EX VIVO VERSUS *IN VIVO* the challenge of generating meaningful results with traditional cell culture

While the use of human cell lines has become a permanent fixture in drug discovery and development, the lingering issue has been in their inconsistent results. This article discusses current attempts to make results from cell culture systems more biologically relevant which, in turn, could lead to an innovative era of transformation in how we use cells throughout the biomedical community.

By Dr James Lim and Dr Fred Aswad

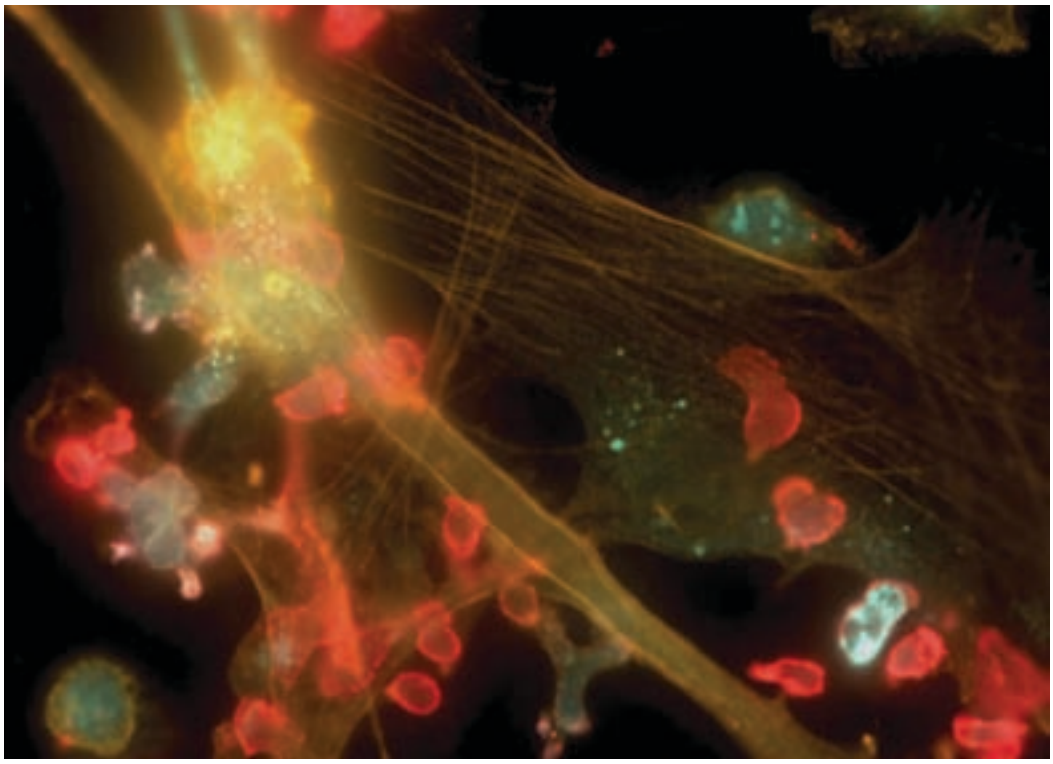
Over the past decade, R&D spending worldwide for drug development has nearly doubled, increasing lead candidates in development by 60%. However, the average number of drugs approved in a given year is lower than it was two decades ago. As it stands, only one in 10 candidates entering human clinical trials will be approved by the FDA across all disease indications. In oncology, success rates are even lower, with only one in 15 drugs gaining approval after entering into Phase I trials.

These dismal approval ratings reflect the challenges of translating preclinical success in cell lines and animal models into predictions of drug efficacy in humans. This could be partially explained by advances in genomic sequencing and bioinformatics that have shed light on the complexity of human disease, in particular for cancer – often defined as an evolving collection of many different subtypes of the disease. Traditional preclinical models often fail to account for this disease heterogeneity, relying on human cell culture for simplified and uniform model systems that have been mainstays in drug development for nearly 60 years.

It is hard to think of a technology that has had a broader impact on modern drug discovery and development than cell culturing. Since the very first human cell line was started in 1951, the ability to immortalise cells has allowed scientists to test for toxicity, conduct high-throughput screens for efficacy, and compare results from different compounds across a spectrum of cell types.

From targeted cell-based assays to massively parallel screens, human cell lines have become a permanent fixture in drug discovery and development pipelines. There are thousands of cell lines available from research companies, and pharma/biotech companies maintain proprietary catalogues of cell lines that span all disease indications.

