Stem cells in culture: defining the substrate

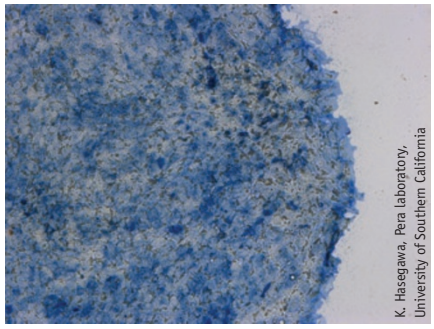
Monya Baker

Efforts to improve stem cell culture are shifting to the surface.

Pluripotent stem cells (PSCs) are expected to perform. For other kinds of 'cultured' cells, the main requirement is robust growth and, perhaps, protein production. PSCs are supposed to grow indefinitely and differentiate as desired, all while maintaining an unaltered genome. When PSCs had been first derived, researchers searching for suitable culture conditions supplied all sorts of undefined biological substances: mouse feeder cells, cow serum and several kinds of supplements. Consequently, standard conditions for deriving, maintaining and differentiating PSCs are inherently variable.

Though companies began selling more consistent, 'defined' media in 2007, most culture systems still require undefined ingredients. There are several reasons why researchers want even more defined conditions. Those hoping to develop cell therapies need to follow strict guidelines from regulatory authorities and usually want to avoid all non-human animal extracts. Researchers who have no intention of making clinical products still want to avoid batch-to-batch variations in biological materials. Furthermore, unraveling influences on a signaling pathway is difficult if unknown components of the culture are affecting it.

Glyn Stacey, head of the UK Stem Cell Bank, lists the research community's shift toward serum-free media as one of the most important improvements to stem cell culture in recent years, but plenty of undefined components other than serum are still routinely used. Most researchers still grow PSCs with 'feeder cells' or in 'conditioned medium' that contains secretions from feeder cells but not the cells themselves. There is considerable room for improvement, says Martin Pera, who directs the stem cell research center at the University of Southern California. "Ultimately you want defined culture systems. You know what's in them, and you don't have any unknown factors that are interfering with your experiments."



Human ESC colonies can be cultured under defined conditions. Here, stained for alkaline phosphatase, a marker for undifferentiated cells.

Increasing definition

PSCs usually grow best when attached to other cells or an extracellular matrix. Both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are traditionally grown in cultures with feeder layers, typically mouse embryonic fibroblasts that have been irradiated or chemically treated so that they can no longer divide. These cells secrete a complex matrix of proteins, which provide cues that keep stem cells healthy.

As PSCs proliferate, they are 'passaged' onto fresh plates with new feeder layers. Feeder cell cultures themselves last only a few months, can vary with every mouse strain, even each dissection, and markedly alter PSCs grown on them, says Lei Bu at the Harvard Stem Cell Institute. Feeder cells derived from human tissues are also used, particularly for developing clinical products, but they are more expensive, harder to maintain and just as variable. There are more serious issues as well. "It's quite common with feeder layers for the odd cell to survive and creep through," says John Masters at the University College London. "It only takes one contaminating cell; within a few passages it can take over a line."

Instead of feeder cells, researchers often use a gelatinous extracellular extract produced by tumors propagated in mice; this is now sold commercially as Matrigel by BD Biosciences and as Geltrex by Life Technologies. Although they are considerably less variable than feeder layers themselves, such extracts still contain unknown components, and researchers often pretest several batches to find one suitable for their cells. The extract is often combined with medium conditioned by feeder cells or with defined medium containing purified proteins and other supplements, and sold by vendors including Life Technologies, STEMCELL Technologies and Stemgent. (A related and somewhat overlapping group of products are 'xeno-free' reagents, which contain no non-human animal products but are not necessarily defined.)

In 2010, the International Stem Cell Initiative Consortium published the results of a multilaboratory effort to test the growth of ten human ESC lines in seven defined culture systems plus a control system including a feeder layer[1]. Only the control system and two of the defined systems (StemPro from Life Technologies and mTeSR1 from STEMCELL Technologies) could maintain most stem cells lines through the length of the study. More defined media have been described since that study began, and product offerings in that area are considered relatively mature. Interest in fully defined culture conditions is markedly increasing, says David Welch, a senior market development manager at Life Technologies. "Initially, we saw customers having the most interest in moving toward the clinic, but we've seen that expand over the past year or so to large academic centers that are looking for more reproducible conditions and also from

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Larry Couture at City of Hope urges establishing consistent, well-characterized reagents over removing animal products.

industrial customers wanting to use iPSCs for screening programs."

