

A grayscale histological micrograph showing a cross-section of tumor tissue. The image features several clusters of dark, densely packed cells, likely representing neoplastic growth, interspersed with lighter, fibrous-looking connective tissue stroma. The overall texture is complex and irregular, characteristic of a tumor's microenvironment.

THE ECOLOGY OF Tumors

By perturbing the microenvironment, wounds and infection may be key to tumor development.

By Paria A. Kenny, Celeste M. Nelson, and Mina J. Bissell

No tumor is an island. Chemical and physical forces exerted by the diverse cellular populations that surround a tumor – its so-called microenvironment – shape development and progression. Manipulating these ‘ecological’ factors is increasingly attractive therapeutically, as Mina Bissell and colleagues discuss in the following pages. And just as the cellular neighborhood is diverse, tumors enriched in these environments comprise a variety of cell types. In this heterogeneous mix, increasing evidence points to so-called cancer stem cells as the root of malignancy. Irving Weissman and Michael Clarke discuss the implications for leukemias on page 35, and on page 37 Peter B. Dirks discusses cancer stem cells in the brain.

Conventional wisdom says that cancer results when somatic cells acquire mutations that cause them to proliferate more rapidly than their neighbors. This makes cancer cells vulnerable to agents targeting dividing cells, such as radiotherapy, cytotoxic chemotherapies, and more recently, targeted therapeutics. But this view of cancer is a gross oversimplification. Tumor cells exist in intimate symbiosis with the rest of the body and co-opt several normal physiological processes to facilitate their growth and progression. The recognition that cancer growth and spread is not solely a tumor cell-autonomous process suggests that additional components of the tumor milieu might prove useful targets for therapy.

Developmental biologists have long recognized that organogenesis proceeds via inductive cues in an exquisite dynamic crosstalk between different tissues of an organ, such as between epithelium and stroma. These are complex dialogs consisting of soluble molecules, including growth factors, cytokines, hormones, and proteases, insoluble factors such as extracellular matrix components, and direct cell-cell interactions. Both normal and cancer cells, but especially the latter, inhabit a complex cellular ecosystem – stromal cells, endothelial cells, lymphocytes, macrophages, mast cells, and other cell types – all of which may interact via juxtacrine and paracrine mechanisms (see “The Tumor in its Natural Environment,” page 32). It is now becoming more widely appreciated that, as with other organs, the biogenesis of the tumor represents an interaction between the tumor epithelial cells and these other cell types.

Like all solid tissues, tumor cells require vasculature to provide oxygen, nutrients, and a means of waste disposal in order to grow beyond 1–2 mm³. Thus tumor cells co-opt the normal physiological processes of angiogenesis to recruit endothelial cells and a blood supply. They may even masquerade as endothelial cells themselves.¹

Since the earliest examinations of tumors by pathologists, cells of the immune system such as macrophages have been observed in the microenvironment of most solid tumors. These were long thought to be evidence of the body’s immune response to the tumor. It has recently been realized that this immune cell infiltration is often an indicator of poor prognosis – particularly in cancers of the breast, prostate, ovary, and cervix – and that these ostensibly well-meaning cells are, in fact, recruited to the tumor site by chemotactic signaling molecules. Macrophages are rich in growth factors, cytokines, and proteases, and these factors are thought to facilitate (rather than impede) tumor progression.²

The nature of the connective tissue stroma in the tumor may also be radically different from that of the host tissue. Whereas

the host organ may have been relatively soft and pliable, tumors are often tough and fibrotic. This stiffness is often the reason that an individual initially notices a tumor. It has recently been shown that these alterations in the mechanical properties of the tissue contribute to the malignant phenotype.³ The production of this desmoplastic reactive stroma by fibroblasts within the tumor microenvironment is in response to signals from the tumor.

