

CORNING

Culture and Assay Systems Utilized for Cancer Stem Cell Research

Review Article

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Introduction

Advances in stem cell biology over the last two decades have provided new insights into cancer biology.¹⁻³ Tumors show marked heterogeneity in morphology, proliferation rates, genetic lesions and therapeutic response. Thus, not all tumor cells are equal. This heterogeneity is even seen in an individual tumor that is clonal. The cellular and molecular basis for tumor heterogeneity represents a fundamental problem for cancer researchers.^{4,5} What controls the tumor cells? Are they responding to external and internal influences or are they a caricature of normal adult tissue that retains a hierarchical organization with stem cells at the top? Recent work with cancer stem cells suggests that there may not be one unifying theory to explain tumor heterogeneity.⁶⁻⁸ Both clonal evolution and the cancer stem cell model may be complicated by the plasticity of the cancer stem cell.^{9,10} Despite these theoretical arguments, the cancer stem cell model is vital to cancer research, especially in explaining tumor heterogeneity. The following overview will focus on cancer stem cells and the culture systems for growing and studying these cells. Particular focus will be on the variety of extracellular matrix substrates used to analyze the growth properties and behavior of cancer stem cells, including both *in vitro* and *in vivo* models.

Definition

It is now believed that most tumors contain a small subpopulation of cells with stem cell properties, namely cells with the ability to perpetuate through self-renewal and the ability to generate diverse mature cell types by differentiation.¹¹ These cells, termed cancer stem cells (CSC), have the ability to produce all of the distinct cell types found in their original tumor. They are defined experimentally by their ability to regrow tumors and are also referred to as tumorigenic cells. It is currently unclear whether CSCs arise from the transformation of stem cells or from the dedifferentiation of mature neoplastic cells.^{8,11,12} Although CSCs usually represent a small fraction of the cells within a malignant tumor, they have the ability to initiate tumors upon transplantation and may be the driving force behind malignancies. CSC rich tumors are associated with higher rates of metastasis and poor patient prognosis.¹³ Furthermore, CSCs have been found to have increased resistance to chemotherapeutic agents.^{14,15,16} Understanding the biology and cellular chemistry of CSCs is necessary for developing more effective therapies to treat cancer.

Experimental Evidence for Cancer Stem Cells

Hematopoietic stem cells have been identified and isolated using specific surface marker profiles in conjunction with fluorescence activated cell sorting.¹ More recently, investigators have identified and used surface markers from embryonic or adult stem cells.¹⁷ These surface markers have been used to isolate cells from tumors that exhibit stem cell-like properties. CSCs were first discovered in the hematopoietic system.^{18,19} In these studies, a CD34+CD38- subpopulation was isolated from acute myeloid leukemia that could form tumors when transplanted into immunodeficient mice (the xenograph model). Using this experimental approach, a large number of CSCs have been identified in solid tumors including breast, brain and colon.^{20,21,22} More recently, CSCs have also been identified in prostate, pancreas, head and neck, lung, skin, liver, kidney, ovary, and bone.^{5,23} CSCs have also been isolated from established cell lines and from genetically modified cells.¹⁷ In each case, a small number of cells were isolated from a tumor using specific cell surface markers. These cells were then tested *in vitro* to demonstrate self renewal and shown to transfer disease into immunodeficient mice by forming tumors *in vivo*.

Although CSCs all share the fundamental properties of self-renewal and the ability to differentiate into a diversity of mature cell types that can recapitulate the original tumor, CSCs derived from different tumor types can exhibit significant variability. The properties that are used to identify and characterize CSCs from one type of tumor may be different from other tumor types. This may be due in part to the fact that CSCs identified with different methods display variable phenotypes. Furthermore, some cells not originally identified as CSCs have been shown to have tumorigenic potential and stem cell features, suggesting that there is a bidirectional conversion between stem and non-stem compartments.⁸ These recent studies have suggested that the characteristics required for a cancer cell to be tumorigenic may not be a set of stable properties (expression of a specific cell surface marker) but may involve dynamic pathways that are governed by the tumor microenvironment.²⁴

