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PHENOTYPIC SCREENING IN THE 21ST CENTURY

Topic Editors
Birgit T. Priest and Gul Erdemli

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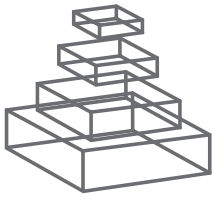

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PHENOTYPIC SCREENING IN THE 21ST CENTURY

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In the genomic era of 1990s-2000s, pharmaceutical research moved to target-based drug discovery which enabled development of a number of small molecule drugs against a wide range of diseases. In many cases however, drugs that arose from genomics failed, questioning the validity of the targets and the suitability of target-based drug discovery as an optimal strategy for all disease states. For monogenic diseases, target-based approaches may be well-suited to the identification of novel therapies. Most diseases, however, are caused by a combination of several genetic and environmental factors and are likely to require simultaneous modulation of multiple molecular targets/pathways for successful treatment. For such diseases, reductionist approaches focusing on individual targets rather than biological networks are unlikely to succeed and new drug development strategies are required.

In search of more successful approaches, the pharmaceutical industry is moving towards phenotypic screening beyond individual genes/targets. However, this requires rethinking of diseases and drug discovery approaches from a network and systems biology perspective. Since returning to the pre-genomics era of screening drug candidates in laborious animal models is not a feasible solution, the industry needs to evolve a new paradigm of phenotypic drug discovery within the context of systems biology. Such a paradigm must combine physiologically and disease relevant biological substrates with sufficient throughput, operational simplicity and statistical vigour. Biomarker strategies for translational medicine, as well as preclinical safety and selectivity assessments, would also need to be revised to adapt to the target agnostic style.

This focused issue aims to discuss strategies, key concepts and technologies related to systems-based approaches in drug development. Design and implementation of innovative biological assays, featuring multiple target strategies, and rational drug design in the absence of target knowledge during the early drug discovery are illustrated with examples. Specific topics include:

- The need for systems-based approaches in drug development
- Phenotypic screening strategies
- Compound libraries (natural product inspired compound collections)

- Target deconvolution and identification
- Target agnostic lead discovery and optimization
- Multi-target approaches and decoding the phenotype (understanding biological interactions and multiscale systems modelling)
- Translational aspects
- Early evaluation of selectivity and safety in a target agnostic manner

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Phenotypic screening in the 21st century

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Keywords: drug discovery, systems biology, label-free detection, phenotypic screening, gene networks

Since the advent of molecular cloning, target based screening has become the norm in pharmaceutical drug discovery. A large number of potential drug targets have been cloned and functionally expressed, and enormous progress has been made in the development, miniaturization and automation of cell based assays on target molecules recombinantly expressed in mammalian cell lines. This approach has delivered many clinical candidates but relatively few new drugs. Target based screening is likely to provide very good drug candidates for monogenic diseases, and the following collection of manuscripts is not meant to discourage the use of target based approaches. However, most of the more prevalent human diseases are most likely multifactorial and require interaction with multiple targets to produce clinically meaningful efficacy. In addition, high potency, selective interaction with a single target may increases the risk of adverse events or be limited by redundancies and adaptive resistance. Here, target agnostic approaches using phenotypic assays may offer significant benefit. Making such approaches viable requires addressing a number of challenges. This e-book attempts to discuss some of these challenges and illustrate recent advances.

Prior to the 1980s, most drugs were discovered using phenotypic assays in live animals or isolated tissues (Swinney, 2013). Most of these drugs interact relatively weakly with a number of targets, and the complete profile of their molecular interactions is not well-known. Examples include most anti-convulsants, diuretics, and vasodilators. Screening *in vivo* typically returns compounds with acceptable ADME properties and access to the target *in vivo*; issues that often frustrate the present target based drug discovery programs.

Screening *in vivo* may be feasible, if a good starting point for Medicinal Chemistry and a clear strategy for differentiation exist. An example is the development of carbamazepine analogs with improved ADME properties (Landmark and Johannessen, 2008). In oncology, mouse xenograft models, based on a patient's tumor cells, have been used to select between drugs (Wu et al., 2012). However, many drug discovery efforts rely on screening to identify starting points for Medicinal Chemistry, and screening *in vivo* in most cases is not commercially or ethically viable. In this case, phenotypic screening relies on an appropriate tissue- or cell-based assay that can be miniaturized and used in combination with high throughput tools. Promising examples include anti-microbial assays, cell proliferation, platelet aggregation, and insulin release from pancreatic β-cells. For CNS diseases involving neuronal networks, selection of an appropriate substrate is especially difficult. The article by Bruni et al. discusses the potential for using zebrafish as a model system, enabling large scale behavior based screens for central nervous system disorders. However, as pointed out by Bruni et al. there are substantial differences in zebrafish and human biology and translation from zebrafish to human may be problematic. In order to limit the reliance on non-predictive animal models, screening strategies utilizing human pluripotent stem cells (hPSCs) are increasingly considered as resources for drug discovery. However, several hurdles need to be overcome before widespread implementation of hPSC-based assays. Viswanathan and colleagues reviewed the recent progress in the culture of hPSCs with emphasis on the importance of the environment surrounding these cells and high content analysis approaches for assay development.

The limited throughput of phenotypic assays compared to most target-based assays necessitates smaller libraries that are optimized with regard to biological and chemical diversity. Wassermann and colleagues discuss strategies for building appropriate, well-annotated compound libraries. Such libraries may also be used to identify pathways underlying the observed effects.

Another challenge involves the identification of a phenotypic end point associated with the disease of interest. In the early phases of drug discovery, phenotypic assays can be used to further our knowledge of the disease process and to identify those end points that are most likely to translate to the clinic. Furthermore, probability of success is greatly increased, if the end point measured can serve as a clinical biomarker. The perspective by Dr. Swinney analyzes the phenotypic end points used in the discovery of new molecular entities that have resulted from phenotypic drug discovery efforts.

An additional hurdle to translation is the large genetic diversity in the human population and the need to identify the right patient population for any given drug. Progress has been made in this regard in oncology. Ross et al. outline the approach taken for cancer and the potential application to other diseases. Similarly, the manuscript by Dr Schadt and colleagues points to the importance of gene networks for complex trait diseases and highlights the importance of understanding these networks in the appropriate biological context. In this regard, the integration of panomic data will be increasingly important.

Label-free technologies can offer advantages for phenotypic screening in that they do not involve assumptions about molecular mechanisms and pathways. The manuscript by Dr. Fang

reviews the use of label-free technologies with a focus on Dynamic Mass Redistribution (DMR). Many label-free technologies offer the potential to be used as live cell kinetic assays. As discussed by Dawson and Carragher, this is especially important for studying drug combinations, since clinically successful combinations require compatible pharmacokinetics of the individual components. High content imaging is an attractive technology in this regard, especially since it is compatible with organotypic co-cultures. High content imaging can be combined with proteomics to identify pathways and biomarkers, and Dawson and Carragher describe some of the advances to increase throughput of proteomics.

We would like to thank the authors for their outstanding contributions and willingness to share their knowledge which made this Special Topic possible. All the manuscripts have been peer-reviewed and we are grateful to the expert reviewers for their valuable comments.

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Discovering novel neuroactive drugs through high-throughput behavior-based chemical screening in the zebrafish

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Most neuroactive drugs were discovered through unexpected behavioral observations. Systematic behavioral screening is inefficient in most model organisms. But, automated technologies are enabling a new phase of discovery-based research in central nervous system (CNS) pharmacology. Researchers are using large-scale behavior-based chemical screens in zebrafish to discover compounds with new structures, targets, and functions. These compounds are powerful tools for understanding CNS signaling pathways. Substantial differences between human and zebrafish biology will make it difficult to translate these discoveries to clinical medicine. However, given the molecular genetic similarities between humans and zebrafish, it is likely that some of these compounds will have translational utility. We predict that the greatest new successes in CNS drug discovery will leverage many model systems, including *in vitro* assays, cells, rodents, and zebrafish.

Keywords: behavior-based drug discovery, zebrafish, phenomics, antipsychotics, screening

INTRODUCTION

At a recent course on neurotherapeutic drug discovery (sponsored by the National Institutes of Health) the keynote speaker joked that if he were trying to be rational, he would not be trying to discover neuroactive drugs. His point was that drug discovery is often much more empirical than rational. Someday, when researchers understand the biochemical mechanisms of psychiatric disease, it may be possible to discover neuroactive drugs based on rational therapeutic hypotheses. Until then, phenotypic assays provide an alternative approach. Behavior-based drug discovery is effective, but it needs to be more efficient.

Researchers can discover new drugs without understanding how they work. Neuroactive compounds including antipsychotics, antidepressants, and anxiolytics are among the top selling prescription drugs (Alonso et al., 2004; Gu et al., 2010; Alexander et al., 2011; Mojtabai and Olfson, 2014; Olfson et al., 2014). We know some details about how these drugs affect different neurotransmitter signaling pathways. But nobody really knows how these simple molecules change our moods, thoughts, and emotions. Target-based approaches to central nervous system (CNS) drug discovery have been largely unsuccessful (Paul et al., 2010). However, we can discover new drugs without understanding the details of how they work (Irwin, 1968; Tecott and Nestler, 2004). Historically, many neuroactive drugs were discovered despite totally incorrect therapeutic hypotheses (Sneader, 2005; Kokel and Peterson, 2008; Enna and Williams, 2009). So, although drug discovery and molecular understanding often go hand in hand—it is mostly in that order.

New technologies are changing how researchers use phenotypic assays to discover new drugs. Low throughput assays have limited the field with small sample sizes, narrow scope and limited hypothesis testing. Many key discoveries were made essentially by chance (Sneader, 2005; Enna and Williams, 2009). Now, high throughput assays are enabling a discovery-based approach that relies more on mathematical modeling and massive amounts of data (rather than theory and luck) to identify new drug leads (Schadt et al., 2009). Automated screening platforms do not need mechanistic theories to generate large data sets and identify correlations between compounds and phenotype. As a result, researchers can focus on discovering drugs and drug mechanisms as separate independent endeavors. Here, we review how this data-driven approach to behavioral phenomics is accelerating the pace of neuroactive drug discovery.

HOW MANY NEUROACTIVE DRUGS ARE THERE?

"How many neuroactive drugs are there?" is a deceptively simple question that can be surprising difficult to answer. Neuroactive drugs are difficult to classify because relationships between compound structure, target and phenotype are often unclear and poorly understood. Structure-based classification is difficult because small structural changes can drastically alter a compound's mechanism of action. Target-based classification is difficult because drug targets are often unknown. Even when *in vitro* targets are identified, their *in vivo* relevance is often unclear. One approach is to classify compounds based on behavioral phenotypes or medical utility. But most phenotype-based classifications are subjective and difficult to quantify. How do we know when a drug is an antipsychotic or an antidepressant (Maher et al., 2011)? There are no known molecular causes or biomarker-based diagnostics for most mental disorders (Javitt et al., 2008) and off-label prescriptions are common (Chouinard, 2006; Alexander et al., 2011). So exactly how many neuroactive drugs are there?

Although the FDA lists thousands of antipsychotics, antidepressants and anxiolytics, most of these compounds fall into just a few structural classes. Consider the antipsychotics. Searching the FDALabel database for "antipsychotic" returns 1,325 hits, but most are mixtures and formulations of identical compounds (U.S. Food and Drug Administration, 2014). The same search in Drugbank returns 42 hits and most are close structural analogs of each other (Kokel and Peterson, 2008). Chemoinformatic algorithms cluster these compounds into a small number of structurally related families (Cao et al., 2008; Backman et al., 2011; **Figure 1**). Like antipsychotics, the antidepressants and anxiolytics show a similar pattern: There are many individual drugs, but most are structural analogs of a handful of prototypes. These data suggest that many drugs seem to discover themselves, due to the exploitation of prototype molecules (Sneader, 1996).

Most neuroactive drug prototypes were discovered during two broad time periods: pre-history and the mid-1900s. Both waves of discovery coincided with the availability of new chemical compounds alongside relatively widespread human and animal experimentation. The first wave of drugs, discovered in prehistoric times, were found by screening (ingesting) natural products in the environment. Compounds like morphine, alcohol, nicotine, and cocaine were identified based on their strange and unexpected behavioral phenotypes. The second wave of drugs, discovered in the mid-1900s, were found by screening synthetic compounds. These drugs, including the first modern anxiolytics, antipsychotics and antidepressants, were also discovered based on unexpected behavioral phenotypes.

Behavioral phenotyping is an essential part of drug discovery, but it is also the bottleneck (**Figure 2**). Prototype discovery often starts with the observation of an unexpected behavioral phenotype. Once a prototype has been identified, medicinal chemists generate structural analogs that themselves often have unexpected phenotypes. Researchers use these compounds to test therapeutic hypotheses and search for mechanistic understanding. When molecular targets are identified, researchers search for new ligands that trigger a new round of behavioral phenotyping. In target-based approaches, behavioral phenotyping is deferred until later in the process. Ultimately, the final step of determining efficacy in humans is also a matter of behavioral phenotyping. The process is incredibly effective and has generated most drugs that we use today. Medicinal chemistry can efficiently generate thousands of structural analogs; technologies for *in vitro* screening are ultra high-throughput. But, decades-old approaches to behavioral phenotyping throttle the drug discovery engine.

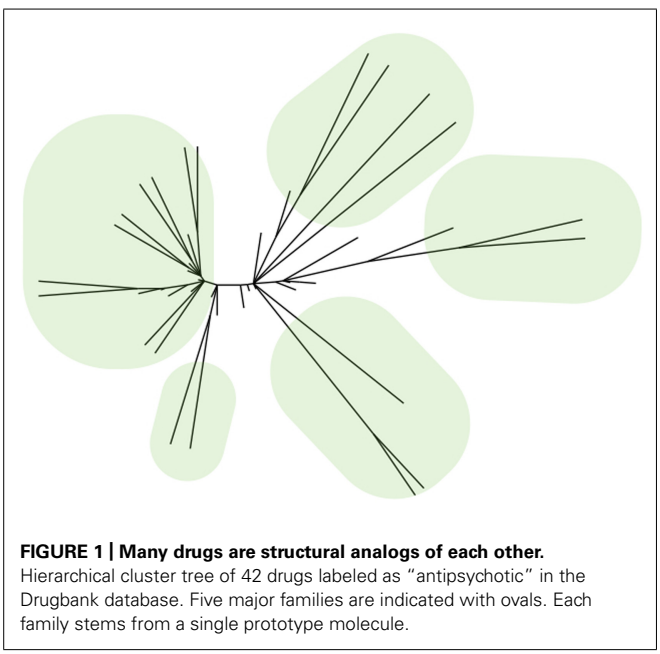

FIGURE 1 | Many drugs are structural analogs of each other. Hierarchical cluster tree of 42 drugs labeled as "antipsychotic" in the Drugbank database. Five major families are indicated with ovals. Each family stems from a single prototype molecule.

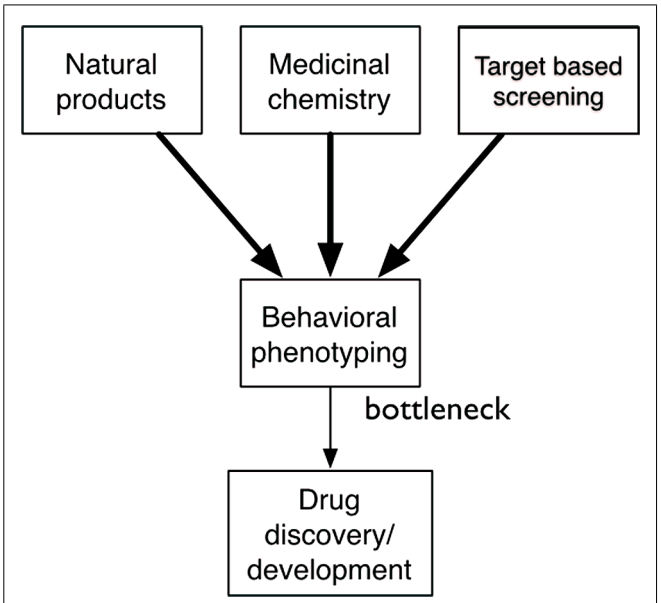

FIGURE 2 | Behavioral phenotyping is a key bottleneck in drug discovery. Although there is an abundance of small molecules from nature, medicinal chemistry, and target-based screening only a very small number are ever tested in behavioral assays, limiting the drug discovery process.

WHAT DO ZEBRAFISH DO?

The zebrafish model system enables researchers to combine complex behavioral phenotyping with high-throughput chemical screening. Like humans, fish are vertebrate animals with complex brains and behaviors. But unlike humans, fish are small enough to fit in 96-well plates and they easily absorb compounds dissolved in the water. These features make zebrafish uniquely well suited for phenotype-based neuroactive drug discovery and enable researchers to scale complex behavioral assays to high-throughput formats.

A frequently asked question about behavior-based drug discovery in zebrafish is "What do zebrafish do?" Personality disorders, depression, and anxiety seem like some of the most complex phenotypes imaginable. Fish do not suffer from these feelings the same way that people do. So, the idea of using fish to discover neuroactive drugs can seem counterintuitive. We tend to think about zebrafish behavior in two different ways: where as some fish behaviors resemble some human behaviors, many others lack obvious human correlates.

Anthropomorphic assays in fish are very powerful. We immediately empathize with familiar behaviors and their circuitry and mechanisms are likely to be relatively well conserved. For example, researchers use circadian cycles in zebrafish locomotor activity to study mechanism that control sleep behaviors in humans (Prober et al., 2006; Zhdanova, 2006; Yokogawa et al., 2007; Rihel et al., 2010). Researchers also use zebrafish behavior to study pain (Prober et al., 2008), fear (Speedie and Gerlai, 2008; Agetsuma et al., 2010; Mathuru et al., 2012), learned helplessness (Lee et al., 2010), feeding (Gahtan et al., 2005; Del Bene et al., 2010; Bianco et al., 2011), courtship (Darrow and Harris, 2004), learning (Valente et al., 2012), vision (Emran et al., 2007), hearing (Gleason et al., 2009), touch (Low et al., 2010, 2011, 2012), social interactions (Pérez-Escudero and de Polavieja, 2011; Mahabir et al., 2013; Qin et al., 2014), anxiety (Stewart et al., 2012), and decision making (Arganda et al., 2012).

By contrast, it is more difficult to empathize with fish-specific phenotypes. The practical implications of understanding fundamental fish behaviors are not always obvious. And it is easy focus on their differences rather then their similarities. Nevertheless, neuroactive drugs affect fish behavior in specific and reproducible ways via conserved molecular mechanisms. Adult zebrafish, differentially change their swimming and three-dimensional tank diving behaviors in response to many neuroactive compounds (Cachat et al., 2011; Grossman et al., 2011; Stewart et al., 2011; Kyzar et al., 2012a, 2013; Williams et al., 2012; Robinson et al., 2013; Stewart and Kalueff, 2014). In larvae, fish specific behaviors like spontaneous swimming (Wyart et al., 2009), the optokinetic reflex (Emran et al., 2007) and photomotor response (Kokel et al., 2013b) can be used to understand neuronal signaling, rapidly identify novel neuroactive compounds and predict their mechanisms of action (Kokel et al., 2010; Rihel et al., 2010). The key challenge is learning how to decode complex patterns of behavior to understand which pathways are being modulated—and how they may affect human health.

A DISCOVERY-BASED APPROACH

Some of the most exciting developments in behavioral phenomics are coming from two very different models: humans and zebrafish. Compared to other animals, human behaviors are probably the most complex, variable and challenging to measure. So it is somewhat surprising that human behavioral phenomics is advancing so rapidly. One reason is that substantial investments by internet technology companies have increased the scale of digital record keeping and chemobehavioral phenotyping. Large medical databases link people's genotype, phenotype and prescription drug records. Researchers are mining these databases to identify unanticipated drug side effects and repurpose drugs for new indications (Dudley et al., 2010).

Human behavioral phenomics is a powerful way to approach drug repurposing, but it cannot be used for chemical screening. Governmental and institutional regulations limit large-scale human studies to compounds that are already approved by the FDA (thankfully). Researchers will need other model organisms, like zebrafish, to systematically discover new molecular entities. Until recently, tools for high throughput behavioral phenotyping were unavailable. But new technologies are changing the drug discovery landscape.

AUTOMATED SOLUTIONS

Automated technologies are making behavior-based chemical screening in zebrafish a more effective, efficient and systematic way to discover neuroactive compounds. Three aspects of automation are changing the field of behavior-based drug screening: robotics, analytics, and academic industrial collaboration. These changes are a small part of larger global trends in computing technology. As sophisticated processors, programming languages, and rapid prototyping tools become more accessible, individual scientists and small academic laboratories are innovating alongside larger biotechnology and pharmaceutical companies.