With defined media commercially available, the focus is now on defining the substrates or surfaces that cells grow on. Doing so could make experiments more consistent over time and across labs. In the past 12 months, vendors have launched an array of more-defined substrate products, and a stream of publications have described growing PSCs on defined substrates. "This is what we're waiting for," says Tenneille Ludwig, who directs media optimization at the WiCell Research Institute. "This is the last piece of the holy grail." However, it is too early to say that salvation has arrived. "Whether a new surface works across a variety of cell lines and applications has to be borne out in time," says Ludwig. Even if a surface performs well, she adds, it may not be affordable.

Surface jobs

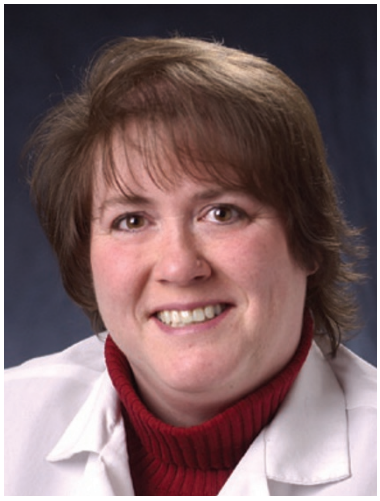
Defined surfaces fall into three broad classes: synthetic coatings, synthetic biomolecules and recombinant proteins. Each has its own advantages and disadvantages: not all can be sterilized or stored easily. Synthetic coatings are often the cheapest to produce, but designing specific biological interactions is more difficult. Recombinant proteins are considerably more expensive, but cell-surface interactions are easier to understand and engineer. Synthetic biomolecules are a sort of middle ground. They can mimic key components of cell-adhesion proteins but manufacturing them and getting them onto a surface can be difficult.

One challenge is that even if the surface is defined, the effects on the cells are not (**Box 1**). To make matters more confusing, PSC lines that thrive on one surface may not grow on another. Often cells require the addition of supplements, such as bovine serum albumin or small-molecule inhibitors to survive on a specific substrate. Some surfaces support maintenance of cells but not their propagation or differentiation; perhaps the toughest hurdle is deriving ESC and iPSC lines on a particular surface.

In contrast, feeder layers and undefined extracts support a wide variety of cell lines. "[Although Matrigel] is a complex material, inherent in that complexity are substances that enable cells to function appropriately," says Marshall Kosovsky, technical support manager at BD Biosciences. "Reproducing everything [a cell needs in its environment] by simply defining a substrate may not always work out," he says.

Researchers who switch to defined conditions for maintaining cells often need to optimize recipes for differentiating them. If defined surfaces can be used to derive and scale-up PSC lines and can be made cheaply enough, worries over shifting PSCs to defined surfaces for differentiation could become a non-issue. "If you've done everything in a defined system from the beginning, you don't have to redefine all your protocols," says Zara Melkoumian, a scientist at Corning Life Sciences who helped develop a new defined substrate called Synthemax.

Several companies are responding to the push for more-defined substrates. BD Biosciences sells versions of Matrigel with reduced amounts of growth factors as well as a partially purified version called BD



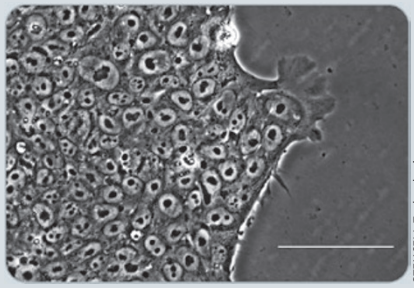
Tenneille Ludwig of the WiCell Research Institute says designing better culture conditions is important, but technicians matter just as much for the stability of cells.

BOX 1 ASSESSING CULTURE EFFECTS

It is easy to tell whether cells grow in new culture conditions; it is much harder to tell whether they change. "We still don't really understand how culture conditions affect the genetic stability of ESCs and iPSCs," says Martin Pera, who directs the stem cell research center at the University of Southern California. "The genetic changes, if they are going to come, pop out at 15 or 20 passages. It's not a situation where you grow them for a week and you know everything is fine."

Too few researchers check even for gross changes such as chromosome number. More subtle genetic and epigenetic changes are much more difficult to detect but can still affect behaviors such as whether a stem cell will differentiate, die or become tumorigenic. The routinely used pluripotency markers are poor indicators of cells' states, says Clive Glover, head of product management at STEMCELL Technologies. "We see morphology change long before we detect a difference in these cell-surface markers." Recently published techniques using genome-wide gene expression may help, but so far these approaches have been used to assess cell lines rather than culture conditions[8,9].

