LIVE CELL IMAGING OF SIGNALING PATHWAYS

SKOV3 Cells having STAT3 Endogenously Tagged with GFP

ENGINEERED CELL LINES Addressing the Complexity

CELL DESIGN STUDIO™ Key Tools for Researchers

AN INTERVIEW WITH Dr. David Drubin
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ENGINEERED CELL LINES
Addressing the Complexity

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AN INTERVIEW WITH
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Sigma® Life Science Global Education Program
Once Upon a Time ...

More than a few years ago, I recruited a post-doctoral student from the prestigious lab of Dr. Pierre Chambon to join my research team in pharmaceutical discovery. To understand tissue development, this young scientist had been disrupting human somatic cell genes in culture using a technique called homologous recombination (HR). Using HR, he was able to completely ablate the production of his particular proteins and then study the effect of their absence. Replacing or removing genes at will in cultured cells was at the time revolutionary and held the promise of rapidly propelling our understanding of what certain cellular proteins did and, for us, which were candidate drug targets. Well, upon arrival and hearing the actual project goals, he enlightened us on the challenge — the near impossibility, as it turns out — of doing this at the scale and within the timelines that discovery-for-profit demanded. He went on to an impressive career in cancer research while the field of eukaryotic gene manipulation plodded along. Fortunately, since then, new technologies have surfaced that make it practical to expeditiously “engineer” many types of user-defined changes into living cells.

One such technological advent is the zinc finger nuclease (ZFN) system. Like the old methods, ZFN technology also relies on the HR biology, as well as another cellular DNA repair mechanism called non-homologous end joining (NHEJ); together or separately, these natural cellular activities can be used to deliberately sculpt the genetics of the target genome and make designer cell lines.

The ZFN system is commercially distributed by Sigma-Aldrich® as CompoZr®, and is now being used by my current team of cell biologists to create genetically engineered cell line products. These ZFN-altered model cell lines are available as both Sigma-Aldrich catalog items and as customer-defined custom products (Cell Design Studio™). In this issue’s companion article, Wemhoff, Holroyd, and Keller give an example of the custom cell line engineering process and how this can be effectively applied to a once perplexing problem in cancer biology.

Complexity Redux

In my earlier Biowire review on cancer, I discussed the application of ZFN-derived cell-based models to study complex diseases. Complexity enters the cell scientist’s world in two places: once at the level of the biological problem being investigated (bio-complexity), and again in the biology of the cell-based system being used as a tool to study the problem (bio-technical complexity). Both are not trivial, both are important, and both are obviously interrelated.

Bio-Complexity

Most complex biological phenomena arise from large arrays of multi-molecular interactions. This complexity is compounded by the natural variation present in the “activities” (kinetics) of a population’s interactants. And it is this variation that is due to the unique genetic constellation present in the individual — whether that is an individual person or an individual cell. Often, the complexity becomes so complicated, that cause-and-effect generalizations about a complex phenotype are woefully insufficient, if not misleading (cf. ).

The challenge of “personalized medicine”, as discussed here in the context of cell line engineering by Wemhoff, Holroyd, and Keller, is a direct result of the necessity to examine the unique genetic complement of an individual’s “interactome”.

The solution to the Bio-Complexity problem, pertinent to the most important biomedical questions, can be addressed through the deliberate fusion of: 1) broad, system-wide analyses (systems biology) and 2) hypothesis-driven investigations of critical mechanisms. Philosophically speaking, it is the co-application of the Baconian data-driven (“omics”) approaches with the hypothesis-driven methods of Galileo to the problem. Because engineered cell lines permit...
the systematic investigation of many independent variables while keeping others controlled, they are superior tools for Galilean-type experiments. This is partly true because ZFN-altered cell lines can be tested in parallel against both other “isogenic” engineered lines and the unmodified “isogenic” parental control cells. Using genetically matched sets of cells is now routine.\(^{10,11}\)

**Bio-Technical Complexity**

Complexity also enters the experimental cell biologist’s realm when they begin to consider the experimental system itself. When using ZFNs to make genetic changes in a cell line, a host of biological situations have an impact on the technology’s facility.

**Chromosomal Clutter: Ploidy**

As Wemhoff, Holroyd, and Keller point out, increases in the number of chromosomal targets per cell (ploidy) will reduce the frequency of engineered mutations detected. In many transformed cell lines, the genetic instability and resultant aneuploidy, which permits the cell’s limitless proliferation, can greatly increase the numbers of individual chromosomes. Theoretically, this increases the number of cells to screen in an engineering experiment; practically, however, it has never prevented us from eventually isolating a full-knockout clonal cell line.

**Problematic Prolificacy: Gene Amplification**

What could prevent success, is if all alleles in a cell needed to be modified and the target sequences had been extensively amplified. For example, in the breast cancer cell line MCF-7, karyotype analysis would suggest that the "q" arm of chromosome 8, at base 116M, is tetraploid; however, a fine copy number variation (CNV) analysis shows that TRPS1, which is located at base 116M, is actually at 10 copies per MCF-7 cell.\(^2\)

**Marred Mending: Homologous Recombination Ability**

In the case of knockin cell lines, where HR is required to insert a donor DNA fragment at the ZFN cut site, it is critical that the cell line be at least moderately proficient in this activity. The human cell line HT29 is an example of a cell without one repair activity; in our studies, while having NHEJ activity equivalent to other lines, it has virtually undetectable HR activity (unpublished).

**Genetic Reshuffling: Mitotic Recombination and LOH**

The goal of most cell line engineering projects is to deliver complete changes in all alleles present (homozygous). In some situations, however, it is not possible to modify all alleles (because complete loss — or gain — affects proliferation or viability); and, in others, the heterozygous state is desired because it is the most biologically relevant (cf.\(^3\)). Regardless, if within a cell there exists both modified and unmodified alleles, the potential for inter-chromosomal recombination is present and can eventually result in both wild-type and homozygous-mutant cell lineages being present in the cell population. Termed loss of heterozygosity (LOH)\(^4\); if a growth advantage results from these genetic rearrangements, then that sub-population will begin to predominate in the culture.

**Expression Paradoxes: Gene Dosage and Compensation**

It is logical to expect, when multiple alleles are present, that inactivating alterations in increasingly larger numbers of alleles would result in a correspondingly decreased expression profile. Often, however, this is not the case. In gene knockout situations where multiple alleles are targeted, knocking out any number of alleles only slightly reduces expression levels; it is only when all alleles are knocked out that expression disappears (cf. BAX/BAK knockouts).\(^5\) It seems, therefore, that for many genes there are compensatory mechanisms that maintain a minimal level of expression.

This is even true when a subset of genes in a functionally related gene family is targeted. In the HCT116 cell line, we inactivated all four copies of the MDR1 gene only to see a significant increase in the BCRP transporter expression; it would seem that the increase in BCRP is to compensate for the loss of function of MDR1 (unpublished).

**Rogue Biology: Synonymous Mutation Suppression and Stop Codon Read-Through**

Other less well-understood phenomena may also confound attempts at gene modification. For instance, sometimes during the engineering of a particular gene, synonymous (translationally silent) mutations are created. While it is usually presumed these have no affect on expression, in fact, sometimes silent mutations drastically reduce the levels of gene products observed.\(^6,7\) This can be true even when the exogenously added synthetic donor DNA sequence is optimized using human preferred codons. As an example, when we used a human codon-optimized green fluorescent protein (GFP) donor DNA to tag an endogenous gene, this completely inhibited expression of the resultant fusion protein; when the optimized codons were replaced with the naturally occurring GFP codons, however, this restored recombinant protein production and fluorescence (unpublished).

