How to pull the blanket off dormant cancer cells

Vivien Marx

When asleep, cancer cells can evade chemo. When they wake up, they can cause cancer recurrence. By deciphering dormancy cues, labs explore how to break this cycle.

Of the many types of cancer cell misbehavior, dormancy—a type of active sleep—is particularly challenging. Dormant tumor cells spend most of their time in cell cycle arrest.

When a tumor sheds cells into a person's bloodstream, the cells land in various microenvironments such as niches in the lung tissue or bone marrow. Upon arrival, the cells may be dormant or become dormant. They can spend months, years, even decades in some niches where they find safe haven and protection from chemotherapy, says Cyrus Ghajar, a cancer cell biologist and translational researcher at Fred Hutchinson Cancer Research Center. One day the cells awaken, grow, spread metastases and, all too often, kill. This, he says, is the challenge of recurrence seen in people with various cancers, including breast cancer, prostate cancer, melanoma and osteosarcoma.

For years, there was a disconnect between the lab and these clinical scenarios, says Julio Aguirre-Ghiso, cancer cell biologist at Icahn School of Medicine at Mt. Sinai. Research focused on oncogenes and their seemingly constant message to cancer cells to proliferate. "But the clinical data suggested this was not the case," he says. Advanced cancer therapies were leading to remission in patients, but recurrence was frequent. Cancer, he realized, didn't always keep growing, so he began to explore what cancer cells did when they didn't grow. He, Ghajar and others are teasing out the molecular mechanisms that underpin dormancy[1,2]. Doing so takes a benchtop of assays including scratch assays for observing cancer cell chemotaxis, sequence analysis for finding mutations that drive cancer cell behavior, three-dimensional (3D) cell culture and engineered environments to model the niche, and intravital imaging, all to gain insight into the cancer cell sleep–wake dynamic.

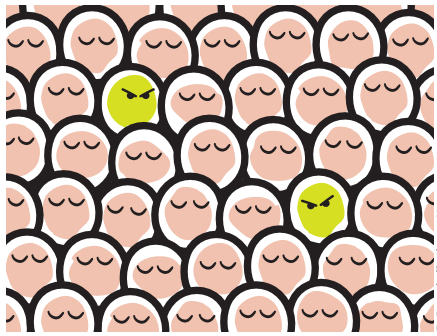
Seed, soil

Not all niches are equally welcoming to cancer cells, as Stephen Paget, a surgeon at West London Hospital, noted in 1889 about the 'seed and soil' hypothesis of cancer[3]. "I think his theory was spot-on," says Aguirre-Ghiso. Cancer cells grow in some locales, die in others, go to sleep in yet others. A cancer cell reaching heart or skeletal muscle tends not to grow, which is like a plant seed falling on concrete, says Ghajar. Cancer rarely afflicts these tissues. Elsewhere, cancer cells fall on more congenial soil.

Quiescent cancer cells resemble adult stem cells, says Aguirre-Ghiso. Instead of making healthy cells, they make tumor cells. In other ways, he says, dormant cells are similar to senescent cells, but they don't appear to enter the irreversible growth arrest of senescence. Both senescent and dormant cells produce factors that seem to sustain their behavior. With dormancy, the immune system, the tumor genome and epigenome, and the microenvironment all play a role; "it's probably a combination of all of these," he says.

The niche sends specific dormancy-inducing signals to cancer cells in their keep. Some of the deciphered signals are BMP-4, BMP-7, TGF-β2 and TSP-1, and the activated p38α/β pathway initiates the dormancy program.

As dormancy research gains recognition, it presents an obvious therapeutic idea: wake up dormant cancer cells and kill them with chemo. Aguirre-Ghiso's response to this is steadfast: unless a chemotherapy can kill each and every single cancer cell, the 'wake-up-and-kill' strategy is not advisable. But, he says, alternative routes will emerge from a thorough understanding of the cues and mechanisms that regulate dormancy.



E. Dewalt/Springer Nature

Tumor cells can be dormant for months, even decades. When they awaken, they can cause tumor recurrence and metastasis.

Cancer cells as cannibals

As he worked on cardiac and bone marrow stem cells, Thomas Bartosh, a stem cell biologist at Texas A&M University, began looking at mesenchymal stem cells (MSCs). MSCs are found throughout the body and they are regenerators: they can, for example, make new blood cells or new bone. Given that MSCs are drawn to a tumor's injury and inflammation signals, Bartosh thought MSCs could be used to kill cancer cells.

