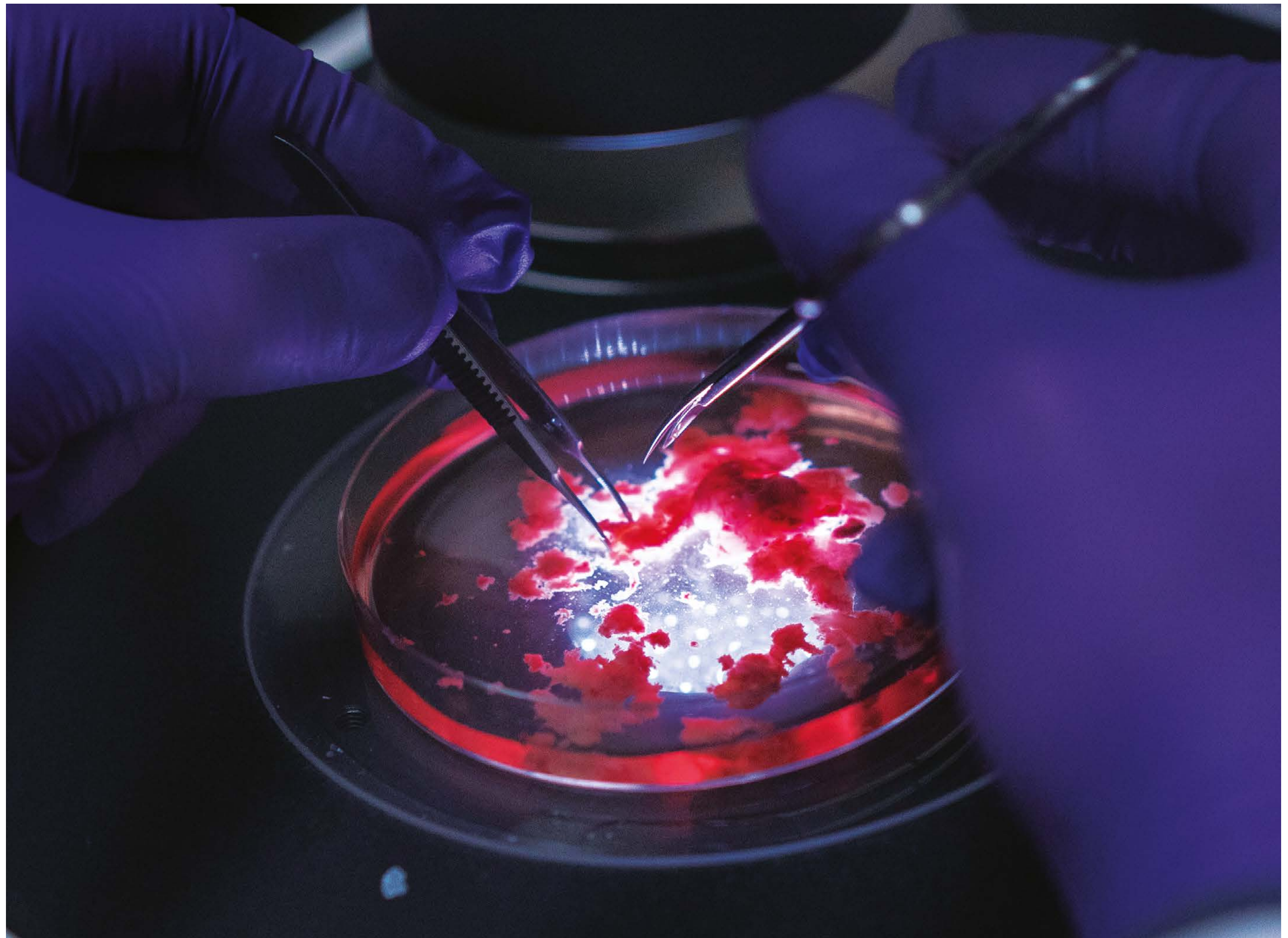

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Tiny pieces of a patient's glioblastoma brain tumour can be used to grow organoid models for studying the role of cancer stem cells in disease.

TRACING CANCER'S ORIGINS

Developmental-biology tools help researchers track elusive cancer stem cells, revealing how tumours grow and evade treatments. **By Jyoti Madhusoodanan**

As Arnold Kriegstein and his team watched, cancer cells sprinted across the culture dish, sometimes travelling 30 times their own length in the hour before they divided. Typically, the only cells that move this fast are certain embryonic stem cells, during fetal brain development. But the cancer cells were from a glioblastoma, a type of brain tumour that is particularly difficult to treat, in part because it spreads so rapidly.

