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Stem Cells and Drug Discovery: The Beginning of a New Era?

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Much of the attention focused on stem cells relates to their use in cell replacement therapy; however, stem cells may also transform the way in which therapeutics are discovered and validated.

Introduction

The attrition rate of drugs is staggeringly high: more than 90% of the drugs tested in clinical trials fail to be approved. This places a significant burden on the health care system. Famously, compounds fail late in clinical testing or even after approval either because of a lack of sufficient efficacy or unanticipated toxicity; many more compounds fail early in the discovery process. How much of this inefficient and expensive pharmaceutical pipeline problem can be rectified by having disease models that will more faithfully represent the actual human diseases so that underlying mechanisms can be better understood and effective and safe medicines derived and validated? In principle, human embryonic stem (ES) cells can be used for just that purpose. ES cells can be derived from patients with specific diseases and protocols can be established to direct the disease-specific ES cells to become the very types of cells affected in the disease. Such disease-relevant cells should be able to drive more predictive drug discovery and toxicity studies.

Reprogramming (Dedifferentiation) Screens

There are many ways in which human ES cells or the progenitor cells generated from them can be used in drug screening (Figure 1). The first step is isolation of ES cells, which is straightforward in the case of the mouse but has been difficult and controversial for human cells. Viable techniques for deriving human ES cells include somatic cell nuclear transfer from human skin biopsies and using embryos from preimplantation genetic diagnosis. However, the recent creation of induced pluripotent stem (iPS) cells has made the production of human ES cells much simpler (Takahashi et al., 2007; see Review by R. Jaenisch and R. Young in this issue), and it is now seemingly quite feasible to make ES cell lines from large numbers of patients with particular diseases. From a screening perspective, lingering concerns over the use of oncogenes and viral vectors to produce the iPS cells will undoubtedly mean that replacement factors will be sought, either small molecules or proteins that can fully or partially reprogram differentiated human cells.

There is a considerable body of research on dedifferentiation (which can be viewed as partial reprogramming of adult cells) primarily in urodele amphibians, which have the ability to regenerate limbs through blastema formation at the site of injury (K.D. Birnbaum and A. Sánchez Alvarado in this issue of *Cell*). This occurs, in part, by cellularization, that is, the fission of multinucleated myotubes into multipotential mononucleated cells (Duckmanton et al., 2005). Understanding dedifferentiation mechanisms in amphibians might help in designing screens to reprogram adult mammalian cells. For example, screening a library containing tens of thousands of diverse compounds culminated in the identification of a microtubule-disrupting molecule, now known as myoseverin (Rosania et al., 2000). This molecule causes mouse C2C12 myotubes to fragment into single cells that apparently continue to retain some of the differentiated properties of the parent cells, such as the expression of myosin heavy chain (Duckmanton et al., 2005). A second round of screening yielded another compound, called reversine, which is able to reprogram the myoblasts to become more like mesenchymal stem cells with the ability to forward differentiate into bone and adipose

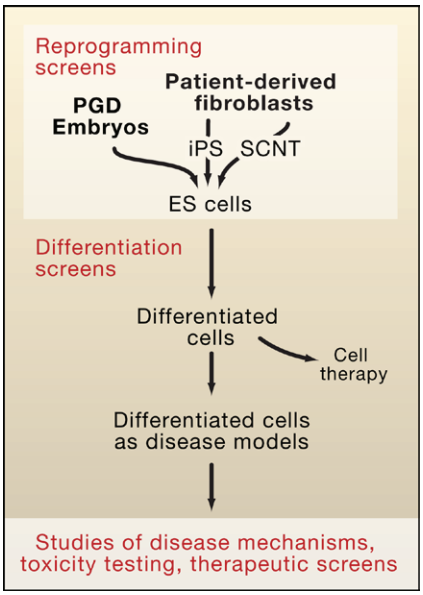

Figure 1. Stem Cell-Based Screening Assays
The goal is to create differentiated cells affected in various diseases from human ES cells and to carry out predictive toxicity testing and therapeutics research in a culture dish. The first step requires isolating disease-specific ES cell lines from patients by reprogramming (dedifferentiating) adult somatic cells using somatic cell nuclear transfer (SCNT), or with a cocktail of transcription factors to produce ES cell-like cells called iPS cells (Takahashi et al., 2007). It will also be of interest to identify other proteins or small molecules that drive reprogramming of adult somatic cells. In the case of SMA and Huntington's disease, disease-specific ES cells could be obtained from human embryos used for preimplantation genetic diagnosis (PGD). Human ES cells then need to be differentiated in culture into cell types that are affected in the disease of interest (for example, nigral DA neurons for Parkinson's disease or medium spiny neurons for Huntington's disease) or that are relevant for toxicity testing (cardiac cells and hepatocytes). Driving ES cell differentiation will also be required to produce cells that can be used therapeutically in cell replacement therapy (for example, pancreatic β cells for treating type I diabetes). The differentiation screens themselves may also produce therapeutic candidates that modulate pathways involved in disease (Hedgehog, Wnt, BMP, etc.) or compounds that modulate cell proliferation.