With all these resources, there is tremendous potential for getting useful, relevant information early in the drug discovery process to help shape the course of a drug candidate. To realise this potential, however, there are many technical and biological challenges that must be addressed along the way. A lingering issue with the use of cell lines has been inconsistent results. The effects are evident everywhere from research papers that cannot



A representative co-culture assay of immune cells (red) interacting with cancer cells (yellow), along with the drug target expressed in green

be reproduced to the failure of even extremely promising drug candidates in clinical trials. While there are countless research steps that can contribute to such inconsistent findings, it has become clear in recent years that cell lines can be contaminated or misidentified far more easily than expected. Studies have also shown that repeated passaging of cells can lead to significant genotypic and phenotypic drift from their original state, often changing profiles enough that their behaviour is no longer representative of how those cells originally behaved in the body. Furthermore, cell lines fail to reflect the diverse array of heterogeneous cell types found in a given tumour, while traditional cell culturing strategies are ill-suited for modelling the dynamic tumour microenvironment.

One area that could help address these concerns and is ripe for innovation is the CO_2 incubator, the very foundation of our ability to culture both canonical immortalised cell lines and primary tumour cells enriched from patient samples. There is mounting evidence that primary cells in particular need carefully tuned environments to survive and maintain their *in vivo* properties (ie expression of drug targets) – and today's incubators do not include the features necessary to create these environments. New incubators with many more customisable settings, such as oxygen and atmospheric pressure control, may be far more effective at keeping primary tumour cells happy by recapitulating the conditions found in their native tumour microenvironment. This kind of advance could help ensure more relevant results from cell-based assays going forward, allowing for more effective strategies in using cell lines to predict success in the clinic more accurately.

The state of cell culture

The very first immortalised human cell line began in 1951 from cells collected from Henrietta Lacks, a patient with an aggressive form of cervical cancer. At the time, coaxing cells to keep dividing *ex vivo* was a major accomplishment; today, there are thousands of such human cell lines around the world that contribute to a thriving field of research.

Despite the advances in immortalising cell lines, the technology used to store these cells has not changed dramatically in the 65 years since the first HeLa cells were cultured. Most traditional incubators keep mammalian cells at a fixed temperature, humidity and CO_2 level, and those features have been relatively constant over the years.

Even with little technical innovation, however, the cell culture field is dynamic and expanding. Recent market research reports have estimated the value of the cell culture market at more than $12 billion with significant growth expected, particularly in the pharmaceutical and biotechnology sectors.

These bullish predictions were based in part on the shift to high-throughput approaches from manual experiments, increased funding and regulatory support for cell-based research and vaccines, and the rising incidence of chronic disease.

In pharma and biotech pipelines today, human cells are used to gather vast data sets about the effects of investigational compounds or interactions between drugs across various cell types and profiles. High-throughput screening can identify lead candidates to use in combination, or can weed out compounds that would fail later without the expense of ushering them through development. With the advent of stem cell-based therapies and immunotherapy, pharma scientists are now deploying primary cells to understand how to alter patients' own cells or to control the differentiation of induced pluripotent stem cells to a cell type of their choosing. For all of these applications, maintaining cells in optimal conditions is critical to producing physiologically relevant results that will have the best chance at leading to new treatments.

Technical limitations with traditional cell culture

Unfortunately for cell-based research, evidence has been growing for years now that cells are typically maintained and expanded under conditions that are not physiologically relevant.

It is widely acknowledged that the microenvironment of a cell plays a major role in its function and behaviour. After all, without differences in environment, it would be difficult to explain how cells with the same DNA take on such different roles, from storing memories to growing hair to tackling foreign invaders.

Mina Bissell, a breast cancer researcher at the Lawrence Berkeley National Laboratory, has generated compelling data showing that cultured cells shed their tissue-specific identities and functions. In a 2005 Cancer Cell paper, she demonstrated that restoring a cultured cell to conditions more reflective of its native environment could reverse the damage. "The cellular identity is not lost permanently, as we have learned that by controlling the

microenvironment of the cells in culture, we can make them 'remember' many of their original tissue specific traits," she wrote.

In a 2007 paper in *Tissue Engineering*, scientists from Brown University showed that even a basic change in cell storage – growing a neuron in three-dimensional conditions rather than the traditional two-dimensional conditions of an incubator plate – had a major biological effect. The 3-D neurons expressed hundreds of genes at different levels compared to their 2-D counterparts, possibly more accurately reflecting the expression of neurons *in vivo*. Other research has proven that even small changes in environmental conditions can stress a cell and alter its gene and protein expression in a matter of minutes.

