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A self-renewal assay for cancer stem cells

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Abstract Cancers of epithelial origin are responsible for the majority of cancer-related deaths in the USA. Unfortunately, although chemotherapy and/or radiation therapy can sometimes shrink tumors, metastatic cancers of epithelial origin are essentially incurable. It is clear that new approaches are needed to treat these diseases. Although cancer cell lines provide invaluable information, their biological properties often differ in crucial ways from de novo cancer cells. Our laboratory has developed a novel mouse model that reliably permits individual cancer cells isolated directly from patients' tumors to be assayed. This will allow the characterization of crucial signaling pathways involved in processes such as self-renewal that are critical for tumor formation by the cancer cells within de novo tumors. These tools should lead to new insights into the cellular and molecular mechanisms that drive human breast cancer growth and invasion.

Keywords Stem cells · Cancer · Self-renewal

Introduction

Although cell lines have led to remarkable advances in our understanding of the molecular and biochemical changes in cancer cells, their use in the identification of effective cancer therapies is somewhat limited [5, 15]. Cell lines are imperfect predictors of drug efficacy in de novo tumors. Several factors likely account for this deficiency. Cancer cell lines are selected from a subpopulation of cancer cells that are specifically adapted to growth in tissue culture, and the biological and functional properties of these cell lines can change dramatically [8, 16, 20, 34]. Furthermore, cancer cells from only a minority of breast cancer tumors will establish cell lines, suggesting a selection bias for cancers capable of growing in tissue culture. Additionally, the phenotypic and functional characteristics of these cell lines can change drastically relative to their properties in vivo. For example, the marker expression of both normal hematopoietic and leukemia tissue culture cells can change rapidly in tissue culture and often does not reflect that of the original stem cells from which they were derived. Even when conditions are devised to permit the proliferation of normal stem cells in culture, the conditions often promote self-renewal or differentiation in a way that prevents the stem cells in culture from recapitulating the hierarchy of cell populations that exist in vivo. Taken together, these observations suggest that the biological properties of cancer cell lines can differ markedly from the cancer cells from which they were derived. This likely explains at least in part why the cell lines are often poor predictors of a drug's efficacy in the clinic.

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An immunodeficient mouse model for solid tumors

Realizing that a reliable assay was necessary to study the self-renewal of dissociated cancer cells, a substantial and ultimately successful effort by our laboratory led to the development of a xenograft assay that measured not just proliferation but also cancer cell self-renewal [2]. This assay appears to be reliable in permitting dissociated cancer cells from patients' tumors to self-renew. The success of this assay is significantly better than tissue-culture assays and other animal models reported to date. Although breast cancer cells often briefly proliferate in certain tissue-culture systems, cells from most tumors do not self-renew in vitro and eventually stop proliferating [9, 10, 18]. Previously developed mouse models were not

efficient when dissociated cells were injected into mice. This model has recently been extended to other epithelial tumors and we have found that, like breast cancer cells, only a subset of the cancer cells was able to form tumors.

Using this model, we found that only a subset of cancer cells isolated from breast tumors was able to form tumors. Using flow cytometry, we found that the $CD24^{-/low}$ $CD44^{+}$ cancer cells were highly enriched such that cancer cells were able to form tumors in the xenograft model (Fig. 1). On the other hand, in most patients who were examined, other populations of cancer cells were depleted of tumor-forming cells.

Stem cells, cancer and self-renewal

Common cancers arise in tissues that contain a large subpopulation of proliferating cells that are responsible for replenishing the short-lived mature cells. In such organs, cell maturation is arranged in a hierarchy in which a rare population of stem cells gives rise to the mature cells that perpetuate themselves through a process called self-renewal [1, 4, 6, 12, 19, 24, 26, 29, 35]. Owing to their rarity, stem cells must be isolated in order to study their biological, molecular and biochemical properties. Several aspects of stem-cell biology are relevant to cancer. First, both normal stem cells and cancer stem cells undergo self-renewal, and emerging evidence suggests that similar molecular mechanisms regulate self-renewal in normal stem cells and their malignant counterparts. Next, it is likely that mutations that lead to cancer accumulate in normal stem cells. Finally, it appears that tumors contain a "cancer stem cell" population with indefinite proliferative potential that drives the growth and metastasis of tumors [2].