The behaviour hinted at similarities between cancer cells and the stem cells that are important in embryonic development, says Kriegstein, a developmental neurobiologist at the University of California, San Francisco (UCSF). "So often, reactivating gene-expression programs normally reserved for embryonic development are a part of how a cell becomes a cancer cell." Kriegstein wanted to investigate that link.

Working with UCSF radiation oncologist David Raleigh, Kriegstein's team split glioblastoma samples into two fractions: one enriched in the fast-moving stem-like cells, the other containing cells that had differentiated into a more mature type of cancer cell. The researchers grew each fraction in separate human brain organoids — simplified structures that mimic organs — expecting to see only the stem-like cells recreate the original tumour. But to their surprise, both fractions reproduced the entire spectrum of cancer cells[1]. "We started with cells that were mostly just one cell type in each fraction, and we ended up with a large heterogeneity," Kriegstein says. "But how did that happen? That remains a kind of a black box."

Since the 1990s, researchers have suspected that stem cells in cancers hold the key to disease recurrence, cancer spread (or metastasis) and resistance to therapies. But cancer stem cells seem to defy characterization. They bear no defining molecular markers; might not exist in every tumour; and, perhaps most frustratingly of all, correlate little with disease

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aggressiveness or treatment outcomes.

"In some cancers, virtually all cells function as stem cells; in others, there's a clear hierarchy of stem cells and more-differentiated tumour cells," says stem-cell researcher Louis Vermeulen at the Amsterdam University Medical Centers. "The debate is really: how many stem cells are in a given cancer, and are they always the same cells?"

To answer those questions, cancer biologists are broadening their toolbox. In addition to improved culture methods, such as the organoids that Kriegstein's team used, researchers are exploiting methods from developmental biology. One is lineage tracing, which is commonly used to track how embryonic cells grow and differentiate into adult tissues. But it can also reveal how a single cancer cell might reconstitute the genetic diversity seen in its tumour parent. Now, cancer biologists are combining that approach with single-cell methods to get a clearer picture of whether — and how — cancer stem cells spur disease.

Expanding cultures

Kriegstein and Raleigh's decision to use organoids was motivated by a lack of good animal models: glioblastomas are hard to culture in mice and rats, for instance. But organoids can also represent human-tissue environments more closely than rodent biology does. And they're surprisingly easy to use, says Aparna Bhaduri, a former postdoctoral researcher in Kriegstein's team. Looking through a microscope, researchers merely pipette tumour cells onto the organoid surface and wait about 45 minutes for tumours to form. "It's so much easier than an animal experiment," Bhaduri says.

Still, organoids cannot replace animals, particularly because the structures lack blood vessels and so can't capture how tumours interact with the circulatory system. They're also highly variable, says Bhaduri, who now leads her own stem-cell biology laboratory at the University of California, Los Angeles. "There's probably more we need to optimize to make sure we're getting all of the heterogeneity that possibly exists in a given tumour," she says.

Compounding the difficulty, organoids have different nutritional and growth requirements from cells in routine culture, which makes them tricky to use for high-throughput studies, says neuro-oncologist Jeremy Rich at the University of Pittsburgh Medical Center in Pennsylvania. His team uses bioprinting — a process similar to 3D printing that uses cells and culture media in place of inert materials — to study how inflammation and the immune system contribute to glioblastoma cell behaviour. Immune dysfunction is a key part of many cancers, but it is hard to study in organoids and animal models: the former have no immune system, and the latter have been engineered to have impaired immunity so that human tumour cells can grow inside them.

Rich and his colleagues print hydrogels using a mix of different cell types and precursor materials, such as hyaluronic acid. When printed, the hydrogel growth medium forms a 3D scaffold seeded with cells that can be used as a tumour model. When Rich and his team tried that approach using glioblastoma stem cells mixed with different combinations of other neural cells, they found that, in the presence of immune cells known as macrophages, the tumour cells expressed genes that correlated with greater invasiveness and drug resistance in people with glioblastoma[2]. They also found that stem cells grown in the context of other cell types mimicked their real-world behaviour more closely, suggesting that the tissue environment plays a part in defining stem cells.

Similarly, cell biologist Silvia Fre at the Curie Institute in Paris has found that, in mice, healthy adult mammary tissues contain only differentiated versions of stem cells that cannot form tumours. But if these same cells are removed from adult tissues, they quickly reactivate multipotency, or the ability to differentiate into discrete cell types, including cancerous ones, reinforcing the tissue environment's crucial role in tumour development[3].