FROM WOUNDS, TUMORS

Harold Dvorak and colleagues at Beth Israel-Deaconess Medical Center and Harvard Medical School first noted the striking similarities between the tumor stroma and the wounding environment in 1986,⁴ and it is now well recognized that wounding and inflammation can stimulate tumor progression. In a series of experiments in chick embryos in the 1980s, our laboratory showed that infection of embryos in ovo with the transforming Rous sarcoma virus (RSV) gave rise to developmentally normal chickens, even though the potent *v-src* oncogene was expressed and active. Cells explanted from these chickens were demonstrably transformed in culture. Injection of adult chickens with RSV gives rise to a tumor at the injection site, even though RSV spreads throughout the body. Wounding a bird after injection at a distant site is sufficient to elicit tumor formation at that site.⁵

Thus, the normal tissue architecture and microenvironment can be considered potent tumor suppressors, even when cells are expressing transforming oncogenes. Disruption of the microenvironment by wounding, inflammation, or when basement membrane is disrupted with proteases, can facilitate the expansion of these mutant cells or indeed cause genomic instability. Using transgenic mouse models, we showed that overexpression of a stromal metalloproteinase, matrix metalloproteinase (MMP)3, leads to tumor formation in the mammary gland by inducing genomic instability in a manner dependent on the production of reactive oxygen species.⁶

These experimental models suggest that a disordered microenvironment can cause or promote tumorigenesis. Epidemiological data indicate that one sixth of human cancers arise in association with chronic inflammation, due to bacterial or viral infection or other problems. For example, the bacterium *Helicobacter pylori* has been convincingly linked to gastric cancer; and hepatitis B, Epstein-Barr virus, human herpesvirus 8, ulcerative colitis, chronic pancreatitis, Barrett’s esophagus, and Hashimoto thyroiditis have been linked to hepatocellular carcinoma, Burkitt lymphoma, Kaposi sarcoma, and carcinomas of the colon, pancreas, esophagus, and thyroid, respectively. Unlike RSV, many of ▶

these agents do not carry a cargo of oncogenes, and the chronic local inflammation is believed to underlie tumorigenesis in each case.⁷ It is known from large autopsy series of individuals who died of causes distinct from cancer, that the majority of people have numerous small indolent tumors that are below the normal threshold of clinical detection.⁸ We propose that the tissue architecture and microenvironment may play important tumor-suppressive roles in these small neoplasms and that microenvironmental perturbations, such as those induced by wounding and inflammation, may in some cases allow such growths to progress to clinically detectable tumors.

THE UNKNOWN TUMOR SUPPRESSORS

While known strong tumor suppressors such as BRCA1/2 and APC underlie familial cancer syndromes, it is believed that a substantial number of low-penetrance alleles remain to be identified, which, in particular combinations, may lead to a familial predisposition. Considerable attention is now being given to polymorphisms in genes encoding proteins present in the tumor microenvironment. Generally these polymorphisms are found in the noncoding regions of the genes and are involved in modulating gene expression.

For example, insertion of a guanine 1,607 base pairs upstream of the transcription start site of MMP1 results in higher expression from that allele. This allele has been shown to correlate with poor prognosis in ovarian and colorectal carcinoma and in malignant melanoma.⁹ Studies of transgenic mice expressing MMP1 are not far advanced, but our lab has shown that overexpression of the related MMP3 results in tumorigenesis in the mammary gland. The contribution of genetic changes in the stroma to epithelial transformation has begun to be studied in transgenic mouse models. In a model of neurofibromatosis, Luis Parada and colleagues have shown that heterozygosity for the NF1 tumor suppressor in nonneoplastic cells facilitates transformation of Schwann cells in which both copies of the gene are deleted.¹⁰ Hal Moses and colleagues at Vanderbilt University School of Medicine in Nashville demonstrated that specific deletion of TGFBR2 in fibroblasts in the stroma leads to transformation of selected adjacent epithelia.¹¹

A considerable body of evidence now suggests that tumor progression represents the coevolution of tumor epithelium and tumor stroma. Mutations in several important oncogenes and tumor suppressor genes, such as EGFR, p53, and PTEN, have been detected in expanding clones of tumor stromal cells. Thus, instead of acting as a lone cellular renegade, a tumor may be more akin to a desperate addict needing to get its fix from the dealers in the neighborhood. It follows then that, while killing the tumor cells is an obvious thing to do, it might also be prudent to target the other troublemakers in the neighborhood and rehabilitate them to restore order in the cellular society.

