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## EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer

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### Abstract

Tumors are cellularly and molecularly heterogeneous, with subsets of undifferentiated cancer cells exhibiting stem cell-like features (CSCs). Epithelial to mesenchymal transitions (EMT) are transdifferentiation programs that are required for tissue morphogenesis during embryonic development. The EMT process can be regulated by a diverse array of cytokines and growth factors, such as TGF- $\beta$  whose activities are dysregulated during malignant tumor progression. Thus, EMT induction in cancer cells results in the acquisition of invasive and metastatic properties. Recent reports indicate that the emergence of CSCs occurs in part as a result of EMT, for example, via cues from tumor stromal components. Recent evidence now indicates that EMT of tumor cells not only causes increased metastasis but also contributes to drug resistance. In this review, we will provide potential mechanistic explanations for the association between EMT induction and the emergence of CSCs. We will also highlight recent studies implicating the function of TGF- $\beta$  regulated non-coding RNAs in driving EMT and promoting CSC self-renewal. Finally we will discuss how EMT and CSCs may contribute to drug resistance as well as therapeutic strategies to overcome this clinically.

### Introduction

Cellular heterogeneity is a histological hallmark of many cancers (Pardal *et al.*, 2003). Despite their clonal origin, tumors are comprised of cells with varied morphological and molecular features. Rare tumor-initiating cells with stem cell-like properties in both hematopoietic and solid malignancies have been identified and classified using distinct cell surface markers. These cancer stem cells (CSCs) can self-renew and differentiate to generate the cellular heterogeneity of the originating tumor (Al-Hajj *et al.*, 2003; Hermann *et al.*, 2007; Lapidot *et al.*, 1994; O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007; Singh *et al.*, 2004). However, whether CSCs are truly the only cells with *de facto* tumorigenic potential remains controversial (Gupta *et al.*, 2009a; Quintana *et al.*, 2008).

Regardless, tumors are clearly histologically heterogeneous, with subsets of cancer cells exhibiting distinct molecular profiles (Gupta *et al.*, 2009a). Furthermore, cells with different molecular characteristics within the same tumor respond differently to anti-cancer therapeutics, leading to drug resistance (Zhou *et al.*, 2009). Cancer cells may also undergo adaptive changes following therapy, exacerbating drug resistance. In epithelial cancers, these adaptive changes may involve, at least in part, epithelial to mesenchymal transitions (EMT) and the reverse process (MET). These are developmental programs that can be usurped by oncogenically transformed cells during tumor progression (Thiery *et al.*, 2009).

Intriguingly, EMT can trigger reversion to a CSC-like phenotype (Mani *et al.*, 2008; Polyak and Weinberg, 2009), providing an association between EMT, CSCs and drug resistance.

Parallels exist between tumorigenesis and the process of wound healing during tissue injury (Dvorak, 1986), both involving the recruitment of mesenchymal stem cells (MSCs), which can differentiate along various epithelial lineages, via MET, and may serve as cell types of origin for a number of cancers. This review addresses the role of epithelial plasticity in cancer. We also relate EMT to CSC biology, an association reinforced by the notion that cancer is a disease of abnormal wound healing and tissue repair, which are accompanied by pathophysiological EMT in adult tissues. We will overview a growing body of evidence suggesting a relationship between EMT, the emergence of cancer stem cells, and drug resistance. This 'axis of evil' in the war on cancer represents a significant challenge for the development of improved cancer therapies but provides new opportunities for drug discovery efforts.

## Epithelial to mesenchymal transition – an overview

EMT is an evolutionarily conserved developmental process (Thiery, 2003). Conventionally, epithelial cells are defined as surface barrier cells with secretory functions that display distinct apical versus basolateral polarity established by adherens and tight junctions (Figure 1A). Mesenchymal cells serve scaffolding or anchoring functions and have multifunctional roles in tissue repair and wound healing. During embryonic development, certain differentiated polarized epithelial cells, upon extracellular cues, undergo profound morphogenetic changes, referred to collectively as EMT. This is typified by the dissolution of cell-cell junctions and loss of apico-basolateral polarity, resulting in the formation of migratory mesenchymal cells with invasive properties. These mesenchymal cells are recruited to specific sites in the developing embryo where they can differentiate via MET to form epithelial tissues at distal locations.

EMT inducers, such as transforming growth factor beta (TGF- $\beta$ ) or receptor tyrosine kinase (RTK) ligands trigger changes in gene expression via complex signaling networks. A paramount consequence of these signaling events is the upregulation of transcriptional repressors, such as the C2H2-type zinc-finger proteins Snail, Slug and Zeb1, Zeb2/SIP1, as well as the bHLH factors Twist and E47 (Peinado *et al.*, 2007). These proteins bind to E-box elements in the promoter of the gene encoding the adherens junction protein E-cadherin, where they recruit histone deacetylases (HDACs) and other co-repressors to facilitate chromatin condensation and subsequent transcriptional repression of E-cadherin expression.