These and a host of other studies call into question the results of *ex vivo* cell studies: can we really make good drug discovery decisions based on information from cells that may not reflect their native biology? The obvious answer is no, which is why many researchers have been trying to determine which conditions are necessary to make cells behave in incubators as they would in the body.

For instance, scientist Gregg Semenza at Johns Hopkins University was the first to discover that some transcription factors are induced by hypoxia. Follow-on research has shown that specific oxygen levels are needed for gene regulation, beginning with embryogenesis and continuing into adulthood, with oxygen levels even critical for transcription factors in cancer cells.

These and other studies demonstrated that levels of oxygen and atmospheric pressure are just as important to cell identity and survival as the features in current incubators, such as CO_2 or temperature. The real headline for all this work has been that cells need custom and tunable conditions, rather than the fixed-state settings characteristic of most traditional incubators.

That finding makes a lot more sense as we deepen our understanding of cells through genomics, high-resolution visualisation and metabolic profiling. It has only been appreciated recently how reliant stem cells are on their microenvironment; even slight perturbations keep these cells out of the niche required to differentiate into a specific cell type. Replicating this process through cell culture has proven extremely challenging, even for stem cell veterans.

The revelation of the importance of a cell's microenvironment may offer an explanation for some of the lack of reproducibility in biological research, with direct ramifications for scientists in pharma and biotech. A study from Amgen of 53 high-profile cancer publications showed that results from 47 of those papers could not be reproduced. At Bayer, researchers assessed internal cancer projects based on published scientific discoveries, determining that less than a quarter of the projects could replicate the previous results. The situation has become discouraging enough that the journal *Nature* last year called for better standards, especially in the use of cell lines. While some of the lack of reproducibility is no doubt attributable to slight differences in experimental procedure, it is becoming clear that at least some of it is due to a research focus on cells with unexpectedly altered profiles that yield results inconsistent with native biology.

Recent innovations in cell culture technologies

With the rampant use of cell lines in drug discovery, it is no surprise that pharmaceutical and biotech companies are investing in improvements to make cell-based results more accurate and relevant. Innovations have come from areas as basic as improved cataloguing for cell lines to next-generation incubators designed to more faithfully represent cells' native microenvironment.

In 2014, for example, Genentech won an industry award for developing a new system to manage hundreds of cell lines. More recently, the company added standards for quality control and contamination testing of these cells. Genentech's gCell system includes a full history for each of its 1,800-plus cell lines, including storage and tracking details, as well as a bioinformatics platform to ensure proper identification for every line. Part of that involved implementing a standard nomenclature system for cell lines and a defined ontology for describing them to improve scientists' ability to search or sort them.

At Bayer, scientists have been evaluating innovative incubators that allow for more customisable settings and make it possible to mimic the native environment of cells. We worked with the new Avatar system from Xcell Biosciences, which has settings for temperature, CO_2, oxygen and atmospheric pressure – the latter component unique to the Avatar system. These settings can be adjusted to mimic various microenvironments found within the body to support the maintenance and propagation of a given cell type. (To illustrate: glioma cells do best with 1% oxygen and 3 pounds per square inch of pressure, while immune cells need 10% oxygen and 2 PSI.)

Previous studies have shown that this instrument can maintain cells at optimal conditions for

prolonged periods to the point where even circulating tumour cells, which are notoriously difficult to culture, can successfully divide and reproduce while maintaining expression and signalling patterns very close to their native profiles. With its small footprint, low gas consumption and stackable configuration, this incubator makes it feasible to determine optimal settings by testing cell growth under many different conditions.

Advancing immunotherapeutic development through co-culture

In a recent comparison of cell behaviour between this next-gen incubator and traditional incubators, we tested cells with three immunotherapy compounds (one approved by the FDA, two investigational) and found significant differences in the outcomes.

We cultured patient-derived CD8+ T-cells with a lung carcinoma cell line – one set in a traditional incubator with standard conditions of 37°C and 5% CO_2, and the other set in an Avatar system using the same CO_2 and temperature settings as well as 2% oxygen and 2 PSI to mimic the microenvironment in lung cancer. Cells were treated with one of the immune checkpoint inhibitor drugs and monitored for 72 hours, far longer than the usual eight hours that would be allocated for this type of experiment in a traditional incubator. Immune T-cells turn over quickly, with a half-life of 24 hours in a standard incubator; but when cultured under low oxygen and high pressure they have a half-life of 72 hours.