Role of the Microenvironment

There is a mutual exchange of information that guides the functional organization of normal tissues through collaboration with stromal cells, epithelial and tissue specific cells.²⁵ Cells communicate with each other via cell junctions, through interactions with the extracellular matrix (ECM) via receptors, and via dynamic interactions with hormones and soluble factors.²⁶ The ECM is composed primarily of glycoproteins, collagens, proteoglycans, and elastin. This material serves to stabilize tissues, promote cell attachment, and modulate cell functionality by specifically interacting with cell surface receptors and activating the associated signaling pathways.²⁷ It has been shown that the ECM plays structural, biochemical and mechanical roles in normal growth and differentiation of stem cells²⁸ and in cancer progression.²⁹

Normal stem cells reside in a “stem cell niche” that maintains them in a stem cell state.² This niche has a complex architecture containing stromal cells such as immune cells and mesenchymal cells, a vascular network, and ECM.³⁰ Like normal stem cells, CSCs are influenced by interactions between the nonmalignant cells that comprise their microenvironment. In fact, recent data suggest that CSCs rely on a similar niche, the “CSC niche,” which controls their self-renewal and differentiation.^{31,32} *In vitro* approaches have been used to investigate the specific, well defined interactions between CSCs and the surrounding stromal cells (detailed below). Syngeneic mouse models have helped clarify the role of the microenvironment in CSCs. Furthermore, signal transduction pathways (eg, Notch, Hedgehog, and Wnt pathways) are regulated by extrinsic signals originating in the stem cell microenvironment or niche.²⁵

This niche may even protect the CSCs from genotoxic insults by promoting a higher rate of DNA repair^{1,4,15,33} This suggests that many cancer therapies will fail if they kill the bulk of the tumor cells but do not eliminate the CSCs.^{7,34} As a result, the CSC microenvironment, including ECM binding sites and associated signaling pathways, is considered a potential target for anti-cancer therapies.¹¹

Strategies for Studying Cancer Stem Cells

CSCs are most often defined by the enrichment of a subpopulation of tumor cells from tumor tissue using specific cell surface markers and isolation with FACS.³ In addition to tumor tissue, CSCs have been isolated from existing tumor-derived cell lines.³⁵⁻³⁷ CSCs have also been isolated from genetic modifications of normal cells.^{8,38,39} Once isolated, the CSC enriched populations are then tested for their ability to self renew and form tumors.

Two *in vitro* assay systems are typically used to demonstrate self-renewal of CSCs: colony formation assays and sphere formation assays. The colony formation assay measures the functional capacity of stem cells and has recently been used to study breast and colon CSCs.^{37,40} Sphere formation (or tumorsphere) assays involve three dimensional (3D) culture systems. There are two main approaches for growing 3D tumorspheres, as a suspension in serum-free media or on a 3D substrate comprised of reconstituted basement membrane (Corning® Matrigel® matrix). Matrigel matrix has been used for tumorsphere formation of CSCs isolated from many solid tumor types as well as tumor derived cell lines and genetically modified cells (Table 1). The major component of Matrigel matrix is laminin, followed by collagen IV, heparin sulfate proteoglycans, entactin, nidogen and growth factors. Based on its physiological composition and functionality, Matrigel matrix effectively models the physical interplay that occurs between CSCs and the ECMs that exist in the tumor microenvironment *in vivo*. This is especially important in light of the recent studies demonstrating the role of the microenvironment in maintaining the CSC niche.^{25,30,77}

Xenotransplantation into immuno-compromised mice is the primary *in vivo* assay used to demonstrate tumorigenicity of CSCs. Many of the CSC transplantation experiments have utilized Corning® Matrigel® matrix as a carrier (Table 1). In a recent study, Quintana, et al., used FACS to fractionate melanoma cells into CSC-enriched CD271+ and CD271- subpopulations.⁶¹