In 3D coculture experiments, Bartosh brought breast cancer cells and bone-marrow-derived MSCs into close contact. The MSCs kept disappearing. "We thought this is strange," says Bartosh. Analysis revealed that "the cancer cells were just eating the MSCs that were there to kill them," he says. Like a monster sated after a meal, the cancer cells entered dormancy after this act of cannibalism. "We tried to hit them with nutrient deprivation and chemotherapy," says Bartosh, but the cells would neither grow nor die. They repeated the experiments with tumor cells implanted into mice and had the

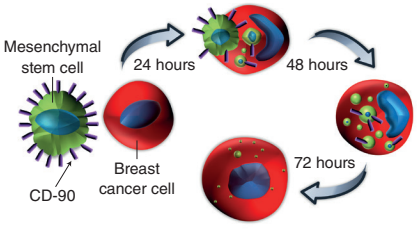
same result: cannibalism by cancer cells followed by dormancy[4].

Cannibalism was first observed in human cancer tissue over a century ago, says Bartosh. In his experiments, he thinks cancer cells cannibalize to survive. A hanging-drop environment with its high cell-to-media ratio leads to nutrient deprivation and hypoxia and the environment turns acidic, all of which challenges tumor cells. He decided to equip MSCs with suicide genes such as inducible caspase and use cannibalism to deliver MSCs to the tumor. An activated suicide gene can destroy the dormant cancer cell from the inside. The work has a long way to go, he says. Using laser capture microdissection, he plans to profile dormant cancer cells and cannibalistic cancer cells and compare their gene expression and metabolic profiles to the results from coculture and mouse experiments.

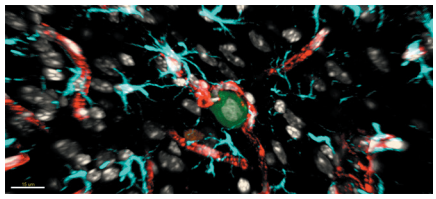
Methods strategies

What Bartosh likes about hanging-drop 3D coculture is that it's informative and reproducible and permits quick screens for pathway analysis such as with pharmacologic inhibitors. He has yet to use organoids. "As you go up the ladder of complexity in 3D culture, it becomes closer and closer to *in vivo*," he says. When moving toward greater complexity, it can be preferable to go straight to the animal model, he says. Next he wants to delve more deeply into cannibalism and cancer dormancy in human tissue and patients with a view to eventual therapies. But basic research comes first. "We can't have those therapies if we don't understand this biology," he says.

To mimic the niche and its cross-talk, scientists might use 3D-embedded assays with substances such as collagen or Matrigel, a gelatinous protein mixture secreted by mouse sarcoma cells. At Dana Farber Cancer Center, Kornelia Polyak uses the 3D on-top assay in which cells are cultured on a surface, a synthetic version of the extracellular matrix (ECM), which is easier to image than cells embedded in hydrogel. Three-dimensional cocultures in an ECM are certainly better than 2D coculture for experiments that look at dormancy, says Polyak. But in her view the interaction of epithelial stromal cells with cancer cells is best studied *in vivo*. *In vitro* assays are necessary when *in vivo* assays are too challenging in terms of logistics or cost, such as, for example, in screening projects. Polyak and her team have applied 3D coculture to look at how cancer cells find "stromal protection" from certain chemotherapeutics[5]. The sensitivity to some drugs is lower when cancer cells are quite close to fibroblasts than when the cells are cultured alone.



In hanging drops, breast cancer cells cannibalize mesenchymal stem cells and then appear to go to sleep. This cannibalism is followed with cell tracker dyes (red, green) and the marker CD90. T.J. Bartosh/adapted with permission from ref. 4.



J. Dai, Ghajar lab, Fred Hutch. Cancer Research Center

Breast tumor cells (green, with two white nuclei) are shown in mouse brain tissue. The dormant cancer cells are wrapped by a blood vessel (red).

Engineering environments to mimic the niche of dormant cancer cells is possible, says Ghajar. But these reconstitutions have to be comprehensive, with built-in genetic information, spatial gene expression patterns, proteomic and metabolomics profiles. "If you have all this information about the niche you care about and now you are able to systematically dissect it in an engineered environment, then yes, I would think it's very useful," he says.

