increased ability to form mammospheres *in vitro*, demonstrating an enrichment of stem cells as well as the capacity to self renew [78]. The mesenchyme-like cells derived from an EMT

are more tumorigenic and have an increased ability to seed tumors in mice, as demonstrated by limiting dilution assays. Further strengthening the association between EMT cells and stem cell properties, the CD44^{high}/CD24^{low} fraction of human mammary epithelial cells exhibits a mesenchymal-like expression profile, with low E-cadherin and increased expression of EMT inducers Snail, Twist, and others [77]. In a non-mammary model, Zeb1 induces expression of stem cell markers and is associated with sphere-formation ability in pancreatic cells [79].

Perhaps the clearest molecular connection between stem cells and the EMT lies in the Wnt signaling pathway. Wnt is important for the maintenance of the stem cell phenotype in both bona fide stem cells in a mouse model [80; 81] and in an EMT in cell lines [82]. In development, it plays a critical role in inducing an EMT [83; 84]; indeed, deletion of the Wnt effector β -catenin leads to lethal defects at gastrulation [85]. In cancer, activation of β -catenin appears to promote tumor progression [86; 87] and is found in the CSC population in a range of cancers [88; 89]. Wnt has similarly been shown to induce an EMT in cell lines via stabilization of Snail [90; 91] and induction of the expression of Slug [92] and Twist1 [93].

Although Wnt can induce an EMT, activation of the canonical Wnt pathway depends on processes central to an EMT. The Wnt effector β -catenin can be localized to adherens junctions, linked to E-cadherin [94; 95]. During an EMT, loss of E-cadherin is seen concomitant with nuclear translocation of β -catenin and induction of its target genes [94; 96]. This demonstrates that the phenotypes shared between stem cells and EMT-derived cells are driven, at least in part, by the same molecular mechanism.

4. The clinical impact of CSCs and EMT

Currently, evidence is accumulating for a model where cancer cells can reactivate an EMT to generate cells with CSC abilities by co-opting molecular programs from development or stem cell maintenance. However, the full extent of the similarities or differences between cancer stem cells derived from embryonic or progenitor stem cells (SC-CSCs) and those derived from differentiated cells through an EMT (DC-CSCs) are not yet fully appreciated, and will need to be explored more thoroughly to obtain an understanding of their impact on the progression and treatment of cancer in the clinic.

Nevertheless, clinical evidence of the association between an EMT state and clinical outcomes is accumulating. In some cancers, EMT markers have been found to be predictors of clinical outcomes. Snail expression is predictive of poor prognosis in hepatocellular carcinoma [97] and breast carcinoma [98]. Zeb2 is predictive of poor prognosis in ovarian carcinoma [98]. And activation of EMT is linked to poor survival in colorectal cancer [99]. Furthermore, it has been hypothesized that tumor aggressiveness of primary breast cancer may correlate with the proportion of CSCs within a corresponding tumor [100].

In addition, stem cell and EMT markers are indicative of local or distant recurrence.

The metastasis of breast cancer is correlated with both Snail [101] and Slug [102] expression. Further strengthening the link between EMT and metastasis in breast cancer, both EMT and stem cell markers are found in circulating tumor cells [103; 104]. Disseminated cells from breast cancer (found in the bone marrow) exhibit an increase in the CD44^{high}/CD24^{low} stem cell marker [105]. Demonstrating the specificity of the connection between EMT and metastasis, Snail expression can lead to metastasis even after the initiating oncogenic signal is lost [106]. Finally, in head and neck squamous carcinoma, induction of EMT is associated with metastasis [107; 108].

5. CSCs complicate therapies

The SC-CSC sub-population within tumors is less responsive to standard therapies (reviewed in [109]). After chemotherapy, it is common to see an increase in the fraction of CSCs [110; 111; 112; 113], although the opposite has also been reported [114]. One explanation for the decreased sensitivity is that chemotherapeutic strategies target dividing cells, and thus are not effective against CSCs [115], which are typically quiescent [116]. Arguing against this model is the observation that CSCs can exhibit increased proliferation [117; 118]. Regardless, an alternative explanation rests on the observation that CSCs express genes that confer chemotherapy resistance (reviewed in [119]). Thus, while it is well established that CSCs have increased resistance to chemotherapies, the mechanism is still under investigation.