These subpopulations were each mixed with Matrigel matrix High Concentration (HC) and then injected into NOD/SCID IL2R g null mice. The results demonstrated that both subfractions of melanoma cells were able to generate tumors *in vivo*, whether enriched for CSCs or not. These findings question the hierarchical model that CSCs are a minority cell type in all solid tumors, but rather may exist as heterogeneous CSC sub-types within one tumor type. Furthermore, these findings suggest that CSCs may be characterized by a unique plasticity that allows for reversible changes of their phenotype. Interestingly, the plasticity of CSCs has been demonstrated recently in breast cancer cells.^{8,51}

The functionality of CSCs may be influenced by the characteristics of the immunodeficient recipient, the site of implantation, the cell carrier (e.g., collagen, Matrigel matrix), the number of input cells, as well as the time *in vivo*.⁹ Since the growth of human tumors in mice is under the control of murine stroma and vasculature, it may be difficult to analyze the effects of the microenvironment on the growth and functionality of human cancer stem cells in this system. Although *in vivo* assays using immunodeficient mice are the gold standard for identifying stem cells, serial transplantation assays with animal models do not lend themselves to highthroughput screening.¹⁷ Alternatively, defined *in vitro* systems allow for the study of CSCs plasticity, the regulation of CSCs by the microenvironment, and may ultimately be used for compound screening and the development of anti-cancer drugs.^{25,49}

Table 1. Cancer Stem Cells/Representative Culture and Assay Conditions

Cell Type	Extracellular Matrix or other condition	Cell Function or Behavior	Cell Type	Extracellular Matrix or other condition	Cell Function or Behavior
BRAIN	Corning Matrigel Matrix	Tumorsphere Formation (3D) (31) Cell Invasion (41) Cell Migration (42) Differentiation (42)	PANCREAS	Matrigel Matrix	Migration (63) Xenotransplantation (64)
	Poly-D-lysine-Laminin	Differentiation (31,43)	KIDNEY	Matrigel Matrix	CSC Microvesicle-induced Angiogenesis (65) Xenotransplantation (66)
	Laminin	Attachment (44)	LUNG	Matrigel Matrix	Xenotransplantation (67)
	Serum-free Suspension	Tumorsphere Formation (3D) (21,43)		Fibronectin	Proliferation (50) Migration (67)
BREAST	Matrigel Matrix	Colony Formation (40) Tumorsphere Formation (3D) (39,8) Cell Invasion (36,45-47) Differentiation (3D) (39,48) Xenotransplantation (20,38,49)	PROSTATE	Serum-free Suspension	Tumorsphere Formation (3D) (68)
	Corning Primaria™	Proliferation (38)		Matrigel Matrix	Tumorsphere Formation (3D) (69,70) Cell Invasion (70,71) Differentiation (70) Xenotransplantation (70,72)
	Fibronectin	Adherence (50) Differentiation (48)	Laminin	Proliferation (50)	
	Collagen I, Laminin	Differentiation (48)	BONE	Matrigel Matrix	Xenotransplantation (73)
	Collagen IV	Differentiation (51)	OVARY	Matrigel Matrix	Xenotransplantation (74,75)
	Serum-free Suspension	Tumorsphere Formation (3D) (48,51)	Serum-free Suspension	Tumorsphere Formation (3D) (75,76)	
	COLON	Matrigel Matrix	Colony Formation (37,52) Tumorsphere Formation (3D) (33,53) Differentiation (3D) (32,53) Xenotransplantation (22,37,54,55-57)		
Collagen		Differentiation (55,58) Proliferation (53)			
Fibronectin		Adherence (50)			
Serum-free Suspension		Tumorsphere Formation (3D) (22,32,33,54,55)			
MELANOMA	Matrigel Matrix	Migration (59) Differentiation (3D) (60) Xenotransplantation (61)			
	Serum-free Suspension	Tumorsphere Formation (3D) (62)			