Similarly perplexing is a phenomenon sometimes seen when gene knockouts are made using ZFNs to either directly introduce stop codons or create deletions that result in a translational frameshift. Usually, it is presumed that stop codons in the new reading frame will terminate translation; however, once again, it is not always that simple. Sometimes, it is possible that the new theoretical stops are ignored when the DNA-sequence context of those stop codons is suboptimal; this phenomenon, known to be exploited by animal viruses, can also occur through the action of natural suppressor tRNAs.\(^7,8\)

**Coda**

If we are indeed only limited by our imaginations, then it is necessary that we appreciate the complexities facing us. For research scientists, who are encouraged to use Occam’s Razor to simplify their conclusions, what they must not do in their experimental design is trivialize the intricacies of the biology or the technology used to understand it. The sophisticated cell line engineer avoids both.
CREATION OF CUSTOM-ENGINEERED CELL LINES BY OUR CELL DESIGN STUDIO™ PROVIDES KEY TOOLS FOR RESEARCHERS

Gregory Wemhoff, Principal Scientist
Erika Holroyd, Scientist
Bradley Keller, Product Manager
Sigma® Life Science, St. Louis, MO
The onset of cancer is generally believed to occur from the accumulation of specific mutations that alter the genetic makeup of the transformed cell. Some mutations may result in over- or under-expression of the target gene, while other mutations may be as subtle as a single base pair change altering ligand/substrate interaction. Early studies demonstrated the loss of TP53 gene expression leads to a significantly higher tumorigenic rate compared to cells with normal expression. Moreover, since TP53 was first recognized as a tumor suppressor, the number of genes identified as playing a role in cancer development has increased dramatically. Recently, the advent of robust, high-throughput, and cost-efficient methods of sequencing DNA has accelerated the rate of identification of specific cancer-relevant mutations and the use of transformed cell lines to study these mutations.

The hypotriploid, transformed human lung epithelial line, A549, has been used extensively to study lung cancer and carries mutations in several genes known to be associated with cancer development such as the epithelial growth factor receptor (EGFR). In the review of Sharma et al., over 40 different mutations associated with EGFR were highlighted. The precise EGFR mutation a patient carries is critical, rendering the tumor more or less susceptible to kinase inhibitors such as gefitinib and erlotinib, and ultimately affecting the choice of treatment. Unfortunately, panels of cell lines that allow investigation of each EGFR mutation or other cancer-relevant mutations are not currently accessible. Using zinc finger nuclease (ZFN) technology, cell lines containing very specific, directed mutations can be created. The basic researcher, as well as diagnostic and therapeutic development programs, need no longer be saddled by the limited availability of relevant cell lines for their studies. Through Sigma Life Science’s Cell Design Studio™ (CDS) custom cell line development group, cell lines containing mutations of interest can be generated.

To initiate an inquiry, a researcher approaches CDS and requests a specific cell line carrying a targeted gene with their modification of interest. CDS then investigates the target cell line to determine clonability (i.e., expansion from a single-cell parent), chromosome ploidy of the target gene, potential target gene amplification, and other key features. Modifications of the target gene can include base insertions, deletions, or nucleotide base substitutions to mimic naturally occurring mutations. Fusion proteins can also be created for the purpose of attaching reporter tags to an endogenous gene locus. Notably, genetic information can be introduced into well-defined locations, rather than relying on random integration events, thus reducing the impact on expression of endogenous genes. Control is paramount, as the genetic alterations can occur on a single allele, leaving the other allele(s) in the wild-type state (i.e., a heterozygous mutation), or all alleles can be altered (i.e., homozygous). Unlike limitations associated with other gene engineering technologies, ZFN-based alterations can alter every allele in polyploid cell lines.
Figure 1. Workflow for a Standard CDS Cell Line Project.

As an example of the CDS process, let’s assume an investigator involved with breast cancer research requires a breast epithelial cell line that carries a particular mutation known as the BRAF mutation. It is well documented that in melanoma and colorectal carcinoma the BRAF gene often carries a single nucleotide change, the V600E mutation, which is thought to promote metastasis. Using a near-normal breast epithelial line, MCF10A, the CDS team first pursues the generation of a ZFN pair that will cut specifically near the site where the V600E mutation is known to occur. Additionally, a donor oligonucleotide sequence is designed having the single nucleotide difference associated with the V600E mutation and flanked by a wild-type (homologous) sequence. Concurrently, investigations are conducted to examine ploidy of the MCF10A cell line, which reveal it is diploid, thus having only two alleles for the BRAF gene target. If not previously examined by the CDS, the cell line is subjected to single-cell cloning to determine the difficulty of generating clonal cell populations. In the case that the cells cannot be grown from single-cell clones, the customer is advised, and it is determined whether a mixed population will serve their needs. The target gene is also investigated (through available literature) regarding the potential impact on cell viability if it is disrupted. This includes understanding if the desired mutation is considered a heterozygous dominant mutation such that, if only a single allele carries the mutation, the oncogenic effect is realized. With the key information in hand, the customer is contacted to determine if they require a homozygous or a heterozygous alteration of their gene target, after which work to create the cell line may commence.

Once available, the ZFNs with the “donor oligonucleotide” carrying the BRAF mutation are nucleofected into the MCF10A cell line, and ZFN activity is confirmed from samples of the cell pool. The cells are then expanded and subjected to single-cell sorting to generate cloned cell lines. Each clonal line is expanded and subsequently screened by sequence analysis to determine which are carrying the desired mutation. The clones screening positive for the mutation are examined further to determine if all alleles carry the mutation or if a fraction of the alleles have been impacted. Specific populations of the clones are then selected for further expansion and cryopreservation.

At numerous steps throughout the CDS process, the customer is advised of the experimental results and must determine if the project should continue to move forward. One possible outcome, after attempting two nucleofections, is the inability to isolate cells carrying the desired mutation due to the complexity of the biology (e.g., gene polyploidy). A customer must then decide if additional nucleofections will be attempted or if they would prefer terminating the current project and attempting a different approach. Varying approaches could include selecting a different ZFN pair targeting a slightly different cut site, designing a different donor oligonucleotide, or perhaps pursuing a different target cell line.

While this example outlines the introduction of a single nucleotide mutation, the insertion of stop codons to shut down gene translation or the generation of out-of-frame insertions or deletions may also be pursued to alter gene expression. Additionally, over-expression of genes or gene amplification can be mimicked by introducing an additional copy in “safe harbor” sites known to have no impact on cell integrity, such as the AAVS1 site. Table 1 illustrates several types of projects the CDS team can conduct for customers.

The ability to use either normal or near-normal cell lines instead of transformed lines allows the researcher to investigate a particular gene target against a near-normal background. This is quite distinct
from using a transformed parental cell line where several genes are known to be disrupted and, in many instances, present in a polyploid state. To that end, our CDS expertise is also available for modification of inducible pluripotent stem (iPS) cells to create a “fit-for-purpose” cell line. In addition to the full range of gene-editing capabilities, characterization of iPS cells such as directed differentiation, copy number variation analysis, and epigenetic analysis can be performed. Just as with the immortalized cell lines, deliverables include the cell line, the ZFN kit (and donor if applicable), and complete project report documentation to support scientific publications.

This article began by outlining cancer generally as the result of “the accumulation” of various gene-altering events. The CDS team has the ability to introduce multiple, distinct modifications into the target cell line. Following the example, once a clone carrying the BRAF-V600E mutation has been isolated, this cell line can then serve as the foundation line to introduce additional mutations. Many cancers carry a loss of PTEN expression in addition to other mutations, and colorectal cancers are often V600E-positive and carry a KRAS mutation. An additional option available through the CDS group is to tag genes with markers such as fluorescent proteins. Cells can then be readily tracked and the migration of cellular proteins monitored through live cell imaging, a method ideal for high-content analysis screening. While the target cell line or target gene may introduce some limitations, the overall possibilities for cell line creation by the CDS are limited primarily by the expressed desire of the researcher. The next step to advancing a treatment or cure for a cancer, or other genetic condition, may first be identified by your desired gene modification in a new cellular disease model developed by the CDS.