Reduced E-cadherin expression causes adherens junction breakdown, and along with other signaling events such as modulation of Rho GTPase function, loss of cell polarity ensues. This is accompanied by increased stability and cytoplasmic accumulation of the adherens junction component  $\beta$ -catenin. In cooperation with Wnt-mediated signaling,  $\beta$ -catenin forms a nuclear-localized transcriptional complex with TCF/LEF that promotes robust gene expression changes. The canonical EMT program is characterized by complex gene expression changes. Thus, epithelial and mesenchymal cells can be distinguished by expression of a number of classical markers that are used to define either epithelial or mesenchymal character (LaGamba *et al.*, 2005). For instance, epithelial markers include adherens and tight junction proteins such as E-cadherin and ZO-1, whereas mesenchymal markers include the extracellular matrix (ECM) component fibronectin and the intermediate filament protein vimentin (Zeisberg and Neilson, 2009). The loss of polarity and gain of motile characteristics of mesenchymal cells during embryonic development has prompted comparisons to metastatic cancer cells during malignant progression (Yang and Weinberg, 2008). Notably, these properties have also been ascribed to normal stem cells and CSCs.

## Operational definitions of cancer stem cells

Cancer stem cells with tumor-initiating potential are often compared to normal stem cells. These are multi- or pluri-potent progenitor cells that can self-renew and divide by asymmetric cell division to give rise to differentiated or committed progenitors (Pardoll *et al.*, 2003). Such properties are characteristic of oncogenically transformed cells. Dick and colleagues performed seminal studies identifying and isolating leukemia-initiating cells (LICs) in acute myeloid leukemia (AML), which exhibited a distinct cell surface antigen expression profile (Bonnet and Dick, 1997; Lapidot *et al.*, 1994). These rare CD34<sup>+</sup>, CD38<sup>-</sup> cells can form large colonies in immuno-compromised SCID mice and exhibit the self-renewal capacities and multipotency ascribed to normal stem cells, suggesting a hierarchical model for CSC differentiation.

More recent studies have isolated tumor-initiating cells with CSC-like properties in a number of solid malignancies, beginning with the isolation of CD44<sup>+</sup>, CD24<sup>-/low</sup> breast cancer initiating cells (Al-Hajj *et al.*, 2003). These cells are thought to exhibit stem cell-like properties because they are capable of reconstituting the heterogeneity of the originating tumor (Figure 2A). These observations were later extended to brain tumors, which exhibit a subpopulation of CD133<sup>+</sup> cells with tumor-initiating capabilities (Singh *et al.*, 2004). CD133<sup>+</sup> tumor-initiating cells were subsequently isolated from colon cancers (O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007) and pancreatic cancers (Hermann *et al.*, 2007). Additionally, hepatocellular carcinomas (HCCs) exhibit a tumor-initiating population that can be identified by expression of another cell surface marker, epithelial cell adhesion molecule (EPCAM), which is expressed in normal epithelial progenitor cells (Yamashita *et al.*, 2009). Other markers include aldehyde dehydrogenase 1 (ALDH1), identified as a specific marker for breast cancer-initiating cells (Ginestier *et al.*, 2007). In summary, CSCs can be isolated or identified by distinct marker expression profiles, which may provide clinical utility. Furthermore, since putative CSCs have been implicated in the etiology of a number of diverse cancer types, the CSC model appears to display a universality that extends beyond leukemias.

## Origin of cancer stem cells

Do CSCs arise from existing stem/progenitor cells or from acquisition of oncogenic lesions in terminally differentiated cells resulting in dedifferentiation to a primitive stem cell-like state (Figure 2)? Both of these scenarios are likely to exist depending on the context. Early studies of CD34<sup>+</sup>, CD38<sup>-</sup> LICs suggested that CSCs arise from normal stem cells that are capable of repopulating the hematopoietic system of SCID mice, since these cells are also CD34<sup>+</sup>, CD38<sup>-</sup> (Bonnet and Dick, 1997). Since tumor-initiating cells from various tissue types display properties ascribed to normal stem cells it was hypothesized that tumors originate from these cells. However, differentiated cells can be artificially induced to acquire the pluripotency of embryonic stem cells (so-called iPS cells) (Takahashi and Yamanaka, 2006; Yu *et al.*, 2007b). This has challenged the long-held dogma that terminal differentiation is irreversible. Notably, iPS cells are tumorigenic, forming teratomas that comprise rudimentary tissues from all three germ layers in SCID mice. In addition, the SOX2 transcription factor, which is required for efficient iPS cell reprogramming is amplified in lung and esophageal squamous cancers, underscoring a role for stem cell factors as oncogenes (Bass *et al.*, 2009; McCaughan *et al.*; Yuan *et al.*). Recent studies have demonstrated that the p16/INK4a tumor suppressor provides a block to efficient iPS cell reprogramming by inducing a senescence response, which may explain its frequent deletion in human cancers, if the CSC model is valid (Banito *et al.*, 2009; Li *et al.*, 2009a; Utikal *et al.*, 2009). Thus, gain of oncogenic activity and loss of tumor suppressor function cause dedifferentiation, suggesting that CSCs may arise from the acquisition of genetic lesions. In

support of this, it has been reported that leukemic stem cells can be generated from committed hematopoietic progenitors through expression of the MLL-AF9 fusion protein (Krivtsov *et al.*, 2006). Thus, oncogenic transformation of partially differentiated cells can lead to the emergence of CSCs.