Specific *In Vitro* Culture Conditions And Assays

3D Culture of Cancer Stem Cell Tumorspheres

Many laboratories have utilized reconstituted basement membrane (Corning® Matrigel® Matrix) to grow tumorspheres *in vitro*.^{8,31,33,39,48,53,69,70} Other studies have grown tumorspheres in suspension using serum-free media.^{21,22,32,33,43,48,51,54,55,62,68,75,76} Regardless of the conditions in which the tumorspheres are initially grown, they can be further cultured *in vitro* in the presence of Matrigel matrix, which allows for their propagation and differentiation. Dontu and colleagues developed a 3D culture system in Matrigel matrix that allowed single cells isolated from mammospheres to generate complex acinar structures (Fig. 1).⁴⁸ Two more recent studies have demonstrated the plasticity of breast epithelial CSCs in a 3D culture environment comprised of Matrigel matrix. Mani and colleagues have shown that breast epithelial cells transformed with the SNAI¹ and TWIST genes, which are known to induce the Epithelial-Mesenchymal Transition (EMT), acquired characteristics of CSCs.³⁹ The EMT is important in development, and it is often activated in cancer invasion and metastasis. Specifically, the transformed, immortalized cell lines exhibited an increased ability to form mammospheres in a matrix composed of Matrigel matrix. These cells looked like CSCs isolated from human tumors and showed an increase in EMT markers. These researchers also demonstrated that single mammospheres from the transformed cell lines could differentiate in Matrigel matrix to form complex 3D structures similar to mammary ducts (Fig. 2).³⁹ These findings illustrate a direct link between the EMT and the acquisition of epithelial stem cell properties. In another study, breast CSCs cultured in 3D using Matrigel matrix were found to exhibit an unexpected degree of plasticity between stem-like and breast cancer cells that do not exhibit stem-like properties.⁸ Using 2D and 3D culture, a subpopulation of mammary epithelial cells was shown to spontaneously differentiate into stem-like cells. Furthermore, the results demonstrated that genetic transformation enhances the spontaneous conversion of non-stem cancer cells to CSCs *in vitro* and *in vivo*. Both of these studies dispute the hierarchical model for CSCs and have the potential to drastically change the strategies used for anti-cancer drug discovery.

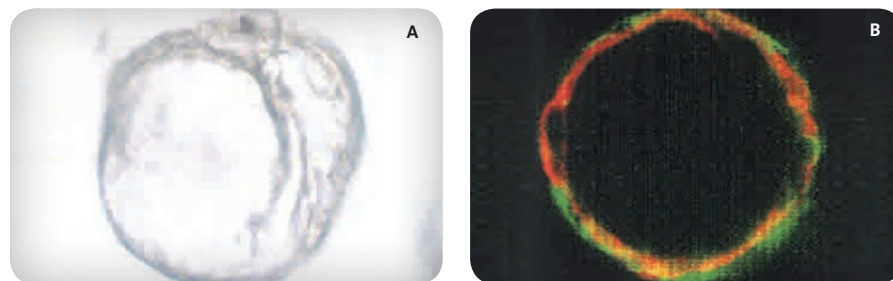


Figure 1. Mammospheres Contain Multipotent Cells Capable of Differentiating into Ductal-Alveolar Structures. (A) Acinar structure generated by a single human mammary epithelial cell isolated from the mammosphere and grown on Corning Matrigel matrix for 3 weeks (3D culture). (B) Acinar structure visualized by immunostaining for myoepithelial lineage (CD10, FITC, green) and ductal epithelial lineage (ESA, TEXAS RED®). Data courtesy of Dr. Gabriela Dontu (originally published in reference 48), University of Michigan.

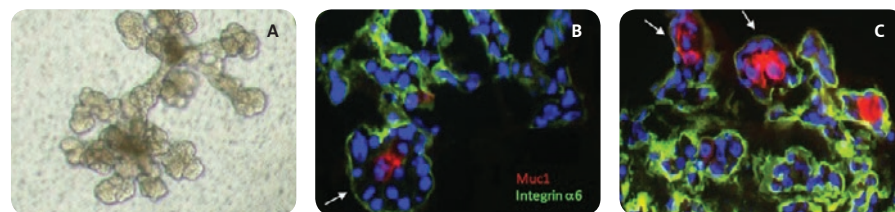


Figure 2. *In Vitro* Differentiation of Mammospheres in Matrigel Matrix Promotes the Formation of Secondary Structures. (A) Phase contrast images of the differentiated mammospheres following culture in Matrigel matrix. (B and C) The differentiated structures were immunostained for Muc 1 (red) and CD49f/integrin 6 (green). Data courtesy of Dr. Robert Weinberg (originally published in reference 39), Massachusetts Institute of Technology.