Table 1. General Types of Projects Conducted by the CDS.

<table>
<thead>
<tr>
<th>TYPE OF CELL MODIFICATION</th>
<th>TECHNOLOGY APPROACH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene Knockdown</td>
<td>shRNA or siRNA</td>
</tr>
<tr>
<td>Gene Disruption (Knockout)</td>
<td>Knockout ZFN</td>
</tr>
<tr>
<td>Knockin at Specific Gene Locus (e.g., SNP, gene replacement)</td>
<td>Custom ZFN and Plasmid or Oligo Donor</td>
</tr>
<tr>
<td>Targeted Integration into Safe Harbor Site</td>
<td>ZFN TI KIT and Plasmid or Oligo Donor</td>
</tr>
<tr>
<td>Random Integration</td>
<td>Plasmid or Lentivirus Construct</td>
</tr>
<tr>
<td>Reporter Gene Fusion Protein</td>
<td>Custom or Off-the-Shelf ZFN and Custom Plasmid Donor</td>
</tr>
<tr>
<td>Bioproduction</td>
<td>Custom ZFN and Donor or Plasmid Construct</td>
</tr>
</tbody>
</table>

References:
An Interview with

Dr. David Drubin

University of California, Berkeley

In this issue, our thought leader is Dr. David Drubin, Professor of Cell and Developmental Biology at UC Berkeley, whose recent live-cell imaging studies dramatically changed our understanding of clathrin-mediated endocytosis in mammalian cells.
Clathrin-mediated endocytosis (CME) enables cells to take up nutrients and regulate the composition of cell-surface lipids and proteins, which in turn controls how the cell responds to environmental signals, differentiates, and grows. CME is the primary route for endocytosis, and alterations of the CME process are implicated in the pathogenesis of some cancers and atherosclerosis, as well as how viruses and harmful bacteria infect cells. David Drubin, Ph.D., Professor of Cell and Developmental Biology at the University of California, Berkeley, is using genome editing and high-throughput live-cell imaging to transform the conventional understanding of CME in mammalian cells.
When we tagged the endogenous clathrin light chain A and dynamin-2 genes with RFP and GFP using the zinc finger technology, we were amazed. The clathrin-mediated endocytosis process that we observed was much more efficient and regular than reported in the literature.

In prior studies in mammalian cells, the process was observed to be extremely heterogeneous, so much so that one couldn’t predict the order and timing of events. There was also significant heterogeneity in the size and shape of the endocytic structures in the mammalian cells. However, when we expressed the tagged proteins at endogenous levels, the process looked much more homogenous and similar to yeast than what had been described previously.

Cellular processes should be studied as close to their natural states as possible. I suspect that, as we see more uses of zinc finger nucleases [for tagging endogenous genes], people will find that they have been inadvertently perturbing the processes that they have been studying.

Tagging two endogenous genes, left under their natural promoters, opened up a data collection boon: unbiased high-throughput screening and analysis.

**Dr. David Drubin:**

The standard technique for expressing fluorescent proteins in mammalian cells is to transiently transfec the genes into the cells. Since different cells will take up different amounts of DNA, there is significant cell-to-cell variation in expression. Typically, people choose the cells that appear normal, and then image and study those cells. That introduces bias.

When we used the ZFNs to fluorescently tag endogenous genes, we produced a clonal cell line in which every cell is expressing the tagged proteins at endogenous levels. This feature allowed us to use software programs to image many different cells repeatedly in an unbiased manner. We did experiments in which we quantified tens of thousands of these dynamic events systematically in every single cell, without the scientist using his or her judgment to say the cell is fairly normal or well behaved.

Previously, it was difficult to predict how the CME process would change in response to a stimulus. Now, with a regular profile for the kinetics of the CME process, Dr. Drubin’s group is exploring how different cargos, stressors, and growth factors, among other things, affect CME.

**Dr. David Drubin:**

Endocytosis also plays different roles in different cell types. For example, in a nerve cell, endocytosis occurs very quickly every time an action potential is fired because the cell has to rapidly take up its neurotransmitters after they are released. We’ve been engineering stem cells that express fluorescently tagged endocytic proteins at endogenous levels. The beauty of the stem cells is that we can differentiate them to become all sorts of different types of cells. We want to know what happens to the process as a cell differentiates into a nerve cell, a liver cell, or a heart cell — and how those changes alter the dynamics and regulation of endocytosis.

We are also examining the clathrin-mediated endocytosis process in finer detail. Using three more zinc fingers from Sigma®, we are adding markers that help reveal how different stages of the pathway are regulated.

**Reference:**

NOVEL REPORTER CELL LINES
FOR DETECTION OF ENDogenous PATHWAY ACTIVITY IN LIVE CELLS

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Hongyi Zhang, Senior Scientist
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John Fetter, Associate Research Fellow
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Sigma® Life Science, St. Louis, MO
Introduction

CompoZr® ZFN (zinc finger nuclease) technology was used to insert the sequence for fluorescent proteins adjacent to endogenous genes for signal transduction proteins to create novel reporter cell lines for compound screening. These cell lines include ones with fluorescent tags on EGF, STAT1, and STAT3. Addition of growth factors to these cell lines activates translocation that can be monitored under live cell conditions.

Receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGFR) are cell-surface growth factor receptors that dimerize and autophosphorylate upon ligand binding (Figure 1). They transduce signals across the cell membrane and thus regulate diverse cell functions. Overexpression of EGFR has been found in various cancers. This has lead to the development of clinically approved therapeutics that target EGFR for colon, lung, breast, pancreas, and head and neck cancers. Overexpression of EGFR, along with that of other kinases, can lead to constitutive activation of the oncoprotein STAT3 (Signal Transducer and Activator of Transcription 3). STAT3, a member of the STAT protein family (STAT1-7), is a signaling intermediate that mediates the action of many cytokines and growth factors (Figure 1). STAT3 is constitutively active in many different cancers including prostate, breast, lung, head and neck, colon, liver, and pancreas, as well as in multiple myeloma and large granular lymphocytic leukemia. Numerous studies have repeatedly demonstrated inhibiting STAT3 results in decreased tumor growth and improved animal survival by inducing tumor cell apoptosis, inhibiting angiogenesis, and enhancing antitumor immune-mediated cytotoxicity. Another STAT family member, STAT1, possesses cancer-inhibitory properties and, once activated, may promote apoptosis in tumor cells (Figure 1). Ideally, the potential inhibitors of STAT3 should be very selective and should not inactivate STAT1.

Utilizing our in-house CompoZr technology with the homologous recombination pathway induced by ZFNs, single-clone-derived EGFR-FP, FP-STAT3, and FP-STAT1 cell lines were created in A549 and SKOV3 cells (Figure 2). Having endogenous gene expression/regulation and preserved protein function for FP-tagged EGFR and STAT3 makes the cell lines valuable for high-content screening of compound libraries to find novel modulators of their activity.

Materials and Methods

A549 and SKOV3 cells (Cat. No. CCL-185 and HTB-77) cells were obtained from ATCC. The SKOV3 cells were cultured according to the product manual and A549 were grown in RPMI-1640, 2 mM glutamine, 10% FBS in 5% CO2, 37 °C.