On the other hand, a recent report that suggests that mesenchymal-like tumors originate from a mesenchymal progenitor cell (Lim *et al.*, 2009). Breast cancers can be subdivided into basal versus luminal subtypes based on hierarchical clustering of gene expression profiles (Sorlie *et al.*, 2001). The basal subtype exhibits a mesenchymal signature whereas the luminal subtype appears to be epithelial (Neve *et al.*, 2006). Lim and colleagues provide evidence that basal subtype breast cancers harboring BRCA1 mutations display a gene expression signature that most closely resembles that of normal luminal progenitor cells in the mammary epithelium. This observation suggests that mesenchymal-like cancers need not develop via dedifferentiation of epithelial-like cells, but instead result from the expansion of pre-neoplastic BRCA1-mutant mesenchymal progenitor cells.

### EMT-induced formation of CSCs

Pathophysiological conditions such as tissue injury or tumorigenesis can trigger differentiated cells to acquire a multipotent stem cell like phenotype through EMT induction (Figure 2B). This may mirror developmentally regulated EMT signaling pathways, such as Wnt, Notch and Hedgehog, which drive both normal and CSC renewal and maintenance (Huber *et al.*, 2005; Malanchi *et al.*, 2008; Peacock and Watkins, 2008). Studies also indicate that metastatic cancer cells, which have presumably undergone EMT, may exhibit a CSC phenotype. For instance, disseminated breast cancer cells found in pleural effusions are enriched for a CD44<sup>high</sup>, CD24<sup>low</sup> CSC-like population (Al-Hajj *et al.*, 2003). Interestingly, CD44 is a  $\beta$ -catenin/TCF-4 target gene, supporting a role for the EMT-associated Wnt pathway in CSC maintenance (Wielenga *et al.*, 1999). In pancreatic tumors, CD133<sup>+</sup> mesenchymal like cells that also express the CXCR4 chemokine receptor, are found predominantly at the invasive front of tumors where they may be primed for metastatic spread (Hermann *et al.*, 2007).

Empirical evidence connecting EMT to the emergence of stem cells has recently been reported by Weinberg and colleagues. Thus, differentiated mammary epithelial cells that have undergone EMT either upon TGF $\beta$  treatment or by forced expression of E-cadherin transcriptional repressors, such as Snail, give rise to CD44<sup>high</sup>, CD24<sup>low</sup> cells, akin to breast CSCs (Mani *et al.*, 2008). Additionally, stem cells isolated from normal breast tissue or breast cancers express a number of canonical EMT markers. Of clinical significance, studies have shown that EMT-associated emergence of CSCs occurs after an immune response, since CD8<sup>+</sup> T-cells can induce dedifferentiation of breast cancer cells, resulting in the formation of CD44<sup>high</sup>, CD24<sup>low</sup> stem cell-like cells (Santisteban *et al.*, 2009). Finally, poorly differentiated and aggressive cancers display a gene expression signature related to that of embryonic stem cells (Ben-Porath *et al.*, 2008). In summary, compelling evidence exists relating EMT to the emergence of a CSC-like phenotype, which may be prerequisites for cancer cell metastasis. A major driving force for these processes is the TGF- $\beta$  signaling pathway, discussed below.

### TGF- $\beta$ modulation of EMT and “stemness”

TGF- $\beta$  family cytokines are mediators of embryonic development and tissue homeostasis in the adult (Heldin *et al.*, 2009). TGF- $\beta$  itself is a regulator of many types of physiological and pathophysiological EMT (Derynck *et al.*, 2001; Thiery *et al.*, 2009). Type I and Type II TGF- $\beta$  receptors (T $\beta$ RI and RII) are dual specificity kinases, exhibiting both serine/threonine (S/T) and tyrosine kinase activities. Upon ligation of a heterodimeric complex of

receptors RI and RII, TGF- $\beta$  triggers a cascade of signaling events initiated by serine phosphorylation of T $\beta$ RI by RII. This triggers autophosphorylation and activation of T $\beta$ RI, which subsequently phosphorylates and activates a number of downstream effector proteins, most notably the Smad family of transcription factors.

T $\beta$ RI activates effector Smads 2 and 3, which subsequently bind to a co-smad, Smad4. The Smad2/3/Smad4 complexes can associate with accessory transcription factors to activate expression of target genes that cause changes in cellular differentiation. Notably, Smads associate with Zeb proteins (Zeb1 and Zeb2/SIP1) to repress expression of E-cadherin during the initiation of EMT (Comijn *et al.*, 2001; Postigo *et al.*, 2003; Verschueren *et al.*, 1999). Interestingly, SIP1 is essential for human embryonic stem cell (hESC) maintenance by Activin, providing further evidence for an association between TGF- $\beta$  signaling, EMT and “stemness” (Chng *et al.*, 2010).

Genetic evidence implicates TGF- $\beta$  signaling as a tumor suppressor pathway, with frequent loss of function mutations, LOH and homozygous deletions affecting a number of pathway components, most notably Smad4 in pancreatic cancers. TGF- $\beta$  has strong growth inhibitory and apoptotic effects resulting in tumor suppression during early stages of tumor progression. However, TGF- $\beta$  can play a pro-metastatic role during later stages of tumorigenesis. Indeed, metastatic breast cancer stem cells that are CD44<sup>high</sup>, CD24<sup>low</sup> strongly express a TGF- $\beta$  pathway signature and become more epithelial-like following pathway inhibition, suggesting a role for TGF- $\beta$  in driving breast cancer EMT and promoting CSC maintenance (Shipitsin *et al.*, 2007).