Robotic solutions are growing to meet nearly every early step of the screening process including fish breeding, sorting, and phenotyping. Robotic aquaculture racks automate feeding cycles and monitor water quality. Specialized breeding tanks produce thousands of synchronized embryos (Adatto et al., 2011). Flow cytometry platforms sort zebrafish into 96-well plates. And imaging platforms automate morphological and behavioral phenotyping (Burgess and Granato, 2007a; Pardo-Martin et al., 2010a, 2013; Ahrens et al., 2012; Engert, 2012; Wittmann et al., 2012). For example, researchers have developed an elegant and powerful (freely available) software package, FLOTE, for automated tracking of precise kinematic events in larval zebrafish (Burgess and Granato, 2007a). The software has already been used to analyze startle modulation, light adaptation, and navigation (Burgess and Granato, 2007a,b; Burgess et al., 2009, 2010; Jain et al., 2011; Fernandes et al., 2012). The software has also been used to find compounds that modulate memory formation in larval zebrafish (Wolman et al., 2011). Although not yet used for drug screening, recent advances in whole-brain functional imaging record patterns of firing activity of individual cells in large populations of neurons (Ahrens et al., 2012, 2013; Kokel et al., 2013b; Muto et al., 2013; Satou et al., 2013; Portugues et al., 2014) and will likely add massive amounts data to the behavioral pharmacology field. As behavioral datasets grow, researchers are applying new analytical approaches to explore, organize, and discover correlations between phenotypic patterns and compound treatments.

Academic-industrial partnerships are improving zebrafish phenotyping and phenotype-based approaches to drug discovery. The innovations flow both ways, from academia to industry and industry to academia. Acquifer (http://www.acquifer.de), a new biotech company with roots in academic automated zebrafish phenotyping, is developing network platforms for managing huge amounts of data from zebrafish phenotypic screens. Commercial imaging platforms, like the Vertebrate Automated Screening Technology marketed is based on academic innovations (Pardo-Martin et al., 2010b, 2013; Chang et al., 2012). When equipment is too expensive, academic bioinstrumentation laboratories are working to develop more affordable do-it-yourself kits (Alper, 2009; Marzullo and Gage, 2012). As sophisticated rapid prototyping tools become more accessible (like 3D printers, open source programming languages, and cheap microcontrollers) the pace of innovation is accelerating.

SCALING BEHAVIORAL DATABASES INTO CONNECTIVITY MAPS

Today, database-linked tools for analyzing gene expression data and behavioral data look very different. Behavioral databases tend to be designed for finding and summarizing data via search field descriptors like compound name, genes name and strain name. For example, the Zebrafish Neurophenome Database (ZND) is a publically available database designed to provide a comprehensive resource of neurobehavioral phenotypes in adult zebrafish (Green et al., 2012; Kyzar et al., 2012b; Kalueff et al., 2013). To search the ZND, a researcher uses drop-down fields to select investigator and drugs of interest to experimental results and drug effects that are often presented as textual descriptions. Similarly, large-scale mouse phenotyping projects like the Mouse Phenome Database (MPD) at The Jackson Laboratory allow users to find, visualize and analyze mouse behavioral phenotypes across different strains and conditions (Maddatu et al., 2012). The MPD stores a large amount of standardized, quantifiable and comparable data (like weight and grip strength). The MPD also provides a variety of tools to analyze results (including tools to find strains that best fit phenotypic criteria). But, as phenotypic databases grow ever larger, they will enable more complex data-driven queries.

Given sufficiently rich behavioral phenotyping, it should be possible to build a connectivity map to systematically identify neuroactive compounds and sort them into phenotypic classes. For example, the Connectivity Map is designed to use gene expression data as a discovery framework by allowing researchers to use gene expression signatures to query the data for closely related perturbagens (Lamb et al., 2006; Lamb, 2007). As a result, one can use the data itself to identify correlations, perform cluster analyses and identify outliers. Analyses that were originally developed for applications like speech recognition and social networking can just as easily be applied to analyzing zebrafish phenotypes. And these analyses allow new questions about large diverse data sets. We imagine that someday soon, it may be possible to query large behavioral databases with BLAST-like and speech recognition tools. This could allow researchers to identify all compounds with similar behavioral phenotypes, link genetic mutants to small molecule treatments and identify new treatments with totally novel phenotypes. Will it be possible to identify just the right pattern of fish behaviors to accurately identify drugs with complex activities in humans (like antipsychotics and antidepressants)? Future studies may provide the answer.

WHAT ARE WE LIKELY TO FIND?

Given that so few compounds have been tested in animals, large-scale behavioral screens are almost guaranteed to identify new neuroactive compounds. These studies will provide high-resolution maps of how small molecules affect the brain and behavior. But what kinds of compounds are likely to be discovered? Are we really likely to identify new compounds with new mechanisms of action? Or just more of the same kinds of drugs we already have? The data supports both arguments.

On the one hand, one could argue that behavior-based drug screening has been saturated: Multiple classes of antipsychotics, antidepressants, and anxiolytics have already been identified. One possibility is that the low throughput non-systematic approaches employed in the past have already identified all the neuroactive drugs worth discovering. Alternatively, it is interesting to speculate that compounds with antipsychotic, antidepressant and anxiolytic effects may be relatively common. If so, large-scale screens would likely identify a variety of new psychotropic drug prototypes with a range of phenotypic and mechanistic profiles, including totally new structures, mechanisms, and phenotypes.

Large zebrafish behavior-based chemical screens are already identifying a variety of new compounds. Some of first neuroactive compounds to be discovered in zebrafish, str1, and str2, were novel acetylcholinesterase inhibitors (Kokel et al., 2010). These compounds were new molecular entities, but they were not first in class compounds. These compounds may provide modest advantages over current treatment options. But identifying novel structures with novel targets and mechanisms would have a greater impact. One potential way to identify compounds with novel mechanisms is to identify compounds that cause outlier phenotypes in behavioral databases. If one compound in ten thousand causes a unique behavioral phenotype, this suggests it may be working through a new (and rare) mechanism of action. For example, a new kind of light controllable rapidly reversible TrpA1 ligand, optovin, was recently discovered in just this way (Kokel et al., 2013a). Several novel light activated molecules have been developed using zebrafish behavioral readouts (Szobota et al., 2007; Janovjak et al., 2010; Levitz et al., 2013). This suggests that truly novel compounds are waiting to be found, if only we use the right methods to look for them.

MODIFIER SCREENS: CHEMICAL AND GENETIC MODELS

Although wild-type phenotypes may be useful for identifying certain compounds, we can also use chemical and genetic tools to model specific disease states. These disease models combine the advantages of unbiased phenotypic screening with readouts that are specifically designed to target certain kinds of compounds. In one recent example, researchers identified a zebrafish mutant (in the Scn1a gene) and then used this model to screen for potential treatments for Dravet syndrome (caused by mutations in the homologous human gene; Baraban et al., 2013). These researchers identified an FDA approved compound that suppressed the fish phenotype, suggesting that the approach may be a powerful way to identify therapeutics for this specific disorder. This work elegantly illustrates the potential for genetic models in zebrafish to identify desperately needed targeted therapeutics with potential utility in humans. One can imagine many variations on this theme. CRISP-Cas technology is revolutionizing zebrafish researchers' ability to efficiently generate knockout and knock-in models (Hwang et al., 2013a,b; Auer et al., 2014). Transgenic overexpression models phenocopy aspects of neurodegenerative and other dominant diseases (Bai et al., 2007; Olson et al., 2010). And, due to the ease of chemical manipulations, researchers have run large-scale modifier screens in chemically treated disease models (Baraban et al., 2005).

WHOLE ORGANISM PHENOTYPING: BLOOD–BRAIN BARRIER AND TOXICOLOGY

Researchers can expand phenotypic readouts to encompass almost any aspect the organism including blood–brain barrier (BBB),

toxicity, and cardiovascular readouts. Larval zebrafish develop a functional BBB with size exclusion and transport pumps including those that are similar to mammals (Jeong et al., 2008). So researchers have some reason to believe compounds with CNS activity in fish may also penetrate the BBB in mammals. Similarly, potentially toxic compounds can be screened for unwanted and unexpected toxic or cardiovascular side effects. One could potentially capture data on zebrafish development, behavior and heart rate simultaneously in a high-throughput and automated fashion. Because researchers can apply diverse phenotyping assays, zebrafish are an exciting model for toxicology in addition to drug discovery.

THE CHALLENGE OF TRANSLATING FROM FISH TO HUMANS

Despite the power of new technologies, there are substantial fundamental challenges to translating CNS drug discovery from fish to humans. Most investigational new drugs fail when they are finally tested (for efficacy) in humans (Paul et al., 2010). There are many reasons why preclinical predictions from any model system would fail to translate, but lack of rigor should not be one of them.

Inefficient animal studies contribute to publication bias, decrease scientific rigor, and limit the drug discovery process. Compared to zebrafish, studies in larger animals, like mice, are relatively expensive and require substantial amounts of test compounds. Due to these costs, some large-animal studies tend to be underpowered, which contributes to irreproducible results (Landis et al., 2012). Zebrafish enable a level of rigor and reproducibility that can be difficult to achieve in larger model organisms, simply because the assays can be easily reproduced on larger scales. Hypotheses can be tested on thousands of animals, rather than just a handful, at small cost in time and other resources. For example, treating a single mouse (at 10 mg/kg) requires approximately 100X more compound than is needed to treat a well of zebrafish (at 10 μM). When researchers increase sample size it becomes easier to find true signals amongst the noise. However, even if new compounds can be discovered with reproducible effects on zebrafish behavior, substantial challenges remain to translate these discoveries for improving human health.

Many compounds work in humans, many work in zebrafish, and some fraction is likely to work in both–although the exact level of overlap is difficult to predict (**Figure 3**). Humans and zebrafish are closely related (Howe et al., 2013), but there are many differences at the phenotypic, neuronal network, and molecular levels. When a new bioactive compound is first discovered in zebrafish, it will be difficult to predict its potential therapeutic utility in humans. Many compounds that appear to work well in mice and other animal models subsequently fail to translate to humans. The same will surely be true of zebrafish. The problem is especially relevant in neuropharmacology, where CNS disorders are poorly understood and difficult to model. Despite the challenges, in the upcoming years we are likely to see at least a few compounds identified in zebrafish screens translate from bench to bedside.

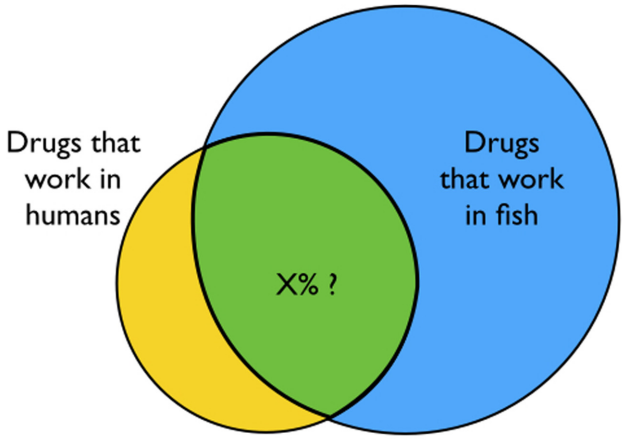

FIGURE 3 | How many drugs work in both humans and zebrafish? Not all drugs that work in zebrafish will also work in humans. Because so many more compounds can be screened in zebrafish, it is likely that some will translate to the clinic. However, the precise level of overlap (indicated by X) is difficult to predict.

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Human pluripotent stem cells on artificial microenvironments: a high content perspective

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Self-renewing stem cell populations are increasingly considered as resources for cell therapy and tools for drug discovery. Human pluripotent stem (hPS) cells in particular offer a virtually unlimited reservoir of homogeneous cells and can be differentiated toward diverse lineages. Many diseases show impairment in self-renewal or differentiation, abnormal lineage choice or other aberrant cell behavior in response to chemical or physical cues. To investigate these responses, there is a growing interest in the development of specific assays using hPS cells, artificial microenvironments and high content analysis. Several hurdles need to be overcome that can be grouped into three areas: (i) availability of robust, homogeneous, and consistent cell populations as a starting point; (ii) appropriate understanding and use of chemical and physical microenvironments; (iii) development of assays that dissect the complexity of cell populations in tissues while mirroring specific aspects of their behavior. Here we review recent progress in the culture of hPS cells and we detail the importance of the environment surrounding the cells with a focus on synthetic material and suitable high content analysis approaches. The technologies described, if properly combined, have the potential to create a paradigm shift in the way diseases are modeled and drug discovery is performed.

Keywords: phenotyping, pluripotent stem cells, microenvironment, high content, single cell

PLURIPOTENT STEM CELLS AND THEIR CULTURE

HUMAN PLURIPOTENT STEM (hPS) CELLS ARE DERIVED FROM EMBRYOS OR THROUGH REPROGRAMMING

Stem cells are defined as cells capable of self-renewal, the capacity to generate identical copies of themselves, and differentiation, the ability to provide cells performing a specific biological function (Smith, 2006).The capacity to differentiate into all lineages sufficient to form an entire organism, and not necessarily extra embryonic tissues, is defined as pluripotency. This property can be demonstrated by showing differentiation into cells from the three germ layers (endoderm, mesoderm, and ectoderm). With no test available for germline transmission of human cells, pluripotency can be demonstrated in immune-deficient mice by the ability to form teratomas. *In vitro,* a number of molecular markers are used as a surrogate for pluripotency; some of these carry functional significance, such as Oct4 and Nanog, whereas others are considered mostly descriptive, such as stage-specific embryonic antigen (SSEA)-4 and Trafalgar antigen TRA-1-60.

Using diverse culture methods, several types of cells have been characterized which broadly fit many aspects of the definition of pluripotency. hPS cells are derived from transient populations of cells isolated from the embryo such as human embryonic stem (hES) cells or are artificially reprogrammed from somatic cells such as human induced pluripotent stem (hiPS) cells. In mice, several populations of pluripotent cells can be derived from pre- and post-implantation embryos. The earlier or naïve cells are the originally described mouse embryonic stem (mES) cells whereas the post implantation cells (epiblast stem cells or epistem cells) represent a later stage of development and are thought to be "primed" to differentiate with potential lineage bias (Nichols and Smith, 2009)

In human, however, hES cells resemble more closely the mouse epistem cells (Tesar et al., 2007; Greber et al., 2010; Ware et al., 2014). Moreover, whilst hES cells fall into the "primed" category with hiPS cells, the characteristics can vary depending on somatic source, reprogramming method and culture system. In recent times, many researchers have attempted to define conditions under which naïve hPS cells can be derived and maintained (Gafni et al., 2013). Importantly, in the human blastocysts there are several different cell populations that can give rise to pluripotent stem cells when explanted and cultured (Niakan and Eggan, 2013). The extensive crosstalk between stem cells and their niche encompasses neighboring cells, soluble cues, and extracellular matrix (ECM) proteins and is key to the maintenance of pluripotency. It is also likely that in the early phases, the surrounding environment plays a major role in instructing cells to enable self-organizing properties as has been reported in the mouse system (Bedzhov and Zernicka-Goetz, 2014). Unsurprisingly then, at each step of the evolution of culture systems, the emphasis has been on recapitulating the "natural" environment, or "niche" (Lutolf and Blau, 2009). Along these lines, the secretion of growth factors by stromal cells has informed the choice of factors and more recently attention has been devoted to mimic the structural and mechanical properties of the natural niche. However, although a tractable model

system, cell culture is artificial by definition and it is not easy to pinpoint what the "natural" conditions are *in vivo* and should be *in vitro*.

DEFINING THE CULTURE: FEEDERS AND MEDIA

Irrespective of the biological differences, expansion of homogeneous starting populations in self-renewing conditions is key to realizing the promise of hPS cells for screening and modeling strategies. hPS cell culture has progressed a long way from the initial derivation and expansion on mouse feeders in medium containing bovine serum (Thomson et al., 1998). Nonetheless, production of large numbers of stable, homogeneous, and undifferentiated cells in standardized protocols is still far from a trivial matter. Mirroring progress obtained a decade in advance with mES cell culture, culture of hPS cells has evolved substantially. In fact it moved from mouse feeders to defined feeder-free systems taking in human feeders, conditioned medium, and complex substrates along the way.

There are some disadvantages associated with each of these variations. The use of feeders brings additional variability to the culture, particularly crucial if the cells are non-human. It has been shown that animal products can modify as well as contaminate hPS cells (Moore, 2006). An important factor for variability in hPS cell yield and viability is the effect of feeder cell density (Heng et al., 2004), often inconsistent across laboratories. A comparison of the literature reveals the use of a large range of seeding densities, from 20,000 to 75,000 cells/cm^2 (Zhou et al., 2009). Human feeders although expensive, hard to maintain and equally variable have allowed the relatively early establishment of clinical grade hES cell lines (Tannenbaum et al., 2012).

Using feeder free cell culture has the advantage of removing the requirement for parallel culture and mitotic inactivation of feeder lines. Yet, it often involves conditioned medium or xenogenic complex substrates. The use of xeno-free, defined products can improve robustness and there are a number of combinations now available that do not contain animal derived components or complex additives such as sera. As implied above, selected culture systems will result in subtly different populations. Whilst still fitting the wider definition of hPS cells, these will respond in different ways to external stimuli. Therefore the lack of consensus about culture systems does pose a hurdle when comparing data between laboratories. Also, it is important to stress that established differentiation protocols will not necessarily transition seamlessly to a different culture system and can therefore represent a high barrier to progressing culture conditions even when long-term gains are significant.

Different media used for the different systems have been thoroughly and recently reviewed elsewhere (Chen et al., 2014). Here, we will briefly describe selected defined media for feeder free culture. A number of defined media for hPS cells are commercially available such as mTeSR1/2 (STEMCELL Technologies), StemPro (Invitrogen), Pluripro (Cell Guidance Systems), PluriSTEM (Millipore), Stemline (Sigma), and Nutristem (Stemgent). Most of these contain bovine serum albumin (BSA) along with a complex mixture of amino acids, trace elements, hormones, and growth factors. Human serum albumin (HSA) is present in TeSR2 whereas derived from it, the more recent Essential 8 (Invitrogen) medium does not contain HSA or BSA and can perhaps be considered a truly defined medium. Most commercially available and homemade media contain both fibroblast growth factor 2 (FGF2) and transforming growth factor β(TGFβ)/Activin A/NODAL at varying concentrations. Some of these media require higher concentrations of FGF2 to maintain the cells, further adding to the cost of culture.

DEFINING THE PHYSICAL CONDITIONS: CELL–CELL CONTACT AND HYPOXIA

Unlike their murine equivalents, hPS cells poorly tolerate being separated to single cells and have historically therefore been propagated as clusters using mechanical or enzymatic methods or a combination of the two. Mechanical passaging methods are least favored when considering the scalability of the culture process. It is difficult to accurately determine cell-seeding densities, as hPS cells are kept in large clumps or colonies, using this technique. Consistent seeding densities are essential to reduce variability in hPS cell culture as they result in higher quality cells and more predictable yield.

Apoptosis induced by dissociation to single cells can be modulated by pharmacological inhibition of specific pathways involved in cell–cell adhesion. For example, the Ras homolog gene family member A (RhoA) acts on its downstream effector, Rho-associated protein kinase (ROCK) and ROCK inhibitors (ROCKi) can be added to the culture just at time of passage or throughout cell maintenance to counteract the stress induced by dissociation into single cells (Watanabe et al., 2007). Preparation of hPS cells as single cells, in the presence of ROCKi allows for more homogeneous populations and these are more amenable to automation. It has been suggested that enzymatic passaging or ROCKi can cause chromosomal abnormalities in hPS cells (Mitalipova et al., 2005). Despite initial discordances, a number of reports have now shown that normal karyotypes can be maintained after prolonged single cell passage demonstrating that single cell passage *per se* does not lead to chromosomal abnormalities (Mitalipova et al., 2005).

Various enzymes are currently used in hPS cell culture, including dispase II, collagenase IV, accutase, TrypLE Express (Invitrogen). Dispase and collagenase allow cells to remain as clusters, whereas accutase and TrypLE Expresss dissociate hPS cells into a single cell suspension. It should be noted, however, that these methods often still require manual removal of differentiated cells prior to enzyme addition which creates an obvious barrier to automation (discussed below). An alternative to enzymatic dissociation is the use of EDTA, which allows the dissociation of colonies to small clusters and works in conjunction with E8 medium on a defined substrate (Beers et al., 2012).

The normal atmospheric oxygen tension, at which hPS cells are generally cultured is 21%. *In vivo*, mammalian oxygen tension on the other hand ranges from 1.5 to 5.3% (Fischer and Bavister, 1993). As for other cell types (Parrinello et al., 2003) there have been attempts to evaluate the biological effect of hypoxia on hPS cells, for example through hypoxia-inducible factors (Mathieu et al., 2013). Low oxygen enhanced clonal recovery of hES cells and reduced the incidence of chromosomal aberrations without altering hES cell pluripotency marker expression (Forsyth

et al., 2006).Moreover, it can improve pluripotency maintenance while reducing the incidence of chromosomal aberrations and reduce the occurrence of spontaneous differentiation (Forristal et al., 2010; Zachar et al., 2010). Despite these interesting findings, logistical problems severely limit the use of low oxygen in most laboratories and dedicated culture chambers have been proposed (e.g., biospherix).