ZFN nucleofections were performed with the Amaxa® Nucleofector® device (Cat. No. AAD-1001) and Nucleofector® Kit T or Nucleofector® Kit V (Cat. No. VCA-1002 or Cat. No. VCA-1003) from Lonza AG according to the product manual. Donor plasmids were designed and constructed in-house.

Fluorescent reporter genes were obtained from Evrogen (evrogen.com/products/TagFPs.shtml). CompoZr ZFNs were designed and manufactured by Sigma-Aldrich®. Unless otherwise indicated, all reagents and materials used in this work were obtained from Sigma-Aldrich (St. Louis, MO, USA).
Figure 2. Schematics of the Three Tagged Loci Showing ZFN Binding Sites/ZFN Cut Site with Respect to the Targeted Integration Site.
The donor (top) has the homology arms of indicated length and the fluorescent protein (FP) sequence (green).
Figure 3. Fluorescence Microscopy and Differential Interference Contrast (DIC) Images of Isolated Single-Cell Clones Expressing the EGFR Gene (Epidermal Growth Factor Receptor, Also Known as ErbB1) Endogenously Tagged with GFP (A549 Lung Carcinoma) or RFP (SKOV3 Ovarian Adenocarcinoma) at the C-Terminus. The SKOV3 line also has the second gene HER2 (Human Epidermal growth factor Receptor 2, also known as ErbB2) endogenously tagged with GFP at the C-terminus. The EGF images were taken 20 (A549 EGFR-GFP) or 10 (SKOV3 HER2-GFP/EGFR-RFP) minutes after addition of 100 ng/ml EGF. Preincubation with 1 mM Tyrphostin AG 1478, a selective EGFR inhibitor, for 20 minutes prior to the addition of EGF blocks the internalization of the fusion protein (shown for A549 EGFR-GFP).

Figure 4. Fluorescence Microscopy and DIC Images of Isolated Single-Cell Clones Expressing the STAT3 Gene Endogenously Tagged with RFP (A549 Lung Carcinoma) or GFP (SKOV3 Ovarian Adenocarcinoma) at the N-Terminus. The IL-6 images were taken 30 (A549 RFP-STAT3) or 20 (SKOV3 GFP-STAT3) minutes after addition of 100 ng/ml IL-6. In cases where cells were preincubated for 1 hour with 20 mM Static, a specific STAT3 inhibitor, the IL-6 treatment did not induce STAT3 translocation (shown for A549 RFP-STAT3).
Fluorescent imaging of cells was done with a Nikon Eclipse TE2000-E inverted research microscope and MetaMorph® software using a 40x/1.3 oil objective. Cells were imaged live in Hanks’ balanced salt solution (Cat. No. H8264) supplemented with 2% fetal bovine serum (Cat. No. F2442). Filter sets were GFP (excitation 450–490 nm/emission 500–550 nm) and RFP/TRITC (excitation 530–560 nm/emission 590–650 nm).

For STAT1 immunolabeling, A549 cells were fixed with 3.7% formaldehyde and permeabilized with 0.1% Triton® X-100 before or 30 minutes after addition of 100 ng/ml IFN-γ. Anti-STAT1 (isoform α) rabbit primary antibody (Cat. No. SAB4300441) was used at 1:50 dilution and TRITC-labeled goat anti-rabbit IgG (Cat. No. T6778) was used at 1:500 dilution.

**Discussion**

Fluorescence detection of proteins traditionally relied on either exogenous promoters or immuno-techniques requiring cell fixation. With ZFN technology, it is now possible to create stable integration of a reporter gene into the genome. Unlike fusion proteins generated with an external promoter, the fusion proteins created using the ZFNs are expressed at their physiological level and thus are more likely to retain the characteristic expression profile of the endogenous proteins in the cell. In contrast to biochemical assays or immunostaining, using a tagged protein under endogenous regulation avoids fixation artifacts and allows detection of the activity in live cells. This work demonstrates successful tagging of three loci that code for important pathway targets: EGFR, STAT1, and STAT3. In wild-type cells, activation of EGFR leads to receptor internalization (Figure 3, top row). The EGFR kinase inhibitor Tyrphostin AG 1478 blocked the receptor internalization (Figure 3, middle row).

With ZFN-mediated gene tagging in knockin cell lines, STAT3’s native gene regulation is conserved, resulting in normal protein expression levels and preservation of protein function. Within 30–40 minutes of IL-6 treatment, the GFP- or RFP-tagged STAT3 was primarily localized in the nucleus of the A549 and SKOV3 cells (Figure 4, top and bottom row). However, after preincubation of the cells with 20 mM Static for 1 hour, the IL-6-induced nuclear translocation of STAT3 was inhibited (Figure 4, middle row).
ZFN-mediated gene tagging of endogenous STAT1 allowed normal expression of the fluorescently tagged protein (Figure 5, middle row). STAT1-GFP expression occurs throughout the cell. The punctate labeling is from autofluorescence of endosomes that can be seen even with the wild-type cell line. Within 25 minutes after addition of IFNγ, the STAT1-GFP was primarily localized to the nucleus (Figure 5, middle row). Immunostaining of wild-type cells showed a similar effect—i.e., that addition of IFNγ caused the STAT1 to concentrate in the nucleus (Figure 5, bottom row). Thus the GFP-tagged cell line shows the same function as the wild-type cell line without the requirement for fixation and immunostaining.

Other tagged cell lines have also been created, including tagged organelle markers and ones that allow monitoring of cell signaling intermediate translocation (Table 1). These cell lines should be useful for live-cell screening assays to find compounds that affect cell structure or signaling pathways.

### References:

### Table 1. Current FP-Tagged Cell Lines.

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<td>Osteosarcoma</td>
<td>LMN1B (N) and ACTB (N)</td>
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<td>BFP-LMN1B (N), GFP-TUBA1B (N) and RFP-ACtB (N)</td>
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<td>Receptor binding domain (activated EGFR)</td>
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<td>Lung carcinoma</td>
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DETECTION AND PREVENTION OF CELL LINE CROSS-CONTAMINATION

Since its inception in the early 20th century, cell culture has become adopted in almost every field of life science research. Soon after the establishment of the first human cancer cell line (HeLa) in the early 1950s, it was realized that vigorous lines could contaminate and overgrow slower-growing cultures. The problem of misidentified cell lines, however, is still prevalent.

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HeLa’s success as a cell line, coupled with its immortality and vigor, led to its worldwide distribution during a period when cell culturists were perhaps oblivious to the ease with which cross-contamination could occur, particularly in the absence of good cell culture practices.

HeLa contamination was recognized as early as 1967. Using Isoenzyme Analysis, Stanley Gartler reported that 18 cell lines of supposed independent origin shared a rare enzyme isoform with HeLa. These included the Chang Liver, Hep-2C, and KB cell lines. Yet in the last decade, there have been over 1,000 citations for these cell lines. It is estimated that 15–20% of cell lines currently in use may not be what they are claimed to be. Astonishing as it may sound, a report in 2004 suggested widespread lack of vigilance in cell sourcing and identity testing. Out of 440 respondents, over one third received their cell lines from other laboratories and almost half did not perform identity testing. There is no excuse for this. A literature search will soon reveal the available resources, with culture collections being particularly proactive on this subject. The European Collection of Cell Cultures (ECACC) for example hosts a comprehensive list of misidentified cell lines on its website: bit.ly/misidcells

Karyotyping, Isoenzyme Analysis, and, more recently, DNA Barcoding are invaluable tools for the detection of inter-species cross-contamination. Short Tandem Repeat (STR) profiling has become the standard for the intra-species identity testing of human cell lines. The assay works on the same principle as the forensic DNA fingerprinting technique developed by Sir Alec Jeffreys. However, labor-intensive Southern blotting has now been superseded by a multiplex PCR-based technique that simultaneously amplifies the polymorphic STR loci in the genome. Each allele occurs in the population with a particular frequency. By amplifying a sufficient number of different alleles and multiplying by the frequency in which each occurs within the population, a unique profile for a cell line is obtained. As recently as 2008, 40 human thyroid cancer cell lines were analyzed by STR profiling. Only 23 unique profiles were obtained and many of the cross-contaminating cell lines were not even thyroid in origin. These cell lines had been previously used for two decades in the field of thyroid cancer research.