But how do cancer cells with intact Smad signaling components metastasize upon TGF- $\beta$  exposure without undergoing cell death? There is precedence for this during wound healing, as TGF- $\beta$  promotes EMT to facilitate the recruitment of inflammatory mediators, which aid in the tissue repair process (Wendt *et al.*, 2009). In order to prevent widespread cytotoxicity, cooperating anti-apoptotic signaling pathways are triggered while TGF- $\beta$  induces EMT and tissue fibrosis. These pathways, notably those involving PI3K/AKT and NF- $\kappa$ B are frequently activated in human cancers, providing the context for the metastasis-inducing properties of TGF- $\beta$  without the accompanying cell death.

TGF- $\beta$ -induced PI3K signaling can block apoptosis potently in mammary epithelial cells treated chronically with TGF- $\beta$ , which leads to a metastable mesenchymal phenotype and circumvention of TGF- $\beta$ -induced cell death (Gal *et al.*, 2008). This effect is dependent on the downstream PI3K effector Akt, which can sequester Smad3 in the cytoplasm following insulin treatment of epithelial cells (Conery *et al.*, 2004; Remy *et al.*, 2004). Interestingly, this is independent of Akt's function as a kinase, which may have clinical implications for the use of Akt kinase inhibitors in certain contexts. PI3K signaling can also circumvent TGF- $\beta$ -induced growth arrest, specifically in neuroepithelial and glioblastoma cells, by blocking the function of a FoxO/Smad2/3 ternary complex that regulates p21/CIP1 expression (Seoane *et al.*, 2004). Thus, the PI3K/AKT axis appears to be a critical antagonist of TGF- $\beta$ -induced apoptosis and cell cycle arrest.

## TGF- $\beta$ , microRNAs and cancer cell plasticity

The functional role of non-coding microRNAs in cancer progression has received tremendous attention since the discovery of the let-7 microRNA, which negatively regulates Ras expression (Reinhart *et al.*, 2000). MicroRNAs regulate gene expression by forming duplexes with the 3' UTRs of target mRNAs, facilitating their degradation by the RISC complex of proteins. They are transcribed as precursors, which are subsequently processed by the DROSHA complex (Baulcombe, 2006).

A role for microRNAs in TGF- $\beta$  signaling has recently been appreciated. Firstly, Smad proteins can directly regulate microRNA processing by binding to the DROSHA complex, which has been shown for mir-21 (Davis *et al.*, 2008). MicroRNAs can regulate TGF- $\beta$ -induced apoptotic and growth suppressive functionality. For instance, TGF- $\beta$  can activate Akt in glomerular mesangial cells by inducing expression of mir-216a and mir-217, which target the PI3K negative regulator PTEN (Figure 3A) (Kato *et al.*, 2009). The E2F1-regulated microRNA mir-17-92, which has established oncogenic properties (He *et al.*, 2005), and mir-106b-25 can block TGF- $\beta$ -mediated growth arrest in gastric cancers by targeting p21/CIP1 and the pro-apoptotic protein BIM (Figure 3A) (Petrocca *et al.*, 2008). Interestingly, transgenic expression of mir-17-92 in the embryonic lung of mice results in the expansion of lung progenitor cells by blocking differentiation (Lu *et al.*, 2007). This occurs by targeting of an Rb-like gene, *Rbl2*. Along similar lines, loss of RB1 expression in fibroblasts results in reprogramming to an embryonic stem cell-like state via upregulation of Sox2, Oct-4, Klf4 and Nanog (Liu *et al.*, 2009). These reprogrammed cells form spheres and are tumorigenic, exhibiting elevated Zeb1 expression and a CD44<sup>high</sup>, CD24<sup>low</sup> CSC-like phenotype. Thus, loss of the Rb tumor suppressor and increased E2F1 activity may stimulate the emergence of CSCs in cooperation with TGF- $\beta$ -mediated changes in microRNA function.

Several reports indicate that TGF- $\beta$  induced EMT in cancer cells is dependent on microRNA function (Figure 3B). Thus, mir-155 regulates epithelial plasticity by targeting RhoA and promoting TGF- $\beta$  induced EMT as a result of the dissolution of tight junctions (Kong *et al.*, 2008). Most significantly, EMT is regulated by the expression of the mir-200 family and mir-205 (Burk *et al.*, 2008; Gregory *et al.*, 2008; Korpala *et al.*, 2008; Park *et al.*, 2008). These microRNAs target Zeb proteins and are downregulated by TGF- $\beta$ , resulting in EMT. Interestingly, cancer cells established from a mouse model of lung adenocarcinoma, driven by oncogenic K-Ras and loss of function p53 mutations, display epithelial plasticity (Gibbons *et al.*, 2009). These cells undergo EMT following TGF- $\beta$  exposure, which is dependent on downregulation of mir-200 with concomitant stabilization of Zeb1 expression.