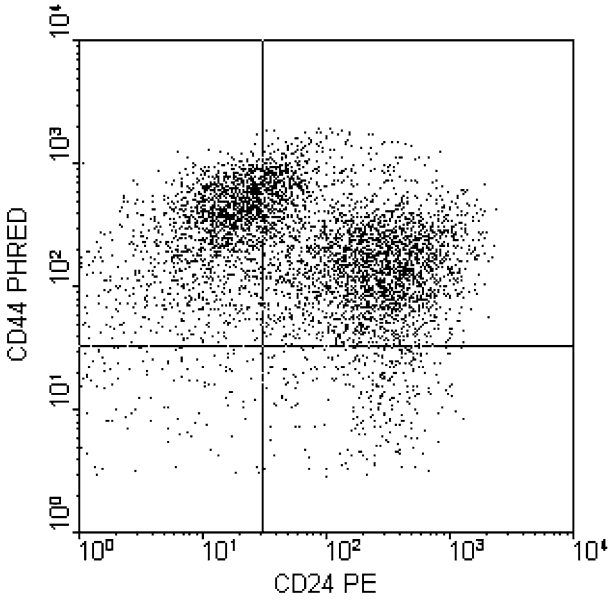

Fig. 1 Flow cytometry of a breast cancer tumor. Pleural effusion cells were dissociated and stained with antibodies against CD44, CD24, and a panel of antibodies that recognize normal cells [2]. The $CD44^{+}$ $CD24^{-/low}$ cancer cells, but not the majority of cancer cells with other phenotypes, had the exclusive ability to form tumors

Genetic regulation of self-renewal in normal stem cells and cancer cells

Maintenance of a tissue or a tumor is determined by a balance of proliferation and cell death [13]. In a normal tissue, stem cell numbers are under tight genetic regulation resulting in a constant number of stem cells in the organ [21, 22, 25]. By contrast, cancer cells have escaped this homeostatic regulation, and the number of cells within a tumor that have the ability to self-renew is constantly expanding, resulting in continuous expansion of the tumor. Thus, the identification of mechanisms in which cancer cells regenerate themselves through a process called self-renewal is critical to our understanding of these diseases. It is becoming clear that self-renewal pathways in at least some of the cancer cells are disrupted, resulting in an expansion of tumorigenic cancer cells. To develop assays to identify these pathways, one must first identify which cancer cells in the tumor are capable of self-renewal.

Several genes that regulate normal stem cell self-renewal play a role in cancer. Recently, it has been shown that Wnt/β-catenin signaling plays a pivotal role in both self-renewal of normal stem cells and malignant transformation [7, 17, 27]. The Wnt pathway (Fig. 2) was first discovered in mouse mammary tumor virus-induced breast cancer where deregulated expression of Wnt-1 due to proviral insertion resulted in mammary tumors [23, 32]. Subsequently, it has been shown that Wnt proteins play a central role in pattern formation. Wnt-1 belongs to a large family of highly hydrophobic secreted proteins that function by binding to their cognate receptor, a dimer of one of the members of the Frizzled family with low-density lipoprotein receptor-related protein 5 or 6, resulting in the activation of β-catenin [7]. In the absence of receptor activation, β-catenin is marked for degradation by a complex consisting of the adenomatous polyposis coli (APC), Axin and glycogen synthase kinase-3β proteins [14, 30].