Just as the ecology of the primary tumor microenvironment is a critical determinant of tumor progression, if the tumor cells are to successfully establish a metastatic colony, a suitable habitat must be found here also. Metastasis is an incredibly arduous proposition for a tumor cell, as the body has so many controls to try to prevent

The Tumor in its Natural Environment

Tumorigenesis doesn't happen in isolation, but rather in the complex milieu of structured tissues and organs. This graphic depicts the development of a generic epithelial tumor. In this microscopic ecosystem the interactions between local participants gradually change, creating a vast network of cross-talking cellular and architectural components that sustain growth. Below are just a few of the interconnected cells and signaling factors that sustain uncontrolled proliferation, angiogenesis, recruitment of immune cells and their accompanying growth factors and cytokines, invasion through the basement membrane, and metastasis. This interconnected web makes attacking "just the tumor" an incomplete therapy.

1. TUMOR CELLS: As growth of transformed epithelial cells progresses out of control these become invasive, breaching the integrity of the basement membrane, invading the local tissue and entering the bloodstream

Produce: Proteases, VEGF, CSF1, TGF-beta, PDGF, other growth factors
Respond to: TGFβ, other growth factors, CXCL12, other cytokines

2. BLOOD VESSELS: VEGF and other tumor-derived growth factors promote growth towards the tumor. Increased vasculature provides oxygen and nutrients and alters the physiology of the microenvironment.

3. MONOCYTES AND MACROPHAGES: As monocytes mature into macrophages, they infiltrate the stroma and tumor in response to chemotactic signals from the tumor.