A role for Zeb1 in modulating CSC biology has now been established (Wellner *et al.*, 2009). Zeb1 represses expression of mir-200c, mir-203 and mir-183, which drives the tumor-initiating properties of pancreatic and colorectal cancer cells by upregulating expression of stem-cell factors Sox2 and Klf4. Another report showed that TGF- $\beta$  regulates EMT through HMGA2, which forms a complex with Smad proteins to recruit repressors such as SNAIL and SLUG (Thuault *et al.*, 2006). HMGA2 is targeted by the prototypical microRNA let-7, which has also been implicated in the self-renewal and tumorigenicity of breast cancer stem cells (Yu *et al.*, 2007a). In summary, microRNAs play a significant role in TGF- $\beta$  induced EMT and subsequent dedifferentiation to a stem cell-like state.

## EMT-associated resistance to cancer therapeutics

Cancer therapy is often associated with two major forms of drug resistance-- *de novo* or acquired. Patients who are initially refractory to therapy display intrinsic or “*de novo*” drug resistance. Patients that initially respond to therapy typically relapse as a consequence of “acquired” drug resistance. The use of so-called “targeted therapeutics”, such as Erlotinib/Tarceva for EGFR-mutation positive non-small cell lung cancer (NSCLC), has led to the elucidation of various drug resistance mechanisms. Approximately 50–70% of cases of acquired drug resistance in NSCLC patients treated with EGFR inhibitors involve genetic changes including T790M ‘gatekeeper’ mutations in EGFR or amplification of the MET oncogene (Engelman *et al.*, 2007; Sharma *et al.*, 2007). How can we explain drug resistance in the remaining cases? A burgeoning body of literature now suggests that reversible epigenetic changes emerging during acquired drug resistance reflect changes in the

differentiation state of the tumor, which is likely to reflect EMT and the emergence of chemorefractory cells with stem cell-like features (Sharma *et al.*; Voulgari and Pintzas, 2009) (Figure 3).

The differentiation state of a tumor may also contribute to *de novo* resistance. For instance, elevated E-cadherin expression is associated with sensitivity to EGFR kinase inhibitors, with drug-resistant cells being more mesenchymal-like (Witta *et al.*, 2006). Urothelial cancers are subdivided into two groups, superficial and muscle-invasive. The latter group, associated with extremely poor prognoses and high mortality rates, exhibits mesenchymal-like properties, underscored by the observation that these tumors express Zeb2/SIP1, which protects cells from DNA damage-induced apoptosis (Sayan *et al.*, 2009). Basal subtype mesenchymal-like breast cancers are also associated with higher mortality rates. Even in epithelial-like luminal breast cancers with HER2/neu amplification, cells with CSC features (CD44<sup>high</sup>, CD24<sup>low</sup>) are resistant to neoadjuvant chemotherapy and HER2 pharmacological inhibition (Li *et al.*, 2008). In fact, chemotherapy leads to an increase in the number of CD44<sup>high</sup>, CD24<sup>low</sup> CSCs, which represents a potentially important mechanism of acquired drug resistance in this setting (Figure 4A). However, some reports also suggest that basal, mesenchymal-like cancers are initially more sensitive to neoadjuvant chemotherapy than epithelial luminal tumors (Carey *et al.*, 2007; Liedtke *et al.*, 2008; Paik *et al.*, 2006; Yauch *et al.*, 2005). Yet, despite the initial responses, patients with basal cancers are given the worst prognoses, suggesting that these mesenchymal-like cancers are more prone to developing drug resistance. It is possible that epithelial-like cancers are initially more sensitive to targeted therapies, such as EGFR and HER2 antagonists, whereas mesenchymal cancers are more sensitive to DNA damaging agents such as doxorubicin.

## Strategies to modulate EMT and eliminate CSCs

Targeting CSCs therapeutically is likely to be challenging, since both bulk tumor cells and CSCs must be eliminated, potentially demanding combination drug therapies (Figure 4B). Since CSCs are molecularly distinct from bulk tumor cells, one can target their activity by exploiting these molecular differences. For instance, cell surface marker expression could be used for antibody-directed therapy to target proteins such as CD133, CD44 or EpCAM (Deonarain *et al.*, 2009). ABC transporters, which are ATP-dependent drug efflux pumps, could be targeted by ATP-competitive agents (Kuhnle *et al.*, 2009; Robey *et al.*, 2007). Another strategy to selectively target CSCs has emerged from high-throughput compound screening efforts. In one study, the antibiotic salinomycin was identified, which preferentially killed breast CSCs (Gupta *et al.*, 2009b). Interestingly, salinomycin induced the differentiation of mesenchymal-like cancers *in vivo*, as assessed by increased E-cadherin and reduced vimentin expression. Thus, a proof of principle has been established for the notion that poorly differentiated cancers with a high CSC content can be treated by facilitating differentiation to a more epithelial-like state.