QUALITY BY DESIGN, SCALE-OUT, AND SCALE-UP

The choice of the culture system has severe consequences for the potential use in different applications and a significant impact on optimization of downstream differentiation protocols. Considerations of required cell number and batch size should be addressed at an early stage to facilitate the efficient translation of protocols and avoid population drift, which will introduce variability (**Figure 1**). These problems can be minimized by establishing master- and working cell banks with limits imposed on the number of passages. Nonetheless, current bench-scale methods described above show intrinsic limitations in terms of variability and yield (Veraitch et al., 2008). Two diverse approaches can be considered to produce large numbers of cells: scale-up and scale-out.

Most 2D culture, providing manual removal of differentiated cells is not required, can be relatively easily scaled out, at least to a degree (e.g., larger or multilayer or stacked flasks) and this may be sufficient to meet certain requirements. Scaled-out systems may be especially useful for culturing multiple different cell lines at once though with high labor costs and variability. This can be addressed in part through the use of automation. Automation has been used in several steps of hPS cells expansion processes often improving consistency although not necessarily reducing process time. The first use of automation to aid hPS cells expansion was based on dissection of hPS cell colonies (Joannides et al., 2006). Subsequently studies were published in which automation was used to monitor hPS cell cultures (Narkilahti et al., 2007), to seed hPS cells and change media (Terstegge et al., 2007), to harvest hPS cells (Haupt et al., 2012) and to carry out high throughput screening as discussed below. To date, only two systems have been described that automate the full PSC expansion process. These are the CompacT SelecT (TAP Biosystems; Thomas et al., 2009) and a custom-built platform which has been tested for mES cells (Hussain et al., 2013) and is currently used to expand and differentiate hPS cells.

Scale-up methods on the other hand commonly use specialized systems such as stirred-tank reactors (STRs), spinner flasks, perfusion systems or wave bioreactors. Due to the adherent nature of hPS cell culture, cells in these systems require a surface to attach to. The use of coated beads in bioreactors can be considered as 2D culture and may not differ significantly from the output of traditional 2D culture. However, the media change dynamics are likely to have an impact on the culture conditions. STRs can contain large volumes, where culture conditions such as pH, oxygen levels, and metabolite concentrations are precisely and carefully controlled in a uniform environment with adequate nutrient levels and oxygenation (Chen et al., 2010a). To circumvent some of these problems, cells can also be microencapsulated in hydrogels in 1.1% calcium alginate capsules, which allow for the cells to remain pluripotent and proliferate for more than 8 months (Siti-Ismail et al., 2008). True 3D expansion of hPS cells in defined medium has also been reported (Zweigerdt et al., 2011) demonstrating the potential of this approach for scale-up. However, aggregated pluripotent culture pose problems and cell damage can be attributed to shear force (Serra et al., 2012).

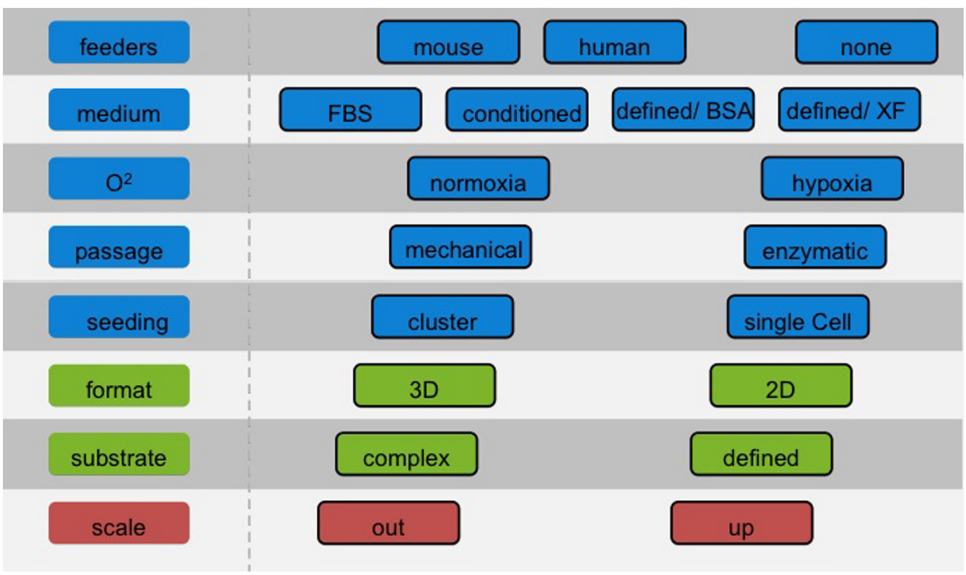

FIGURE 1 | Choosing the right cell culture conditions for hPS cells. The number of cells required for a screening campaign is typically less substantial than for cell therapies. Nonetheless, it is of paramount importance to choose the appropriate cell culture conditions beforehand, with a clear view of the route and the end-points to achieve. Adapting culture protocols at a later stage may be problematic as cells may respond distinctly to established differentiation protocols.

Overall, the use of scale-up systems brings considerable advantages for culture control; pH and dissolved oxygen tension can be monitored and controlled throughout the cell expansion process, which cannot be done in static culture. Perfusion systems permit the removal of waste products and addition of fresh media as required.

For feeder-free culture of hPS cells (including on microcarrier beads) the most commonly used substrates are gelatinous extracts such as Matrigel (BD Biosciences) or Geltrex (Life technologies). These are undefined extracellular matrices derived from Engelbreth–Holm–Swarm (EHS) sarcoma and are susceptible to high batch-to batch variability. Although the use of these substrates can eliminate feeders from the culture system, certain components remain unknown. More defined substrates that can support single cell passage are fibronectin, vitronectin, and laminin. Laminin-521 (Rodin et al., 2014) or laminin-511 E8 fragment (Nagakawa et al., 2014) have also been proposed. Alternative xeno-free substrates, such as CellStart (Invitrogen) and Synthemax (Corning), are also available albeit at a high cost.

THE ROLE OF THE MICROENVIRONMENT AND SYNTHETIC SUBSTRATES

Culture conditions (density of surrounding cells, soluble factors, substrates) should be considered as a whole to capture the complexity of soluble signals and the surrounding environment. Soluble factors such as Wnts and FGFs regulate stem cells self-renewal, some membrane-associated proteins such as cadherins form adherens junctions involved in cell positioning and anchoring (Lutolf et al., 2009) while integrins, bind to other components of the ECM, including fibronectin, vitronectin, laminin, and collagen to promote cell adhesion and differentiation (Fuchs et al., 2004).

Several lines of research have recently attempted to focus on the effect of substrates on the proliferative behavior of stem cells. In tissue stem cells, previous studies have reported that engineered surfaces with precise ligand affinity, density, and tethering, spatial arrangement of surface chemistry, topologies, and matrix stiffness can elicit cell responses ranging from self-renewal to differentiation(McBeath et al., 2004; Engler et al., 2006; Dalby et al., 2007; Khetan and Burdick, 2010; Unadkat et al., 2011; Kilian and Mrksich, 2012; Trappmann et al., 2012; Viswanathan et al., 2012). Such synthetic substrates are valuable tools to dissect cell matrix interactions *in vitro*.

These design principles can be extended to determine substrates that contribute to optimal self-renewing conditions as well as materials that direct hPS cells into specific lineage differentiation. In this section we describe the main components of cell–cell and cell–ECM interactions that may guide the design of new synthetic materials. Moreover we discuss matrix properties that can affect hPS cell self-renewal and differentiation and mechanotransduction pathways that are important in these processes. Finally, we review synthetic tools to study cell material interactions, with a view on the potential application for screening materials using hPS and image analysis (**Figure 2**).

MIMICKING THE EXTRACELLULAR MATRIX (ECM)

Cell–cell and cell–ECM interactions are mediated by integrins, cadherins, or polysaccharides such as glycosaminoglycans (GAGs). These molecules transmit biophysical cues and environmental cues across the cell membrane to intracellular signaling pathways involved in cell fate decisions. Integrins are heterodimeric proteins involved in adhesion and bi-directional signaling containing α and βsubunits. Combinations of these (24 have been described) may take place, determining the ligand specificity and affinity for specific ECM motifs such as the tri-peptide RGD (Arginin–Glycin–Aspartic acid).Their adhesion strength is modulated by activation or clustering, which anchors stem cells to their niche (Ellis and Tanentzapf, 2010). hPS cells have been shown to express a range of integrin chains including α_3, α_5, α_6, and β_1. Furthermore hES cells also express α_2, α_{11}, α_V (Braam et al., 2008; Meng et al., 2010), and hiPS cells, α_7, α_V, and β_5 (Rowland et al., 2010; Jin et al., 2012). GAGs are long unbranched polysaccharides whose chemical functionality determines the type such as heparin, chondroitin or others. GAGs are other mediators of adhesion to the ECM and are abundant on the surface of hPS (Sun and Fu, 2013). Peptide sequences derived from vitronectin (Klim et al., 2010) that bind to heparin have been used as synthetic feeder-free substrates for the maintenance of hPS cell pluripotency and like other ECM components can be readily conjugated to any synthetic surface that are now commercially available (Synthemax, Corning).

CELL–CELL INTERACTION, CELL SHAPE, CYTOSKELETAL TENSION, AND TOPOGRAPHICAL CUES

Important mediators of cell–cell interactions are proteins from the cadherin family. Cadherins play many roles in cell recognition, cell sorting and strengthening of cell–cell adhesions. They also operate as signaling receptors that modulate cell behavior or drive cell-upon-cell locomotion because they are force-resistant (Niessen et al., 2011). There are three classical types of cadherins that have been most extensively studied: the epithelial (E-cadherins), vascular endothelial (VE-cadherins), and neural (N-cadherins). E-cadherins are involved in calcium-dependent cell–cell adhesion in both epithelial and embryonic stem cells, and are integral for hES cell self-renewal and survival (Xu et al., 2010). E-cadherins are also utilized to identify hES cells as markers of undifferentiated state (Li et al., 2012). They also interact with ROCKs to regulate the function of the actin-cytoskeleton and promote hES cell clonogenicity (Li et al., 2010).

Human mesenchymal stem cells (hMSCs) provided early proof demonstrating that the shape of the substrate used to culture cells could strongly influence cell fate and tissue architecture. A decrease in plating density (or larger fibronectin islands) increased cell spreading and area and induced osteogenic differentiation; conversely an increase in plating density (or smaller fibronectin islands) generated rounded less spread cells and induced adipogenesis. The RhoA-ROCK signaling pathway was implicated in the adipogenic–osteogenic switch. Pharmacological inhibition of RhoA and its effector ROCK has shown to disrupt the cytoskeleton and affect hMSC differentiation mediated by the matrix (McBeath et al., 2004). A more recent study demonstrated that cell shape and cytoskeletal tension rather than the area, dictated hMSC lineage commitment (Kilian et al., 2010). For example, micro-patterned islands with the same area but of different shapes exhibited high or low cytoskeletal

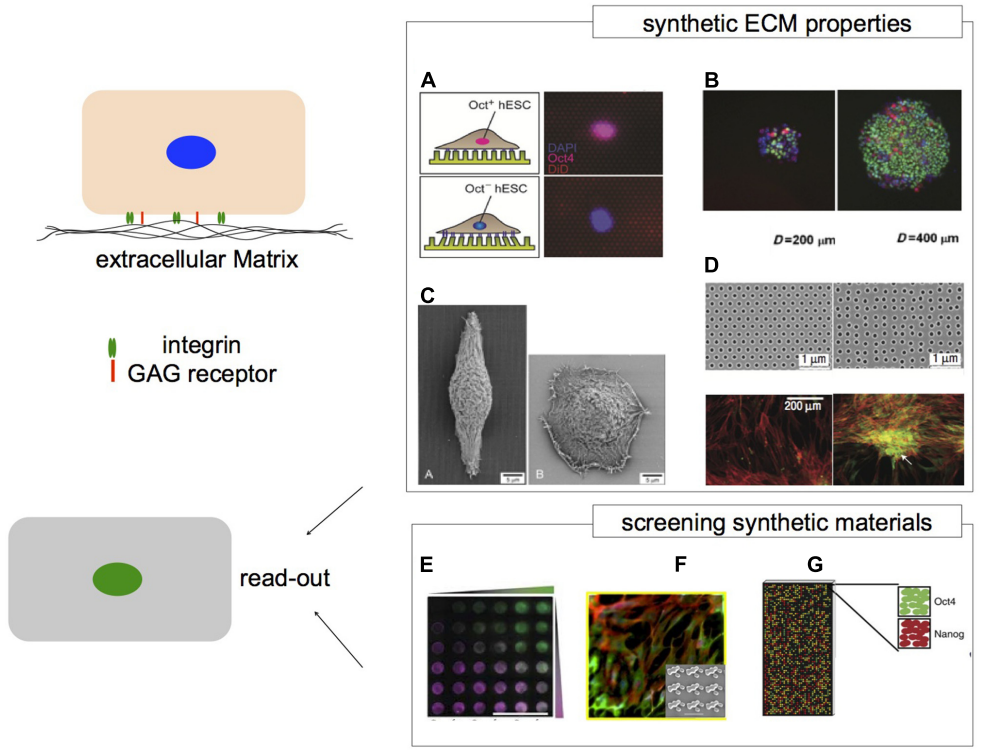

FIGURE 2 | Microenvironments and their impact on hPS cells. Cells respond to the surrounding microenvironment via cell–cell contacts and cell–matrix contacts. The ECM provides structural and chemical cues. Synthetic ECM niches can recapitulate aspects of ECM properties to regulate cell behavior. **(A)** Human embryonic stem cells that are $Oct4^+$ and $Oct4^-$ respond differently to PDMS micro-posts with varied stiffness. **(B)** hES cells grown on larger adhesive islands of 400 μm have greater levels of Oct4 expression via Smad1 signaling. Nuclei depicted in blue, Oct4 in green and pSmad1 in red. **(C)** Micro-grooved substrates (left) can alter epithelial cell morphology by contact guidance compared to flat surfaces (right). **(D)** Symmetry of nanoscale topography with semi-random geometry induces osteogenesis of hMSCs compared to hexagonal geometry. Immunofluorescence represents cytoskeleton in red and osteopontin in green. High content analysis can be applied to screen a wide array of biomaterials to associate specific properties with biological response. Platforms such as **(E)** PEG based hydrogel arrays of protein concentration gradients and PEG stiffness. FITC- and rhodamine labeled BSA gradients are represented in green and magenta, respectively. **(F)** The Topochip platform can be used to vary surface topography of the same material chemistry. **(G)** Spotted polymer arrays of varied substrate mechanical properties for long term culturing of hES cells. **(A)** Image adapted from Sun et al. (2012b). **(B)** Peerani et al. (2007), image adapted with permission from John Wiley and Sons, Copyright 2007. **(C)** Teixeira et al. (2003), image reproduced with permission from *Journal of Cell Science*, Copyright 2003. **(D)** Dalby et al. (2007), image reprinted with permission from Macmillan Publishers Ltd.: *Nature Materials*, Copyright 2007. **(E)** Gobaa et al. (2011), image reprinted with permission from Macmillan Publishers Ltd.: Nature Methods, Copyright 2011. **(F)** Unadkat et al. (2011), image adapted with permission from the *National Academy of Sciences*, Copyright 2011. **(G)** Zhang et al. (2013) image reprinted with permission from Macmillan Publishers Ltd.: *Nature Communications*, Copyright 2013.

contractility resulting in osteogenesis or adipogenesis, respectively.

Though much of what we understand of cell-shape induced differentiation has come from adult stem cells, similarities with hPS cells are beginning to emerge. By patterning Matrigel islands of 200, 400, and 800 μm, bone morphogenic protein (BMP) mediated small body size/mothers against decapentaplegic (Smad1) signaling maintained hES cell pluripotency on the largest islands that supported large, densely packed colonies (Peerani et al., 2007).The dependence of pluripotency on the size of the niche highlights in this case the role of soluble factors secreted by the hPS cells. Mechanotransduction was here not implicated, yet the biophysical signals of the microenvironment in controlling cell shape can be affected by the colony size. UV/ozone patterned vitronectin substrates have been used to study hPS cell shape and morphology at the single cell level both in the presence and absence of ROCKi (Pryzhkova et al., 2013). Patterns that can stimulate cell polarity are crucial to dissect phenomena such as cell shape induced epithelial to mesenchymal transition (EMT), which is a key step in hPS cells differentiation. EMT has also been involved in a study showing that fibroblasts cultured using parallel microgrooves or aligned nanofibers on poly(dimethyl siloxane; PDMS) present an increase in reprogramming efficiency (Downing et al., 2013).

Stem cell growth and differentiation can also be affected by micro- and nano-topographic cues such as grooves, ridges or pits. Grooved and ridged topographies for example, can induce cell alignment and elongation through contact guidance (Teixeira et al., 2003) for a number of specialized cells including differentiation of hES cells (Chan et al., 2013) and hiPS cells toward neuronal lineages (Pan et al., 2013). hES cells cultured on PDMS gratings with 600 nm feature-height and spacing also generated cell

alignment in the presence of soluble factors (Gerecht et al., 2007). The polarization of gamma-tubulin complexes (GTCs) on nanotopographies may play a role in mediating topography-induced changes in cell morphology, as GTCs can govern cytoskeletal function and assembly of filamentous actin. However, changes in hES cell shape and morphology governed by actin assembly directing eventual cell fate was not investigated.

Apart from contact guidance, the size, spacing, and orientation of nanotopographies can directly affect cell response resulting from topographies. Surface topography can also alter cell behavior indirectly from changes in the conformation of surface adsorbed proteins. Nanoscale topographies for example, arranged in a square planar geometry induced hES cell differentiation toward the mesenchymal lineage in the absence of growth factors (Kingham et al., 2013). Unlike ECM patterned islands that promote cytoskeletal tension mediated differentiation, it has been suggested that stem cell adhesion to surface topographies is mediated by the modulation of integrin clustering and focal adhesion formation (Biggs et al., 2010; Sun et al., 2012a).

Cell adhesion to the ECM can result in the recruitment, organization, and clustering of integrins and the formation of focal complexes maturing into focal adhesions and providing direct anchorage (via vinculin, talin, and paxillin) to the actin cytoskeleton. Furthermore, integrin mediated adhesion can activate tyrosine kinase and phosphatase signaling to modulate downstream signals that determine cell fate (Vogel and Sheetz, 2006). For example in hES cells cultured on nano-roughened and smooth glass substrates (Chen et al., 2012) maintenance of pluripotency was found to depend on an interplay between focal adhesion formation, cell–cell contacts and cytoskeletal rearrangements mediated by non-muscle myosin IIa (NMMIIa) on flat surfaces. On the other hand, nanotopographic pillars of hexagonal versus honeycomb arrangements (Kong et al., 2013) and nanopillar gradients of varied spacing (Bae et al., 2014) supported $Oct4^+$ cells and maintained E-cadherin expression. Moreover, focal adhesion kinase (FAK) inactivation led to a more dynamic reorganization of the cytoskeleton on the topographies of the lowest diameters. Thus, only nascent focal complexes or disrupted focal adhesions rather than mature focal adhesions were observed. These latter studies suggest that selected nanotopographies can be used to maintain pluripotency.

Despite the fact that there is inconsistency in discerning which subset of surface topographical or chemical features eventually dictates hPS cell response, there are early indications that integrins may play a role in hPS cell fate decisions. Moreover, it has been widely demonstrated that integrin-mediated adhesion to the ECM is crucial for hPS cell survival. However, it is still unclear if and how initial adhesion events activate downstream signaling cascades involved in EMT and lineage commitment. FAK activation has been suggested to act upstream of the Rho/ROCK (Bhadriraju et al., 2007) and mitogen-activated protein kinase (MAPK; Salasznyk et al., 2007) signaling pathways, both of which have been implicated in cell shape induced differentiation. Thus, the perturbation of FAK or other focal adhesion complexes or anchor proteins will need to be further explored, in particular its effect on E-cadherin expression and pluripotency.

hPSC MECHANOSENSING AND SUBSTRATE RIGIDITY

The inherent sensitivity of hPS cells arises from the relatively poor understanding of cell–cell and cell–substrate interactions underlying the maintenance of pluripotency. It is now known that hPS cells undergo dissociation-associated apoptosis and inhibiting RhoA/ROCK mediated, NMMII-dependent cytoskeletal tension enhances hPS cell survival (Ohgushi et al., 2010). As RhoA/ROCK mediated cytoskeletal tension is an important factor in mechanotransduction (McBeath et al., 2004) the cytoskeletal hyperactivation of hPS cells upon dissociation and in conjunction with loss of cell–cell contacts (through loss of E-cadherin expression) suggests that the mechanical properties of the stem cell environment may indeed be key to determining cell fate decisions. P120 catenin, an Armadillo-domain protein implicated in cell–cell adhesion is stabilized by NMMII and this process has been shown to be necessary for E-cadherin dependent mechanical tension and maintenance of pluripotency in hESCs (Li et al., 2010). When cultured on polyacrylamide (PA) gels of 8.5 kPa, continued inhibition of NMMIIa by blebbistatin markedly down-regulated E-Cadherin expression. In another study, hES cells pluripotency was maintained when hES cells were cultured on shorter, stiffer vitronectin coated PDMS microposts and the expression of Oct4 paralleled that of E-cadherin (Sun et al., 2012a). Cell–cell contacts alone, however, may not be involved in sensing matrix rigidity. For example, GAG mediated hES cell adhesion (Klim et al., 2010) to PAgels (Musah et al., 2012) also showed that hES cells preferred adhering to stiffer gels (10 kPa) and when cultured on softer gels (0.7–3 kPa) no longer expressed the pluripotency markers Oct-4 and SSEA-4. Here it was proposed that only the stiff PA gels promoted YAP/TAZ (Yes associated protein/transcriptional coactivator with PDZ binding motif) localization in the nucleus while cells cultured on softer gels exhibited low levels of cytoplasmic (i.e., inactive and the degraded form of) YAP/TAZ. YAP and TAZ serve as mechanosensors and transcriptional regulators required for cell differentiation influenced by substrate stiffness (DuFort et al., 2011) though others have suggested that TAZ functions in hES cell self-renewal (Varelas et al., 2008). The precise mechanisms of YAP/TAZ mechanotransduction in hPS cells is still unknown and more work will be required to unravel a potential role in hPS cell mechanosensing. Adhesion to ECM proteins is highly dependent on integrin-mediated adhesion. It is unclear whether the latter can convey mechanical signals.