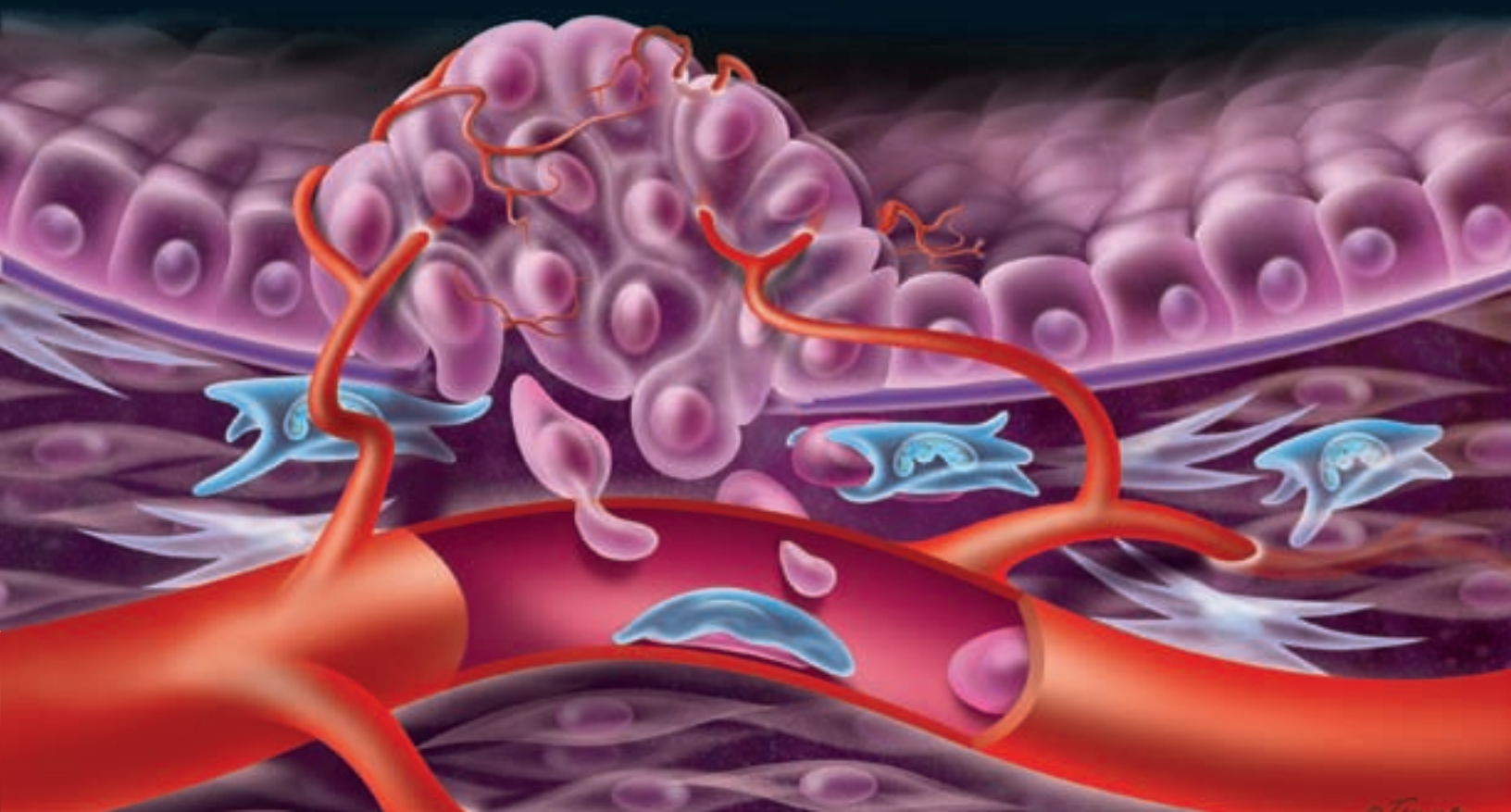
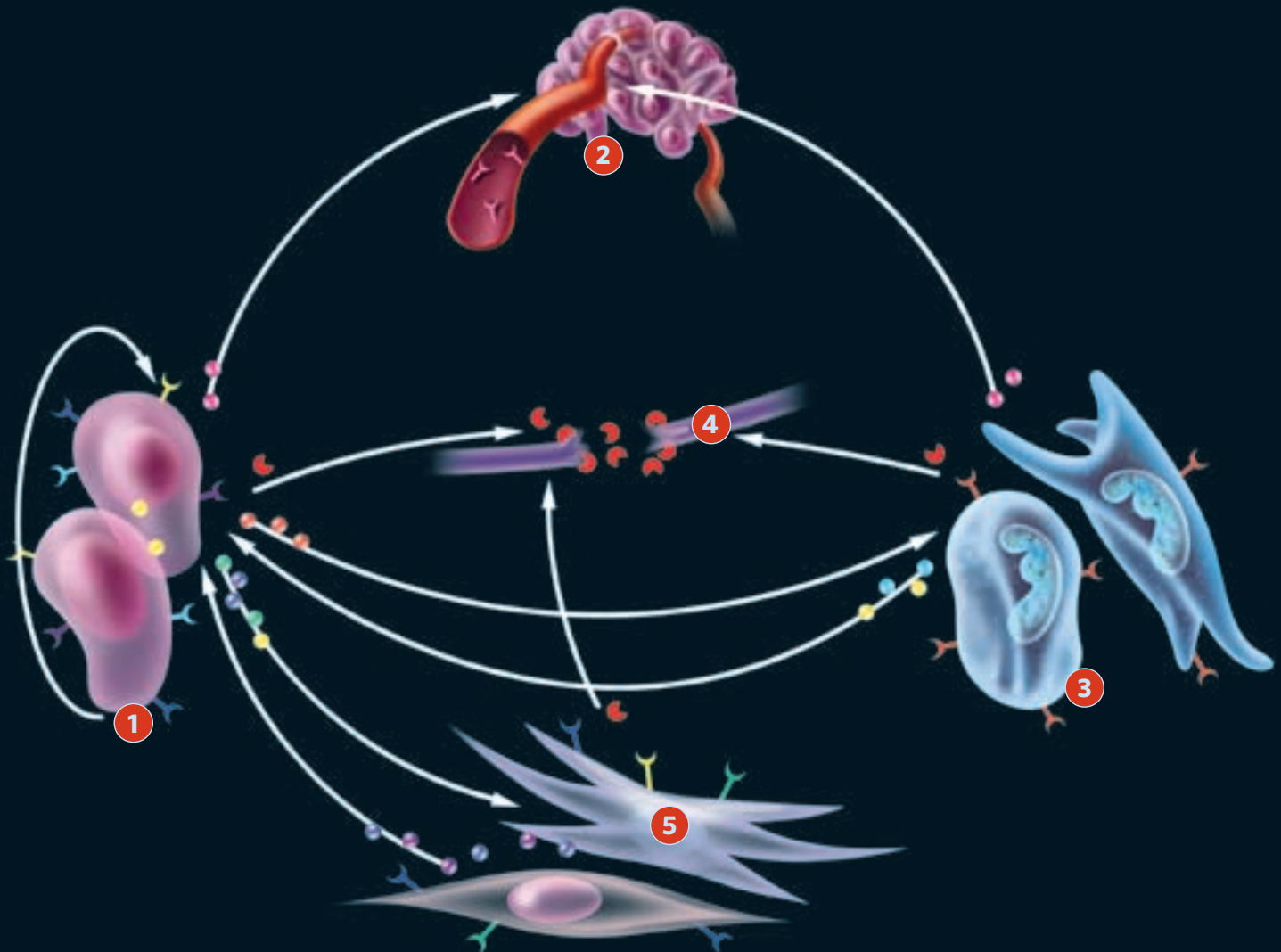
Produce: Proteases, VEGF, EGF, other growth factors
Respond to: CSF1