> **"Looking at cancer through this prism of stem cells has just really transformed the ability to understand it."**

Simpler culture models can be illuminating, too. In a 2018 study[4], Vermeulen and his colleagues used a simple model system called a spheroid culture, in which a person's tumour cells are grown in a free-floating 3D culture in a dish, as well as tumour xenografts — when human cancer cells are implanted in an immunodeficient mouse and allowed to grow as a tumour. These approaches revealed that human colon-cancer cells become increasingly stem-like the closer they get to the edge of a tumour. When the team took cells that are not proliferative from a tumour's centre and transplanted them to the edge, the cells acquired markers of proliferation. The authors concluded that human colon-cancer stem cells are defined not by their intrinsic gene-expression patterns, but by their location. "I was very surprised to learn that the tumour environment is the dominant factor that defines a stem cell," Vermeulen says. "Which cells behave like stem cells changes all the time depending on their position in the tumour."

Capturing cellular trajectories

This interplay of environment and cellular identity means that cancer cells might look stem-like under some experimental conditions but not in others, or might express different sets of genes depending on their neighbours. They also lack universal surface markers, making it even trickier to tag and study them. But researchers have devised a range of alternative strategies to track the cells' trajectories, many of which are borrowed from the developmental-biology toolset.

To study stem cells in embryonic mammary glands, Fre and her team used a strain of mice called Confetti, so named because the cells can express four different fluorescent reporters. When the researchers treated animals with a chemical to induce reporter-protein expression at different times during development, the proteins were activated in various locations. Using fluorescence microscopy, the team could then see where cells of different lineages ended up in adult tissues. Vermeulen and colleagues have used a similar fluorescence-based approach to understand how the environment controls colon-cancer stem cells in cell culture studies[5].

Genetic barcodes are another option for tracking cells when they acquire mutations and diverge into different subgroups. The approach gives each population of cells a fixed genetic barcode; as the populations divide, the barcodes evolve. By sequencing all the barcodes in the population and comparing them, researchers can then work out how the different cells relate to one another, and their relative contribution to the growth of the tumour.

Early variants of this approach relied on static barcodes carried inside lentiviruses, used as a way to insert the sequences into a pool of cells at random. Now, the gene-editing tool CRISPR is improving the process.

In CRISPR-based lineage tracing, researchers insert an array of CRISPR target sequences into cells' genomes. The Cas9 enzyme then periodically cuts into these targets, triggering DNA-repair processes and leaving a genetic scar that acts as a unique identifier for a cell and its progeny. Unlike lentiviral barcodes, this system generates unique barcodes dynamically, potentially every time the cells divide, allowing researchers to reconstruct how different cells and their progeny are related[6]. "Changes accumulate over time," says stem-cell biologist Alexander van Oudenaarden at the Hubrecht Institute in Utrecht, the Netherlands. "It's fundamentally different from the lentiviral barcodes that were used earlier."

Another approach couples the sequence for a fluorescent protein to a repetitive piece of DNA — a long repeat of cytosine and adenine bases that cells see as problematic. As cells divide, they periodically 'repair' this repetitive sequence by trimming it, ultimately bringing the sequence for the fluorescent protein into a position in the genome where it can be expressed. This fix happens once in every 10,000 cells or so, Vermeulen says,

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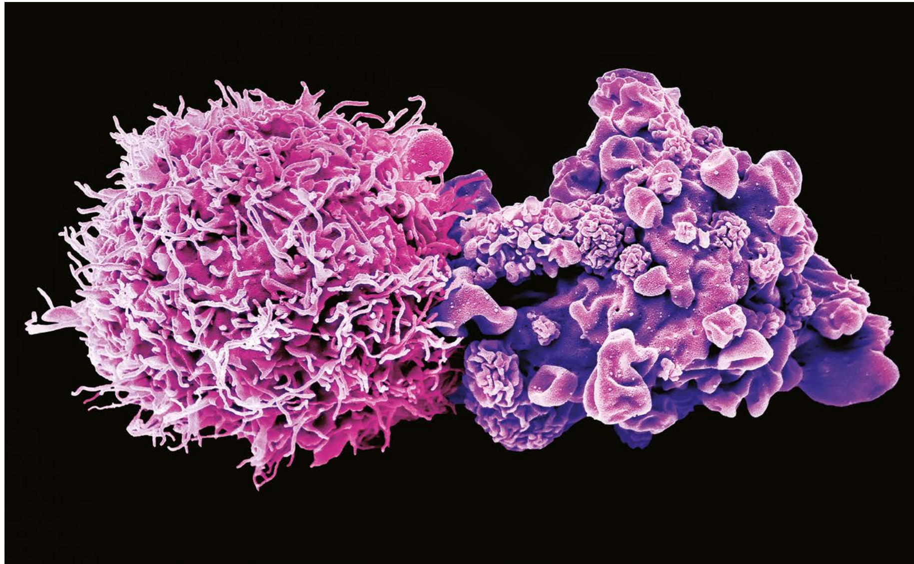
STEVE GSCHMEISSNER/SPL



Cancerous cells from a glioblastoma, an aggressive type of brain tumour.

sending up a tiny genetic flare that's visible under the microscope. The advantage, he says, is that this sort of fluorescent label doesn't require a chemical to activate it. "It's a way of lineage tracing that leaves the cell completely untouched," he says.