This can be reinforced by the results with HDAC inhibitors, such as n-butyrate, which can induce the differentiation of erythroleukemic cells (Leder *et al.*, 1975; Riggs *et al.*, 1977). HDAC-dependent histone deacetylation, which is elevated in some cancers, is required to repress expression of epithelial genes such as E-cadherin during EMT. Furthermore, HDACs can affect the activity of non-histone substrates, such as HIF-1 (Jeong *et al.*, 2002; Kong *et al.*, 2006) and NF- $\kappa$ B (Ashburner *et al.*, 2001), which have been implicated in driving EMT and drug resistance. Thus, HDAC inhibition could be a useful strategy to induce the differentiation of mesenchymal-like cancer cells and CSCs, which could trigger apoptotic responses or chemosensitize these cells to other therapies. Several HDAC inhibitors, such as Trichostatin A (TSA) and Vorinostat, have now entered clinical trials for various cancers either as single agents or in combination with targeted and conventional therapeutics (Lane

and Chabner, 2009). Finally, activation of the AMP-activated kinase (AMPK) with the diabetes drug metformin results in selective killing of cancer stem cells in combination with chemotherapy (Hirsch *et al.*, 2009). This suggests that targeting mTOR activity, which is negatively regulated by AMPK, may be a strategy to block the renewal of CSCs.

The feasibility of manipulating epithelial plasticity is reinforced by studies showing that depletion of Zeb1, either chemically or by RNAi, in mesenchymal-like cells results in a partial epithelial metaplasia and either gain of K-Ras dependency or drug sensitivity (Li *et al.*, 2009b; Singh *et al.*, 2009). Thus, inhibitors of TGF- $\beta$  pathway components that promote Zeb1 expression could prove effective in sensitizing chemoresistant mesenchymal-like tumors to anti-cancer therapeutics. Epithelial trans-differentiation could also be achieved by re-expression of mir-200 family members, which may limit the expansion of CSCs by virtue of Zeb1 blockade. Effective delivery of therapeutic microRNAs has been achieved in mice with liver cancer, so this may eventually prove to be feasible in humans (Kota *et al.*, 2009).

The PI3K/PTEN/mTOR signaling pathway plays a prominent role in mediating drug resistance. Loss of the PTEN tumor suppressor in many malignancies is associated with extremely poor prognoses and *de novo* chemoresistance (Keniry and Parsons, 2008). A direct relationship has been demonstrated in breast cancers that exhibit PTEN loss, since these tumors are resistant to the HER2/Erb2-targeting antibody trastuzumab (Herceptin) (Nagata *et al.*, 2004). In fact, PTEN activity is directly responsible for the growth inhibitory effects of trastuzumab. PI3K-mediated chemoresistance in cancer cells may be a function of TGF- $\beta$  pathway activation, and resultant CSC maintenance. Indeed, *HER2* amplified breast cancer cells, upon TGF- $\beta$  exposure, lose sensitivity to HER2 inhibition as a result of PI3K activation by the ErbB3 protein (Wang *et al.*, 2008).

Recent reports have linked PI3K activity directly to CSC expansion and maintenance. For example, the mTOR and stat3 pathways cooperate to promote the proliferation of breast CSCs (Zhou *et al.*, 2007). PTEN loss in hematopoietic progenitor cells leads to myeloproliferative disorder followed by leukemia as a result of preferential expansion of leukemia-initiating stem cells (LICs) with the accompanying loss of normal hematopoietic stem cells (HSCs) (Yilmaz *et al.*, 2006). Therapeutically, rapamycin mediated inhibition of mTOR, which is hyperactivated as a consequence of PTEN loss, results in loss of LICs and gain of normal HSCs. PI3K/AKT signaling is also required for the maintenance of CD44<sup>+</sup> and CD133<sup>+</sup> prostate cancer CSCs (Dubrovskaya *et al.*, 2009). PTEN-deficient CSCs are likely to be highly refractory to therapeutics. In medulloblastomas, where radiation therapy results in the eradication of bulk tumor cells, therapy-resistant PTEN-deficient CSCs, displaying elevated Akt activity, remain viable (Hambardzumyan *et al.*, 2008). In summary, inhibition of PI3K/Akt/mTOR signaling components may prevent CSC self-renewal and circumvent CSC-mediated resistance to cancer therapeutics.

## Concluding remarks

Since cancers are heterogenous, future drug discovery efforts aimed at increasing patient survival will undoubtedly need to consider the plasticity of cancer cells. At least one source of such plasticity is EMT and the emergence of dedifferentiated cells with CSC-like properties. Several key signaling pathways contribute to this process, namely TGF- $\beta$  and Wnt, known inducers of EMT and promoters of stem cell maintenance. Recent studies have implicated microRNA functionality in these processes, and the dysregulation of microRNA expression is likely to be a major contributing factor in the etiology of some cancers.

Targeting EMT pathways and CSC maintenance is a promising therapeutic strategy. This appears to be feasible, since several studies have successfully shown that pharmacological agents can modulate the differentiation state of a tumor. Moreover, CSCs can be eliminated



or functionally antagonized by inducing their differentiation. Thus, “differentiation-inducing” agents such as salinomycin or HDAC inhibitors may have therapeutic value. Targeting the TGF- $\beta$  and Wnt pathways provides additional means to eliminating CSCs. These pathways can strongly activate anti-apoptotic signaling such as those mediated by PI3K and NF- $\kappa$ B. Thus, PI3K or Akt inhibition to block EMT and the emergence of CSCs may prove useful. Finally, since a role for microRNAs in mediating EMT and CSC maintenance is now becoming fully appreciated, therapeutic delivery of microRNAs may represent yet an additional strategy to potentially disrupt this axis of evil in the war on cancer.