Small-molecule inhibitors of various signaling pathways can help keep cells alive and retain a pluripotent state. Combinations of small molecules that inhibit differentiation allow mouse ESCs to grow without an otherwise crucial protein supplement and have been used to produce ESCs from rats and a variety of mouse strains[10,11]. More recently, Hong Wu and colleagues at the University of California, Los Angeles used a feedback system borrowed from engineering to identify a cocktail of three small-molecule inhibitors that could, even in defined culture conditions, allow researchers to passage human ESCs as single cells rather than as clumps[12]. They also showed that the cells maintained this way have normal karyotypes and give rise to teratomas consisting of the three germ layers. But as it is unclear how tweaking signaling pathways might change the cells, stem cell scientists are anxious about adding small-molecule inhibitors to their cultures, says Larry Couture, senior vice president of the Center for Applied Technology Development at the research hospital City of Hope in Duarte, California, USA. "We all want less apoptosis, but we [add inhibitors] with a certain amount of angst," he says.



STEMCELL Technologies

Human ESCs grown under defined conditions. Scale bar, 100 μm.

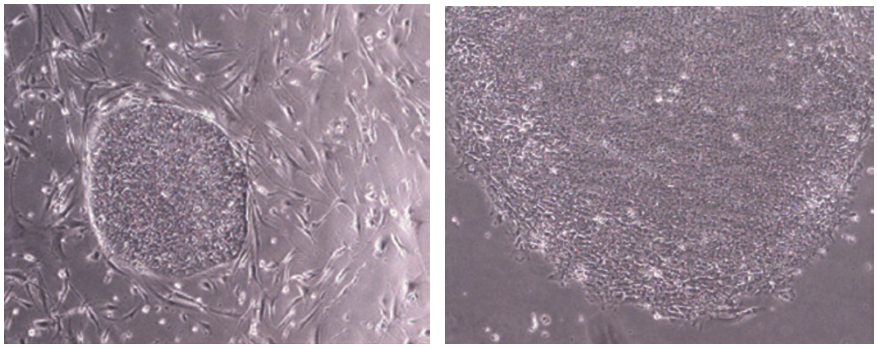
Cells themselves secrete unknown components into the medium, says Mick Bhatia, who directs the stem cell institute at McMaster University. "You can have different conditions with the same cells, and after 12 hours, the medium is completely different." To make matters even more complicated, PSCs in a culture are much more heterogeneous, genetically, epigenetically and functionally, than most people realize. "What mostly people report and look for are markers associated with pluripotency, but that only measures the small fraction of cells that are likely to be self-renewing," he says. Worse, he suspects that optimizing culture systems for pluripotency markers could result in cells that resist differentiation. Finding the best markers to assess stem cell quality, he says, will depend on desired endpoints.

Laminin/Entactin. Last October Thermo Fisher launched a product, Nunclon Vita, that uses a modified polystyrene plastic on which cells can be placed directly. Chemical groups added to the plastic provide a hydrophilic surface that allows for the accumulation of glycoproteins and other components, a sort of *in situ* assembly of an extracellular matrix, explains Tom Cummins, director of research and development at the company. With the addition of a small molecule known to boost cell survival, the human ESC line H1 can grow without a feeder layer or additional substrate for a dozen or so passages in conditioned medium. The company is currently developing media formulations to accompany the surface and testing the system on more cells, says technical field scientist Cindy Neely. "We're striving for simple reproducible systems that will apply for the vast majority of cells."

Gary Smith, Joerg Lahann and colleagues are developing synthetic polymers at the University of Michigan–Ann Arbor. After screening several potential polymers, they identified one with the ungainly name of poly(2-(methacryloyloxy)ethyl dimethyl-3-sulfopropyl) ammonium hydroxide (PMEDSAH) on which stem cell lines could attach and proliferate[2]. Two human ESC lines grew well for 15 or more passages in medium conditioned by human cells, and one did so in defined medium. Research to improve the surface can address a "multitude of questions," says Smith. Rigidity, composition and patterns can all be readily altered on an artificial surface, and varying conditions can be used to understand cells' growth requirements and interactions with their microenvironments.

Another approach is coating surfaces with recombinant proteins known to support ESCs. In 2008, Life Technologies launched a substrate known as CELLstart, which is made of a few (undisclosed) human recombinant proteins and is part of the Cell Therapy Systems brand, which provides increased documentation including 'traceability' through the

Martin Pera at the University of Southern California says researchers are still trying to understand how culture conditions change cells.



BD Biosciences

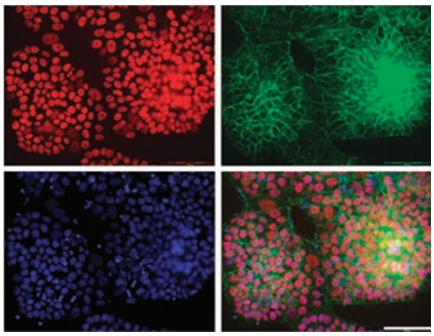
Phase-contrast images of human ESCs grown on mouse embryonic fibroblast feeder layer (left) and on BD Matrigel Matrix using mouse embryonic fibroblast–conditioned medium (right).

manufacturing process to support development of clinical products.