Ghajar and his team use what he calls "organ-like culture" to reconstitute the dormant cancer cell microenvironment. He has built "high-fidelity microvasculature in culture" in which he puts breast cancer cells to sleep through environmental cues. "When we do that time and time again, we are able to steer fully malignant tumor cells into a quiescent state in culture and for a durable period of time," he says. Mouse models are helpful but ideally he wants to work with human tissue specimens. That's a challenge in the dormancy field: "the specimens aren't there," he says—not the primary tumor samples, but healthy tissue likely to harbor dormant tumor cells is lacking.

Tissue could come from a bone marrow biopsy taken during breast cancer surgery. It might also be a sample from someone who died of metastatic disease. The dormant tumor cells from uninvolved tissue could teach labs many lessons, says Ghajar about when cells sleep, stay asleep, wake up, grow or don't grow.

Together with John Condeelis of Albert Einstein College of Medicine, Aguirre-Ghiso uses intravital imaging approaches to study the behavior and dynamics of cancer cells in live tumor and solitary disseminated tumor cells. They can alter a tumor microenvironment in targeted ways and, using fluorescent protein sensors, multiphoton microscopy and a device slightly smaller than a rice grain called Induction Nano Intravital Device (iNANIVID), image the behavior of a tumor's single cells[6]. The device is inserted into the tumor and can, for example, be loaded with drugs, potential drugs or hydrogel with various biochemical cues to then track the chemotaxis, growth or cell cycle arrest of cancer cells.

Condeelis has also developed a window for imaging lung tissue over extended periods of time in a live mouse[7]. It can be used to track single cells and small groups of cells and return to these cells in repeated imaging sessions. Unlike the more invasive vacuum-stabilized imaging windows that can be used for only a few hours, this window has been used to track events in the lungs of mice for a month.

Lung is a common site of metastasis and where dormant cancer cells wake up, says Aguirre-Ghiso. Longitudinal data captured with this type of imaging could reveal a play-by-play of dormancy and metastasis and potentially show the association of cancer cells with blood vessels. It could track how frequently cancer cells interact with immune cells and how these interactions change the behavior of cancer cells as they wake up and expand into a lesion or head into the bloodstream and spread tumors. Imaginably, he says, one could devise experiments such as with mice lacking a certain receptor or an experiment with cells lacking certain genes.

Dana-Farber Cancer Institute

The interaction of epithelial stromal cells with cancer cells is best studied *in vivo*, says Kornelia Polyak.

Aguirre-Ghiso and his team use multiple assays: single-cell gene expression profiling to characterize tumor cells in

3D cell culture and xenograft experiments with mice. The team uses tissue organoids to expand cells in different stages of cancer progression and also single-cell culture. What risks an inaccurate model, he says, is if labs work with a hanging drop or an organoid experiment and perform *in vivo* experiments much later. Work in animals is harder and more expensive but it is more telling about events in patients. He sympathizes with choosing a reductionist approach to deconvolute variables. He likes to start from a strong *in vivo* finding in animals or patients, model in animals and then "walk back" to understand the mechanism.

Educating cancer cells

Aguirre-Ghiso and colleagues will soon be starting a dormancy-related clinical trial in prostate cancer. The idea is to "reprogram cancer cells into dormancy to see if we can keep them from awakening and keep them sleeping," he says. "It's a kind of an education of a cancer cell." Two approved drugs—a demethylating agent and retinoic acid, which induces differentiation—would act on a 'node' that is crucial to the maintenance of dormancy. The reprogramming leverages the power to rewrite a cancer's epigenome and the reprogramming is reinforced by the morphogen. Together, the effect could be to "convince" the cancer cell to stay in growth-arrest mode, thus thwarting metastasis.

This node in question is an important one in dormancy. In healthy cells, the orphan nuclear receptor NR2F1 is part of cell lineage determination in response to biochemical cues such as retinoic acid[8]. For example, when NR2F1 is upregulated, prostate cancer patients who have received androgen-blocking treatment stay free of metastasis for longer.