PATHWAYS REGULATING hPSC PROLIFERATIVE BEHAVIOR

Generally, MAPK, protein kinase B (PKB), and nuclear factor κ-light-chain-enhancer of activated B cells (NFκβ) signaling are involved in supporting viability and pluripotency of hPS cells. PKB can cascade through the MAPK signaling pathway resulting in hES cell differentiation. The NFκβ signaling cascade is also involved in cell survival (Armstrong et al., 2006). As mentioned above, inhibition of the ROCK pathway can prevent anoikis of hPS cells when dissociated to single cells; however, manipulation of this pathway has been utilized in other settings. Differentiation can be initiated by RhoA and ROCK activation of myosin light chain kinase (MLCK) controlling the processes of cytoskeletal tension and stress fiber development. This phenomenon has been widely studied in

hMSC differentiation. RhoA/ROCK can induce cells to undergo fluid-flow-induced osteogenesis, while on the contrary, the inhibition of this pathway triggers adipogenesis and chondrogenesis (Arnsdorf et al., 2009). The Wnt/β Catenin signaling pathway is needed to preserve and support the pluripotency of hES cells. The actions of Wnts are growth-factor like, and can control asymmetric cell division, cell proliferation, migration, and polarity. Wnts have the ability to enhance the process of somatic cell reprogramming to generate iPS cells. The interaction of β Catenin with transcription factors Sox2, Klf4, and Oct4 can only occur via the Wnt pathway, triggering the upregulation of Nanog, which demonstrates the involvement of this pathway in cell reprogramming, maintenance of the cell in a pluripotent state, and ability for self-renewal (Kuhl and Kuhl, 2013).The Wnt pathway also promotes pluripotency and can be activated by the addition of lithium chloride as in the mTeSR medium formulation. Other signaling pathways involved in pluripotency and self-renewal are transforming growth factor-β (TGF-β), which signals through Smad2/3/4, and FGF2 which signals to its receptor, FGFR to activate the MAPK and PKB pathways (James et al., 2005; Vallier et al., 2005).

TOOLS TO STUDY SUBSTRATE EFFECTS ON CELL BEHAVIOR

Advances in synthesis and fabrication techniques have allowed for a wide range of materials suitable for applications in cell biology. Fabrication of synthetic matrices may be produced from either "top-down" or "bottom-up" approaches. As this encompasses a large body of work, the reader is directed to several reviews that summarize the types of materials used in matrix mediated stem cell differentiation (Stevens and George, 2005; Sands and Mooney, 2007; Lutolf, 2009). The most common of these materials and fabrication techniques used in cell biology for exploring the cell–materials interface are briefly discussed here.

Hydrogels are polymer networks mimicking many aspects of the native ECM and are readily hydrated. They can be easily manufactured and can be tuned to the desired elastic and viscous moduli, making them attractive for studying mechanotransduction. Most hydrogels are often composed of cell/protein inert chemistries, for example poly(ethylene glycol; PEG) and require functionalization to promote cell–material interactions. The surface functionality of PEG or PEG macromer hydrogels, can be modified by conjugating peptides or proteins to the backbone of PEG for example, via Michael-type additions requiring PEG macromers end functionalized for example with acrylate or vinyl sulfone groups that readily react with thiols (Metters and Hubbell, 2005). Other methods of conjugation may also be implemented (Liu et al., 2010). Such hydrogels can also be modulated in stiffness by tuning the cross-linking density or the molecular weight of the PEG macromer. In general, hydrogels offer an easy starting point for developing defined synthetic niches. For example, the covalent attachment of peptides such as the integrin binding sequence RGD or GAGs (Klim et al., 2010; Musah et al., 2012) or matrix metalloproteinases (MMPs; Jang et al., 2013) to result in 2D or 3D scaffolds have been used for hPS cell propagation. Other hydrogels based on hyaluronic acid (Gerecht et al., 2007) that can bind to cells via CD44 surface receptors have also been used to culture hES cells albeit using feeder conditioned medium.

Topographical features have been fabricated using a range of lithographic techniques such as electron beam-, photo/UV- or dip-pen lithography or through microcontact printing. The advantages of these methods are that features can be fabricated in a highly reproducible manner and can be highly ordered spatially. Microcontact printing (μ-CP) utilizes an elastomeric PDMS stamp consisting of the desired features, which is then used to transfer "inked" material onto a substrate. In this way, many materials such as individual ECM proteins are patterned for single cell studies (Mrksich and Whitesides, 1995; Ruiz and Chen, 2007). Photo/UV- and electron beam-lithographies on the other hand can be used to produce topographical features such as grooves, pits, and islands in the micro- and nano-meter length scales that can be ordered or disordered over large areas providing a plethora of tools for studying fundamental cell biology.

FINDING THE RIGHT NICHE: SCREENING BIOMATERIALS USING hPS CELLS

As hinted above, it is now increasingly accepted that biophysical cues arise not only from surface chemistry but also from topography. In combination with soluble factors, these can have a profound influence on determining cell fate of hPS cells. Engineered biomaterials are therefore studied to recapitulate biological complexity (i.e., combining key components of matrix properties, heterogeneity, and complexity) and understand the relationships between the physical and chemical properties of the material and its interaction with cells. To this end, screening approaches with materials in high throughput or "materi-omics" (Cranford et al., 2013) have been attempted such as the "Topochip" platform (Unadkat et al., 2011). In this study, photolithography techniques were used to generate more than 2000 unique microscale topographies (though the possibilities are far greater) by combining the primitive shapes; circles, rectangles, and triangles. Here, surfaces that promoted osteogenic differentiation of cultured hMSCs in the absence of soluble growth factors were investigated. Protein-based microarrays by robotically spotting various ECM proteins have been previously used to study hES cell–matrix interactions (Flaim et al., 2005). Additionally, others (Gobaa et al., 2011) have developed a hydrogel microwell array that combines both and physical properties to encapsulate both adherent and non-adherent cells. Such platforms have been used to probe cell–cell interactions and cell–materials interactions that drive osteo- and adipogenic differentiation of hMSCs and may be adapted to the study of microenvironments affecting hPS. The first progress in this direction involves the use of polymer arrays with inkjet printing to combine acrylate and acrylamide monomers and generate thermo-responsive hydrogels (Zhang et al., 2013). Such stimuli responsive matrices were used to mechanically disperse cells as an alternative to enzymatic dissociation while supporting hES cell proliferation and pluripotency in culture.

The increased throughput and the development of comprehensive structure–function methodologies in these cutting edge studies will allow quicker identification of the most relevant synthetic substrates for specific responses (Mei et al., 2010; Saha et al., 2011). Although many materials technologies have advanced to produce a vast selection of topographies, chemistries, and

combinations, a challenge currently faced is how to predict and quantify stem cell responses at the single cell levelusing engineered microenvironments (Treiser et al., 2010; Vega et al., 2012).

STRATEGIES FOR HIGH CONTENT ANALYSIS AND DISEASE MODELING

Together with cell culture and liquid handling, the technologies available for microscopy, image analysis, and computing have undergone an extremely rapid progress in recent times. Collectively, the confluence of outputs from such distinct fields has brought to life the discipline of high content analysis (HCA). Cells can be readily examined in real time or in cytochemistry endpoint assays. Acquired images are processed and groups of pixels are computationally segmented into defined "objects"capturing imaged cells, nuclei, or subcellular organelles (**Figure 3**).This allows quantification of proliferative behavior, morphology changes, and expression of proteins such as lineage or functional markers. Importantly this can now happen upon exposure to up to several thousands conditions per week so that the term high throughput can be appropriately used for HCA approaches as well. Complex datasets are acquired and interrogated using proprietary or open source computational tools as detailed elsewhere (Singh et al., 2014). The value of these methods in discovering new chemical entities has been demonstrated (Swinney and Anthony, 2011).

Biologically significant assays may help ensure that toxic or non-effective drugs fail *in vitro*, in the pre-clinical phases and not in the clinic, with huge benefits for the cost of the discovery process and for the patients. The field has developed substantially using cancer cell lines, typically well suited for cell based assays but of unclear biological relevance (Wilding and Bodmer, 2014). On the other hand, more relevant primary cells are often not suitable due to phenomena such as replicative senescence, differentiation, spontaneous immortalization or transformation in culture. The focus on identification of optimal cell types for HCA has highlighted hPS cells and their derivatives as an attractive alternative for a number of reasons. First, the capability to self-renew and generate a consistent starting population of cells over a number of passages reduces variability of the starting population. Secondly, together with robust differentiation protocols, hPS cells can be used to produce the high number of progenitors or differentiated cells required for screening. Additionally, patient-derived iPS cells offer unique tools to study the range of physio-pathological mechanisms involved in selected diseases at the cellular level and to identify drugs that benefit specific cohorts of patients. It is also worth to note that in some cases, diseases can present with a block of differentiation (Kuhlmann et al., 2008). Assays that are built around the specific differentiation protocols may in principle be used to screen for drugs that bypass the differentiation blocks to develop therapeutics.

HIGH CONTENT ANALYSIS APPROACHES USING HUMAN PLURIPOTENT STEM CELLS

We will not discuss further studies aimed at isolating compounds that improve reprogramming as these are discussed elsewhere (Chen et al., 2010b; Yang et al., 2011; Li and Rana, 2012). In reviewing HCA screens using hPS cells with a reasonable throughput we will start by focusing on survival and self-renewal (**Table 1**). Distinguishing between effects that are limited to prolonged survival or true long-term maintenance of self-renewal can be difficult. Visualizing hPS cell colonies formed on feeders, TRA-1-60 staining and DNA dyes were chosen as a suitable marker of pluripotency (Barbaric et al., 2010a,b). Imaging pipelines were here used to "erode" and "dilate" in parallel the segmented nuclei in order to accurately quantify on one hand the percentage of undifferentiated cells and to remove on the other from the analysis confusing data regarding feeder cells (Barbaric et al., 2011). This strategy isolated the antihypertensive drug pinacidil as a promoter of hES cells survival. Independently, the same molecule

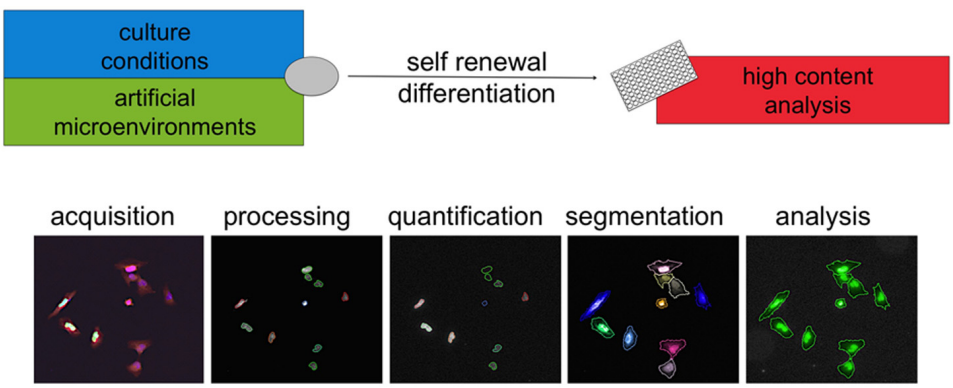

FIGURE 3 | High content analysis. High content analysis involves a series of steps to convert images into data. Cells in different conditions are imaged via microscopy. Images are then processed (for example in contrast) and then analyzed using computer algorithms. Segmentation refers to the definition based on pixels intensity of "objects" such as cells, nuclei, or other subcellular structures. Via cytochemistry, the expression of specific markers can be elicited and the levels quantified. These measures can be combined with morphological parameters and objects can be included or excluded from further analysis. This approach is now increasingly applied to the use of new artificial microenvironments to develop specific conditions allowing self-renewal or differentiation. This is likely to synergize with the advances in the understanding of human pluripotent stem cells culture and bring fast and substantial contribution to disease modeling and drug discovery.

Table 1 | High content analysis chemical screens using human pluripotent stem cells.

Source of cells	Culture conditions	Number of cells	Read out	Imaging device	Number of conditions	Proposed pathways	Reference
hES (SA461)	Single cells, fibronectin, FF, MEF-CM	5000 per 96 well	"Percent Activation" from DAPI	InCell 1000	1200+, 4100+, 15000	ROCK	Andrews et al. (2010)
hES (Shef4)	Colonies on MEFs	6000 per 96 well	Tra 1-60, Hoechst	InCell 1000	1040	ROCK	Barbaric et al. (2010b)
hES (HSF1, H9)	Single cells on MEFs, gelatin, DM	Approx 5000 per 384 well	Oct4, Hoechst	Image Xpress micro	1280+, 504	ROCK and PKB	Damoiseaux et al. (2009)
hES (H9)	Single cells on Matrigel, MEF-CM, automation	6000 per 384 well	Oct4, Hoechst	Incell 3000	2880	TGFβ, wnt, FGF2	Desbordes et al. (2008)
hES (BG01, WIBR3)	Single cells on polymer, MEF-CM	Low density, 40 cells mm^{-2}	Oct 4, SSEA-4	iCys laser scanning, AFM	496 (materials)	–	Mei et al. (2010)
hES (HEUS9)	Single cells on Matrigel, DM	4000 per 384 well	ALP, compact colony morphology	Inverted micro-scope	50,000	E-cadherin	Xu et al. (2010)

Abbreviations: PKC, protein kinase C; AP, alkaline phosphatase; MEF, mouse embryonic fibroblast; CM, conditioned medium; FF, feeder free; DM, defined medium; FGF-2, fibroblast growth factor 2.

was found using hES cells feeder free with conditioned medium; here the authors showed pinacidil as well as other compounds to be structurally related to classic ROCK inhibitors (Andrews et al., 2010). Similar approaches have been tried elsewhere and identified compounds that improved survival by inhibiting ROCK or protein kinase C (Damoiseaux et al., 2009; Sherman et al., 2011). Notably, automation and culture in 384 wells was applied to hES cell culture in a single-cell dissociation protocol using conditioned medium and quantitation of the pluripotency marker Oct4 together with a nuclear dye to isolate compounds promoting differentiation or expansion (Desbordes et al., 2008; Desbordes and Studer, 2013).In line with the importance of cell–cell contacts and cell–ECM contacts described above, it is interesting to note that a central role for signaling involving E-cadherin, RhoA/ROCK pathway and integrins in survival has also been proposed based on HCA screening (Xu et al., 2010). A different angle was taken in (Ben-David et al., 2013) to use HCA to identify compounds that selectively eliminate hPS over differentiated cells for future cell therapeutic application to mitigate the risk of teratomas. Over 50,000 compounds were screened against undifferentiated hES on matrigel with mTeSR1. Cytotoxic compounds were then tested on nine different cell types with very stringent criteria. A selective inhibitor, PluriSIn #1 was identified this way. Very few attempts have been reported to challenge hPS against a substantial number of materials as screening conditions. Among these, in Mei et al. (2010), combinations of monomers were screened on a primary array for colony formation using transgenic Oct4-GFP hES cells flow sorted and seeded in near-clonal density. The results were then compared with an analysis of physical characteristics of the materials. Surface roughness, indentation, elastic modulus, and wettability were included. Self-renewal ability of cells was showed to be dependent on adsorbed proteins, surface chemistry and the geometry of the spot the cells were occupying. Several hit polymers coated with vitronectin in mTeSR1 were suggested as the most advanced culture conditions. It is likely that future studies will include chemistries and materials as it has been attempted for MSC. Altogether, the majority of these studies use end-point assays that require fixation and staining of cells. An alternative or complementary HCA tool is live imaging. This has proved effective in iPS-derived neural cells (Danovi et al., 2010) and also as a tool to distinguish cells that are genuinely reprogrammed (Chan et al., 2009). Because of the substantial improvement in the field and its power, we predict that live imaging will raise in importance using hPS cells for screening and will be applied in synergy with end-point assays and the use of artificial microenvironments to model diseases.

HCA, hPS CELLS, DISEASE MODELING, AND DRUG DISCOVERY

Disease modeling is possibly the most immediate potential application of hPS cells to therapy. The majority of studies have used selected small number of compounds in hypothesis testing experiments. We will cover selected cases and refer the reader to recent reviews for a more exhaustive perspectives (Maury et al., 2012; Robinton and Daley, 2012) and for neural diseases (Xu and Zhong, 2013; Imaizumi and Okano, 2014).

Seminal studies on Spinal Muscular Atrophy (Ebert et al., 2009) offered the proof of principle that it is possible to obtain reprogrammed cells from patients suffering a specific disease. The authors also reported a disease phenotype: patients-derived cells proved impaired in their neuronal differentiation and gave rise to neurons that were smaller in size, lacking the SMN protein and specific nuclear gems structures. Selected compounds,

valproic acid and tobramycin could rescue this phenotype. Ectopic expression of SMN was later shown to rescue a similar phenotype (Chang et al., 2011). Amyotrophic Lateral Sclerosis was also the subject of recent attention. Screens using motoneurons from both wild-type and mutant SOD1 mouse model allowed the isolation of hits compounds. Kenpaullone was then validated using cells differentiated from patients-derived iPS cells and improved the survival of motoneurons more than compounds that recently failed in ALS clinical trials (Yang et al., 2013; see also Makhortova et al., 2011; Burkhardt et al., 2013; Chestkov et al., 2014).

Alzheimer has also been modeled (Yagi et al., 2011) and drug screening platforms have been proposed (Yahata et al., 2011; Xu et al., 2013). A recent study pointed toward the limitation of classic HCA assays in 2D for this disease. Neurons from iPS-derived neuroepithelial cells were derived. From these, the detection of canonical features of the disease was possible in 3D but not in classic assays (Zhang et al., 2014). Schizophrenia has been investigated extensively in terms of its genetic etiology with no conclusive consensus. A number of differences in phenotype and gene expression were observed in cells derived from patients versus healthy individuals (Brennand et al., 2011). Importantly, key cellular and molecular traits were shown to ameliorate with an antipsychotic, loxapine. Other important studies were reported for dementia (Almeida et al., 2012) and familial dysautonomia (Lee et al., 2009, 2012). Parkinson's disease (PD) is particularly difficult to model using current hiPS cells. Cells reprogrammed from a patient that showed triplication of the alpha-synuclein locus and their comparison with those derived from an unaffected first-degree relative represent a significant step forward (Devine et al., 2011). The pathological aggregation of alpha-synuclein present in PD-affected neurons was recapitulated in iPS-derived cells. Then the authors turned to yeast to identify early pathogenic phenotypes and showed that a small molecule (NAB2) and its target Nedd4 could rescue the disease phenotype (Chung et al., 2013). Another important study focusing on PD offers a fascinating insight in the differences and similarities between senescence and aging while proposing an interesting option to model late-onset diseases overcoming the "rejuvenation" that is triggered with reprogramming (Miller et al., 2013).

Beside neurological diseases, several attempts modeling cardiac diseases were undertaken. Cardiomyocytes derived from patients carrying LEOPARD syndrome, an autosomal dominant developmental disorder (Carvajal-Vergara et al., 2010) showed larger higher sarcomeric organization and preferential nuclear localization of NFATC4 in the nucleus when compared with cardiomyocytes derived from hES cells or hiPS cells from healthy sibling donors. Congenital long QT syndrome is a familiar arrhythmia. Cardiomyocytes derived from hiPS from type 2 long QT syndrome patients showed significant prolongation of the action-potential duration and were used to evaluate the effects of channel blocker drugs (Itzhaki et al., 2011) reviewed in (Friedrichs et al., 2013). In some cases gene abnormalities have been corrected in hiPS cells opening interesting prospects for both cell therapy and disease modeling (Yusa et al., 2011; see also Choi et al., 2013). Despite the impressive progress in the field, very few studies have successfully recreated features of the disease in a cell-based assay robust enough to be screened with a substantial number of conditions on hiPS-derived cells.

CONNECTING THE DOTS

In order to allow HCA, several requisites are necessary as summarized in (Engle and Vincent, 2014). Cell robustness and reproducibility, in vitro differentiation, reasonable throughput, relevance, assay characteristics, screening cascade design are all of paramount importance to achieve meaningful disease modeling. We envision that in the near future, synthetic materials and sophisticated HCA analysis including bright field label free live imaging will enrich the palette of tools available. There is a growing awareness that the understanding of pluripotent stem cells, the definition of culture conditions, the engineering of optimal substrates and the development of appropriate HCA pipelines can be combined toward disease modeling.