cells. Interestingly, additional chemical modification and affinity chromatography with immobilized compounds suggested that reversine might have more than one essential intracellular target, including the structurally dissimilar MEK1 kinase and nonmuscle myosin II heavy chain (Chen et al., 2007). A self-renewal screen carried out with mouse ES cells by the same lab produced a hit, SC1, which also seemed to have the unusual property of binding to two structurally different intracellular targets (Chen et al., 2006). It will be important to understand whether the success of these screens actually depends on having library compounds that are not absolutely specific for single targets.

Differentiation Screens

Directed Differentiation

Dedifferentiation or reprogramming screens start with differentiated cells and attempt to identify factors that make them less differentiated. However, one of the greatest challenges in creating large numbers of different kinds of disease-relevant cells is controlling differentiation, that is, inducing stem or progenitor cells to become more differentiated. The stem cell literature has many descriptions of "home run" experiments in which ES cells were treated with a concoction of factors, with the hope that large numbers of cells would undergo multiple steps of differentiation to produce the desired end product. Recently, research has focused on the more rational approach of inducing cells to undergo sequential stages of differentiation in a tissue culture environment, thereby recapitulating aspects of normal embryonic development (see Review by C.E. Murry and G. Keller in this issue). For example, working on mouse spinal cord development, Tom Jessell's group and others (reviewed in Briscoe and Ericson, 2001) showed that specification of neural cells during development was achieved in stages by controlling the expression of particular sets of transcription factors in response to precise gradients of extracellular factors. Each type of spinal cord progenitor cell that appeared was defined by a particular set of transcription factors. Wichterle et al. (2002) managed to successfully reproduce these observations in cell culture. They used mouse ES cells, expressing green fluorescent protein (GFP) under the control of a motor neuron-specific promoter, and grew them as embryoid bodies (EBs). When the EBs were treated with two factors known to be important in motor neuron development, first retinoic acid and then with an activator of sonic Hedgehog signaling (Frank-Kamenetsky et al., 2002), functional motor neurons that expressed GFP appeared.

Numerous groups have tried to carry out equivalent studies, starting with ES cells or progenitor cells. For example, the laboratories of McKay (Lee et al., 2000), Sasai (Watanabe et al., 2005), and Studer (Perrier et al., 2004) have used biologically relevant combinations of soluble factors or cell lines producing those factors with reasonable success. However, this systematic approach has not always worked. Andersson et al. (2006) were unable to derive midbrain dopaminergic (DA) neurons from mouse ES cells although they essentially followed the Wichterle et al. protocol (modified to include FGF8, an essential inducer of differentiation along the DA neuronal lineage). Importantly, they were able to produce these cells by transiently expressing the homeobox transcription factor Lmx1a in mouse ES cells and then adding the relevant extracellular factors.

The key issue is how to find a general approach for directing ES cell differentiation. Certainly, it makes sense to reproduce the steps in the process of making a fully differentiated cell, with each step along the differentiation pathway achieved using a separate chemical screen. So, for example, mouse ES cells could be differentiated into DA neurons by biasing them first toward a neural progenitor fate ($Sox2^+$), then early DA neural progenitor ($Raldh^+$, $Otx2^+$), followed by late DA neuron progenitor ($Lmx1a^+$, $Nurr1^+$), and finally DA neuron (TH^+, $AADC^+$). Or, ES cells could be directed to become pancreatic β cells with initial endodermal induction ($Sox17^+$), then early pancreas ($Pdx\text{-}1^+$), pancreatic endocrine ($Ngn3^+$), and then mature β cells secreting insulin ($insulin^+$). What is required is knowledge of the set of transcription factors or other cell-specific markers for each stage of development and a way of identifying molecules that will stimulate each step of the differentiation process.

Directed Differentiation with Small Molecules

This type of sequential differentiation model has not yet been carried out from beginning to end. In other words, no one has yet used a series of screens to identify sets of small molecules (for example) that will generate fully differentiated cells from ES cells with high efficiency. However, various labs have identified factors that stimulate at least partial differentiation. For example, Ding et al. (2003) used mouse P19 embryonal carcinoma cells expressing tubulin α1-luciferase as a reporter of neural differentiation to screen a large library of diverse small molecules. There was one hit, a pyrrolopyrimidine, and analogs of this molecule were prepared; a more active compound, TSW119, was identified and an effective affinity ligand synthesized. This culminated in the identification of GSK-3β as a specific binding partner for this class of neuron-inducing molecule. Of course, it could easily be the case that TSW119 inhibits other kinases and it is those other kinases, or perhaps GSK-3β in combination with one or more other kinases, that regulate the appearance of neural cells. However, given that GSK-3β is a well-known negative regulator of Wnt signaling, this result raises the possibility that the Wnt pathway, which is one (but not the only) regulator of GSK-3β activity, controls neural differentiation. Several studies have implicated the Wnt signaling pathway in aspects of self-renewal and differentiation, not always with consistent results (for instance, see Sato et al., 2004).