Similar to SC-CSCs, DC-CSCs have also been shown to be resistant to therapies in cell line and xenograft models. This has been reported in cell lines from a number of different organs including colon [120], ovary [121], and breast [122]. Resistance has been linked to the expression of the EMT inducers Snail or Slug [123; 124], Zeb1 [79; 112; 125; 126; 127], and Twist [122]. The resistance to chemotherapies are linked to activation of anti-apoptotic pathways. Twist can lead to the upregulation of Akt2 expression, increasing the anti-apoptotic signal [122]. P53-induced apoptotic genes have been shown to be repressed by Twist [128] and Slug [129]. In mouse models, it has also been reported that chemotherapies may increase metastasis by activation of an EMT through a TGF- β -dependent pathway [130].

For targeted therapies, there is also evidence in cell lines that the EMT state of a cell can determine its response. In various types of cancers, the presence of EMT markers is correlated with resistance to Egfr inhibitors [131; 132; 133]. And for Ras inhibitors, EMT markers have been found to be a better predictor of sensitivity than Ras mutation [134].

Finally, both SC- and DC-CSCs have been shown to be resistant to radiation therapy in cell line models. This is seen in breast stem cells [135] mediated through the Wnt pathway [136]. And expression of EMT inducers renders breast and ovarian cells resistant [124].

Although CSCs can be resistant to a wide range of therapies, a potential approach to restore sensitivity in the DC-CSC sub-population may be by reestablishing the epithelial state. In lung cancer cell lines, restoration of E-cadherin can decrease the resistance to kinase inhibitors [137]. Loss of the EMT inducer Twist can reduce the degree of multidrug resistance in breast cancer cells [138]. Also, overexpression of the Zeb1 repressor miR-200 family members can also restore drug sensitivity across a range of cell types [79; 139; 140; 141; 142], as well as overexpression of the Snail repressor miR-34 [143]. These results show that the epithelial and mesenchymal states, even within the same genetic background, represent distinct signaling programs that confer differing responses to therapy. Consequently therapeutic strategies must be developed that can target the two populations.

6. Therapeutic strategies to target CSCs

Prior results demonstrate strongly that both SC-CSCs and DC-CSCs have profoundly different responses to a wide range of therapies, as compared to the non-stem population of the tumor. The evidence strongly supports the notion that therapies must specifically target the CSC population to produce a robust reduction in metastasis. There are four potential therapeutic strategies being investigated : 1) selectively kill stem cells, 2) selectively kill mesenchymal cells, 3) force differentiation of stem cells, 4) force differentiation of mesenchymal cells (by inducing an MET).

The first of these strategies, using small molecules to kill stem cells, is difficult for many reasons. Stem cells are hard to identify, they are hard to reach with small molecules, and there are a relatively limited number of known CSC specific targets because of a lack of knowledge of the biology across the diversity of tissue stem cells [144]. Furthermore, few stem cell specific drug targets are known because it is not yet fully understood how the signaling of a cancer stem cell differs from bona fide normal stem cells [145].

To address the limited understanding of CSC pathways, another approach is to find drugs that are cytotoxic against the CSC phenotype, even if the molecular target is unknown. As an example, metformin was originally identified as a diabetes drug, but is now being investigated for cancer therapy because it has recently been found to be cytotoxic against CSCs in xenograft models of breast, prostate, and lung cells [146; 147]. As another example, salinomycin was identified from an unbiased screen for small molecule compounds that specifically kill stem cells [148]. These examples show two potential avenues, either by repositioning a drug from another disease or unbiased screening, for identifying novel therapies for CSCs.

In contrast to SC-CSCs, there is a wealth of potential targets for DC-CSCs. For one, there is already a vast knowledge of the pathways that drive the activation of an EMT, as reviewed above. Secondly, EMT-inducing pathways overlap with growth-signaling pathways that can be pharmacologically inhibited with drugs. Finally, activation of these pathways in adult tissues is aberrant, and thus therapies may not affect normal cells. It is unknown the extent that these EMT targets may also be effective in SC-CSCs, but at this point, there is not yet a theoretical framework to support such an approach.

To block known EMT pathways, many therapies are currently being explored (reviewed in [149]). To give some examples, a Wnt gene expression signature was seen in basal-like breast cancers, and inhibition reduces tumor growth in xenograft models [150]; inhibition of Hedgehog (HH) [151] can block metastasis in xenograft models of pancreatic cancer cell lines [152]; the Src inhibitor dasatinib is effective against mesenchymal-like breast cell lines [153], and inhibition of mTOR can decrease the number of CSCs in mouse xenograft models of human pancreatic cancers [154].