Recently, several projects have been launched aimed at establishing multidisciplinary frameworks to characterize several hundreds lines derived from patients and/or healthy individuals. Some examples include the California Institute for Regenerative Medicine (CIRM), the New York Stem Cell Foundation (NYSCF), the Harvard Stem Cell Institute iPS Core Facility, the hPS cell database at the National Institute of Health (StemCellDB NIH), all based in the United States. In Asia, the China IPSCs program and the Japan Science and Technology (JST) agency among others also hold stem cells programs. European initiatives include the European Bank for induced pluripotent stem cells (EBiSC), The Innovative Medicine Initative (IMI)-funded StemBancc, and the hiPS cells initiative (HipSci). Combined these programs will generate hiPS cell lines from approximately 10,000 individuals. HipSci was established in November 2012, headed by Prof. Fiona Watt (London) and Prof. Richard Durbin (Cambridge). Engagement of the clinical genetics community, open access model of data sharing and collaborative cell phenotyping are key features of the project. A bank of several hundred iPS cell lines is being generated and extensive genome, epigenome, proteome, and phenotype analysis is being carried out at the partnering centers. The project aims to develop a baseline analysis for iPS lines from healthy individuals and valuable assays for rare diseases for which calls for proposals have been launched.

In conclusion, the knowledge required to capture the complexity of the field is broad in spectrum; the technology needs to remain focused on the development of relevant complementary tools while exploring the synergies between these. Our goal in this review is to transmit a sense of the diverse backgrounds required for this purpose. An impressive set of resources is being devoted through innovative platforms and bridging between governmental, academic, and commercial partners to expand the core of competencies around stem cells, artificial microenvironments, and HCA. Our hope is this will soon allow to harness the full potential of hPS cells to model diseases and to develop therapeutics.

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The value of translational biomarkers to phenotypic assays

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Phenotypic assays are tools essential for drug discovery. Phenotypic assays have different types of endpoints depending on the goals; (1) empirical endpoints for basic research to understand the underlying biology that will lead to identification of translation biomarkers, (2) empirical endpoints to identify undesired effects related to toxicity of drug candidates, and (3) knowledge-based endpoints (biomarkers) for drug discovery which ideally are translational biomarkers that will be used to identify new drug candidates and their corresponding molecular mechanisms of action. The value of phenotypic assays is increased through effective alignment of phenotypic assay endpoints with the objectives of the relevant stage in the drug discovery and development cycle.

Keywords: drug discovery, phenotypic screening, molecular mechanism of action, MMOA, biomarkers, target-based

INTRODUCTION

The goal of the paper is to provide awareness that a key feature of phenotypic assays for drug discovery is the relationship of the measured endpoint to a biomarker that translates to the desired clinical response. In the early research phase phenotypic assays can be used to increase understanding of the disease and to identify potential translational biomarkers, while in the application phase in which the underlying knowledge of the disease is translated to treatments phenotypic assays should be aligned with translational biomarkers. Examples of drug discovery strategies show that the phenotypic endpoints for many of the successful strategies used previous knowledge that effectively translated to clinical outcomes.

Phenotypic assays measure a phenotype in a physiological system. The term "phenotypic assay" includes all preclinical assay formats that use physiological systems, e.g., animals, cells, and biochemical pathways. Phenotypic assays make few assumptions as to the molecular details of how the system works and provide an empirical method to probe effects in physiological systems. The phenotype most relevant to the practice of drug discovery is a phenotype that directly translated to the clinical disease (translational biomarker).

Phenotypic assays have always played an important role in drug discovery. Much of early pharmacology and drug discovery was based on phenotypic assays. Phenotypic assays were used to identify leads that provided the desired efficacy. In his nobel lecture entitled "Selective inhibitors of dihydrofolate reductase" George H. Hitchings Jr. stated "Those early, untargeted studies led to the development of useful drugs for a wide variety of diseases and has justified our belief that this approach to drug discovery is more fruitful than narrow targeting"(Hitchings, 1988). In the last decades of the 20th century the emphasis of drug discovery changed to a more reductionist, target-based approach and phenotypic assays were primarily used to confirm efficacy and evaluate safety. The hope was that the molecular and genetic revolution would provide numerous new medicines due in part to the capabilities to identify many new drug targets. Though not explicitly stated, the idea was that the drug targets would be biomarkers for the disease. Accordingly, a new paradigm of drug discovery emerged in which the target was the biomarker for disease. In this paradigm the central features are (1) identification of a molecule that binds to that target and (2) optimization of the biopharmaceutics properties such that the drug concentrations in the body are sufficient to ensure that the drug is bound to the target through-out the dosing interval. This target-based paradigm was envisioned to provide a more rational approach to drug discovery, analogous to a design and engineering approach. It is well documented that this approach has not produced the desired results and in fact productivity has dramatically decreased (Scannell et al., 2013).

Phenotypic assays in animals have always been required to evaluate the safety of a drug substance. In the last few years there has been a reemergence of interest in using phenotypic assays to drive discovery. Swinney and Anthony (2011) analyzed the discovery strategies for new molecular entities (NMEs) that were approved by the U.S. Food and Drug Administration (FDA) between 1999 and 2008. Of the 259 agents that were approved, 75 were first-in-class drugs with new molecular mechanism of action (MMOAs), and out of these, 50 (67%) were small molecules and 25 (33%) were biologics. The results also showed that the contribution of phenotypic screening to the discovery of first-in-class small-molecule drugs exceeded that of target-based approaches—with 28 and 17 of these drugs coming from the two approaches, respectively—in an era in which the major focus was on target-based approaches. A more recently analysis by Swinney and Xia (2014) of the 102 NMEs approved between 1999 and 2012 for rare diseases showed a similar trend of success with phenotypic strategies; for first in class NMEs there were 15 that used phenotypic drug discovery (PDD), 12 that used target-based drug discovery (TTD) and 18 for biologics.

What is required to realize the full value of PDD in the 21st century? There are many aspects that are important including the quality of the assays, the sources of drug substance and the

Abbreviations: MMOA, molecular mechanism of action; PDD, phenotypic drug discovery; TDD, target-based drug discovery.

strategies to move compounds forward through development despite incomplete knowledge of their mechanisms of action. Another important feature is the choice and predictability of the phenotypic endpoint, which is the focus of this short report.

THE DRUG DISCOVERY AND DEVELOPMENT CYCLE

The approval of a medicine to treat an unmet medical need involves an iterative cycle of testing and learning. **Figure 1** describes some of the important phases in the process. The process of discovery and development of a new medicine is initiated in response to an unmet medical need to treat a disease. Physiological, genetic, and chemical knowledge provide an understanding of the disease. This knowledge will lead to the identification of translation biomarkers that are used to evaluate the effectiveness of a potential medicine. This is the research phase (12–3 o'clock). Phenotypic assays run in the research phase are extremely important to the understanding of the underlying biology and to help identify translational biomarkers.

The knowledge obtained in the research phase is used to inform the discovery phase (3–6 o'clock). The available knowledge informs drug discovery strategies which are used as starting points for the practical process of discovering a new medicine. TDD is associated with modulating a specific gene product known as the target, PDD is a strategy driven by assays which measure phenotypes associated with the disease. Ideally these phenotypes will be the associated with the translational biomarkers. These two strategies are primarily focused on small molecules and are medicinal chemistry intensive, in contrast to biologics which use recombinant proteins and antibodies as therapeutics. It should be noted that the knowledge to choose a strategy is generally incomplete, however the more iterations that occur in the drug discovery/development cycle the more complete the knowledge and the better chance that a molecule will make it to registration. The discovery strategies will result in a lead molecule, ideally with activity against the translational biomarker. The molecule will work by a MMOA that provides an optimal therapeutic index. These molecules will then be optimized for biopharmaceutics properties and safety to provide a drug candidate. At this point the process of drug discovery is complete and the molecule should succeed or fail based on its own merit.

The left hand of the circle (from 6 to 12 o'clock) is the development phase of drug discovery which involves testing for safety and efficacy in humans leading to registration. Multiple iterations are generally required before a medicine with sufficient efficacy at a safe dose is discovered, tested in humans, and registered.

HYPOTHESIS, MMOA, AND BIOMARKERS

Phenotypic assays have always been required to evaluate safety and recently, their value to identify efficacious molecules and their mechanisms of action for first-in-class medicines has been reevaluated (Swinney and Anthony, 2011; Kotz, 2012; Lee et al., 2012; Lee and Berg, 2013; Swinney, 2013a; Swinney and Xia, 2014). A major challenge in the identification of safe medicines is to identify MMOAs that provide both sufficient efficacy and safety (Swinney and Anthony, 2011; Swinney, 2011, 2013a). These MMOAs could be thought of as "pharmacological hot spots." Due to the dynamic complexity of physiology both at the molecular and systems level it is difficult to *a priori* predict the exact interactions and molecules that will elicit a safe, therapeutically useful response, Empirical phenotypic assays provide an unbiased approach to identify the "pharmacological hot spots."

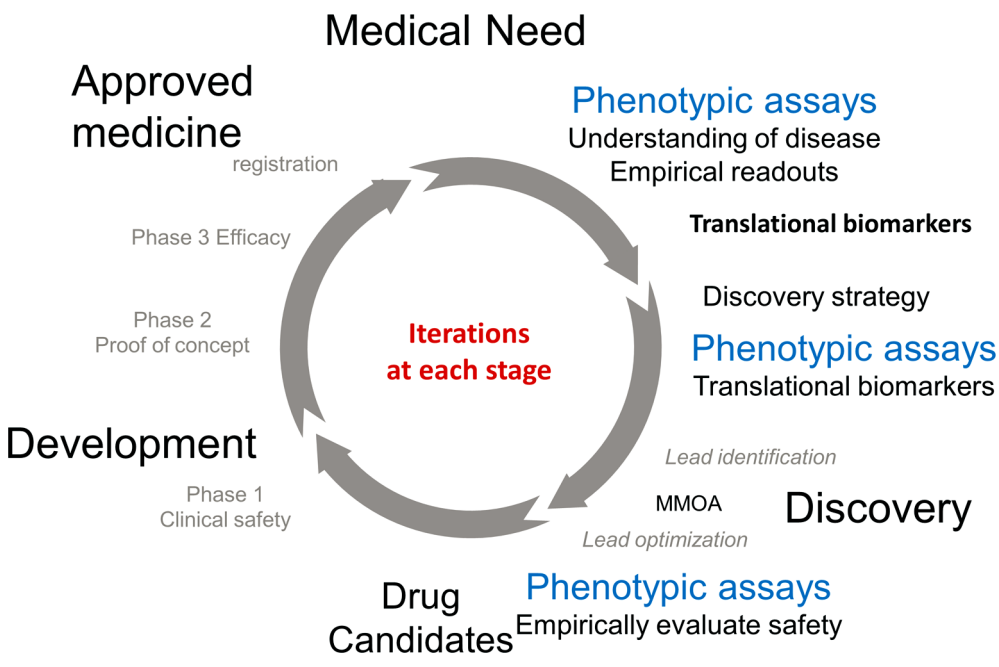

FIGURE 1 | Drug discovery research and development cycle.

In our earlier work we noted that in the target-based approach, drug discovery is generally hypothesis-driven, and in this case, there are at least three hypotheses that must be correct to result in a new drug (Swinney and Anthony, 2011). The first hypothesis applies to all discovery approaches: the hypothesis that activity in the preclinical screens used to select a drug candidate will translate effectively into clinically meaningful activity in patients. The other two related hypotheses are that the target selected is important in human disease and that the MMOA of drug candidates at the target in question is one that is capable of achieving the desired biological response. Successful first-in-class, TDD requires the time and resources to investigate all three hypotheses, and in particular, the importance of hypothesis-testing to identify an appropriate MMOA may be an underappreciated challenge, that – if neglected –could contribute to increased attrition for such approaches. In other words, it is clearly difficult to rationally identify the specific molecular interactions, from all the potential dynamic molecular interactions, that will contribute to an optimal MMOA. Thus the key biochemical nuances important for translation of the molecular interaction between a drug and the target to an optimal pharmacological response could be missed with target-based approaches. One value of the phenotypic approach is the unbiased identification of the MMOA.

PHENOTYPIC ASSAYS FOR DRUG DISCOVERY

The endpoints for phenotypic assays can be anything that can be accurately measured and range from a systems end point such as blood pressure and seizures, to specific biomarkers including

Table 1 | Discovery biomarkers for phenotypic assays of NMEs approved by US FDA between 1999 and 2008 (Swinney and Anthony, 2011).

Generic name	Discovery biomarker/assay	MMOA
Aripiprazole	Dopamine sensitive assays in animals	Partial agonist D2 receptor
Azacitidine	Cell based assays show effects on differentiation	Irreversible
Caspofungin Acetate	Inhibition of glucan synthesis *in vitro*	Non-competitive
Cilostazol	Blood platelet aggregation	Inhibitor
Cinacalcet Hydrochloride	Increased in Ca+2 in bovine parathyroid cells	Allosteric activator
Daptomycin	Cytotoxicity in antimicrobia	Unknown
Docosanol	Viral infection assays	Unknown
Ezetimibe	Cholesterol lowering in animals	Transporter slow kinetics
Fulvestrant	Binding followed by animal studies	Antagonist induced degradation
Levetiracetam	Audiogenic seizure susceptible mice	Unknown
Linezolid	Random screening against bacterial disease in plants	Conformational trap
Lubiprostone		Unknown
Memantine Hydrochloride	Originally identified in early 1960s as anti-diabetic	Uncompetitive fast kinetics
Miglustat	Glycolipid biosynthesis in HL-60 cells	Reversible inhibitor
Nateglinide	Hypoglycemic effects in fasted normal mice	Fast kinetics
Nelarabine	Cell based assays required for activation	Chain terminator
Nitazoxanide	Antimicrobial	Redox/irreversible
Nitisinone	Compounds originally discovered in screening against plants	Irreversible
Pemirolast Potassium	IgE induced anaphylaxis in animals	Unknown
Ranolazine	Animal models	Unknown
Retapamulin	Antimicrobial assays against resistant organism	Allosteric inhibitor
Rufinamide	Animal anticonvulsant	Unknown
Sinecatechins/green tea extract	No screening herbal/evaluated in humans	Unknown
Sirolimus	Screened in antimicrobial assays	Conformation inhibition
Varenicline	Focused approach culminating in animal assays	Partial agonist Nicotinic receptor
Vorinostat	Cell based assay/cytodifferentiation	Enzyme inhibitor
Ziconotide	Intra-cerebral injection into mice	Ion channel equilibrium kinetics
Zonisamide	Animals models of epilepsy	Unknown

blood cholesterol and glucose for hyperlipidemia and diabetes, respectively. Current technologies in genomics and high content analysis allow measurement of many different markers of activity. For drug discovery it is important to understand and differentiate if these markers are translational biomarkers related to the clinical disease.

A closer look at the discovery biomarkers for the 28 NME's categorized as phenotypic in the analysis of how medicines were discovered by Swinney and Anthony (2011) is shown in **Table 1**. As previously reported 10 of the medicines were identified in animal studies (Swinney, 2013b). The phenotypic endpoints for the studies were well correlated with clinical indications. Levetiracetam, rufinamide, and zonisamide were identified in well-establish models for anti-convulsant activity and aripiprazole in dopamine dependent activity known to be associated with anti-psychotic behavior. Ziconotide was discovered in a model for pain and ranolazine in animal model measuring anti-anginal and anti-ischaemic effects. The endpoints for ezetimibe, nateglinide, and pemirolast were blood cholesterol, blood glucose, and cutaneous anaphylaxis, respectively. Nitisinone is used to treat tyrosineamia type 1 and was originally developed as a herbicide and repurposed for the rare disease when safety studies demonstrated an effect on tyrosine metabolism (Swinney and Anthony, 2011; Swinney, 2013b).

The phenotypic end points for those discovered using cell based assays provide examples where cell death was used as the phenotypic marker. These included azacitidine, daptomycin, linezolid, nelarabine, retapamulin, and sirulimus, all were approved for use as either anti-infective or anti-cancer therapies. Vorinostat was discovered by its ability to induce cytodifferentiation and growth arrest. The phenotypic markers for docosanol and cilostazol were viral replication and platelet aggregation, respectively. For the discovery of varenicline mesolimbic dopamine levels were measured and for fulvestrant the estrogenic effects.

The phenotypic marker for cinacalcet was an increase in calcium [Ca^{+2}] in bovine parathyroid cells. The investigators were looking to agonize a calcium receptor. Miglustat was prepared and tested to interfere with glycoprotein synthesis and was repurposed for the Gaucher's disease, a glycosphingolipid storage disorder.

The phenotypic readouts for all these NMEs were well validated markers of physiological functions. Cell death, anti-convulsant activity, calcium activation, platelet aggregation, viral replication, blood cholesterol, and glucose levels all translate to clinical disease. In the course of the iterative R&D cycle these biomarkers have become validated translational markers used to align drug discovery with clinical development (**Figure 1**).

FUTURE TRENDS

The goal of drug discovery is to identify medicines that can benefit patients at safe doses. The challenge to achieve this goal is to identify medicines that will be safe and efficacious prior to testing in human studies, in preclinical studies. The major point highlighted in this short paper is the importance of translational biomarkers for PDD and to point out the difference between phenotypic assays that are used to investigate the underlying disease biology. Both are important, the research assays can be used to identify translational biomarkers and the discovery assays apply this knowledge to identify new medical treatments. There is a great need for validated translational biomarkers to guide drug discovery in order to identify safe and effective medicines prior to clinical evaluation. This is key to decreasing attrition and increasing productivity of pharmaceutical research.

The reality is that the more relevant the system is to physiology the better it will predict the clinical success. Associated with this complexity is the feasibility of obtaining predictive information. Phenotypic assays that translate effectively to human disease will always be required for the reasons described above, including the ability to identify an optimal MMOA and derisk safety. Unfortunately predictive phenotypic assays and relevant biomarkers are not available for most human diseases. One of the hopes for the genetic revolution was to identify specific genotypes, genes, and targets that could be used to guide preclinical drug discovery to identify new medicines. This approach has not been as widely successful as hoped. Aligning these efforts to identify translational biomarkers for phenotypic assays should increase the successful discovery of new medicines.

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In vitro clinical trials: the future of cell-based profiling

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The drug discovery process classically revolves around a set of biochemical and cellular assays to drive potency optimization and structural-activity relationship models. Layered on top of these concepts is the inclusion of molecular features that affect final drug use, things like: bioavailability, toxicity, stability, solubility, formulation, route of administration, etc. Paradoxically, most drugs entering clinical trials are only tested in a handful of human genetic backgrounds before they are given to people. Here we review efforts and opine on the use of large scale *in vitro* cellular and *in vivo* models that attempt to model human disease and include diversity found in the human genetic population. Because hundreds to thousands of individual assays are needed to scratch the surface of human genetic diversity, sophisticated high throughput automation technologies or pooling and deconvolution strategies are required. Characterization of each model needs to be extensive to enable non-biased informatics based modeling. Such approaches will enable deep understanding of genetic to pharmacological response and result in new methods for patient stratification in the clinic. Oncology medicines and cancer genetics have been paving the way for these approaches and we expect to see continued expansion to other fields such as immunology and neuroscience.

Keywords: *in vitro* clinical trials, cell line profiling, cell panels, genetically defined models, iPS derived cell models, high throughput screening

INTRODUCTION

When a drug discovery project starts, a project team must conduct an important thought experiment: if a perfect molecule that meets all of the team's criteria was suddenly available, what patients would be selected and what clinical assays would be used to demonstrate efficacy. In genetically well-defined diseases this can be a conceptually straight-forward task. For example in monogenic, recessively inherited diseases, like sickle cell anemia or spinal muscle atrophy, patients are readily identifiable by symptoms in the clinic and confirmed genetically. A homogenous population of patients provides the best chance of achieving a high signal to noise readout in the clinical trial. This is because all the patients should be similar in their molecular pathology and patients treated with a targeted drug should, in an ideal scenario, be obligate responders.

Cancer, at a fundamental level, is more heterogeneous, but patient selection strategies have made tremendous strides in the last decade. An example is the use of Gleevec and other Abl kinase inhibitors in chronic myelogenous leukemia (CML) patients that harbor the Philadelphia Chromosome translocation creating a BCR-Abl gain of function fusion protein. Prior to the use of Abl inhibitors, approximately 10,000 people in the US died each year due to CML; that number has dropped to less than 500 people per year since the introduction of these drugs to the market. Other examples of therapies "targeted" to selected cancer types are abundant and include B-RAF, C-KIT, p53-MDM2, c-MET, JAK1, and EGFR. In many cases these therapies lead to a remission, not a cure, and this is likely a reflection of the genetic heterogeneity of the original tumor and a result of the subsequent selective pressure under drug treatment that enable resistant cells to continue to grow. Ultimately, this will lead to drug resistance. New next gen sequencing (NGS) technologies are tremendously impacting the field's understanding of the spectrum of mutations and underlying heterogeneity in a tumor.