Each of these strategies has its pros and cons. Some CRISPR sequences are more prone to scarring than others, for instance, introducing bias into a theoretically unbiased process. And both microscopy and sequencing-based strategies require advanced computational and technical skills. Still, coupled with single-cell RNA sequencing, the labels provide powerful tools to assess the relative importance of individual cells in a tumour.

"If a tumour is driven by cancer stem cells, only a few labelled cells will proliferate and become large clones," Vermeulen points out. "But in a tumour that depends on many cell types, most cells will expand. When the data are put into a mathematical model, you can actually identify to what extent it's one mode of growth versus the other."

Doubled-up detection

Such models can provide a more complete picture of how tumour cells grow and change. But they also require new computational algorithms. Models that have conventionally been used to infer phylogenetic relationships between cells can't handle the large amounts of information generated when a lineage-tracing data set is combined with one from single-cell RNA sequencing.

It's a problem that developmental biologists have long struggled with, says geneticist Jay Shendure at the University of Washington in Seattle, whose group developed one of the first CRISPR systems for simultaneous lineage tracing and RNA sequencing[7].

When it comes to lineage tracing in cancer studies, the biggest problems are technical: recovering sufficient amounts of barcode and handling missing data. Lineage-tracing studies often have gaps, because some cell populations disappear or the amounts of barcode sequence in a sample are too small to process. Algorithms can struggle to handle these gaps, Shendure says, so it's crucial to maximize the yield and stability of the RNA sequence that encodes the barcode. "You need relatively high rates of recovery," he says. "If you put *x* cells into a protocol, you want to get a relatively high fraction of them back."

In a study published this year[8], UCSF cancer researcher Trever Bivona and his colleagues simultaneously tracked lineages and changes in RNA expression in lung cancer cells that had been transplanted into animals. Their Cas9-based tool enabled them to follow, in real time, how genetic changes drove cancer cells to seed tumours in distant tissues — the process of metastasis.

The team captured lineage and gene-expression data for more than 40,000 mouse cells from 6 different locations in animals' bodies, and found that cells moved back and forth between various genetic states several times before committing to a distinct, differentiated path.

To analyse these voluminous data, Bivona's collaborators — biologist Jonathan Weissman at the Whitehead Institute in Cambridge, Massachusetts, and computer scientist Nir Yosef at the University of California, Berkeley — developed a suite of tools called Cassiopeia, which helps to reconstruct lineages on the basis of CRISPR–Cas9 barcode data[9]. They and others have made their analytical tools freely available to other researchers (see go.nature.com/2ptezwd).

For her part, Bhaduri frequently turns to a toolset named Seurat[10], developed by statistician Rahul Satija and computational biologist Aviv Regev when they were at the Broad Institute of Harvard and MIT in Cambridge, Massachusetts. The Seurat tools allow Bhaduri to simultaneously analyse changes in gene expression and variations in the number of copies of a particular gene in single cells.

Whatever toolset researchers choose, Bhaduri recommends that people who are new to such analyses rely on tutorials and work through courses provided by algorithm developers. Those who have developed their own in-house analytical software, such as Vermeulen and others, typically collaborate with biostatisticians to do so.

Still, better tools are needed, Shendure says. "As the number of cells in a phylogenetic tree grows, the number of possible arrangements increases exponentially," he says. "We're going to need richer tools before we can fully realize the potential of this line of inquiry."

Still complicated

Taking a developmental-biology lens to the cancer stem-cell problem has begun to reveal the many complex forces that drive cancer, and the plethora of routes that cells take towards tumour formation. "Looking at cancer through this prism of stem cells has just really transformed the ability to understand it," says Rich.

The field still lacks a precise definition of a cancer stem cell, but such clarity might be unnecessary. Recognizing the importance of stem-cell-like properties in tumours, and how cell microenvironments can nudge them to gain these traits, might be sufficient to lead to new therapies.

Some traits are characteristic of cancer cells and embryonic tissue, such as the sprinting behaviour seen in glioblastoma stem cells or the multipotency of breast-cancer stem cells. The absence of such traits in healthy adult tissues could make them ideal drug targets, because therapies that block these behaviours in cancer cells should leave normal cells unharmed. And blocking these behaviours — whether or not they are exhibited by cells that can be defined as cancer stem cells — is the eventual goal of these research efforts.

"As of yet, we haven't really had a strong success on the therapeutic side, showing that if we target cancer stem cells, we see improvements in patient survival," Rich says. "That's the one piece that's missing."

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