As an alternative to targeting pathways, there has been success in inducing SC-CSCs to differentiate. This mechanism was first used on acute promyelocytic leukemia (APL), where *trans* retinoic acid was effective in inducing terminal differentiation, leading to a 90% clinical response rate [155].

While a differentiation-based strategy has been effective against SC-CSCs, it has seen less success when applied to DC-CSCs. Here, the analogous approach is to induce a MET therapeutically, leaving epithelial cells that are then sensitive to conventional therapies [89]. Since EMT is a reversible process, it may be possible to pharmacologically activate the MET process, restoring the cells to a sensitive epithelial state. However, forcing differentiation experimentally can increase the number of metastatic nodules produced when mice are injected with a melanoma cell line [156]. An explanation is that this differentiation process may induce an MET in DC-CSCs that have already seeded distant organs, promoting metastatic progression. A mouse model has shown that as EMT promotes the escape of cells from the primary tumor, an MET that confers epithelial characteristics can increase the number of metastatic deposits [157]. Thus, while inducing differentiation in APL using retinoic acid was effective, a similar approach in solid tumors might aggravate the disease, suggesting that eliminating DC- and SC-CSCs may require different strategies. Disentangling these issues will be required to effectively deploy this strategy in the clinic.

7. Final Perspectives

The identification of EMT has shown that the phenotypes of cancer cells can be transient and mutable, and more importantly, that this plasticity can potentially serve as a mechanism to produce stem cell properties. It was not previously appreciated the role that the plasticity of the epithelial cells in cancer can have both in determining the potential for recurrence, metastasis, and therapeutic response of the disease [158]. While there is still uncertainty as to the driver of the plasticity, whether it occurs primarily through microenvironmental factors or acquired genetic mutations, it is now accepted that changes in cellular phenotype is necessary for cells to survive the diverse environments required in metastasis [145].

While combinations of therapies are typically thought to alter signaling within a single type of cell, there are now efforts to begin constructing combinations to target different populations of cancer cells [159]. Over the last years, we have been developing an understanding of the differences in the phenotypic profiles between the stem-like and non-stem-like subpopulation of tumors. What is still unclear is the source of these stem cells, whether they originate from a classical progenitor stem cell, or from a differentiated epithelial cell through an EMT. Nevertheless, there is a large amount of evidence that an EMT has a role to play in cancer. There is emerging clinical evidence that treatments targeting EMT pathways are likely to improve clinical outcomes.

However, another implication of the plasticity of the EMT state is that the plasticity itself, while it can confer aggressiveness to the disease by creating a new stem cell, may also be its Achilles heel. It may be possible pharmacologically to take advantage of this phenotypic fluidity and generate cells that are responsive to the traditional therapeutics. A deeper understanding of the mechanisms that can potentially induce an MET in these cells may introduce a new class of drugs.

Currently, there are two main types of CSCs being investigated, SC-CSCs and DC-CSCs. The evidence is accumulating that both can have clinical impact. Although the data remain incomplete, the likelihood is that cancers of every epithelial tissue have the capacity both to harbor SC-CSCs and to generate DC-CSCs. However, the specific roles that they play in the development of cancer, or whether they are both required for tumor progression and metastasis is still unknown.

Many fundamental questions regarding the CSCs remain unresolved. Do some cancers metastasize due to SC-CSCs, while others due to DC-CSCs? Or, do they both contribute to the metastasis in the same tumor? In other words, are there one or two or more distinct subpopulations of CSCs, and what are their characteristics? While in this review we have not distinguished among the DC-CSCs, emerging models propose sub-categorizations that arise due to different mechanisms in the induction of EMT [145]. And then finally, are there therapeutics that can effectively eliminate all types of CSCs, or will individualized strategies be required? Addressing these issues will be necessary to developing therapeutic approaches that can result in effective and lasting elimination of the disease (Figure 1).

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Figure 1.

Solid epithelial tumors are comprised of differentiated cells and cancer stem cells. The cancer stem cells may originate from either multipotent progenitor cells or be produced through an epithelial to mesenchymal transition, a mechanism of phenotypic plasticity in a tumor. Activation of an EMT in differentiated cancer cells confers stem cell properties. While standard chemotherapy regimens reduce the differentiated cell population, they are less effective against the stem cell populations. The remaining cells possess the capacity to reproduce the tumor, leading to recurrence. Therefore, generating a durable response will require therapies that target the differentiated cancer cell subpopulations as well as all existing cancer stem cell populations. Understanding the characteristics and therapeutic profiles of the distinct stem cell populations are active areas of investigation.