Wnt proteins are expressed in the bone marrow, and activation of Wnt/β-catenin signaling by Wnt in vitro or by expression of a constitutively active β-catenin in vivo expands the pool of early progenitor cells and enriched normal transplantable hematopoietic stem cells in tissue culture and in vivo [27]. The inhibition of Wnt/β-catenin by ectopic expression of Axin, which targets β-catenin for ubiquitination and degradation, leads to the inhibition of stem cell proliferation both in vitro and in vivo. Other studies suggest that the Wnt/β-catenin pathway mediates stem or progenitor cell self-renewal in other tissues [32]. Higher levels of β-catenin are seen in keratinocytes with higher proliferative potential than those seen in keratinocytes with lower proliferative capacity [11]. Like their normal hematopoietic stem cell

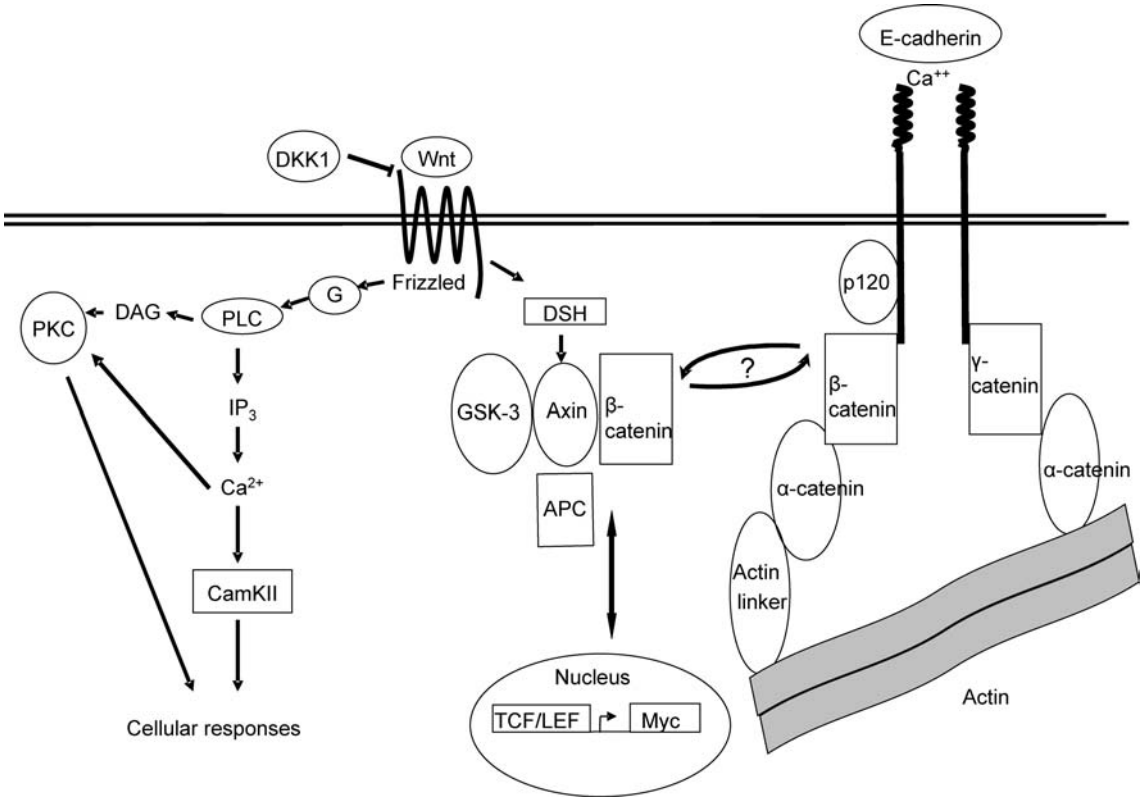

Fig. 2 Wnt signaling cascades. In normal cells, E-cadherin and α-catenin link β-catenin to the cell membrane and the cytoskeleton. In the canonical Wnt pathway, Wnt ligands bind to Frizzled receptors utilizing the LRP 5/6 co-receptors. DKK1 is an extracellular inhibitor of Wnt signaling that blocks the LRP 5/6 co-receptors. Ligand-bound Frizzled receptors activate Dishevelled (*DSH*) by an unknown mechanism, inhibiting the glycogen synthase kinase-3 (GSK-3)/Axin/adenomatous polyposis coli (APC) complex that targets β-catenin for degradation via the ubiquitination pathway in the absence of Wnt signaling. β-Catenin translocates to the nucleus, where it interacts with TCF/LEF transcription factors to initiate transcription of target genes. In the alternative pathway, the binding of Wnt ligands to Frizzled and LRP 5/6 co-receptors activates heterotrimeric G proteins (G). The ? indicates that disruption of the E-cadherin/β-catenin complex may release β-catenin from the adhesion complex and allow it to activate the canonical Wnt-signaling pathway. Activation of G proteins results in the formation of inositol-1,4,5-triphosphate (IP_3), which triggers the release of intracellular Ca^{2+}. This triggers the activation of the Ca^{2+}-sensitive enzymes such as calmoldulin-dependent protein kinase II (*CamKII*) and protein kinase C (*PKC*), which leads to cellular responses (*DAG* diacylglycerol)