Co-Culture of Cancer Stem Cell Tumorspheres

Co-culture systems have been used to better understand how CSCs are regulated by their micro-environment. In a recent study, two approaches were used to examine brain CSCs in the presence of primary human endothelial cells (PHEC).³¹ When these cells were cultured together in Corning® Matrigel® matrix, the PHECs formed vascular tubes (Fig. 3).³¹ This result was only observed using the CD133+ subfraction (enriched with CSCs), but not the CD133- subfraction. Since normal neural stem cells are maintained by soluble factors secreted by endothelial cells, the authors then tested if endothelial-secreted factors were able to maintain brain CSCs using a cell culture insert model. In this system, the PHECs were cultured in the apical compartment (Falcon® inserts, 0.4 µm), and brain CSCs in the basolateral compartment. This insert system allows for the exchange of diffusible factors, but not cells, between chambers. The experiment demonstrated that PHECs allow for the maintenance of self-renewal and the undifferentiated phenotype (tumorspheres) of brain tumor CSCs. In addition, tumorsphere differentiation was demonstrated on a substrate composed of poly-D-lysine/laminin (Corning BioCoat™ coverslips). Taken together, these experiments demonstrate how the niche microenvironment participates in the regulation of CSC behavior.

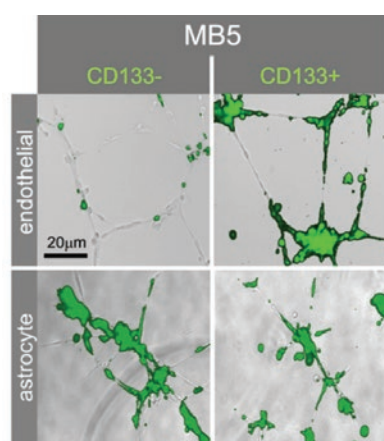


Figure 3. CD133+ Brain Tumor Cells Associate with Endothelial Cells in 3D Matrigel Cultures. Overlay of phase contrast and autofluorescence photomicrographs of unlabeled endothelial cells (top panels) or astrocytes (bottom panels) that were cocultured with CD133- or CD133+green fluorescence-labeled primary medulloblastoma cells (MB5). Data courtesy of Dr. Richard Gilbertson (originally published in reference 31), St. Jude Children’s Research Hospital, Memphis, TN.

Cancer Stem Cell-Mediated Cell Invasion Assays

In vitro cell invasion assays have been used to examine the interaction of cancer stem cells with stromal cells in order to better understand the stem cell niche in tumors. Previous studies have shown that mesenchymal cells may be recruited to the sites of developing tumors and stimulate tumor growth via the production of IL-6. Recently, Lui et al. demonstrated that bone marrow-derived mesenchymal stem cells (MSCs) exhibit invasive behavior in the presence of breast CSCs when cultured in invasion chambers precoated with growth factor-reduced Matrigel matrix.⁴⁶ Specifically, they showed that breast CSCs cultured in the basolateral chamber increased mesenchymal cell invasion from the apical chamber. This effect could be blocked with anti-IL-6 antibody. These results indicate that MSC invasion towards breast CSCs is mediated via the IL-6 signaling pathway, which supports the conclusion that cytokine networks regulate CSC interactions with stromal cells.

Previously, Charafe-Jauffret and colleagues used BioCoat Matrigel Invasion Chambers to demonstrate that IL-8 increases the invasiveness of CSCs that were isolated from breast cancer cells lines.³⁶ Specifically, they demonstrated that the Aldefluor+ subpopulation (enriched for CSCs) had a 6 to 20 fold increase in invasion through Matrigel matrix as compared to the Aldefluor- subpopulation (not enriched in CSCs). The addition of IL-8 to the culture system significantly increased the invasion of the CSCs, but had no effect on the Aldefluor- subpopulation. They concluded that the IL-8 pathway, reported by others to play a role in metastasis, is involved in CSC invasion. More recently, McGowan, et al. have used invasion assays to demonstrate the involvement of the Notch signaling pathway in maintaining the stem cell-like phenotype of breast CSCs.⁴⁷ This study demonstrated that silencing of Notch1 with shRNA significantly reduces the ability of breast cancer cells to invade through a barrier of Matrigel matrix. Taken together, these examples highlight key experimental systems currently used for studying the regulation of CSCs by signal transduction pathways *in vitro*.