In dormant cancer cells, NR2F1 appears to coordinate quiescence and it regulates pluripotency genes, says Aguirre-Ghiso. In addition to its role in dormancy in prostate cancer patients it has also been implicated in estrogen-positive luminal breast cancer grown in mice. Both cancers have a tendency to metastasize to bone. It is likely, he says, that some but not all disseminated tumor cells follow the same dormancy program. Microenvironment factors will differ but he, too, is hopeful this work can lead to new approaches to address metastasis in many cancers.

As Condeelis explains, tumor cells, such as certain types of breast cancer, are disseminated through a 'doorway' in tumor blood vessels that acts as a kind of 'educational' passageway. The cells passing through this doorway are enriched for dormant tumor cells, he says. This passage likely programs the cells for dormancy and shapes their ability to resist chemotherapy, sleep, wake up, enter the bloodstream and invade organs. Studying what happens in this doorway and how to inhibit it is a high priority also for clinical trials, he says.

Aguirre-Ghiso and his team have been exploring how tumor cells' maturity affects their behavior. Disseminated tumor cells were once believed to be replete with mutations. In fact, he notes, cells disseminated early in the development of breast cancer, before metastasis, contain fewer genetic abnormalities than those from patients with metastatic breast cancer. Tumor cells disseminated early sleep for a long time. In mice, the team found dormant breast cancer cells a year after dissemination. That might sound like a short time span, he says, but translated to human life span that's 25 years. Such observations, he says, underscore the importance of understanding the cross-talk between the tissue and tumor cells.

Studying cancer cells in the context of their microenvironment was pioneered by Mina Bissell, a researcher at Lawrence Berkeley National Laboratory, and her findings are now being recognized, says Ghajar. A parallel development has been the increased resolution at which cancer can be studied, revealing details about the tumor genome landscape and tumor cell heterogeneity. "I don't think anyone who really understands cancer doubts that the microenvironment is important," says Polyak. "Especially not nowadays when immunology is the most exciting and hopeful area in cancer research and treatment."

Ghajar, a former postdoctoral fellow in Bissell's lab, points to her 'tumor reversion model' showing that the conversion

enviromantic/E+/Getty

Some 'soils' are more welcoming to dormant cancer cells than others.

B. Jungmayer/Fred Hutchinson News Service

Cyrus Ghajar and his team study dormancy by reconstituting the dormant cancer cell environment.

of cancer cells to healthy cells can happen both *in vitro* and *in vivo* with the correction of a few signaling defects in the microenvironment. While in her lab, he began wondering how this plasticity indicates overlap between quiescence in stem cells and quiescence in tumor cells and he set out to build on work by, among others, Bissell, Ann Chambers at the University of Western Ontario and her data on the persistence of single tumor cells in tissue, and Christoph Klein of the University of Regensburg with the idea that metastasis is not a linear process. Disseminated tumor cells, dormant or not, are likely evolving separately from the primary tumor, says Ghajar. "The primary tumor that we see is an inaccurate snapshot of what is going on in other organs," he says.

Like quiescent stem cells, tumor cells are closely associated with the vasculature. Shahin Rafi at Weill Cornell and others showed that "the endothelium wasn't just plumbing," says Ghajar, which further spurred his experiments. The vasculature is part of what helps turn tumor cells quiescent and make them chemo-resistant. He expects many more patterns to emerge about dormancy-inducing factors. Some cancers and organs will have cues in common but some tissues, such as brain, likely have cues all their own and "the tissue specificity is extremely important to understand." Before advancing therapies, the cancer community must understand the global ramifications and potential negative of dormancy-related approaches also in a tissue-specific way. "What works on one tissue may not work in another," he says.

Some microenvironments are good to cancer cells. "I come down hard on the environment side of things," says Ghajar. "I think that they are protected by the niches that they are in," he says. Deep understanding of niches will lead researchers to an understanding of how deprivation of certain environmental survival cues also in the face of chemo could kill these cells. A therapy could reduce or even eliminate residual disease and prevent metastasis.