At this moment in time there is no standard STR protocol for the unique identification of non-human cell lines. ECACC is unique among culture collections in having uniquely fingerprinted its nonhuman cell lines with Jeffreys probes. There are initiatives in place to replace this technique with up-to-date molecular methods.

Cell culture collections have started to forge close relationships to tackle the issues of misidentification and are working together to publish a standard (American National Standards Institute [ANSI] ANSI-0002) for the authentication of human cell lines, using STR profiling and a free-to-use online interactive database containing the STR profiles of almost 2,700 human cell lines for users to compare their STR data. Providing the closest matches, the database allows the identification of a cell line or alternatively gives confidence in the uniqueness of a novel cell line: bit.ly/strcells

In his 2007 article entitled “Eradication of cross-contaminated cell lines: A call for action,” Roland Nardone proposed that cell line authentication should be conditional for the receipt of grant funding and for the publication of research findings. Although authentication is not yet a requirement for all funding or publication, things are slowly moving in this direction with a number of journals adopting an authentication policy. Requests for ECACC’s STR profiling service have increased significantly over the last two years. Occasionally, driven by a requirement from a journal to provide evidence of cell line identity prior to publication, samples are submitted after project completion. Often in these cases, the news is not good, and sadly there are more than enough examples of inability to publish due to the unwitting use of the wrong cell line. It is in the opinion of these authors that neglecting to perform regular identity testing constitutes poor cell culture practice and that the promotion and insistence on identity testing should be an integral part of cell culture training.
While considering the major advances in identification methods and the need for regular testing, it is worth reflecting on the good practices that can minimize the chances of cell line misidentification. Cell lines should be sourced from reputable suppliers who can provide certificates of authenticity. The gifting of cell lines should be avoided and novel human cell lines must be authenticated to ensure that they are indeed unique.

A continuous cell line has the capacity to multiply indefinitely, and if the cell line is not subject to a controlled cell banking regime of Master and Working Cell Banks then the chances of contamination, cross-contamination, and genetic and phenotypic instability increase with each successive subculture.

There are two main routes to cell line misidentification. Firstly, the accidental inoculation of one cell line with another. A faster-growing line, introduced by a single drop of cell suspension or the accidental reuse of a pipette, will soon completely displace the original culture. To avoid such scenarios, a policy of clearance and segregation is recommended as follows:

- Only one cell line should be handled in a bio-safety hood at any one time, and the workspace thoroughly cleaned between cell lines.
- Dedication of equipment to single cell lines — bottles and aliquots of cell culture medium and other reagents — must be enforced.
- Bottles and aliquots should be clearly labeled and never shared between cell lines.
- Waste pots must be regularly emptied and replenished with a disinfectant effective against both microbes and cell cultures.

The second and possibly more common route of cross-contamination is the accidental mislabeling of a flask or other container. Repeated hand-labeling can rapidly lead to cell line name corruption with letters being confused with numbers, etc. Throughout its cell banking operation, ECACC uses pre-printed labels detailing the cell line name and other relevant information. All labels are removed from spent flasks, reconciled, and attached to the cell culture records.

The regular checking of cell line morphology and growth characteristics by phase contrast microscopy and comparison with reference images can give an early indication of problems. Identification of unexpected morphology or growth characteristics in a cell line soon after resuscitation from frozen storage could be an indication of either mislabeling the vials at the time of cryopreservation, or even the removal of the wrong vial from storage. Printed labels including the cell line name in its correct format must have adhesive suitable for low temperature storage, as inappropriately adhered labels will detach, leaving an unlabeled vial in the inventory. Users then have to rely on the accuracy of the inventory records and confidence that the unlabeled vial contains the expected cells. A combination of regular identity testing and vigilance of good cell culture practices is required to provide valid cell cultures to application and to assure that research is acceptable for peer review. Ignorance of this guideline will lead to an undesirable legacy and invalid research.

(see references at PREVENTION on page 34)
Introduction

Programmed cell death is a fundamental process important in development, as well as a principle mechanism of tumor suppression. Apoptosis is triggered in non-malignant cells as a protective mechanism to remove damaged and unhealthy cells that may harm the body. Many chemotherapy treatments are based on the induction of programmed cell death\(^1,2\). Cells undergoing apoptosis are morphologically distinct from necrotic or autophagic cell death. Cells dying via apoptosis are characterized by cell shrinking, nuclear condensation and fragmentation, membrane blebbing, and finally separation of the cellular components into apoptotic bodies, which are then engulfed by phagocytes.

Apoptosis is thought to occur through two main pathways: the intrinsic pathway where the death signal arises from within the cell, and the extrinsic pathway, which involves the activation of cell surface receptors by an extracellular death factor. The intrinsic pathway can be triggered by various intracellular stimuli including DNA damage, growth factor starvation, and oxidative stress. These signals lead to mitochondria outer membrane permeability, resulting in mitochondrial protein leakage, which in turn induces apoptosis through activation of members of the cysteine-aspartic acid protease (caspase) family. Caspases are found in the cell as inactive proenzymes, cleavage of which produces active proteases\(^3\). For the intrinsic pathway, the initial enzyme activated is caspase-9, followed by the activation of caspase-3, 6, and/or 7. The extrinsic apoptotic pathway is stimulated by binding of a death ligand to a specific receptor, initiating signaling leading to caspase-8 activation. Caspase-8 subsequently either directly activates caspase-3 or induces mitochondria outer membrane permeability to trigger the cascade of caspases-3, 6, and/or 7, leading to cell death\(^4\).

In this study, we analyzed the ability of three small molecule antibiotics derived from specific microorganisms, to induce apoptosis in Jurkat
Figure 1. Apoptosis Induction with Ikarugamycin (Cat. No. SML0188), Zapotin (Cat. No. Z4652), or Siomycin A (Cat. No. S6076). Jurkat cells were left untreated (A), or treated for 24 hours with one of the following: 1 μM Ikarugamycin (B), 5 μM Ikarugamycin (C), 10 μM Zapotin (D), 20 μM Zapotin (E), 5 μM Siomycin A (F), or 10 μM Siomycin A (G). Cells were stained with Annexin V-FITC Conjugate and Propidium Iodide (Pi) using Apoptosis detection kit (Cat. No. APOAF) and analyzed by flow cytometry. Cells, which were stained by Annexin V-FITC, are cells in early apoptosis (bottom right quadrant), while cells stained with both Annexin V-FITC and Pi (top right quadrant) are cells undergoing late apoptosis (FL1, Annexin V-FITC; FL2, Pi).

Methods and Results Analysis
Jurkat cells were grown to a density of 1 x 10⁶ cells/ml and were either left untreated or treated with increasing concentrations of the following antibiotics: Zapotin (Cat. No. Z4652), Siomycin A (Cat. No. S6076), and Ikarugamycin (Cat. No. SML0188). Cells were then analyzed for apoptosis induction using two methods:

1. **Staining with Annexin V**: After 24 hours of incubation with the indicated molecules, cells were washed and stained with Annexin V-FITC Conjugate and Propidium Iodide (Pi) using Apoptosis Detection Kit (Cat. No. APOAF) following the kit technical bulletin, and analyzed by flow cytometry. The level of apoptosis

The early stages of apoptosis involve cellular changes that include loss of phospholipid asymmetry. At the onset of apoptosis, phosphatidylserine translocates from the internal to the external side of the plasma membrane. The phosphatidylserine thus becomes available to bind to Annexin V in the presence of calcium. The Annexin V-FITC Apoptosis Detection Kit detects apoptotic cells by flow cytometry utilizing Annexin V-FITC as a fluorescent probe to detect early apoptotic phosphatidylserine binding. Propidium iodide binding to DNA is also monitored to indicate compromise of the cell membrane and progression in apoptosis.