A number of conceptually similar screens have been prosecuted. For example, a diverse library of 100,000 compounds was screened against P19 embryonal carcinoma cells expressing luciferase downstream of the atrial natriuretic factor promoter to look for inducers of cardiac myocytes (Wu et al., 2004a). A diaminopyrimidine, cardiogenol, scored in this screen and was shown to induce expression of multiple markers of the cardiac lineage. The target of this small molecule has not yet been identified. A bone differentiation screen of a small molecule diversity library looked for increased alkaline phosphatase activity in multipotent 10T1/2 fibroblasts. This was followed by chemistry to produce analogs of the hits in the screen, yielding a molecule termed purmorphamine

(Wu et al., 2002) that was shown subsequently to be a Hedgehog pathway agonist (Wu et al., 2004b).

These screens all involve fairly large-scale testing of small molecule diversity libraries and use enzyme reporter readouts. The compound collections resemble those commonly used by pharmaceutical companies. Although these and similar studies have provided useful reagents and observations, hits from these compound screens often do not provide immediate information relating to compound target or mechanism of action, and so follow-up chemistry is required. This presents a problem for many investigators who have access neither to high-quality diversity sets nor to the type of chemistry expertise that may be needed to pursue hits from the primary screens.

Other types of compound libraries can be considered for finding molecules that induce cell differentiation. For example, many academic labs have taken to screening so-called "bioactive" collections comprised mainly of known drugs and pharmacologically active agents. These small sets of 1500–5000 compounds have become widely used for several reasons. One is the relatively new interest in repurposing drugs (finding new uses for old drugs) among academic groups interested in translational research because this can accelerate the identification of therapeutics for orphan diseases (see, for example, North et al., 2007). However, there are other advantages to using these libraries for differentiation screens. The collections are readily available and, under favorable circumstances, hits may provide useful reagents for inducing cell differentiation in the laboratory without the need for further chemistry. In addition, classes of molecules that induce a specific differentiation step may provide mechanistic information. For example, if a number of different calcium channel blockers score in a particular cell differentiation assay, this would suggest several avenues of additional experimentation. Saxe et al. (2007) screened bioactives in a neurogenesis assay, starting with primary neurospheres prepared from embryonic rat brain and using a chemiluminescence assay to measure compounds that increased the total amount of βIII tubulin, a neuronal marker. One hit was the orphan ligand L-serine-O-phosphate, which may stimulate differentiation by binding to metabotropic glutamate receptors. Diamandis et al. (2007) tested a somewhat different small collection of bioactives searching for inhibitors of neural stem cell proliferation, which they detected, again indirectly, using a standard MTT assay. These investigators observed that agonists and antagonists of several different neurotransmitter receptors were antiproliferative agents (and, interestingly, exerted similar antimitotic effects on brain tumor stem cells).

Another set of "perturbagens" worthy of consideration for differentiation assays include morphogens or other protein inducers and substrate- or cell-associated factors. Embryonic development is regulated largely by a small number of ligand-activated signaling pathways, including those of TGF-β/BMP, FGF, Wnt, Notch, and Hedgehog. Investigators developing small-scale (nonrobotic) differentiation protocols usually test several of these protein factors, whereas those designing robotic screening protocols routinely focus on small molecules and not on proteins. This is curious because, although the number of active signaling pathways may turn out to be small, the embryonic signaling environment is defined by having each relevant ligand present at the correct time, in the proper amount, and in appropriate combinations with other factors in order to have cell fate accurately specified. It may be that dialing in the correct concentrations and combinations of these ligands could increase the efficiency of cell differentiation substantially. Such a problem could ideally be solved by robotic screening, which can test suitably large combinations of these factors.