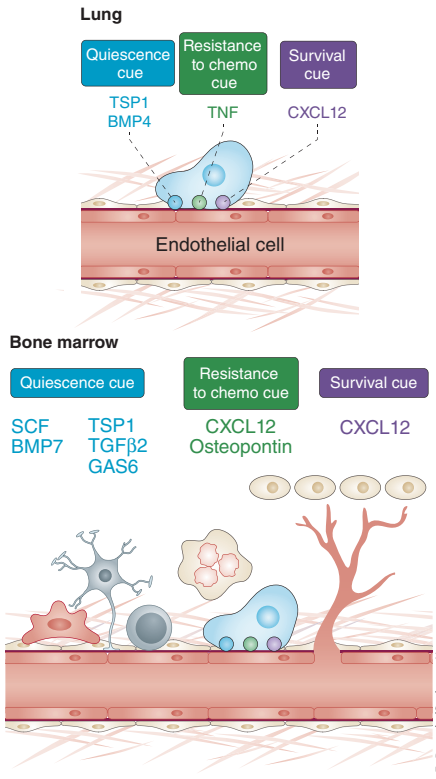
Confronting dormancy

Dormant tumor cells are chemotherapy-resistant not just because they are not actively dividing, says Ghajar. But how exactly dormant tumor cells resist chemo is "still poorly understood," he says. Many chemotherapies are thought to disrupt cancer's rapidly dividing cells. "But the more that they're studied, the more that we realize they actually function through multiple mechanisms," he says. He hopes dormancy research can lead to a therapy that targets the microenvironment of a dormant cell, sensitizes it to chemo and kills the cells without waking them up.

Among other researchers, Robert Gatenby, who studies the evolutionary dynamics of cancer at H. Lee Moffitt Cancer Center, advocates an approach sometimes called adaptive therapy, says Polyak. It's "basically using evolutionary approaches to treat cancer." Treatment usually selects for the most resistant and most aggressive cancer cells. Clinicians use the maximum tolerated dose in the hopes of killing all cancer cells, "but this many times does not work, so in fact leads to the outgrowth of resistant populations." Using environmental constraints to restrain tumor, such as by activating immune responses, "could certainly work and are worth exploring," she says.

A dormancy-related therapy, says Ghajar, has the potential to root out residual disease and avoid the wait for potential recurrence. He sees lessons for the dormancy field, for example, in the experience with HIV and antiretrovirals. A dormant cancer cell does not hide in another cell as HIV does, but the virus takes cover in a microenvironment that sustains it, and it, too, is in a niche with an ECM and cell–cell contacts, hormones and other biochemical cues, he says.

Even though labs studying dormancy have different views on how to apply the research therapeutically, there needn't be a battle within the field about which is better, says Ghajar, because multiple strands might work together. In some cancers and depending on individual risk factors and disease stage, it can be better to keep dormant cells asleep, whereas in others the better strategy might be to kill the sleeping cells in their niche.



Microenvironments can support tumor cells (blue), also in tissue-specific ways. Here, some known cues in bone and lung tissue. Adapted with permission from ref. 2.

To weigh therapeutic scenarios, basic biology and clinical data must be considered, says Ghajar. Many women with breast cancer face eventual bone metastases. When bone marrow is examined at diagnosis and dormant cells are discovered, in the next five years these women are far more likely to experience metastasis. But as many as two-thirds of these women will not experience metastasis. Such observations are challenging for trial design, he says. Treating all women with dormant tumor cells risks overtreating too many people who would not have experienced metastasis. Scientists also still need to learn to distinguish dormant tumor cell types.

Some tumor cells will stay asleep and cause no harm; others will wake up and cause damage. Dormancy researchers hope that their work can dramatically reduce or even eradicate cancer recurrence and metastasis.

Vivien Marx is technology editor for *Nature Methods* (v.marx@us.nature.com).

1. Sosa, M.S., Bragado, P. & Aguirre-Ghiso, J.A. *Nat. Rev. Cancer* **14**, 611–622 (2014).
2. Ghajar, C.M. *Nat. Rev. Cancer* **15**, 238–247 (2015).
3. Paget, S. *Lancet* **133**, 571–573 (1889).
4. Bartosh, T.J. *et al. Proc. Natl. Acad. Sci. USA* **113**, E6447–E6456 (2016).
5. Marusyk, A. *et al. Cancer Res.* **76**, 6495–6506 (2016).
6. Williams, J.K. *et al. Intravital* **5**, e1182271 (2016).
7. Entenberg, D. *et al. Nat. Methods* **15**, 73–80 (2018).
8. Sosa, M.S. *et al. Nat. Commun.* **6**, 6170 (2015).