4. BASEMENT MEMBRANE: This fibrous protein envelope provides important cues to maintain tissue-specific differentiation of normal epithelia. It separates the epithelium from the stroma and forms a barrier to local tissue invasion by cancer cells. Proteases produced by the different cells of the tumor microenvironment can digest it.

5. FIBROBLASTS AND MYOFIBROBLASTS: Collagen producing fibroblasts in the stroma develop into muscle-like myofibroblasts when stimulated by tumor-derived factors, producing the tactile stiffness of tumors and aiding the invading cells.

Produce: Proteases, TGFβ, CXCL12
Respond to: TGFβ, PDGF, other growth factors

- Proteases
- CSF1 (colony stimulating factor 1)
- CXCL12 (chemokine CXC motif ligand 12)
- EGF (epidermal growth factor)
- PDGF (platelet derived growth factor)
- VEGF (vascular endothelial growth factor)
- TGFβ (transforming growth factor β)
- Other Growth Factors



TUMOR MICROENVIRONMENT-TARGETED THERAPIES AND THEIR TARGETS

Class	Examples	Current status
Heterotypic cell-signaling mediators	Infliximab (monoclonal antibody against TNF α), etanercept (soluble TNF α receptor)	Infliximab is approved in US for Crohn disease and ulcerative colitis (both predispose to colorectal cancer). Etanercept is approved in US for rheumatoid arthritis. Both are in clinical trials for efficacy in cancer
Nonsteroidal anti-inflammatory drugs (NSAIDs)	Aspirin	Large observational studies of hundreds of thousands of individuals have associated frequent aspirin use with significantly reduced incidence of colorectal cancer, in which cyclooxygenase 2 is often highly expressed. ^{1,2}
VEGF targeting therapies	Bevacizumab	Approval in the US for treatment of colorectal cancer in combination with 5-fluorouracil
Aromatase inhibitors	Anastrozole, letrozole, exemesthane	Class has been shown to be more effective than tamoxifen at preventing recurrence after surgery for breast cancer in postmenopausal women, where aromatase in stromal cells plays a crucial role in biosynthesis of estrogen, which in turns promotes tumor growth in estrogen-positive tumors
Bisphosphonates	Zoledronic acid	Demonstrated utility in solid tumors metastasizing to bone. If treatment is started when bone loss due to multiple myeloma is first detected, zoledronic acid significantly reduces the incidence and delays the onset of skeletal complications. The drug achieves high local concentrations by binding to bone hydroxyapatite, leading to loss of osteoclasts and, consequently, an attenuation of bone resorption.
Cryptic peptide fractions of extracellular proteins that inhibit angiogenesis	Canstatin and tumstatin (from collagen IV), endostatin (from collagen XVIII), angiostatin (from plasminogen), restin (from collagen XV)	Collagen XVIII is located on chromosome 21. Individuals with Down syndrome who bear three copies of this chromosome have 50% more serum endostatin, and experience epithelial malignancies at one tenth the rate of age-matched individuals of normal genotype. While endostatin has shown efficacy in preclinical models, the early results of clinical trials in human patients were not very encouraging. Nevertheless, anecdotal reports of a Chinese Phase III study using a modified endostatin are considerably more promising.

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 2. M.J. Thun et al., "Aspirin use and reduced risk of fatal colon cancer," *N Engl J Med*, 325:1593-6, 1991
 3. Y. Sun et al., "Results of phase III trial of Endostar (rh-endostatin, Y-16) in advanced non-small cell lung cancer (NSCLC) patients," *Proc ASCO*, 23:7138a, 2005.

this invasion of its integrity. To establish a metastatic colony successfully, a tumor cell needs to: (1) digest the basement membrane, the barrier to local tissue invasion, (2) migrate to a blood or lymphatic vessel, (3) access the vessel, (4) evade the immune system and survive in the circulation, (5) escape from the blood vessel at a distant site, and (6) survive and proliferate in the new organ. Stephen Paget proposed the "seed and soil" hypothesis more than 100 years ago.¹² His observations of metastatic spread from primary breast cancers led him to believe that metastatic colonies were not randomly distributed and that such colonies would grow only if the "seeds" fell upon a "congenial soil." In other words, in order for metastasis to become clinically relevant, the tumor cells need to find a suitable microenvironmental niche in the new host tissue.

MICROENVIRONMENT-TARGETED THERAPIES

The foundation for therapies directed at the tumor microenvironment is the concept that the tumor may be dependent on many local factors extrinsic to the tumor cells themselves. These cells, unlike the neoplastic cells, are generally genetically stable and are thus thought less likely to become resistant to therapy.

The process of angiogenesis is essential for both the establishment and the maintenance of the tumor, and therefore, the tumor vasculature represents an especially attractive target for therapy. Despite the fact that angiogenesis is a complex process with many soluble signaling proteins playing both pro- and anti-angiogenic roles, some success in the clinic has been reported using a recombinant humanized antibody against vascular endothelial growth factor, the prototypic pro-angiogenic molecule. The US Food and Drug Administration approved this drug, Avastin, in 2004 for treatment of metastatic colorectal cancer in combination with 5-fluorouracil. Its efficacy in several other epithelial tumor types is currently

being evaluated in further Phase III clinical trials. In addition to Avastin, a number of other anti-angiogenic small molecules, siRNAs, monoclonal antibodies, and decoy receptors are being evaluated. For more examples of microenvironment-targeted therapies, see the table above.