Other "common" diseases, like Alzheimer's disease, Type II Diabetes, and Schizophrenia are thought to be at least partially driven by common genetic variants. The genetics are complicated but the prevailing theory is that small changes in gene regulation, likely at the level of mRNA, slightly predispose an individual for a disease. As subtle genetic variations accumulate in an individual, that person's risk of developing the disease also increases. Due to this, genetic stratification in common disease populations is extremely difficult. Nonetheless, common variant research has identified examples where genetic testing of patients can be used to select patients for clinical trials. These include people who are homozygous for a common variant in APOE, ApoE4, whom are 10 times more likely to develop Alzheimer's Disease relative to persons not harboring the ApoE4 variant. An excellent review by Plenge et al. (2013) draws connections between common variants and disease understanding; it outlines strategies for using genome wide association studies and other human genetic data to select drug targets and stratify patients.

Cancer therapeutics have already benefitted from the use of genetically defined human cell panels. This approach should be applicable to monogenic neurological disorders and eventually to other indications where predictive cellular models can be

generated. The goal of "*in vitro* clinical trials" will be to change the arc of drug development, from a paradigm where many therapies are dropped between optimization and candidate selection (**Figure 1**). Human cellular models will help bridge this gap (as illustrated in **Figure 1**, dashed line), speeding the development of new drugs and our understanding of human disease. In this Technology Report, we aim to highlight past examples of cell panel screens and point to future applications of the approach that will extend past cancer to rapidly evolving areas like neurobiology.

GENETIC STRATIFICATION WITH CANCER CELL LINES – HISTORICAL PERSPECTIVE

In the late 1980's the national cancer institute (NCI) began profiling a set of approximately 60 human cancer cell lines in what became known as the NCI-60 panel (Shoemaker, 2006). The profiling platform was the first to enable researchers with drug candidates to find out whether their molecule of interest displayed any selectivity towards certain cancers and to focus a molecule in indications based on pre-clinical data. What was remarkable about the effort was the realization that cancer cell lines could capture some of the diversity of patient cancers and that with brute force effort, one could emulate a small scale clinical trial resulting in some predictive value. The NCI-60 was also pioneering from a technical perspective, establishing rigorous methods for genetically distinguishing cell lines from each other and enabling methods for large scale data analysis across many cell lines and compound treatments so that overlapping bioactivity between small molecules could be easily observed. Some of the major findings that have emerged from the NCI-60 effort include the link between TGFα-PE38 and EGFR expression, halichondrin B being identified as a microtubule inhibitor, and bortezomib/Velcade^{TM} as a proteasome inhibitor. Additionally, the recognition that cells could pump out a small molecule via the multidrug resistance pump (e.g., MDR and PgP) and that this could be modeled across cell lines could only be appreciated in the context of a panel of cell lines; the NCI-60 effort was truly pioneering in this approach.

The evolution of approaches to measure genomic chromosomal aberrations led Garraway and Sellers to look for genetic amplifications across the NCI-60 panel of cell lines as a potential means to identify new oncogenic drivers (Garraway et al., 2005). This work led to the discovery of microphthalmia-associated transcription factor (MITF) as a lineage specific oncogenic transcription factor in melanoma. Using single nucleotide polymorphism (SNP) arrays, the NCI-60 cell lines, spanning nine different cancer lineages, were assayed for chromosomal alterations. Hierarchical clustering was used to organize the cell lines by copy number alteration and the observation was made that the cell lines largely clustered by cancer type leading the authors to postulate that the chromosomal alterations driving these clusters might contain lineage-specific cancer genes. One of the most striking clusters was chromosome 3 (3p13–3p14) in the melanoma lineage. In their subsequent studies the authors used gain-of-function and loss-of-function approaches to demonstrate that MITF is capable of cooperating with mutant BRAF to transform normal cells and that MITF knockdown causes loss of viability in MITF copy-gain melanoma cells. This study served as another example of the early use of cell panels and their utility in leading to significant biological discovery that would not have been possible with individual disease models.

The approach taken by Garraway and Sellers was further extended in a report by Solit et al. (2006) where the genetic composition of the NCI-60 cell lines was compared against compound activity profiles, allowing the authors to link MEK inhibition to BRAF mutant melanoma. Subsequent studies have led to new applications such as that of Neve et al. (2006). Using a panel of 51 breast cancer cell lines and Trastuzumab, they identified an important relationship between HER2 amplification status and response to Trastuzumab treatment (Neve et al., 2006).

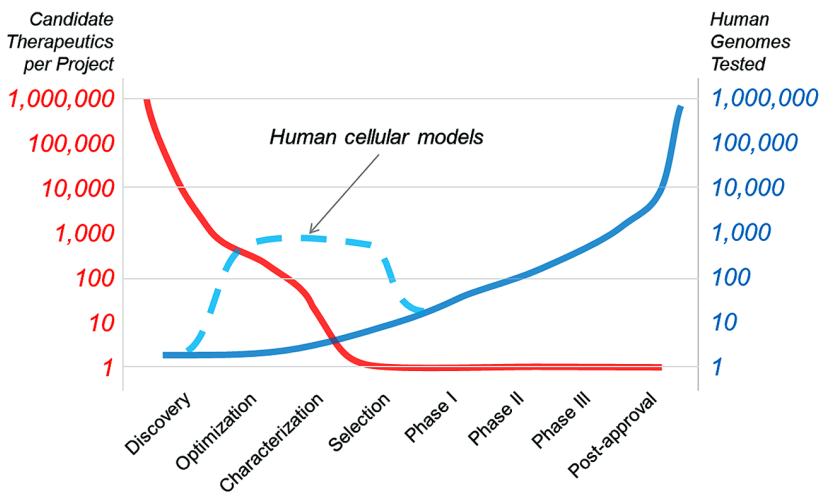

FIGURE 1 | The goal of "*in vitro* clinical trials" is to use genetically characterized cell panels in an effort to reduce the number of potential therapies dropping out of clinical development before entering clinical trials and to better inform patient stratification.

Pushing the cancer cell panel approach further, McDermott et al. (2007) established a panel of 500 cancer cell lines and tested 14 kinase inhibitors across this panel of lines. Their data confirmed the established relationships between EGFR, HER2, MET, and BRAF inhibitors and cell lines with the respective mutant gene. As had been observed previously at the NCI, the cell panel approach allowed for compounds with similar bioactivity profiles to be grouped together, revealing previously unappreciated biological activity and/or compound polypharmacology. In a subsequent manuscript McDermott et al. (2008) expanded their cell panel to 602 cell lines and detailed the relationships between ALK inhibitors and ALK mutant cancers. More recently several teams have established large cell line panels of > 500 cancer cell lines and have published profiling results of a sub-set of the compounds screened. These efforts include those of MGH/Sanger group (Garnett et al., 2012) and the Broad/Novartis Cancer Cell Line Encyclopedia effort (Barretina et al., 2012). There are several good reviews on the development of these large scale cancer cell panels (Shoemaker, 2006; Sharma et al., 2010; Caponigro and Sellers, 2011) and their application to patient selection and pharmacological compound analysis.

TECHNICAL CONSIDERATIONS FOR THE SUCCESSFUL APPLICATION OF CELL PANELS AND OPPORTUNITIES FOR IMPROVEMENT

Many of the same challenges that were faced by the NCI-60 team in the 1980's still exist today. First among these is the quality control (QC) of cell line identity. As has been the case since the inception of the cancer cell line screening panel, knowing the absolute identity of each cell line screened is critical for all subsequent analyses. Early on, HeLa cell contamination was mitigated by cytogenetic testing of cell lines via chromosome banding (Shoemaker, 2006). This process was also able to tease out cell lines derived from a common origin, adding an important layer of information for future data analyses. Since this time, technology in the field has evolved, starting with restriction length polymorphism analysis, on to DNA fingerprinting, then spectral karyotyping, and most recently, short tandem repeat (STR) or SNP testing. Other practices for QC, important in the past and still relevant today, are mycoplasma testing of all cell lines at each iteration of a panel screen and limiting the experiment to a common media type for tissue culture. In recent years, high-precision automation and the use of 1536-well format cellular assays have vastly increased the throughput at which cancer cell line panels can be screened. While this is powerful in light of the volume of data generated, which can lead to more interesting correlations of compound-cell activity, the requirement for appropriate QC measures will be even more important.

Much like the technology for cell line QC, assay readouts have also advanced over the last 30 years. NCI's pioneering work led to the development of a high throughput screening assay using sulphorhodamine B as a read out (Shoemaker, 2006). This assay proved robust and conveniently included a fixation step before reading, which was highly desirable before the advent of modern automation equipment. Other early examples of large scale screening used 96-well plates and a cell-fixation assay with a fluorescent nucleic acid stain (McDermott et al., 2007). These protocols were modernized as part of the Cancer Cell Line Encyclopedia (CCLE) effort, when industrial-scale screening systems were brought to bear on the cell profiling challenge. In the published CCLE screen Novartis adapted the assay to 1536-well format and used Cell Titer Glo (Promega) as a one-step luminescent assay for cell viability (Barretina et al., 2012). However, even in this modern interpretation of the NCI-60 assay some methods remain the same, such as fixed seeding densities and standard media for all cell lines tested.

While proper QC is essential for yielding good data, selection of the ideal metrics for comparing compound response profiles across cell line panels is central to turning good data into impactful data. Today's myriad methods for genetically characterizing cell lines offer many possibilities for dissecting the details of compound activity and cell-line responsiveness. Early efforts in this area include COMPARE for NCI-60 compound profiles (Shoemaker, 2006) and CellMiner (Shankavaram et al., 2009) for compound-genetic feature comparisons. Beyond the genetic feature correlations, there are a number of other parameters that can add to the complexity, such as dose-response curve IC50 (or crossing point), curve inflection point, activity area (or area under the curve), and maximal activity, frequently being utilized (Barretina et al., 2012). Most recently, Fallahi-Sichani et al. (2013) have demonstrated that incorporation of the concentration-response curve slope, area under the curve, and maximal effect can also yield insights into the mechanism of cell-death and also identify cell-to-cell variability in drug response.

Further insight can be gained by collecting more complex genetic information. Recently Jaffe et al. (2013) conducted global chromatin mass spectrometry profiling of 115 cancer cell lines. A common internal standard allowed for comparison of relative methylation and acetylation levels of all lysine residues on histone H3 between cell lines. This led to the observation that a set of cell lines had increased histone 3 lysine 36 dimethylation and that these lines contained either a t(4;14) translocation or a previously unknown coding mutation in the histone 3 lysine 36 methyltransferase NSD2, which was later shown to be an activating mutation. The t(4;14) translocation is known to drive high expression of NSD2, providing further support for the overlapping histone methylation profile of these cell lines. Increasing the depth of characterization of cancer cell lines will likely lead to further novel observations such as those described here.

MOVING BEYOND CANCER CELL LINES

Even at the earliest stages of the NCI-60 cell panel project, the importance of transitioning from cancer cell lines to animal models of cancer was recognized. Limitations to cancer cell lines include inherent biases towards specific cell signaling and growth pathways that favor growth in cell culture. For example PI3Kalpha, RAS, and BRaf mutations are well represented in cancer cell lines. However, some pathway mutations are not well represented in cell lines, for example the hedgehog pathway, via smoothened (SMO) and GLI1/2/3 mutations do not appear in any known cell line and no cell line is responsive to SMO antagonists (ref: http://www.ncbi.nlm.nih.gov/pubmed/20881279 and

unpublished results). Another example are IDH1 and IDH2 gain of function mutations that are commonly found in Glialblastoma and AML in clinical settings, yet very few, one or two, cell lines harbor these activating mutations. Therefore, the new generation of IDH1 inhibitors cannot be tested in standard cell proliferation models.

Two new approaches have capitalized on this idea, instead performing panel based screening directly in mice. These are referred to as "mouse avatars" and mouse "co-clinical trials" and both were recently reviewed by Malaney et al. (2014). The "mouse avatar" approach (also known as "xeno-patient trials") utilizes patient derived tumor xenograft models (PDTX), which rely on implantation of patient tumor samples into immunocompromised mice for study with pharmacological tool compounds. Tumor samples are genetically characterized both when removed from the patient as well as at intervals after transplantation into mice. While PDTX models have been generated for most major human cancers many challenges still remain for this platform to be widely adopted (cost and engraftment difficulty); however, we anticipate that the approach will continue to grow in prominence.

The second concept of mouse "co-clinical trials" involves using genetically engineered mouse models (GEMMs) in parallel with on-going human clinical trials as a way of anticipating response in the human arm of the trial (Nardella et al., 2011). Several co-clinical trials with GEMMs have been initiated recently with mixed results. In a successful example, Chen et al. (2012) have tested the use of the MEK inhibitor selumetinib in combination with docetaxel in a KRAS-mutant lung cancer trial. This effort led to the observation that the combination therapy outperformed monotherapy in cases where KRAS mutation alone or KRAS and p53 mutation were both present; however, mice with KRAS and LKB1 mutation were resistant to the combination therapy. This data suggests that patients in the clinical trial should be screened for LKB1 mutations in parallel as they may become resistant to therapy. This result, and others like it, could have a significant impact on the human arm of the co-clincial trial and could inform patient stratification for future human trials as well. We anticipate that co-clinical trials will be focused in nature compared to more exploratory cell line panel screens, but could impact the shape of human clinical trials and influence their outcomes while the trial is in progress.

USING CELLULAR MODELS IN NON-ONCOLOGY FIELDS

Beyond oncology indications, immunological profiling of patient derived blood cells represents a fruitful and relatively straightforward method to profile drug candidates across human genomic variation. From blood draws, peripheral blood mononuclear cells (PBMCs) can be isolated and are commonly used in experiments; large numbers of patient samples can be profiled with this ready source of cells. A key issue to using PBMCs is that they are a mixed cell population representing many of the various immunological lineages, including B and T cells, macrophages, etc. Until recently this represented a significant hurdle because only a few bulk attributes could be used to monitor response to compounds thus the cellular and mechanistic resolution was not nearly high enough. Advances in single cell analytical techniques have changed the landscape and now enable researchers to examine individual cellular responses.

Bodenmiller et al. (2012) recently described a clever proof of concept experiment using a new instrument called a CyTof. The CyTof is essentially a cell sorter that measures mass tags rather than fluorescent tags typical of a FACS instrument. The higher resolution of the mass spectrometer enables a 10–50x improvement in the number of tags that can be read per cell, and this enables identification of each cell type in the sorting along with multiple signaling pathway readouts on a per cell basis. In their study, PBMCs from eight patients were collected and separated into 14 cell types and further sub-division is possible. They simultaneously measured well characterized signaling cascade markers in each cell, like phospho-ERK and phoso-STAT5 in the presence and absence of different immuno stimulatory treatments such as LPS and Interferon-gamma. The data clearly show that cell type specificity is achieved, for example only cell types that express TLR4 respond to the LPS stimulation. What makes the study exceptional is that they then look at the responses across all these condition in the context of about 30 drugs or drug-like tool molecules. The authors generally see similarities between the different patient samples, but do note occasional differences, suggestive of environmental or genetic differences.

While the Bodenmiller study was not powered or designed to explore the genetic or environmental factors that may give rise to these subtleties, it is not a far stretch to imagine hundreds of patients or even longitudinal studies examining *ex vivo* response of PBMCs to various stimuli and drug candidates. Differential response between subjects could be explored in more depth with the goal of correlating and attributing genetic or environment factors (for example allergies that immuno activate cells and immuno inhibitory anti-histamines) to mechanistic changes in cell specific signaling pathways. Additionally, improvements are being made in single cell mRNA detection technologies and these are an attractive alternative to the CyTof technology due to enhanced flexibility of nucleic acid detection technologies (Ståhlberg and Bengtsson, 2010; Shalek et al., 2013). From these types of studies, predictive and stratifying biomarkers could be built and deployed to build clinical trials with better signal to noise ratios.

iPS DERIVED CELLS

Induced Pluripotent Stem Cell technology has created a massive paradigm shift in disease modeling. Originally described by Takahashi et al. (2007), the introduction of a handful of "reprograming" transcription factors can revert easy to acquire fibroblasts and blood cells to a pluripotent state. These pluripotent cells can then be differentiated into many different cell types and cell structures. Neural progenitors and various types of neurons turn out to be one of the more straight-forward cell types to make and these neuronal models were simply not available to the general research community previously; most researchers use rodent derived primary neurons as a cellular model. Heart, liver, eye, and many other cell types can also be generated. These cells can be grown is a more traditional 2D cell model and also in 3D organ like precursors, including recent reports of cerebral organoids, also known as mini-brains (Lancaster et al., 2013). These multicellular structures are often limited in growth simply by the lack of blood

innervation and lack of oxygenation. This is a highly active field of research and we should expect many innovations in the future.

Because iPS use relatively standard cell culture techniques there are already many efforts in academia and commercial ventures to scale up collections. A particularly good and recent review written by McKernan and Watt (2013) details many ongoing large scale collection efforts. Summing all the efforts listed in that review there are currently ∼150 cell lines available from public institutions, but there could be more than 20,000 cell lines from 10,000 individuals available in the next 3–5 years. With good data ascertainment for both clinical characterization and genetic profiling, these collections could be enormously valuable.

Most iPS and neuronal disease modeling to date has focused on highly penetrant monogenic diseases. In these experiments researchers collect cells from diseased and normal control patients, reprogram them to iPS states and differentiate them into various neuronal subtypes. Typically characterization of the neurons leads to discovery of a difference in phenotype that reflect the disease state and that, in turn, enables a platform for further characterization and functional screening. Examples here include Pheland–McDermid syndrome (Shcheglovitov et al., 2013), Timothy Syndrome (Paşca et al., 2011; Yazawa et al., 2011; Krey et al., 2012), ALS (Di Giorgio et al., 2008; Dimos et al., 2008), and there are many others. Expanding these collections across large numbers of monogenic diseases offers an opportunity to standardize the QC and neuronal differentiation procedures. Universal assay formats, such as mRNA readouts for cell types or neuronal activity, or activity measures using a system like the MANTRA (Hempel et al., 2011), can be used when pharmacologically profiling. For example, a collection of 250 iPS derived cortical neurons from 25 monogenic diseases (10 lines per disease) could be profiled across hundreds of drugs or drugs candidates using mRNA activity markers. Questions could be asked like what drugs increase the levels of specific synaptic activity markers such as Arc or c-Fos. When contextualized across large numbers of cell lines and compounds, this should reduce potential biases for interpretation and lead to robust results. Although these numbers of iPS cells are considered extremely large for most labs, groups like the New York Stem Cell Foundation (http://www.nyscf.org/) have successfully automated many of the processes associated with culturing stem cells and offer hope on the path to achieving assays scale with iPS derived cells.

One criticism of the this approach is that it is conceptually limited to severe monogenetic disease where penetrance is very high in patients and the expectation is that distinctive iPS derived neuronal phenotypes are related to the disease. Polygenic diseases with high heritability, like schizophrenia, present a much greater challenge. The genetic variants associated with these "common" diseases are thought to exert only a small change to disease risk, and presumably cellular phenotype. Defining what the disease associated variants do at a functional level is a key driver for current research (see commentary by Edwards et al., 2013). Many, if not most, are thought to exert subtle mRNA expression level changes, often confusingly called eQTLs. Because these affects are small and because specific gene regulation can be biased by genome background, hundreds, maybe thousands or tens of thousands of neurons will need to be molecularly and then pharmacologically profiled. Luckily these large scale iPS collections are now coming together. Additionally because common variants are present at rates > 5% throughout the population there is no need to collect harder to get disease patient samples and instead healthy volunteers can be used.

CONCLUSION

Using large numbers of human cellular models is a proven method to identify patients and the fundamental genetics responsible for disease and drug response. These models have been used for decades to characterize mid to late stage pre-clinical drugs in oncology (e.g., the NCI-60). In the last 5–7 years it has been shown by the CCLE and similar efforts that expanding the numbers of cellular models by 10-fold dramatically improves the resolution of the genetic models and enables discovery of the fundamental biology of tumor growth. Similar strategies can be used for immunological profiling of blood cells or neurological profiling of iPS derived neurons. Scaling up these processes will involve significant investments in infrastructure, methods, and QC to achieve reliable models, especially in the nascent iPS derived neuron field. Improvements in sequencing and genetics technologies are far exceeding the ability to build animal models and we believe that these human models will be more predictive by reflecting the patient diversity presented by people in the clinic.