The Caspase 3 Fluorimetric Assay Kit measures the level of caspase-3 activity in cell lysates. The kit is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by caspase-3, resulting in the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety.
induction is assessed by depicting cell staining by PI (y-axis) vs Annexin V (x-axis) (Figure 1). The lower left quadrant (PI and Annexin V negative cells) shows the percentage of live cells. On the lower right quadrant are cells stained with only Annexin V, which are cells undergoing early apoptosis. Cells stained by both Annexin V-FITC and PI (top right quadrant) are cells undergoing late apoptosis/necrosis. Cells stained with only PI (upper left quadrant) are necrotic/non-viable cells.

2. Caspase-3 Activity: Cells were incubated for 4, 8, or 24 hours with the above-mentioned molecules, washed, and analyzed for caspase-3 activity using the Caspase 3 Assay Kit, Fluorimetric (Cat. No. CASp3F) following the kit technical bulletin (Figure 2). Results are presented as fluorimetric units per mg protein.

Results and Conclusions

After testing these two methods of apoptosis detection, it is evident from the data shown that all three antibiotics tested are potent apoptosis inducers (Figure 1 and Figure 2). Cells treated with 1 μM of Ikarugamycin for 24 hours showed induction of apoptosis with 38% of the cells being in late apoptosis (Figure 1B). Elevating the concentration of Ikarugamycin increased apoptosis induction resulting in nearly complete apoptosis in all cells (98%, Figure 1C). Treatment of Jurkat cells with either 10 μM or 20 μM of Zapotin induced apoptosis in 46% and 54% of the cells, respectively (Figures 1D and 1E), while Siomycin A treatment with 5 μM and 10 μM induced apoptosis in 55% and 51% of the cells, respectively (Figures 1F and 1G).

Since all three antibiotics induced apoptosis at different potency following 24 hours of treatment, we wanted to test whether this difference may not be detected if tested at different induction times. Therefore, Jurkat cells were treated for 4, 8, or 24 hours. As shown in Figure 2, Jurkat cells treated with each of the antibiotics for the indicated times and concentrations underwent apoptosis. Interestingly, apoptosis kinetics diverted between the various antibiotics as Ikarugamycin reached its maximum activity at 24 hours post-treatment with 5 μM, while Siomycin A maximal induction was detected 8 hours post-treatment with 5 μM. However, treatment with 50 μM Zapotin for 8 hours seemed to induce caspase-3 activity more efficiently than treatment with the other antibiotics tested (Figure 2).

Discussion

In this study, we wanted to compare the ability of three antibiotics to induce apoptosis. We found that, under the tested conditions, all three antibiotics induced apoptosis in Jurkat cells. However, they differed in the kinetics of their activity and in their intensity, with Zapotin and Ikarugamycin being the more potent inducers.

The apoptotic activity of the three antibiotics has been previously demonstrated to induce cleavage of caspase-9, 8, and 3 in treated cells.
Abstract
To efficiently expand cells and develop robust cell-based models for in vitro drug screening, in vivo-like cell culture conditions — such as dynamic perfusion and 3D growth — are required. Here, we show results on a study in which we cultured CompoZr® Zinc Finger Nuclease-modified U2OS osteosarcoma cells under 3D perfusion cell culture conditions. CompoZr Zinc Finger Nuclease (ZFN) technology was used to insert the gene encoding red fluorescent protein (RFP) into U2OS’s genome to report the presence of β-actin (U2OS RFP-ACTB). These cells regularly show an epithelial-like morphology when grown on 2-dimensional (2D) tissue culture plates. However, U2OS RFP-ACTB cells maintained in 3D perfusion bioreactor cell culture conditions showed different cell morphology, enhanced β-actin-RFP expression, cell proliferation, and collagen deposition when compared to U2OS RFP-ACTB growing in 3D static cell culture conditions. Therefore, the 3D perfusion bioreactor is a better platform for in vitro cell culture and cell-based models.

Introduction
Current cell-based assays for drug screening are mostly based on oversimplified cell culture platforms. For instance, both standardized cell lines and primary cells are grown in 2D dishes under static conditions, creating a simplified cellular model without the complexity of target organs or tissue. A recent study that compared a 3D in vitro model, a mouse model, and a traditional 2D model showed the 3D model has a better genotypic correlation to spontaneous tumor formation found in vivo. Besides 3D cell culture conditions, perfusion is another factor of interest for drug screening models. 3D static growth is sometimes not good enough because static cultures deliver suboptimal nutrition/waste exchange conditions that may limit cell growth. There is, therefore, a need to develop a 3D dynamic perfusion cell-based platform for growing optimal cell cultures.
As the first step towards developing an in vivo-relevant, cell-based, 3D perfusion cell culture platform, Sigma® Life Science and 3D Biotek have jointly developed a method to maintain and amplify ZFN-modified U2OS osteosarcoma reporter cell lines. Using ZFN technology, a reporter gene coding for red fluorescent protein (RFP) can be integrated into the locus encoding β-actin, a cytoskeletal protein expressed during cancer progression and metastasis.

The purpose of this study is to show that 3D perfusion cell culture conditions provide an optimal cell culture platform for cell expansion and in vitro disease model creation.

Material and Methods
1. Instrument: 3D Perfusion Bioreactor (Cat. No. Z687502), 3D Biotek, New Jersey, USA.
3. 24-well 3D Insert™ Poly-(ε)-caprolactone µ(PCl) (3030) (300 µm fiber diameter and 300 µm fiber-to-fiber spacing), 3D Biotek, New Jersey, USA.
4. Cell proliferation was determined using the DNA Quantitation Kit, Fluorescent Assay (Cat. No. DNAQF).
5. 3.8% formaldehyde-fixed 3D Insert™ PCL scaffolds were stained using 0.05% Direct Red 80 dissolved in picric acid to detect collagen deposition. Picric Acid (Cat. No. 239801) and Direct Red 80 (Cat. No. 365548).
6. Fluorescent images of adherent U2OS RFP-ACTB were taken with a Nikon Eclipse TiS100 adapted with an epi-illuminator (Lumen 200) and filter chromarhodamine (excitation D540/25x, emission D605/55m), NextDayScience, Inc., Finkburg, MD.
7. Data processing was done using Prisms 4.0 (2005), GraphPad Software, Inc.
8. Cell seeding in 3D Insert PCL scaffolds for static and perfusion cultures: 5 x 10^5 U2OS RFP-ACTB cells in 130 µl were seeded on PCL scaffolds (Figure 1A, 1B) for static and perfusion cultures, respectively. After seeding, the initial 130 µl cell suspension was allowed to infiltrate the scaffold in plates (3D Static) and inside the perfusion chambers (3D Dynamic) for 3 hours in the incubator (37 °C, 5% CO₂ and 95% humidity). After 3 hours, 370 µl of medium were added to the static culture (37 °C, 5% CO₂ and 95% humidity). The bioreactor was set at 2.0 rpm (33 µm/sec) for 24 hours and 2.5 rpm (43 µm/sec) for long-term cell culture in an incubator (37 °C, 5% CO₂ and < 20% humidity).
Results and Discussion