As it turned out, the extra time in addition to the custom hypoxia and pressure settings provided new insight into how these drugs affected target cells. In the next-gen incubator, the FDA-approved therapy had a strong effect during the first day but quickly lost efficacy as the target cells were able to adapt and recover, which may explain why nearly half of patients who take this drug suffer a relapse after initially responding well to it.

There were also unexpected results for the two investigational compounds. One was less effective in the new incubator than it was in the traditional incubator; this may indicate that the drug will have less effect in human trials than preclinical results have suggested. The second candidate was something of a sleeper hit, with little initial activity followed by increased effectiveness over time, peaking in the Avatar system long after the traditional cell culture study would have ended.

One particularly interesting finding from the study was the heightened T-cell activity in the next-gen incubator compared to the traditional one.

References

Bissell, Mina J and LaBarge, Mark A. Context, tissue plasticity, and cancer: Are tumor stem cells also regulated by the microenvironment? Cancer Cell. 2005 Jan; 7(1): 17-23. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2933216/#R8.

Li, Grace et al. Genomic and Morphological Changes of Neuroblastoma Cells in Response to Three-Dimensional Matrices. Tissue Engineering. May 2007, 13(5): 1035-1047. http://online.liebertpub.com/doi/abs/10.1089/ten.2006.0251.

Iyer, Narayan V et al. Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1α. Genes & Dev. 1998. 12: 149-162. http://genesdev.cshlp.org/content/12/2/149.full.

Amgen/Bayer study. http://www.reuters.com/article/us-science-cancer-idUSBRE82R12P20120328.

Glenn Begley article on Amgen/Bayer. http://circres.ahajournals.org/content/116/1/116.short.

Nature call for better standards (April 2015). http://www.nature.com/news/announcement-time-to-tackle-cells-mistaken-identity-1.17316.

Genentech award. http://www.bio-itworld.com/2014/9/15/gcell-gives-genentech-corporate-memory-cell-line-experiments.html.

Clinical development success rates for investigational drugs. http://www.nature.com/nbt/journal/v32/n1/full/nbt.2786.html.

Immunotherapy has been dogged by reports that only a small fraction of patient-derived T-cells are active against cancer when reintroduced to the patient. Based on this study, we hypothesise that perhaps T-cell activity is reduced during the manufacturing process, and that culturing these cells under more accurate physiological conditions could keep them healthier and better able to attack cancer when reintroduced to the patient.

The promise of immunotherapy is a key motivator to improve upon cell-based research. The immunodeficient mouse models traditionally used for preclinical testing of cancer drug candidates make it impossible to properly test whether these therapies can stimulate the native immune system. Gleaning relevant biological information from human co-culture models instead of lab mice would be a welcome alternative for immunotherapy pipelines.

While there is much research that must be done to follow up on this preliminary study, the results did make it clear that cells behave differently under defined oxygen and pressure levels than they do in a fixed incubator environment. In our experience, cells lived longer and appeared to maintain fitness better when hypoxia and pressure were tailored to match their native environment.

The goal

These and other efforts to advance cell culture by making results more biologically relevant offer hope that the drug discovery field is heading toward a future where cell screening generates better data than we get today from cells and animal models combined.

Cell-based assays are cheaper, faster, and less contentious than animal models. Being able to rely on results from them could help pharma and biotech companies lower costs and accelerate the pace of drug development. As we all know, the goal is to fail compounds as early as possible so that resources are funnelled only to the drug candidates most likely to be safe and effective for patients. If preclinical results all came from reliable human cells, these compounds might see better success rates during clinical trials.

With a greater understanding of how various factors affect cell function, there will no doubt be increased attention for cell culturing and other components involved in the extensive use of cell lines in drug discovery and development. Pharma and biotech researchers can look forward to creative approaches and new technologies that should help solve the challenges that have to date made cell studies less accurate and reproducible than needed. After decades of stasis in cell culturing, it is truly exciting to be on the cusp of an innovative era that could transform how we use cells throughout the biomedical community. DDW

Dr James Lim *is Co-Founder and Chief Scientific Officer of Xcell Biosciences, which was formed based on his previous research involving fluorescent imaging of living single cells. Dr Lim received his PhD from The Scripps Research Institute and completed postdoctoral work at Harvard Medical School and Lawrence Berkeley National Laboratory.*

Dr Fred Aswad *is Director, lead discovery in the Biologics Research US division of Bayer, where he has worked in increasingly responsible positions since 2007. Dr Aswad holds a BS in microbiology from UCLA, a PhD in immunology from USC, and a JD from Santa Clara University School of Law.*