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Evolving toward a human-cell based and multiscale approach to drug discovery for CNS disorders

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A disruptive approach to therapeutic discovery and development is required in order to significantly improve the success rate of drug discovery for central nervous system (CNS) disorders. In this review, we first assess the key factors contributing to the frequent clinical failures for novel drugs. Second, we discuss cancer translational research paradigms that addressed key issues in drug discovery and development and have resulted in delivering drugs with significantly improved outcomes for patients. Finally, we discuss two emerging technologies that could improve the success rate of CNS therapies: human induced pluripotent stem cell (hiPSC)-based studies and multiscale biology models. Coincident with advances in cellular technologies that enable the generation of hiPSCs directly from patient blood or skin cells, together with methods to differentiate these hiPSC lines into specific neural cell types relevant to neurological disease, it is also now possible to combine data from large-scale forward genetics and post-mortem global epigenetic and expression studies in order to generate novel predictive models. The application of systems biology approaches to account for the multiscale nature of different data types, from genetic to molecular and cellular to clinical, can lead to new insights into human diseases that are emergent properties of biological networks, not the result of changes to single genes. Such studies have demonstrated the heterogeneity in etiological pathways and the need for studies on model systems that are patient-derived and thereby recapitulate neurological disease pathways with higher fidelity. In the context of two common and presumably representative neurological diseases, the neurodegenerative disease Alzheimer's Disease, and the psychiatric disorder schizophrenia, we propose the need for, and exemplify the impact of, a multiscale biology approach that can integrate panomic, clinical, imaging, and literature data in order to construct predictive disease network models that can (i) elucidate subtypes of syndromic diseases, (ii) provide insights into disease networks and targets and (iii) facilitate a novel drug screening strategy using patient-derived hiPSCs to discover novel therapeutics for CNS disorders.

Keywords: stem cell-based screening, systems biology and network biology, drug discovery screening, complex disease mechanism, high throughput biology

INTRODUCTION

The disease burden on society is increasing at a dramatic rate. Focusing specifically on central nervous system (CNS disorders), the prevalence is growing at an alarming rate, with one in sixty-eight in the U.S. having some form of autism (Baoi, 2014), 1.8 million estimated to suffer from schizophrenia (SZ), and Alzheimer's Disease (AD) affecting more than five million in the U.S. today, with projections of a 40% increase in the number of AD cases in the next 10 years (Hebert et al., 2013). The costs of these diseases are staggering, both in financial and human terms. In 2002, the overall estimated cost of SZ was $62.7 billion with 36% attributed directly to health care expenses, though most costs are non-health care related, such as decreased productivity. By 2050, if healthcare costs for AD remain unchanged, the entire Medicare budget will be consumed by the treatment of those with AD (Alzheimer's Association, 2014).

Considerable effort and resources are being expended on drug discovery research aimed at developing novel therapeutics that would address the unmet medical need across a broad spectrum of diseases. However, less than one out of every eleven drug discovery programs makes it to market (Cummings et al., 2014). The success rate for CNS disorders is even lower. Though many factors may contribute to the high rate of attrition, the major drivers for CNS disorders, are inadequate efficacy or margins of safety (Kola and Landis, 2004). In this review, we will exemplify the issues of CNS drug discovery and provide a perspective for changing the current paradigms within the

context of the neurodegenerative disease, AD, and the psychiatric disorder, SZ.

For SZ, existing treatments target a very limited number of putative mechanisms, which treat some of the symptoms of SZ in some of the patients some of the time. Although several new pharmacologic interventions have been tested clinically in the last decade, none have shown medically relevant efficacy required for approval by regulatory authorities. Similarly, the field of therapeutic development to slow the progression of AD is littered with clinical failures of multiple pharmacological mechanisms and treatment modalities. These failures have had somewhat sobering effect on the field, particularly since many of the interventions were founded on human genetics- and pathology-informed amyloid hypothesis. A bright spot and beacon of hope may be the field of cancer therapeutics, where personalized treatments with far superior efficacy than traditional chemotherapies have been developed successfully. It behooves us to understand the primary reasons for the emerging successes in cancer treatments in order to adapt the paradigm to drug discovery for CNS disorders.

We will begin this review by discussing key factors that have likely contributed to the clinical failures for novel drugs, discuss the cancer research paradigms that have led to drugs with superior efficacy and diagnostic tests, and offer a perspective on the application of emerging technologies and tools that could improve the success rate of novel therapies. Specifically, we will focus on two potentially game-changing paradigms: (1) advances in human induced pluripotent stem cell (hiPSC)-based disease models and (2) multiscale predictive modeling. Together, these approaches can enable a more integrative biology approach to deriving insights into disease mechanisms, upon which drug screening and development may be founded in the near future. Although these approaches may be adapted broadly to many CNS indications, this review will focus primarily on SZ and AD.

CHALLENGES FOR DRUG DISCOVERY IN NEUROSCIENCE

First, the selection of a target for novel drug therapies requires an in-depth understanding of disease biology, from the etiological factors to pathophysiological mechanisms, and their relationship to disease progression and duration. It is insufficient to move forward with only the knowledge that a particular target is expressed in the brain or that a specific DNA variant is associated to a neurological disease. For common, complex trait diseases, a more systems oriented view is emerging in which human diseases are demonstrated to be emergent properties of biological networks, not the result of changes to single genes (Schadt, 2009; Schadt et al., 2009; Califano et al., 2012). Hence, rather than repeating the mistakes of the past, it is imperative to understand the biological context in which the susceptibility gene/gene networks and gene products operate to give rise to the disease, before beginning high throughput drug discovery screens. As the next step, we will need insights into the effect of the implicated gene network on cellular/physiological pathways in order to determine whether a novel therapy should augment or suppress, either fully or partially, the function of the disease-associated network.

Secondly, other than rare or orphan diseases that are caused by Mendelian mutations, common CNS disorders are syndromic diseases diagnosed primarily by non-specific and blunt clinical diagnostic tools, often based on patient reported symptoms. Thus, both the so-called clinically diagnosed SZ or AD patient population represents a diverse patient population with respect to etiologies and associated pathophysiological mechanisms, giving rise to similar sets of clinical symptoms but with distinct rates of disease progression. The clinical diagnostic tools, such as the Diagnostic and Statistical Manual (DSM) criteria for mental disorders or Mini-Mental State Examination (MMSE) for dementia, have served us well and led to approval of several drugs. However, the poor specificity and sensitivity of these tools require that they be supplemented with objective, diagnostic biomarkers with which to classify or enrich patient populations that are more homogenous in either their etiological or pathophysiological factors, or disease stage, being targeted by drugs.

Third, chronic diseases have an additional inherent issue related to adaptive biological mechanisms that emerge with chronicity of the disease or drug treatment. For neurodegenerative disorders, there is also the complicated factor of loss of resiliency in surviving neurons affected by the disease process. Thus, interventional strategies have to be designed to be specific to the stage of the disease, and when possible, to target primary prevention. Therefore, an understanding of adaptive molecular mechanisms as well as objective biomarkers that can monitor the biologic processes associated with the disease stage or progression and adaptive processes induced by drugs or disease will be required to improve drug discovery and development.

Finally, drug screening paradigms need to evolve so that disease biology mechanisms are monitored in cellular and animal models that more faithfully recapitulate human disease biology. Similarly, the systems biology/disease network approach will require that end-points of drug screens may have to be multi-parameter or phenotypic in nature, rather than those based on ease or throughput considerations alone. Thus, we must facilitate the development of cell-based systems derived from patients with disease as well as normal controls, in which the cell types are directly relevant to those implicated in human disease, so that we may garner insights into disease biology and design effective screening paradigms. Given that the large-scale generation and integration of panomic data has enabled the construction of complex gene networks that provide a new framework for understanding the molecular basis of disease (Ideker et al., 2002; van't Veer et al., 2002; Schadt et al., 2003; Barabasi and Oltvai, 2004; Bystrykh et al., 2005; Ghazalpour et al., 2005; Schadt, 2005; Lum et al., 2006; Wang et al., 2006; Chen et al., 2008; Emilsson et al., 2008; Zhu et al., 2008), it is now possible to take a data driven, network-based view of diseases, which in turn enables the elaboration of a network-based view of drug discovery and development, one that is fundamentally different from current methods (Chen et al., 2008; Emilsson et al., 2008; Yang et al., 2009; Zhong et al., 2010a,b; Califano et al., 2012; Zhu et al., 2012; Zhang et al., 2013a; Kidd et al., 2014; **Figure 1**). Systems biology approaches seek to presume less knowledge, capture more information, and interpret the information in a more data driven way.

The four points discussed above require translational approaches that begin with patient-centered research to derive

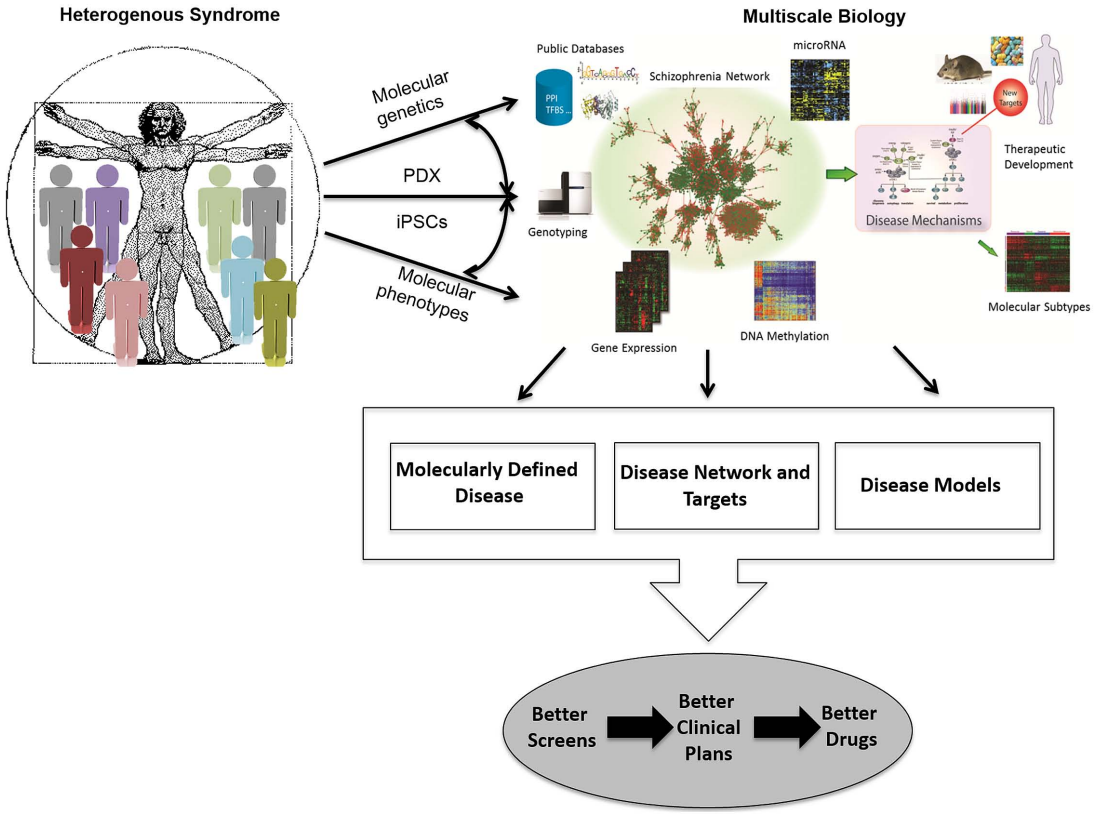

FIGURE 1 | Schematic for how multiscale biology can lead to therapeutic development. A network-driven framework to integrate diverse types of data that lead to multiscale models of disease that can then be used to elucidate disease mechanisms, stratify patient populations, and develop novel therapeutics. PDX, patient-derived tumor grafts; iPSCs, induced pluripotent stem cells.

insights into disease biology. Of all the major therapeutic areas, cancer drug discovery research has led the way in translating the explosion of human genomic data into new therapeutics despite each clinically diagnosed malignancy being a heterogeneous mixture. Hence, we begin by reviewing the cancer drug discovery paradigm.

CANCER AS AN EMERGING MODEL OF MODERN DAY DRUG DISCOVERY

Drug hunters have been able to take advantage of two unique features of cancer biology. Firstly, the accessibility of tumor tissues facilitate their study through genomic and phenotypic approaches as well as provide patient-derived pre-clinical *in vitro* and *in vivo* model systems, which are being shown to more faithfully recapitulate important aspects (notably uncontrolled proliferation) of the disease, and in many cases can be formatted for high throughput screening (Cheung et al., 2011b; Barretina et al., 2012; Dar et al., 2012; Garnett et al., 2012; Hirabayashi et al., 2013). This has resulted in a second distinguishing feature of cancer biology: although cancer encompasses a large diversity of distinct malignancies, each can be defined by an even larger diversity of driving mutations. Importantly, the driver mutations are not random, but instead reflect different core biological processes and signaling pathways central to the onset and progression of the tumors and thereby provide a rich source of new drug targets. Thus the taxonomy of cancers is being redefined on the basis of molecular markers.

Multi-center genome sequencing endeavors (Stratton, 2011) have revealed 100s of cancer causing mutations, discoveries which shed welcome light on vital nodes in the otherwise largely cryptic underlying disrupted networks (Eifert and Powers, 2012; Alexandrov et al., 2013; Cancer Genome Atlas Research Network, 2013, 2014; Cancer Genome Atlas Research Network et al., 2013a,b; Kandoth et al., 2013; Zhang et al., 2014a). These growing lists of cancer genes become immediate opportunities for therapeutic intervention. When the encoded product of an oncogenic mutation belongs to a conventionally druggable protein class, this information can lead to very rapid development of novel and effective drugs. For example, translocations fusing *EML4* to the tyrosine kinase gene, *ALK*, were discovered in lung cancer biopsies in Rikova et al. (2007), Soda et al. (2007). The EML4-ALK fusion protein results in a constitutively activated catalytic domain and is a key driver of the uncontrolled proliferation of cancer cells with this lesion. So it was postulated that ALK-directed tyrosine kinase inhibitors might be able to nullify the oncoprotein. Crizotinib, a tyrosine kinase inhibitor already in clinical testing for MET kinase-driven tumors, was known to inhibit also ALK kinase and trials were therefore extended to

target EML4-ALK-positive non-small cell lung cancer (NSCLC) patients. Efficacy results in these patients were dramatic, leading to FDA approval of crizotinib in 2011, an unprecedented short duration (less than 4 years) after the first reports of the fusion in patients. Cancer sequencing projects have revealed driving mutations in genes encoding other druggable oncoproteins and, much like crizotinib, drugs targeting several of these have shown considerable promise in clinical trials for patients whose tumors bear the relevant mutation (**Table 1**) although, as described below, the development of resistance has plagued each of these drugs.

Some of the most frequently mutated cancer genes, however, including oncogenes such as MYC and RAS, as well as tumor suppressor genes, do not encode readily druggable proteins. In principle, it may simply be a matter of following the known signaling pathways from the cancer gene product until we reach a tractable drug target, but the further we stray from the mutated node the more likely the intervention will not be effective due to redundancies and divergent branches in the relevant networks. Inhibitors of the druggable GPCR target, Smoothened, have shown encouraging promise in cancers with loss of function mutations in the tumor suppressor PTCH, which encodes an upstream regulator of Smoothened (Berman et al., 2002; Rudin et al., 2009; Von Hoff et al., 2009). Similarly, MEK inhibitors have proven effective in treating *BRAF*-mutant melanoma, where the defective oncogene is directly upstream of, and activates, MEK in the MAPK pathway. Conversely, however, MEK inhibitors have not thus far proven very effective in treating cancers with mutations that activate the oncoprotein, RAS, just one extra step further upstream of RAF in the canonical pathway. What has become evident now is that a comprehensive understanding of the entire defective network, rather than a single canonical pathway, is necessary to identify new drug targets for cancers harboring these undruggable oncogenes and tumor suppressors (Solit et al., 2006; Lito et al., 2014; Schmit et al., 2014; Sun et al., 2014).

Molecular networks can be interrogated with empirical approaches that take advantage of the fact that cells from cancer biopsies can be propagated as established cell cultures, as rodent xenografts, or as patient-derived tumor grafts (PDX). Genetically characterized cancer cell lines and tumor grafts have been established from human disease specimens and generally preserve the genomic features observed in the disease. In many cases, mutated oncogenes and tumor suppressors that drive disease in animal models are also known to be critical for the growth and survival, in culture, of human cancer cell lines with the same mutations. This offered the opportunity to use proliferation assays as a facile but disease biology-relevant phenotypic assay on human cancer cell lines to identify vulnerabilities particular to the network reconfigured by specific mutations. A large number of *in vitro* and *in vivo* screens based on this principle have reported potential new targets, which take advantage of synthetic lethality, non-oncogene addiction and co-lateral vulnerabilities in cancer (Whitehurst et al., 2007; Turner et al., 2008; Luo et al., 2009a,b; Astsaturov et al., 2010; Rehman et al., 2010; Cheung et al., 2011b; Muellner et al., 2011; Kumar et al., 2012; Muller et al., 2012; Toyoshima et al., 2012; Riabinska et al., 2013; Mair et al., 2014). Such approaches have revealed vulnerabilities in cancer cells whose primary driving mutations are not directly druggable. For example PARP inhibitors were demonstrated to be synthetic lethal *in vitro* with mutations in the tumor suppressors *BRCA1* and *BRCA2* (Farmer et al., 2005; McCabe et al., 2005) and clinical testing has established the same genetic dependence on tumor sensitivity to the PARP inhibitor olaparib in patients (Fong et al., 2009).

Using high throughput screening methodologies coupled with statistical methods, large panels of genetically characterized tumor cell lines can be assembled to identify gene–drug interactions in an unbiased manner and thereby identify either drugs or drug–drug combinations effective for the treatment of cancers with the relevant mutation (Barretina et al., 2012; Garnett et al., 2012). In an analogous manner, tumor cell panels are being tested to identify the context of dependencies on the entire human transcriptome using a hairpin dropout whole genome RNAi screen (Cheung et al., 2011b).

These developments over the last decade have demonstrated that the 'one gene – one drug' paradigm is leading to progress in the treatment of multiple cancers and is likely to continue to bear some fruit as new mutations and drugs emerge. However, it is also clear that the efficacy of new targeted therapeutics in cancer is too often short-lived due to the eventual, sometimes rapid, emergence of drug resistance due to adaptive biological processes induced by the disease or drug treatment (Yauch et al., 2009; Barber et al., 2013; Das Thakur and Stuart, 2013; Lord and Ashworth, 2013; Niederst and Engelman, 2013). The mechanisms of resistance to the modern cancer pharmacopeia are being deciphered. Hence, one approach to tackle the drug resistance problem is to continue along the same path, looking to exploit new vulnerabilities that emerge in the resistant clones (O'Hare et al., 2011; Cortes et al., 2012; Yadav et al., 2012; Friboulet et al., 2014; Hata et al., 2014; Traer et al., 2014). Resistance to imatinib, a very effective inhibitor of BCR-ABL for the treatment of chronic myelogenous leukemia, most often occurs due to point mutations in the target kinase that prevent drug binding. Three drugs, designed specifically to inhibit these imatinib-resistant enzymes, have since been approved and CML is now a well-managed disease for the majority of patients (O'Hare et al., 2011). However, in the case of resistance to epidermal growth factor receptor (EGFR) and

Table 1 | Driver genes identified from cancer sequencing studies have led directly to drug targets and subsequent therapeutics that have shown significant promise in clinical trials.

Genotype	Drug	Reference
PML-RARα translocation	ATRA	Dermime et al. (1993)
HER2 amplification	Trastuzumab	Cobleigh et al. (1999)
KIT mutation	Imatinib	Demetri et al. (2002)
EGFRL858R	Gefitinib, erlotinib	Paez et al. (2004)
BRAFV600E	Vemurafenib	Joseph et al. (2010)
EML4-ALK	Crizotinib	McDermott et al. (2008)
RET	Vandetanib	Wells et al. (2010)
BCR-ABL	Imatinib	Druker (2008)
ROS	Foretinib	Davare et al. (2013)

BRAF inhibitors, distinct mechanisms of resistance can emerge in different clones from the same primary tumor – a situation that likely will not be feasibly solved with gene–drug pairs, even if drugs directly targeting the new mutations that arise in each metastasis could be discovered. Instead, the disrupted networks need to be more completely understood to identify downstream or parallel pathways common to all the resistant clones. Here again, cell lines and PDX models derived from drug-resistant tumors can be used to screen for drugs or by RNAi methods, to identify targets that will be effective in dealing with multiple mechanisms of resistance (Huang et al., 2012; Konieczkowski et al., 2014; Sun et al., 2014). High throughput, unbiased drug–drug combination screens are also feasible, at least *in vitro*, to identify drug cocktails that are predicted to more effectively treat and prevent the emergence of drug resistance (Bajrami et al., 2012; Roller et al., 2012).

Other ways to address the rapid ability of cancer cells to evolve drug resistance are emerging. Targeting cancer by avoiding the cancer cells altogether and instead developing drugs that target the other physiologic processes essential to cancer progression has shown considerable promise. Thus discovery of drugs targeting angiogenesis, inflammation, and immune checkpoints does not benefit from the wealth of smoking gun targets informed by somatic mutation data in tumor cells, and preclinical models of these aspects of disease are more complex than proliferation-based assays. But exciting progress has been made, nevertheless, with spectacular results in some cases (Robert et al., 2011; Topalian et al., 2014) and unbiased screening approaches are being explored in the search for new targets in each of these areas (e.g., Zhou et al., 2014). In addition, work done in fruit fly to demonstrate multifactorial targeting of tumors by simultaneously hitting multiple pathways that serve as key drivers of the cancer has not only demonstrated that targeting of individual signaling pathways in a given cancer will have short-lived efficacy, but that multifactorial targeting of the tumor can greatly diminish chances that tumor cells will evolve to defeat the cocktail of drugs that get used in these cases (Cagan, 2013; Das and Cagan, 2013). Here, the use of PDX models provides a more holistic way of studying how tumors may evolve in response to different types of therapy. In this way, a more systems oriented approach can be employed in which PDX models are used as patient avatars to establish the most effective combination of treatment specific to that individual's tumor. These more progressive approaches that seek to model a patient's tumor in systems that can be rapidly screened for therapies that will be most effective for that individual, represent the new generation of precision medicine strategies that hold promise in transforming how we diagnose and treat disease (National Research Council (US) Committee on A Framework for Developing a New Taxonomy of Disease, 2011).