THE WAY FORWARD

Over the past four decades, the extracellular matrix (ECM) has evolved in our opinion from an uninteresting, inscrutable, insoluble gemisch of proteins known as ground substance to the present recognition that the intact ECM and basement membrane play major roles in establishing and maintaining cell fate, tissue architecture, and patterns of tissue-specific gene expression. Accordingly, signaling by ECM and its receptors are also becoming attractive targets of therapy, especially as it is clear that ECM receptors and growth factor receptors collaborate to maintain tissue specificity. Monoclonal antibodies to integrins are being tested in a number of studies, and the tumor data in rodents as well as in 3D assays are encouraging.¹³

Although widespread recognition of the fundamental contribution of the microenvironment to tumor progression has been slow in coming, these concepts are now firmly established. Many research groups are now focused on the identification of the critical mediators of tumor and stromal cell interactions, and this is reflected by increasing numbers of drugs targeting microenvironmental factors. As additional pathways by which ECM molecules and their receptors impart information to cells are identified, the insights derived are likely to pay dividends to our understanding of both normal and tumor biology. ■

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Leukemia and Cancer Stem Cells

By Irving Weissman and Michael Clarke

Cancers and normal tissue stem cells have much in common: Both have self-renewal capacity, and both develop into differentiated progeny. But do true cancer stem cells exist? We believe that they do and that this realization will have a major impact on the understanding and treatment of cancers. Putative cancer stem cells can be recognized by three attributes: They constitute a homogenous cell population; they, on their own, can initiate cancer; and they both self-renew and undergo differentiation into nontumorigenic progeny.

Many normal tissues start with stem cells. In a tightly regulated sequence, daughter cells undergo successive quantal steps in differentiation and have limited self-renewal capacity. This is an idea that has been around for a long time. For example, chromosome-marking experiments supporting the existence of stem cells for the hematopoietic system were published in 1967. Yet it took 20 years for the definitive demonstration of hematopoietic stem cells (HSC), namely long-term regeneration of multiple lineages of donor-derived blood cells in lethally irradiated mice.¹ And it took another 12 years to complete the isolation of the downstream blood cell progenitors, all of them non-self-renewing.

This schema set the stage to examine hematopoietic neoplasms (leukemias and lymphomas). Somewhere in the hierarchy of stem and progenitor cells lie cancer stem cells. These progress, through multiple genetic and gene-expression events that are proto-oncogenic, to full-blown cancer.

Identifying cancer stem cells has been difficult. Looking at fixed tissues and surmising schemes of cell transitions proved to be a recipe for confusion, as was the adoption of molecular pathway morphologies. The first serious attempt to isolate a cancer stem cell was the system that John Dick and his colleagues developed. They transferred cells from patients with acute myelogenous leukemia (AML)

successfully into immunodeficient mice.³ These leukemia-initiating cells shared part of the phenotype of normal HSC; that is, they were CD34⁺38^{low}, but true human HSC are additionally Thy1⁺ and lacked blood lineage markers. We sought to determine if the leukemia stem cells (LSC) were derived from HSC or progenitors.

We used cells from patients with AML that bore the aml1/eto chromosomal translocation, which plays a proto-oncogenic role in these leukemias. To our surprise, the true HSC had the aml1/eto chromosome, but lacked the potential to produce leukemia blast cells in culture, yielding only normal-looking myeloerythroid colonies. The Thy1⁺ progenitors were the LSC.⁴ This proved to be consistent with the clinical data: Many of these treated patients with leukemia were healthy for as long as 150 months, yet their marrows contained detectable normal HSC with the aml1/eto chromosomal translocation. We interpreted these findings to mean that the aml1/eto translocation was probably necessary but not sufficient for the full AML disease.

Several independent events are required for the progression of chronic to acute leukemias in mice, and in mouse and human myelopoiesis, only HSC self-renew. Our interpretation (see figure, next page) is that most or all proto-oncogenic events short of acute leukemia occur in a succession of HSC clonal progeny; had such early events initially occurred in progenitors, they would be lost as the progenitor lifespan was completed. However, the emergence of the acute leukemic clone could occur at the HSC or progenitor level when the self-renewal pathway genes are activated. To give an example, in a particular group of patients (bearing bcr/abl translocations) the chronic phase leukemia is at the level of HSC, producing myeloid, erythroid, and B lymphoid cells. But when myeloid blast crisis emerges, it is progenitors that are responsible, mainly from the granulocyte-macrophage stage of hematopoiesis.⁵