Furthermore, invasion assays have been utilized to study how CSCs modify their tumor environment by triggering angiogenesis through the release of microvesicles (MVs). MVs have been implicated in cancer progression, and tumors are known to release large amounts of MVs.⁷⁸ Grange and colleagues isolated a renal CSC+ subpopulation (CD105+) and then derived MVs from the CSCs.⁶⁵ The MVs from the CD105+ subpopulation, but not MVs from the CD105- subpopulation, were shown to increase the angiogenic phenotype of human endothelial cells. Moreover, the CSC-derived MVs increased the invasion of endothelial cells through cell culture inserts coated with Corning® Matrigel® matrix. This paper demonstrated that MVs from renal CSCs trigger an angiogenic switch and formation of a pre-metastatic niche which may be involved in tumor progression and metastasis.

3D Differentiation Assays of Cancer Stem Cells

Colon CSCs that were isolated using the CD133 marker^{22,56} have been shown to exhibit differentiation *in vitro*.³³ This work investigated the differentiated properties of colon CSCs grown as spheroid cultures (tumorspheres) in serum-free media. The spheroid cells could be forced to differentiate *in vitro* into large polygonal colon cells when grown as adherent cultures on collagen-coated flasks with 10% serum. When the tumorspheres were grown in 3D in the presence of serum in Matrigel matrix, the resulting colonies were organized in a complex structure reminiscent of a colonic crypt (Fig. 4).³³ Others have recently adapted this *in vitro* 3D culture system using Matrigel matrix to propagate normal intestinal crypts^{79,80} and CSCs derived from colorectal cancer-derived cell lines.³⁷

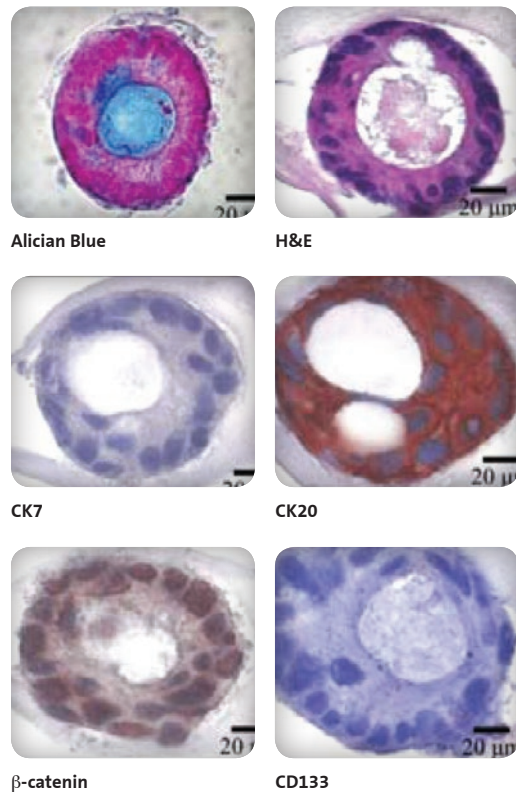


Figure 4. Colon Cancer Spheroids Cultured in the Presence of FBS in Corning Matrigel Matrix Organize in a Complex Structure Reminiscent of a Colonic Crypt. Alcian blue, H&E staining, and immunohistochemical analysis of CK7, CK20, b-catenin, and CD133 performed on paraffin-embedded sections of spheroids cultured in Matrigel matrix for 20 days. Data courtesy of Dr. Giorgio Stassi and Dr. Jan Paul Medema (originally published in reference 33), University of Palermo and Academic Medical Center, Amsterdam, the Netherlands.