Similarly, several investigators have explored growing undifferentiated cells on small numbers of inducer or feeder cell lines or on extracellular matrices to good effect, but only recently has attention been drawn to the advantages of combinatorial testing of substrate-associated and soluble factors. Flaim et al. (2005) measured the effects of combinations of extracellular matrices (in this case, all in the same cell culture medium) on the ability of mouse ES cells to differentiate toward hepatocytes and found some striking differences among the various combinations. Soen et al. (2006) looked at microarrays of human primary neural precursor cells grown on substrates containing various combinations of matrix components with bound morphogens. They also found significant variation in the biasing of cells toward neuronal or glial lineages under the different conditions. Thus, it seems an excellent idea to comprehensively probe these types of protein factors along with some of the small molecules in sequential differentiation assays. Other types of assays such as siRNA assays or screening ORFeome libraries will become more popular once suitable reagents become available, but the usefulness of screening these types of libraries has already been demonstrated (Pritsker et al., 2006; Ivanova et al., 2006).

Another item worthy of discussion concerns the way in which cell differentiation is measured. Most differentiation assays conducted to date have been population studies in that they measured the total amount of luciferase activity or of individual protein markers in each test well. In a sense, these studies are conceptually similar to the industry standard high-throughput assays, which are often conducted in cell-free form (e.g., enzyme assays) but are meant to accommodate the large number of assays and impressive number of compounds that are generally tested in pharmaceutical and biotechnology companies. A different approach, gaining in popularity in both academia and industry, is high content analysis or microscope-based analysis. This kind of assay, which can be automated, provides information concerning multiple properties of individual cells and is well suited to studies of differentiation where the cell populations tend to be nonhomogeneous. For example, even weak hits in a differentiation screen can be detected by counting the number of individual cells expressing a relevant marker, often by means of a fluorescent protein or antibody label. In addition, multiple markers can be used, and quantitative data on a per cell basis can be obtained. High content analysis also reveals whether or not particular compounds are toxic or induce changes in cell shape, such as the extension of processes from neuronal cells or even false positives that occur due to cell shrinkage. Thus, high content screening seems likely to play a very important role in attempts to regulate cell differentiation. Perhaps this approach, especially when combined with biologically or medically relevant cells, will provide a greater number of new therapeutics.

Disease Mechanisms and Toxicity

When it becomes possible to produce all differentiated cell types from mouse or human ES cells, many avenues of experimentation become possible. For instance, in many diseases, certain cell types are affected more than others: in Spinal Muscular Atrophy (SMA) and Amyotrophic Lateral Sclerosis (ALS), motor neurons degenerate; in Huntington's disease, striatal medium spiny neurons die; and in Parkinson's disease, a subset of DA neurons in the substantia nigra are lost. However, even a global neurodegenerative disorder such as Alzheimer's disease affects certain populations of cells, such as entorhinal cortical neurons, more than others. This differential susceptibility is particularly perplexing in the case of SMA and Huntington's disease, which are genetic disorders, as all cells carry the same mutation. Having access to appropriately differentiated neurons should help in providing a more comprehensive understanding of the selective cell death that accompanies these diseases even in cases, such as ALS, that are predominantly sporadic, rather than familial.

It is already possible to make large numbers of motor neurons from ES cells and studies of disease mechanism are underway. Di Giorgio et al. (2007) cultured motor neurons generated from mouse ES cells isolated from an ALS mouse model and showed that their rate of death is faster than that of ES cell-derived motor neurons from normal mice and that the death rate can be accelerated further by coculturing them with astrocytes obtained from the same mice. Astrocyte-induced death of mutant ES cell-derived motor neurons is readily amenable to even a large-scale screening campaign aimed at finding survival-enhancing compounds. My lab has isolated ES cells from a mouse SMA model (Sinor and L.L.R., unpublished data) and induced them to differentiate into large numbers of motor neurons and is currently conducting a high content screening campaign to identify drugs that will prolong the survival of the motor neurons. Until now, most SMA studies have been conducted on fibroblasts, which are phenotypically normal, and it will be important to determine if screening in motor neurons produces better therapeutic candidates. The overall goal of this type of approach is to study the pathogenic mechanisms of these degenerative diseases in the cells that are actually affected in the disease.

Most pharmaceutical scientists view the lack of adequately predictive toxicology during preclinical testing as a huge problem in effective development of new drugs. Eliminating lead compounds with true hepatotoxicity and cardiotoxicity is of special concern. The ability to derive ES cells from a wide and representative spectrum of the human population and having valid protocols to differentiate them into hepatocytes and cardiac myocytes should provide straightforward assays for analyzing certain aspects of drug metabolism and for assessing probable side effects. A key question is how mature the differentiated cells need to be before they can faithfully represent their adult counterparts (see, for example, Khetani and Bhatia, 2007).

It is now realistic to imagine creating disease-specific human ES cell lines from large numbers of patients. Applying fundamental principles of developmental biology should enable screens to be designed using a wide variety of ES cell-derived cell types at different stages of differentiation, allowing diseases to be modeled more faithfully. These stem cell-based screens may well provide more efficient and predictive methods for discovering and testing different classes of therapeutics.

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