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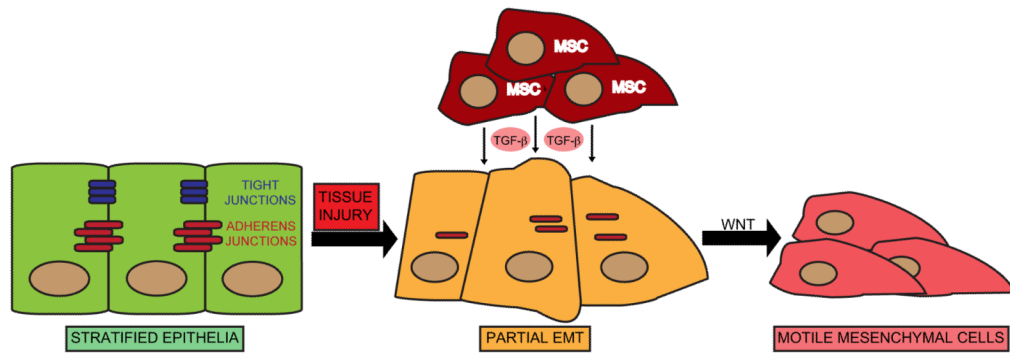
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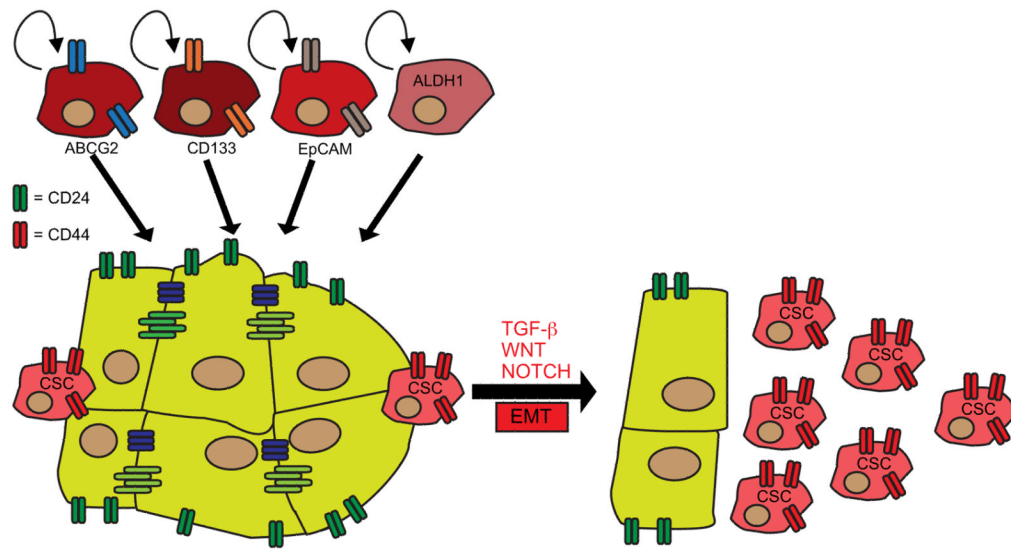
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**Figure 1.**

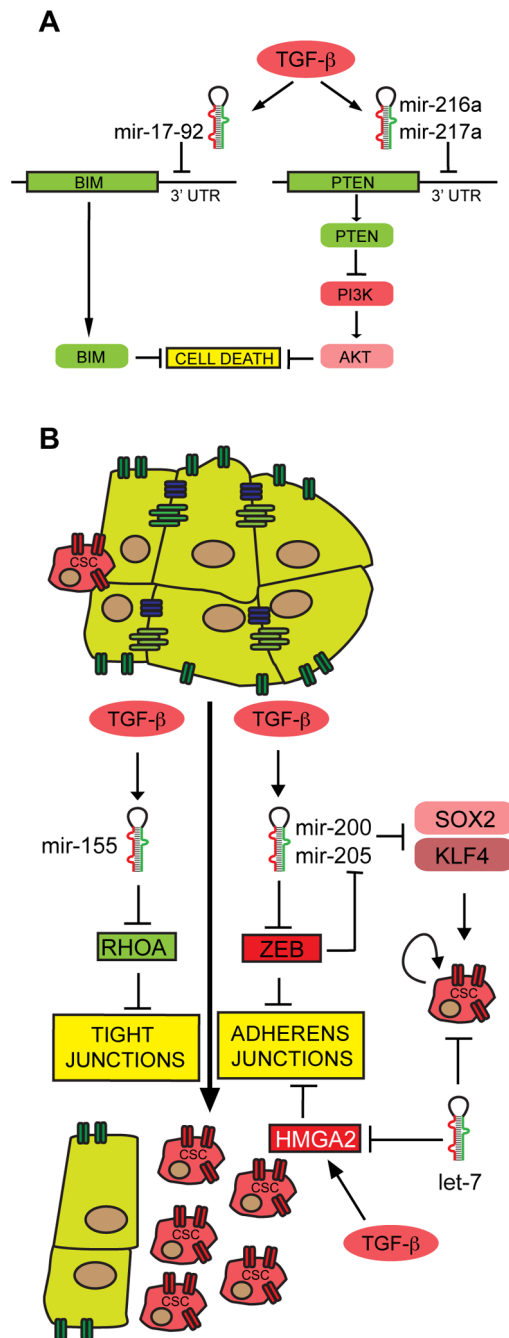
(Panel A) EMT in the context of pathophysiological conditions, such as during the repair of injured tissue. Tissue injury promotes the recruitment of mesenchymal stem cells (MSCs), which can secrete EMT-inducing cytokines such as TGF- $\beta$ , resulting in dissolution of cell-cell contacts and accompanying morphogenetic changes. In cooperation with additional EMT inducers such as Wnt, epithelial cells transform to a fully mesenchymal phenotype, associated with increased motility and invasiveness through basement membranes.