U2OS RFP-ACVB cells cultured as a monolayer on tissue culture plates have a cobblestone-like morphology with significant basal cytoskeletal stress fiber formation (Figure 2A). It has been extensively documented that in vivo-like conditions such as 3D geometry, surface roughness, and medium perfusion can exert significant morphological changes on cells, giving rise to more relevant in vivo characteristics. U2OS RFP-ACVB cells growing under 3D perfusion conditions showed a more spindle-like morphology and recurrent cytoplasmic projections (Figure 2B, 2C, white arrows). In addition, osteosarcoma cells grew away from the PCL fiber surface as adherent cell agglomerates (Figure 2D, white arrows). The complex cell morphologies seen in 3D Perfusion are the direct effect of 3D perfusion culture conditions. Besides cell morphology differences, cells grown in 3D static and 3D perfusion culture conditions showed marked differences in proliferation, β-actin RFP expression, and collagen deposition. Even though cell proliferation plateaued for both 3D static and 3D perfusion at later times, there was a significant difference in cell proliferation as early as day 5. At day 5, the 3D perfusion culture was 2.8-fold higher than the 3D static culture (Figure 3, p < 0.0001). The enhanced proliferation seen in 3D perfusion conditions at days 5 and 10 may be explained as the result of enhanced nutrient/waste transport.

One of the advantages of U2OS RFP-ACVB cells is their adhesion may be monitored using the RFP-tagged β-actin signal in real time. Quantification of the normalized cumulative RFP fluorescence was done in the center of the 3D Insert PCL scaffolds. RFP signal was better in the center of the scaffolds than at the periphery under static culture conditions. However, this was not the case for cells cultured in scaffolds maintained under perfusion conditions, as RFP fluorescence was homogeneous throughout the scaffolds. In terms of RFP expression, there was a statistically significant difference of about 2.7-fold between the 3D perfusion culture and the 3D static culture as early as day 5 (Figure 4, p < 0.0001). Control PCL Insert™ cultured in medium was used to normalize for background noise. The fold difference was calculated from the cumulative fluorescence of 4% of the insert’s area. Thus, the fold differences in β-actin expression between the 3D perfusion culture and 3D static culture could be far greater if the total surface area of the 3D insert were taken into account. Thus, enhanced β-actin RFP may be representative of enhanced cell proliferation and increased adhesion of U2OS towards deposited extracellular matrix (ECM).

Even though osteosarcoma is an aberrant form of normal bone cells, they still secrete collagen5. In this study, it was found that U2OS RFP-ACVB growing in 3D Insert PCL scaffolds under both static and perfusion conditions showed a positive stain for collagen deposition.

Figure 3. Proliferation of Osteosarcoma in 3D Static and Perfusion Conditions. U2OS RFP-ACVB DNA content was measured in scaffolds maintained in both 3D static and 3D perfusion scaffolds. The 3D perfusion scaffolds caused significant differences in cell proliferation as early as day 5 in culture, with a DNA content of 12,760 +/- 217.2 ng (+/- SEM, n = 4), as opposed to 3D static with a value of 4,517 +/- 162.8 ng (+/- SEM, n = 4) unpaired t-test (p < 0.0001). There was no significant difference in proliferation at later times for each cell culture condition.

Figure 4. The Effect of 3D Perfusion on β-Actin RFP Expression. U2OS RFP-ACVB β-actin RFP expression was quantified from fluorescent images of scaffolds maintained under 3D static and 3D perfusion cell culture conditions. The 3D perfusion culture caused significant differences in cell proliferation as early as day 5 in culture, with an RFU value of 163,200 +/- 10,570 RFU (+/- SEM, n = 7), as opposed to 3D static with a value of 62,020 +/- 2450 RFU (+/- SEM, n = 7) unpaired t-Test (p < 0.0001). There was no significant difference in RFP RFU at later times for each cell culture condition. The top inserts are representative fluorescent photographs matched to the average RFU value for their respective cell culture conditions at days 5 and 10.
Figure 5. 3D Perfusion Enhances Cell Adhesion and Collagen Deposition. U2OS RFP-ACTB had differences in collagen matrix deposition. 3D PCL scaffolds were stained with Direct Red 80 to determine the presence of collagen. The stain coverage was complete in scaffolds cultured under 3D perfusion as early as 5 days in culture. However, scaffolds cultured under 3D static conditions had less staining coverage than 3D perfusion. The pattern of collagen deposition correlated visually with the pattern of RFP fluorescence seen in the scaffolds — i.e., at the center for the 3D static culture, and at the center and the periphery for the 3D perfusion culture. (bar = 150 μm; 3D PCL scaffold radius = 7.4 mm).

However, there were striking visual differences in the total area of staining (Figure 5). At day 5, the collagen deposition in the static culture was restricted to the center of the scaffold, correlating with the localized/minimal expression of the β-actin RFP signal. In contrast, 3D-perfusion-cultured U2OS RFP-ACtB showed homogenous collagen distribution throughout the scaffolds, correlating with the homogenous β-actin RFP fluorescence seen throughout the 3D Insert.

Based on the results obtained in the presented study, we concluded that U2OS RFP ACTB osteosarcoma cells grew more efficiently under 3D perfusion cell culture conditions.  

For more information on the 3D Bioreactor, visit sigma.com/3dbioreactor  
For more information on the 3D Scaffolds, visit sigma.com/3dbiotek

References:
Colorectal Cancer

Colorectal cancer (CRC) is one of the most common types of cancer. Each year, approximately one million new cases are detected, and approximately 600,000 deaths can be attributed to this disease worldwide. Today, surgery is the only curative treatment for colorectal cancer, but adjuvant treatment may significantly improve patient survival. For adjuvant treatment to be successful, however, it is important to correctly identify patients who will benefit from treatment. Since adjuvant treatment is recommended for patients with stage III and high-risk stage II disease, it is of utmost importance to find biomarkers that can separate high-risk disease from low-risk disease.

Finding a Biomarker in the Human Protein Atlas

Podocalyxin (PODXL) is a transmembrane protein that is involved in cell-cell interaction. In normal tissue, the protein is expressed in the kidney glomeruli where it may play an anti-adhesive role in the kidney podocytes (Figure 1). The PODXL protein is overexpressed in several types of cancer, e.g., breast, prostate, and testicular cancer.

PODXL was originally identified as a potentially interesting testicular cancer biomarker on the Human Protein Atlas. When further investigated in other selected cancer forms, PODXL was later found to be particularly interesting as a prognostic biomarker in colorectal cancer. High PODXL expression was shown to be an independent predictor of poor prognosis.

Figure 1. Immunohistochemical staining using Anti-PODXL (HPA002110) in normal human kidney shows strong membranous staining of cells in glomeruli on the Human Protein Atlas.
PODXL
The Malmö Diet and Cancer Study (MDCS) is a prospective cohort study designed with the aim of investigating dietary factors that might lead to an increased risk for certain types of cancer. Between 1991 and 1996 there were 28,098 persons enrolled in this ongoing study. By the end of 2008, 626 cases of CRC had been registered. From these CRC cases, a tissue microarray was constructed where PODXL expression could be evaluated based on immunohistochemistry in 536 tumors.

Half of the tumors showed no PODXL staining, 37% showed weak or moderate staining, and 13% showed strong PODXL staining. See Figure 2 for IHC images of tumors showing strong membranous PODXL staining. A clear correlation could be found between high PODXL expression and advanced T-stage, N-stage, M-stage, low differentiation grade, and presence of vascular invasion. There was no association between PODXL expression and age, gender, or tumor location.

A high PODXL expression correlated with a shorter colorectal cancer-specific survival (CCSS) (Figure 3A), indicating that PODXL is a prognostic biomarker for identifying patients with a poor prognosis.