counterparts, enforced expression of an activated β-catenin increased the ability of epidermal stem cells to self-renew and decreased their ability to differentiate. Mice that fail to express TCF-4, one of the transcription factors that is activated when bound to β-catenin, soon exhaust their undifferentiated crypt epithelial progenitor cells, further suggesting that Wnt signaling is involved in the self-renewal of epithelial stem cells. Normally, β-catenin is anchored to the cell surface and the cytoskeleton by E-cadherin and α-catenin (Fig. 2). In the fruit fly, disruption of this interaction leads to increased stem cell proliferation and expansion of the stem cell pool, probably secondary to loss of polar cell divisions [36].

The catenins have been implicated in cancer. Activation of β-catenin in colon cancer by inactivation of the protein degradation pathway, most frequently by mutation of APC, is common [31]. Expression of certain Wnt genes is elevated in some other epithelial cancers, suggesting that activation of β-catenin might be secondary to ligand activation in such cancers [3]. There is evidence that constitutive activation of the Wnt/β-catenin pathway may confer a stem/progenitor cell phenotype to colon cancer cells. Inhibition of β-catenin/TCF-4 in a colon cancer cell line induced the expression of the cell-cycle inhibitor $p21^{cip\text{-}1}$, and also induced the cells to stop proliferating and to acquire a more differentiated phenotype [33]. Enforced expression of the proto-oncogene c-*myc*, which is transcriptionally activated by β-catenin/TCF-4, inhibited the expression of $p21^{cip\text{-}1}$ and allowed the colon cancer cells to proliferate when β-catenin/TCF-4 signaling was blocked, linking Wnt signaling to c-*myc* in the regulation of cell proliferation and differentiation.

The role of the mutations in the components of the canonical β-catenin pathway in breast cancer is less clear [9]. Unlike colon cancer, in breast cancer, mutations in APC or β-catenin resulting in stabilization of the protein are rare. In colon cancer, these mutations result in the accumulation of the β-catenin in the nucleus. Of the 24 breast cancer cell lines studied, only the DU 4475 cells contained a mutant APC gene [28]. Despite the rarity of mutations in APC or β-catenin, in some cases of breast cancer, the β-catenin is located in the nucleus rather than