These proteins are complex, containing several subdomains as well as post-translational modifications. In the summer of 2010, researchers at the Karolinska Institute described a substrate made from a recombinant version of one of the proteins of the laminin family[3], which are the first matrix proteins expressed in the embryo and one of the main components of Matrigel. The team showed that three human ESC lines and two iPSC lines could be cultured in defined medium. Other researchers at the Tokyo Institute of Technology and Medical College of Wisconsin reported growing human ESCs and iPSCs in defined media on plates coated with the cell-adhesion protein E-cadherin fused to an antibody fragment, specifically, the immunoglobulin gamma Fc domain[4].

Surfaces can also be coated with biomolecules considerably smaller than full proteins. One example is Synthemax, recently commercialized by Corning Life Sciences. The substrate is made of peptides that recapitulate the binding domains of known matrix proteins[5]. It is available on six-well plates, and flasks can be stored at room temperature for as long as two years, explains Melkoumian. It is sold as a highly documented 'therapeutic grade' substrate for developing cell therapies and as a less costly research grade; the price of the latter, she says, is roughly equivalent to that of plates coated with mouse feeder cells, if the labor expenses of dealing with feeder cells are considered. So far the surface has been tested on a handful of human ESC lines for up to 20 passages, and tests on iPSCs are underway.

Another peptide-inspired approach has been described by Laura Kiessling at the University of Wisconsin, Madison and colleagues. Using particles that self-assemble on gold, they screened over 500 combinations of peptides that had been reported to interact with cell-surface receptors and



Human ESCs adhere and grow on peptide-displaying surfaces (reprinted from ref. 6). Scale bar, 200 μm.

identified two that allowed a variety of human ESC and iPSC lines to grow without a feeder layer in defined medium[6]. What is more, says Kiessling, analyses of cell-surface markers and gene expression showed that cells grown on defined surfaces behaved more uniformly than those grown on Matrigel. Another advantage is that peptides can be derivatized (for example, with the small molecule biotin, which binds very tightly to the protein streptavidin), and this means that researchers should be able to make their own defined surfaces. "The good thing from our point of view is that it seems like people could just try it," says Kiessling. "You can just take your streptavidin-coated surface, put your biotinylated peptides on it and grow the cells."

The most radical approach is getting rid of substrate coating all together. After observing neural progenitor cells maintained as floating spheres in culture, Benjamin Reubinoff and colleagues at Hadassah-Hebrew University Medical Center adapted the technique and managed to derive and maintain human ESCs as suspension cultures[7]. They supplemented neurobasal medium with KnockOut Serum Replacement (from Life Technologies) along with growth factors and proteins commonly found in extracellular matrix and could grow the cells for several passages, albeit at a slower expansion rate than observed on feeder layers. Though some researchers are suspicious of changes that occur as cells adapt to suspension culture, Larry Couture, director of the Center for Applied Technology Development at the research hospital City of Hope in Duarte, California, USA is evaluating the technique in his facility and is enthusiastic about its potential for making large quantities of cells. "We can differentiate cells right out of suspension culture, and the epigenetic profile is very similar to [cells grown on] Matrigel," he says.

Couture hopes to make large volumes of cells for clinical products; his goal is to make consistent batches of specialized cells, not to tease apart signaling pathways. A hard-core basic researcher will be extremely cautious about moving cells from one substrate to another, he says. "We can show that there are epigenetic shifts as cells grow on different substrates, and they don't always shift back."

That is not necessarily a drawback, however: many scientists believe that the use of different substrates may be an ideal way to promote differentiation into specific lineages. After a given amount of propagation, cells could be transferred to a series of plates that coax cells into ever more specific categories of tissue. Surfaces offer considerably more control over cells' microenvironments and so could better mimic the niches in which stem cells differentiate. "We have to stop thinking about the medium and the surface as two separate entities. We have to think about the whole environment of the cells," says Clive Glover, head of product management at STEMCELL Technologies. Manipulating medium is easier, he says, but "in the next 2–3 years, it's really going to be surfaces that are pulling us ahead."

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Monya Baker is technology editor for *Nature* and *Nature Methods* (m.baker@us.nature.com).

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In the version of this article initially published, a reference was incomplete. The error has been corrected in the HTML and PDF versions of the article.

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In the version of this article initially published, the image descriptions were swapped. The error has been corrected in the HTML and PDF versions of the article.