Patients with tumors expressing high levels of PODXL who were treated with adjuvant chemotherapy (CT) had similar survival to patients with PODXL-low tumors. Untreated patients with PODXL-high tumors had shorter survival than all the other patient groups (Figure 3B). These results suggest patients with tumors expressing high levels of PODXL would benefit from adjuvant chemotherapy.

Summary
• There is a great need today for novel biomarkers capable of distinguishing between different types, stages, and forms of colorectal cancer.
• Potential cancer biomarkers can be identified on the Human Protein Atlas web portal (proteinatlas.org).
• By the use of Prestige Antibodies®, PODXL was found to be a prognostic biomarker in colorectal cancer, with high PODXL expression being an independent predictor of poor prognosis.
• Analyzing PODXL expression could stratify patients into those that should receive chemotherapy and those that may be spared adjuvant treatment.

Discover more at sigma.com/prestige

Figure 2. Immunohistochemical staining (using HPA002110) of PODXL protein in colorectal tumors with an increasing proportion of cells showing strong membranous staining in the tumor samples from left to right.

Figure 3. A) Kaplan-Meier analysis of colorectal cancer-specific survival of patients with tumors expressing no, weak/moderate, or high levels of PODXL protein. B) Kaplan-Meier analysis of colorectal cancer-specific survival, where patients were divided into groups according to whether they received adjuvant chemotherapy (CT) or not, as well as the level of tumor PODXL expression (high or low).

The Human Protein Atlas
The Human Protein Atlas is a public web portal managed by an academic project that aims to map the human proteome in a period of 10 years. Today, more than 700 IHC, WB, and IF images are presented for each of 15,600 antibodies against human targets, covering more than 60% of the human proteome.

(see references at PRESTIGE on page 34)
Cell culture has been traditionally conducted on flat plastic substrates to study cell and tissue biology. These two-dimensional (2D) substrates are inadequate in fully capturing the complexities and intricacies presented to the cells in vivo. The ability to transition from these flat surfaces to textured substrates that provide three-dimensional (3D) environments has long been an unmet need of researchers around the world. However, it is now possible to conduct more accurate experiments and obtain results that closely shadow what is observed in a living organism through the nanofiber-coated multiwell plates developed by Nanofiber Solutions (Figure 1). These well plates not only mimic the complex topography of the extracellular matrix (ECM) found in vivo, but they also allow for live cell imaging (Figure 1) and easy post-process analysis.

The significance of this cell culture innovation cannot be fully grasped unless some recent research comparing 2D culture and 3D culture has been examined. Recent cancer studies done by Abbott’s group have revealed breast tumor cells cultured in 3D that have become malignant can be made to return to their original state when an antibody against β-integrin is added to the system. Furthermore, Yamada’s group discovered fibroblasts cultured in 3D had high motility, divided at a faster rate than those cultured in 2D, and assumed an asymmetric shape that is characteristic of fibroblasts found in living tissue. These differences in cell structure and behavior, which may seem insignificant on first glance, can actually have a tremendous impact on basic cell biology research, and the discovery and development of medical treatments and drugs. For instance, Wang et al. discovered both normal and malignant breast cancer cells had high expression of adenoviral receptors in 2D culture, while only malignant cells carried a large number of receptors in 3D culture. The implications of the study are groundbreaking because the results from the study opened the gates to using gene therapy for cancer treatment. If simply changing the way the cells are cultured can have such an impact on the different options we pursue to treat a disease, one is left to wonder: How many viable avenues for treating a disease have we foreclosed already due to misleading results obtained from 2D cell culture? This type of predicament can be entirely avoided in the future with the use of nanofiber substrates, which are not only ideal for 3D cell culture, but also amenable to customization according to the needs of particular cell types or research areas. So far, nanofiber substrates have been successfully adopted and used for general cell culture, drug discovery and toxicity assays, stem cell expansion and controlled differentiation, production of monoclonal antibodies, and tissue engineering.

With respect to general cell culture, nanofiber plates provide a far more realistic representation of the native tissue than any other substrate type. Images provided in Figure 2 attest to the nanofibrous environment found in vivo and the close resemblance of synthetic nanofibers. As
one example, the nanofibrous texture of the lung ECM is very closely mimicked in size, shape, and appearance by the nanofiber scaffolds, while tissue culture polystyrene (TCP) has no resemblance whatsoever.

An advantage of using nanofiber substrates is they can also be produced in a highly aligned orientation. The orientation of the culture substrate plays an important role in the study of cell behavior whose native environments consist of highly aligned ECM such as white matter in the brain, central nervous system, skeletal muscle, and cardiac tissue. For instance, human mesenchymal stem cells (hMSCs) were observed to assume a compact morphology on randomly oriented nanofibers and an elongated morphology on aligned fibers, which helped them differentiate into functional cardiomyocytes (Figure 1). In Figure 3, human lung cancer cells adopt three very distinct morphologies when cultured on TCPs, randomly oriented nanofibers, and aligned nanofibers. The different cellular morphologies greatly influence the cellular behavior, which is why it is so critical to culture cells in an environment that can accurately reproduce the 3D nanofibrous environment found in vivo.

The nanofiber scaffolds are also very useful in drug discovery and toxicity assays because assays conducted on these scaffolds reveal drug sensitivities that often go undetected in other in vitro models. For example, glioblastoma cells are highly invasive in vivo and their migration can easily be quantified on the nanofiber substrates. The cell cultures revealed dispersion of glioma cells was significantly inhibited by STAT3 inhibitors on both the nanofiber scaffolds and brain slices (the current gold standard for glioma migration), but not on 2D surfaces using traditional "scratch assays". These results clearly demonstrate the inadequacies of existing culture methods and a potential new high-throughput in vitro drug discovery technology.

Nanofiber scaffolds have the potential to pave the way for tremendous scientific discoveries by not only providing in vitro 3D culture models, but also by facilitating regenerative medicine and the synthesis of artificial organs. Since the body naturally consists of a nanofibrous environment (Figure 2), the synthetic nanofiber scaffold structure lends itself well to the tissue engineering of hollow organs such as blood vessels, trachea, esophagus, intestines, etc. In fact, the first synthetic nanofiber human trachea was implanted in November 2011 in Sweden. The patient, from Maryland, was a 30-year-old man with a rare form of tracheal cancer that required the removal of his entire trachea. The removed organ was replaced with a nanofiber trachea that was infused with the patient’s bone marrow stem cells. Figure 4 shows the artificial trachea after fabrication and then just before it was implanted.

As illustrated in the many previous examples, nanofiber scaffolds are the future of scientific research and innovation. Not only do they provide realistic in vitro models for cell culture, but they also function as viable materials for tissue engineering and regenerative medicine. Nanofiber products are changing the way life science research is performed; they are the future of patient care throughout the world.

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Figure 2. A) Decellurized Mouse Lung; B) Tissue Culture Polystyrene (TCPS); C) Nanofiber Scaffold.

Figure 3. Row A) Shows Cells on TCPS, Row B) Shows Cells on Randomly Oriented Nanofibers, Row C) Cells on Aligned Nanofibers. The first column shows scanning electron microscope images. The second column is stained for vinculin (red), tubulin (green), and the nucleus (blue). The third column is stained for phosphorylated paxillin (red), actin (green), and the nucleus (blue). All fluorescence images were taken with a confocal microscope and imaged through the nanofiber scaffold demonstrating the ability for high-resolution microscopy.

Figure 4. Synthetic Nanofiber Trachea after Fabrication at the Nanofiber Solutions Facility in Columbus, OH, and then after Being Perfused with the Patients’ Stem Cells Immediately before Implantation at the Karolinska Institute.

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