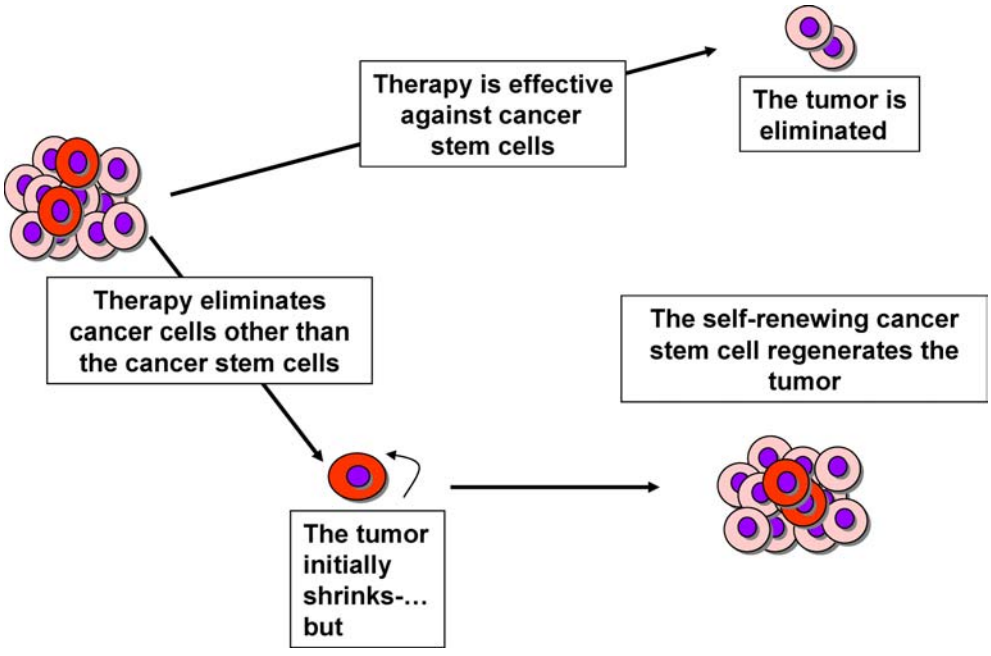

Fig. 3 Effect of cancer treatments. When patients with solid tumors are treated, their tumors often shrink. Since the bulk of the tumor is not composed of the cancer stem cells, the therapies must eliminate the nontumorigenic cells. In the case of testicular cancer, even though platinum-based therapies do not always eliminate all of the cancer cells, the cancer stem cells are usually eliminated and most patients are cured. However, in most solid tumors, the therapies likely spare a significant number of the cancer stem cells and the residual cancer stem cells regenerate the tumor

the outer membrane. In these cancers, c-*myc* and cyclin D1, downstream targets of β-catenin, are overexpressed and this is associated with poor prognosis. Studies using cell lines suggest that in some cancer cell lines the β-catenin pathway is activated by autocrine secretion of Wnts. In these cells, growth is inhibited using the soluble Wnt inhibitor DKK1 and the growth of some breast cancer cell lines is inhibited.

Implications of cancer stem cells

An axiom in the treatment of tumors is that remission is, in general, more difficult to achieve with each relapse. Obtaining initial remission as dictated by current surveillance methods only results in the patient succumbing to disease in relapse. Metastasis is also a difficult hurdle to cross in many clinical settings. In most cancers, the presence of metastasis at diagnosis dictates more aggressive therapies, and lower disease-free survival rates, as seen in sarcomas and neuroblastomas. In the cancer stem cell model, it is the tumorigenic cancer stem cell that escapes chemotherapy and metastasizes to a new location to cause distant tumor recurrence. Our current approaches to the cure are dependent on a few qualities that tumors exhibit. Surgical approaches are successful in tumors in which metastasis is not an issue and where the tumor can be removed en bloc. Many chemotherapeutic drugs and radiation treatments depend on cells that divide and proliferate at high rates. Antibody therapy is dependent on the presence of an effective antigen. Under the cancer stem cell model, it becomes clear that present therapy would not be effective at targeting cancer stem cells. If cancer stem cells exhibit qualities of other stem cells, their low rates of division and proliferation would help them to avoid chemotherapy and radiation. Certain antigens currently targeted by biologic therapies may not be expressed on cell surfaces until the cells are more mature. Current therapy may be good at producing initial tumor burden reduction, but if the cancer stem cells are spared, relapse is inevitable (Fig. 3).

Conclusions

Cancers arise in tissues that contain a stem cell population. Since stem cells are often the longest-lived cells in an organ, mutations leading to cancer often accumulate in the stem cell pool. Tumors of many, if not all, organs contain a cancer stem cell pool that has the exclusive ability to drive tumor growth. These cells may or may not be derived from normal stem cells. Regardless of the cell of origin, the cancer stem cells rely on self-renewal pathways present in normal stem cells to maintain themselves and expand. Clearly, there are mutations in the self-renewal pathways in cancer stem cells. Targeting these aberrant self-renewal pathways in cancer cells may result in more effective cancer therapies.

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