**Figure 2.**

(Panel A) Cancer stem cells (CSCs) with tumor-initiating capability can be identified by the expression of a distinct set of marker proteins, such as the ABC family transporter ABCG2, CD133, EpCAM or ALDH1. These CSCs can self-renew and differentiate into a number of cell types to generate the heterogeneity of the originating tumor. (Panel B) EMT can induce a CSC-like phenotype. Inducers of EMT such as TGF- $\beta$ , Wnt or Notch cause cells to acquire a CD44<sup>high</sup> CD24<sup>low</sup> phenotype, reminiscent of breast CSCs.

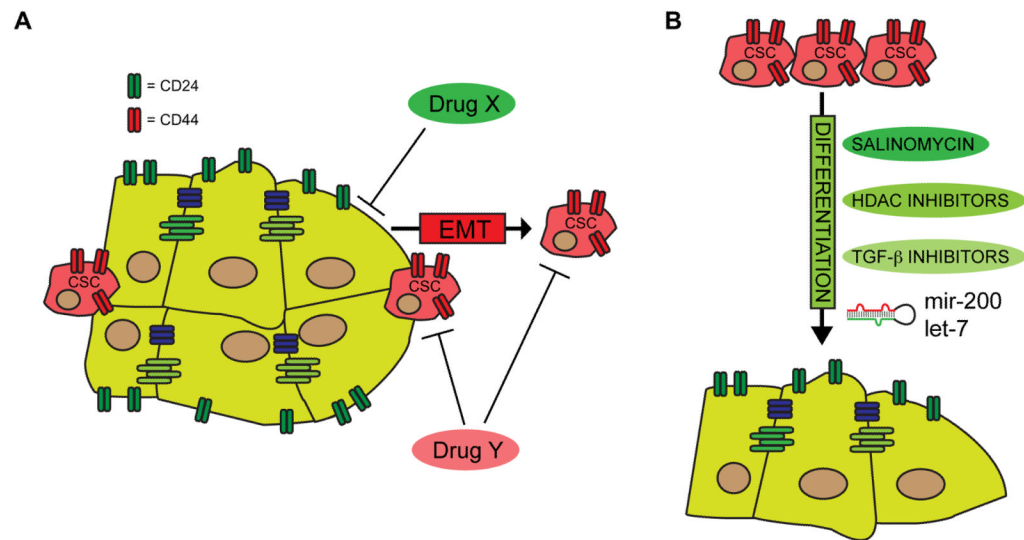




**Figure 3.**

(Panel A) TGF- $\beta$  induced cell death can be blocked by concomitant upregulation of microRNAs. Mir-17–92 can block expression of the pro-apoptotic protein Bim. Pro-survival PI3K signaling can be activated ablation of PTEN expression via mir-216a and mir-217a. (Panel B) Regulation of EMT and CSC “stemness” by microRNAs. TGF- $\beta$ -induced EMT can be potentiated by expression of mir-155, which targets RhoA, resulting in breakdown of tight junctions. TGF- $\beta$ -mediated repression of mir-200 family members and mir-205 results in increased Zeb protein expression, leading to E-cadherin repression and breakdown of adherens junctions. Zeb expression also represses mir-200/mir-205 expression, which is associated with CSC renewal via upregulation of the mir-200 targets Sox2 and Klf4. Finally,

TGF- $\beta$  can upregulate the activity of HMGA2, which can aid in E-cadherin repression. HMGA2 is negatively regulated by the let-7 microRNA, a microRNA that can block the self-renewal of breast CSCs. Thus, let-7 mediated depletion of HMGA2 provides a link between TGF- $\beta$  signaling, EMT and the emergence of CSCs.



**Figure 4.**

(Panel A) Effective cancer therapy may require combination treatments. A putative “Drug X” may selectively kill bulk tumor cells that are well-differentiated. This could be combined with “Drug Y” to kill cells with a CSC phenotype. Cancer cells treated with Drug X may undergo EMT to acquire CSC-like properties, in which case co-administration of Drug Y would kill both pre-existing CSCs and those that emerge via treatment with Drug X. (Panel B) A novel strategy to eliminate CSCs could be to induce their differentiation, which could lead to intrinsic apoptotic cell death or could sensitize the resulting differentiated cells to existing therapies. This differentiation switch could be facilitated with compounds such as salinomycin, HDAC inhibitors or TGF- $\beta$  pathway inhibitors. Finally, differentiation could also be promoted by administration of therapeutic microRNAs, such as mir-200 family members.