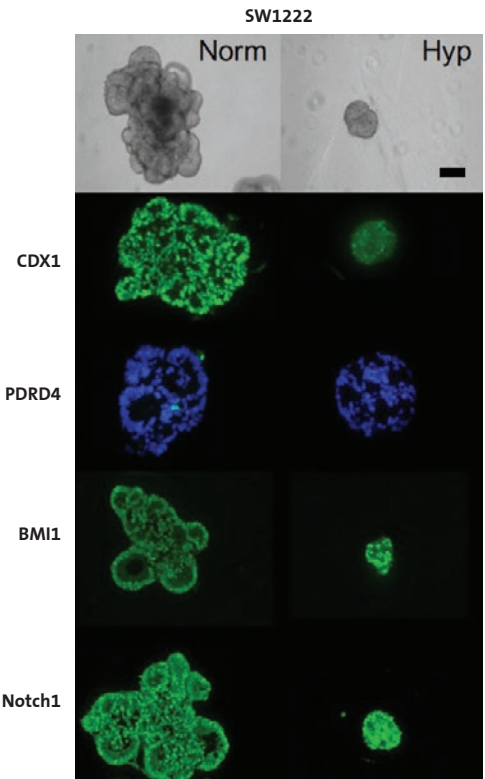


Figure 5. Hypoxia Prevents Differentiation of Colorectal Cancer Cells and Maintains a Stem-like Phenotype. Light microscopy and immunofluorescence of SW1222 grown for 4 weeks in Matrigel matrix under normoxia and hypoxia (1% oxygen). (Magnification: 20x objective; scale bar: 200 μm.) Data courtesy of Dr. Walter Bodmer (originally published in reference 52), University of Oxford, UK.

In a subsequent study using colon CSCs, Vermeulen and colleagues demonstrated that CSC differentiation is controlled by an extracellular signal input utilizing *in vitro* differentiation in the presence of growth inhibitors.³² Single cell clones of CSCs were isolated and shown to form tumors *in vivo* when xenotransplanted into mice and were also shown to exhibit multilineage differentiation *in vitro* when cultured in growth factor-reduced Corning® Matrigel® matrix. Importantly, they showed that the *in vitro* differentiation of colon CSCs could be directed by inhibiting phosphoinositide 3-kinase (PI3K) signaling. These findings have provided clues to the regulatory pathways that govern CSCs *in vivo*.

Yeung et al. have demonstrated that colorectal CSCs derived from cell lines were able to differentiate under normal oxygen conditions *in vitro* using Matrigel matrix.^{52,37} Previous studies have demonstrated that hypoxia can induce the CSCs phenotype in Glioma stem cells⁸¹ as seen by the smaller, rounder, less differentiated colonies grown under hypoxic conditions. In this report, hypoxia inhibited the differentiation of these CSCs and increased their clonogenicity as seen by the smaller, rounder, less differentiated colonies grown in 1% oxygen (Fig. 5).⁵² Furthermore, this study implicated the Notch1 and CDX1 ligands in controlling the differentiation of these cells. Taken together, these findings help to explain why hypoxia is associated with a more aggressive tumor and poor clinical outcome by showing that hypoxia leads to an increase in the proportion of CSCs in a tumor.

Future Directions

The distinct roles of CSCs in cancer progression can be studied by a variety of complementary *in vitro* approaches. Investigation of CSCs offers the possibility of generating novel targets for cancer that may overcome drug resistance and effectively combat the process of tumor cell metastasis.¹² Because CSCs appear similar to normal stem cells, great care must be taken to protect normal cells when patients are exposed to anti-cancer treatments. New and improved experimental approaches, and especially *in vitro* assay systems, will allow scientists to micro-dissect the exact relationship between the various cell types within a tumor and their microenvironment.

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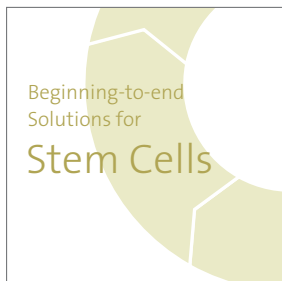
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