These same model systems can be used to reveal the adaptive processes the can follow drug treatment, and often dampen drug efficacy, and to therefore suggest combination therapies that counteract them. For example, BRAF inhibitors can be very effective in melanoma patients with *BRAF* mutant tumors, but have been much less effective in *BRAF* mutant colorectal cancer and in Ras mutant tumors. Activation of c-Raf caused by BRAF inhibitors in tumors with activated Ras has been shown, paradoxically, to stimulate, rather than inhibit MAPK pathway signaling and is suspected of causing new skin cancers that have been observed as a frequent side effect of BRAF inhibitors (Hall-Jackson et al., 1999; Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010). This model predicts that nullifying signaling downstream of c-Raf with MEK inhibitors should prevent the skin lesion side effect and also improve efficacy. The combination of the MEK inhibitor, trametinib, and BRAF inhibitor, dabrafenib, was approved for the treatment of melanoma in 2014. In *BRAF* mutant colorectal cancer it has been learned that negative feedback pathways are stimulated by the constitutive activation of the MAPK pathway conferred by *BRAF* activating mutations and that these pathways act to diminish signaling through the EGFR. A consequence of inhibition of BRAF, therefore, is reactivation of EGFR mediated signaling which diminishes drug efficacy (Corcoran et al., 2012; Prahallad et al., 2012). Based on these discoveries, clinical trials are underway testing combined inhibition of BRAF and EGFR in colorectal cancer (NCT01750918). Similarly MEK inhibitors are only poorly effective in *KRAS* mutant cancers. RNAi-based screens were used to identify new targets that, in concert with MEK inhibition, augment the antiproliferative activity of MEK inhibitors in *KRAS* mutant colorectal cancer cell lines, revealing that c-Raf knockdown, or c-Raf inhibitors, were able to potentiate the activity of MEK inhibitors in *KRAS* mutant tumor cells (Lito et al., 2012; Lamba et al., 2014). Approved Raf kinase inhibitors, such as dabrafenib and vemurafenib, are ineffective inhibitors of c-Raf and so clinical testing of this hypothesis will have to await the emergence of true c-Raf inhibitor drugs. These approaches are beginning to reveal the adaptive processes the can follow drug treatment, and often dampen drug efficacy, and suggest combination therapies to counteract them.

To summarize, the transformative success of the cancer drug discovery and development may be attributable to four key factors: (i) de-risking target selection through identification of driver mutations or disease networks through multi-center studies on patient-derived tumor specimens, (ii) reducing patient heterogeneity by implementing molecular definition of disease taxonomy rather than clinical diagnosis alone, (iii) addressing drug resistance by targeting disease networks associated adaptive processes induced by drug and/or disease, and (iv) incorporating cellular and animal models with greater predictive validity in drug screens.

MULTI-CENTER GENETIC STUDIES AND USE OF hiPSCs TO DERIVE INSIGHTS INTO DISEASE BIOLOGY

Tracking with the great advances in cancer drug development that have benefitted from the genomics revolution, forward genetics strategies to elucidate the complexity of human disease have been accelerated as the cost of assaying nucleic acid sequences continues to drop exponentially and ever bigger cohorts of diseased individuals are assembled. Whether performing a genome-wide association study or whole exome/genome sequencing studies in case/control cohorts or families segregating diseases of interest, the quest has been to identify specific genes, pathways and networks that are critical for disease onset, progression and severity and

thereby rationalizing the selection or prioritization of molecular targets or pathways for drug discovery.

A genetics case study for schizophrenia

Schizophrenia is a complex and heterogeneous disorder with an estimated heritability of about 80% (Sullivan et al., 2003). Much like cancer, many types of DNA variations, [single nucleotide polymorphisms (SNPs), copy number variations (CNVs), and small exonic missense and nonsense mutations) as well as epigenetic and/or environmental factors contribute to the risk of SZ. The genetic risk factors for SZ include both rare variants conferring large relative risks (e.g., CNVs) as well as common SNP variants, the latter (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014) with modest individual effect sizes (Purcell et al., 2009).

Copy number variations are ubiquitous in the population (McCarroll et al., 2008); it is now widely held that in addition to a number of fairly uncommon syndromes, they also contribute to more common disorders such as SZ. In fact, though linkage studies have been unsuccessful in identifying highly penetrant genes (Ng et al., 2009), a large body of work (reviewed Malhotra and Sebat, 2012) defined the following principles across >10,000 SZ samples: (1) genome-wide rates of large (>100 kb), rare (<1%) CNVs are elevated, (2) rates of *de novo* CNVs are elevated 2–50 fold, (3) CNVs generally contain many genes and confer large relative risks (2–50), (4) specific sites of CNVs are often also found in multiple additional neurological diseases, and (5) CNVs are enriched in neuronal functions, particularly those that are involved in synaptic activity and neurodevelopmental processes. CNVs represent a polygenic burden of rare disruptive mutations, one that is particularly enriched in gene sets including the voltage-gated calcium ion channel and the post-synaptic density (Purcell et al., 2014).

While individually penetrant CNVs are only found in a minority of patients (perhaps 5–10%), common DNA variants (minor allele frequency >5%) are significant contributors to the heritability of SZ, accounting for ∼30% of the variance in liability (Lee et al., 2012). It is now widely held that SZ risk also involves 1000s of common alleles of very small effect (Purcell et al., 2009). The earliest convincing evidence for a contribution to common variants in SZ included the major histocompatibility complex (MHC; Purcell et al., 2009; Shi et al., 2009; Stefansson et al., 2009), subsequent work also implicated the microRNA (miR)-137 as well as four of its targets (Ripke et al., 2011). Recent GWAS of ∼38,000 SZ patients and ∼115,000 controls by the Psychiatric Genomics Consortium Schizophrenia Group have now identified 108 genome-wide significant loci, most of which are novel, but individually have small effects (relative risk ranging from 1.09–1.17; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). In fact, it was recently estimated that more than 6300 common SNPs collectively account for at least 32% of the genetic risk for SZ (Ripke et al., 2013). Interestingly, these loci have begun to implicate pathways: in addition to at least one target of current neuropharmacology (the dopamine receptor *DRD2*), for the first time, critical glutamatergic genes such as *GRM3* and *GRIN2A*, and calcium channel subunits (*CACNA1C* and *CACNA1I*) have been associated with SZ.

Studying *de novo* point mutations is also a powerful tool; it was recently shown that small *de novo* mutations affecting one or a few nucleotides are overrepresented among glutamatergic post-synaptic proteins (Fromer et al., 2014). The location of rare disruptive loss-of-function mutations, enriched in glutamatergic and calcium signaling, have been shown to overlap with SZ-associated CNV (Purcell et al., 2014). Similarly, common and rare variants can overlap: a novel variant at 16p11.2 (rs4583255[T]; odds ratio = 1.08) substantially increases risk of psychosis (Steinberg et al., 2014).

However, genetic data on their own are not sufficient to garner insights into disease biology for several reasons. Although disease-associated loci are identified, the causal genes are not always known. Even when the genes implicated are known, an understanding of the functional relevance of the genetic variant, whether it is an activating or inhibitory variant, has to be experimentally derived to design drug discovery strategy. For SZ, the functional implications of the DNA variants in glutamatergic genes or calcium channels remain to be elucidated. Both animal model and clinical studies indicate that SZ is associated with hyper-glutamatergic neurotransmission, at least early in the disease (reviewed by Poels et al., 2014). It is critical to know whether disease-associated GRM3 or GRIN2A SNPs predispose to SZ by increasing glutamatergic neurotransmission at a vulnerable developmental stage. The importance of such functional data is exemplified by the recent failure of two glutamate targeting ligands in Ph3 trials. First, a GRM2/3 agonist, LY2140023, from Eli Lilly and Company, which is predicted mechanistically to reduce glutamatergic neurotransmission. Although, in a Phase 2 study, this compound significantly reduced the symptoms of SZ (Patil et al., 2007), it failed to do so in subsequent larger Phase 3 studies (Adams et al., 2013, 2014). Similarly, on January 21, 2014, Roche announced that the GlyT1 inhibitor, bitopertin, failed to meet its primary end-points in two Phase 3 trials in SZ. What then is the significance of glutamatergic pathway replicably associated to SZ by genetic studies? From the clinical studies we cannot conclude whether the targets were wrong or the patient population was wrong. One key factor to consider is patient heterogeneity. Thus only a minority of patients carry DNA variants in glutamatergic genes (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014) but the drug studies did not stratify patients on the basis of gene variants or glutamatergic imaging or physiological markers. Using the cancer example, two paradigms need to be adopted to leverage the genomic data. First, to refine the taxonomy of SZ and other CNS disorders on the basis of molecular markers. This will allow identification of patient populations most likely to respond to a drug mechanism. Second, use model systems to understand functional biological implications of gene variants, as detailed below.

hiPSC as a model system to translate genetic findings into functional insights

In order to understand the complex network interactions contributing to the entire genetic risk in any given patient, and between patients, one must be able to study the full genetic background, even without knowing all the risk alleles contributing to

the disease. Today, hiPSC-based models for many CNS disorders have been established, by reprogramming patient somatic cells into hiPSCs, and subsequently differentiating these stem cells into different types of neurons (Dimos et al., 2008; Park et al., 2008; Baek et al., 2009; Ebert et al., 2009; Hotta et al., 2009; Lee et al., 2009; Soldner et al., 2009; Marchetto et al., 2010; Nguyen et al., 2011; Pasca et al., 2011). This type of technology has made it possible to connect genetic data to biological insights, elucidating molecular and physiological changes in different neural cell types, something that was incredibly difficult or even impossible prior to hiPSCs.

A number studies of psychiatric disorders have reproducibly demonstrated that even small patient hiPSC cohorts can reveal robust and repeatable neural phenotypes, meriting further investigation. For example, many groups have generated hiPSCs from Rett Syndrome patients, and consistent with post-mortem patient studies, all have reported that neuronal soma size is reduced compared with controls. Additionally, other disease-relevant phenotypes such as reduced spine density, decreased neuronal spontaneous calcium signaling and decreased spontaneous excitatory and inhibitory post-synaptic currents have been reported (Marchetto et al., 2010; Ananiev et al., 2011; Cheung et al., 2011a). Timothy syndrome (TS) is caused by a mutation in the L-type calcium channel Ca(v)1.2 and associated with heart arrhythmias and ASD. TS hiPSC derived cortical neural progenitor cells (NPCs) and neurons show aberrant calcium signaling, (Pasca et al., 2011) ameliorated by treatment with roscovitine, a cyclin-dependent kinase inhibitor and atypical L-type-channel blocker (Pasca et al., 2011). Though early proof-of-concept studies of hiPSC neuronal pathology focused on diseases characterized by both the loss of function of a single gene product and rapid disease progression in early childhood (Ebert et al., 2009; Lee et al., 2009; Marchetto et al., 2010), many groups have recently extended these studies to complex genetic psychiatric disorders.

To date, SZ has lacked a human cell-based platform that incorporates the heterogeneity of this complex genetic disorder with which potential therapeutic compounds might be identified by high throughput screening.

hiPSC-based studies of schizophrenia

For a discovery made in 2006, that transient expression of just four factors (OCT3/4, KLF4, SOX2, and c-MYC) is sufficient to directly reprogram adult somatic cells into an induced pluripotent stem cell (iPSC) state (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007), Shinya Yamanaka was awarded the 2012 Nobel Prize in Medicine. With this revolutionary advance, hiPSCs are now be routinely generated from patient skin or blood cells, owing to the relative ease of tissue access, and are believed to be capable of differentiating into every cell type found in the adult (Maherali et al., 2007; Meissner et al., 2007; Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007). Because hiPSCs can be derived from adult humans, after the development of disease, hiPSCs represent a potentially limitless source of human cells with which to study the onset and progression of neurological disease, even without knowing which genes are interacting to produce the disease state in an individual patient.

In previous publications, we directly reprogrammed fibroblasts from four SZ patients into hiPSCs and differentiated these disorder-specific hiPSCs into forebrain NPCs (Brennand et al., 2014) and neurons (Brennand et al., 2011). Gene expression comparisons of our hiPSC-derived NPCs and 6-week-old neurons to the Allen Brain Atlas indicate that our hiPSC neural cells, from controls and patients with SZ, resemble fetal rather than adult brain tissue (Brennand et al., 2014), indicating that hiPSC-based models may not yet be suited for the study of the late features of this disorder. SZ hiPSC NPCs show evidence of aberrant migration and increased oxidative stress (Brennand et al., 2014), while SZ hiPSC neurons showed diminished neuronal connectivity in conjunction with decreased neurite number, PSD-95 and glutamate receptor expression. Key cellular and molecular elements of the SZ phenotype were ameliorated following treatment of SZ hiPSC neurons with the antipsychotic loxapine (Brennand et al., 2011). Others have also reported that SZ hiPSC neural cells show increased oxidative stress (Paulsen et al., 2011; Robicsek et al., 2013), aberrant responses to environmental stresses (Hashimoto-Torii et al., 2014) and have reduced synaptic maturation (Robicsek et al., 2013; Wen et al., 2014; Yu et al., 2014; Zhang et al., 2014b).

Until recently, functional differences in SZ hiPSC neurons had not been identified, likely owing to the heterogeneity in hiPSC neuronal culture. Now, the first phenotypic characterization of a single neuronal subtype (hippocampal dentate gyrus granule neurons) shows reduced neuronal activity and spontaneous neurotransmitter release in SZ hiPSC-derived neurons (Yu et al., 2014). This demonstration that functional deficits can be detected in live human neurons in vitro convincing shows that phenotypic assays (if not molecular comparisons) must be conducted in specific and defined neuronal subpopulations.

A growing body of evidence links SZ with abnormal functioning of dopaminergic, GABAergic and glutamatergic neurons. Although pharmacological modulation of dopamine transmission helps manage the positive symptoms of SZ for some patients (Weinberger, 1987; Kessler et al., 2009), emerging evidence indicates that aberrant dopamine transmission is most likely downstream from dysfunctional GABAergic and glutamatergic neurons of the prefrontal cortex (Wen et al., 2010; Demjaha et al., 2013). hiPSCs can now be differentiated to cortical pyramidal (Espuny-Camacho et al., 2013), interneuron (Maroof et al., 2013; Nicholas et al., 2013) and midbrain dopaminergic fate (Chambers et al., 2009; Kriks et al., 2011), providing multiple avenues for studying SZ in precisely defined subpopulations of neurons. Efficient protocols to differentiate hiPSCs into dopamine neurons have been systematically optimized and yields now exceed >80% (Chambers et al., 2009; Kriks et al., 2011). After neural induction, DA specification occurs recapitulating the activation of Sonic hedgehog (SHH) and Wnt/β-catenin signaling that patterns dopaminergic neurons in the floor plate region of the ventral midline. Recently published methods to generate GABAergic neurons are similar, reiterating embryonic development of the ventral telencephalon via the inhibition of WNT signals and timed exposure to SHH signals (15,16). Differentiation to glutamatergic fate occurs in the absence of bone morphogenetic

protein (BMP), Wnt/β-catenin and TGF-β/activin/nodal pathways (Mariani et al., 2012; Shi et al., 2012). Protracted cortical differentiation (50–70 days) seems to mimic human developmental temporal patterning, resulting in sequential specification of cortical layer identity (Shi et al., 2012; Espuny-Camacho et al., 2013).

The ability to rapidly induce neurons, rather than rely on protracted differentiation protocols, would clearly be advantageous when considering systematic comparisons of 100s of SZ patients or high throughput screening of 1000s of potential therapeutics. Mouse (Vierbuchen et al., 2010) and human (Pang et al., 2011) fibroblasts can be induced in less than 6 days into iNeurons, via lentiviral (LV) overexpression of just *BRN2, ASCL1, and MYTL1* – with the addition of *NEUROD1* in human cells. Though rapid, the process is inefficient, occurring in just 2–4% of the original fibroblasts, and generates relatively immature neurons unable to form synapses on their own. The addition of key microRNAs improves the process, resulting in mature neurons capable of forming fully functional synapses in pure cultures (Ambasudhan et al., 2011; Yoo et al., 2011). Yields remain at approximately 10%, with substantial variability between fibroblast lines, and the temporal and spatial identity of iNs, relative to the human brain, is unresolved. The ability to generate neuronal populations of a specific sub-type would be ideal for cell-based studies. Already, using pools of cell type specific transcription factors, human fibroblasts can be induced into midbrain dopaminergic neurons (Caiazzo et al., 2011). Though faster than hiPSC reprogramming and subsequent neuronal differentiation, of primary consideration is that this methodology transforms precious primary patient-derived cells into terminally differentiated neurons, limiting the cellular material available for studies.

In comparison to methods of growth factor-directed differentiation, or fibroblast derived iNeurons, inducible LV overexpression of NGN2 in hiPSCs rapidly induces pure populations of functional excitatory neurons, with a transcript profile indicative of cortical layer II/III neurons, in as little as in 21 days (Zhang et al., 2013b). We predict that similar methods for rapid and directed induction of a variety of pure neuronal subpopulations will soon be ubiquitous. While one might fear that this progression toward faster and more defined neuronal induction will bypass normal neural development, potentially limiting the ability to observe early phenotypes such as neural migration, specification or maturation, we note recent evidence that iNeurons derived from patients with an autism-associated neuroligin-3 (NLGN3) mutation perfectly recapitulated the molecular and synaptic defects observed in the Nlgn3 mouse model (Chanda et al., 2013).

To date, most hiPSC studies have been conducted on cells derived from a handful of cases and controls, typically around 3–6 patient lines. However, efforts are underway to make the process of converting somatic cells into stem cells more uniform, efficient, and cost-effective. Thus one can anticipate a time in the near future where 100s of cell lines representing 100s of individual patients may be studied to understand disease biology for common disorders such as SZ. In concert with functional genomics, high throughput electrophysiology, imaging and integrative systems biology approaches, this platform could provide insights into common and unique mechanisms of syndromic diseases upon which new drug discovery paradigms may be founded. Thus analogous to the oncology field, hiPSCs is poised to provide a cellular model platform that could enable personalized medicine for psychiatric indications. Additionally, these cell lines and associated phenotypes will form a powerful platform for drug-screening assays with direct relevance to disease biology.

MULTISCALE BIOLOGY APPROACH TO UNDERSTANDING DISEASE BIOLOGY AND IDENTIFYING THERAPEUTIC TARGETS

Given the enormous amount of panomic data that have been generated to characterize common human diseases, this data can be integrated in order to build predictive network models of normal and disease states, which can elucidate the key biological drivers of the disease state. To fully understand complex neurological diseases, we must link molecular biology to physiology (Schadt, 2009; Schadt et al., 2009; Califano et al., 2012). Multimodal models can be used to identify disease signatures, by using the networks to organize the signatures according to the subnetworks (and the biological processes that they define), which are associated with disease (Schadt et al., 2005, 2008; Chen et al., 2008; Emilsson et al., 2008; Yang et al., 2009, 2010; Zhong et al., 2010a,b; Zhu et al., 2010; Ambasudhan et al., 2011; Greenawalt et al., 2011; Wang et al., 2012). Ultimately, integrating diverse, large-scale data provides a path to predict which drug effects might best counteract the molecular networks underlying disease (**Figure 2**).

Building and applying multiscale network models

Integrative network models utilize panomic data to derive causal relationships among 1000s of intermediate molecular traits and between molecular and higher order physiological traits associated with disease (Barabasi and Oltvai, 2004; Zhu et al., 2012). In this context, networks are represented graphically as nodes and edges, where nodes represent individual molecular and clinical features (gene expression levels, metabolite levels, protein states, methylation levels, biochemical measures, and so on) and edges represent the interactions among these variables. How molecular traits and disease traits causally relate to each other can be modeled using pairwise causality tests (Schadt et al., 2005; Millstein et al., 2009) or probabilistic graphical models, such as RIMBANet (Zhu et al., 2004, 2007, 2008, 2010, 2012), in which all available traits are considered simultaneously. A number of studies performed by us and others, in a variety of species, have demonstrated that predictive networks like Bayesian networks can capture fundamental properties of complex systems in states that give rise to complex phenotypes (Jansen et al., 2003; Lee et al., 2004; Zhu et al., 2004, 2007, 2008, 2012; Schadt et al., 2008; Zhang et al., 2013a). The available molecular data that informs on disease, derived from different tissues in different states, providing the necessary ingredients to reconstruct causal network models of disease (**Figure 1**).

High dimensional panomic data will increasingly be generated in hiPSC derived neurons, with 100s or even 1000s of samples generated from disease cohorts now possible for (relatively) low costs. This type of panomic data permits the construction of

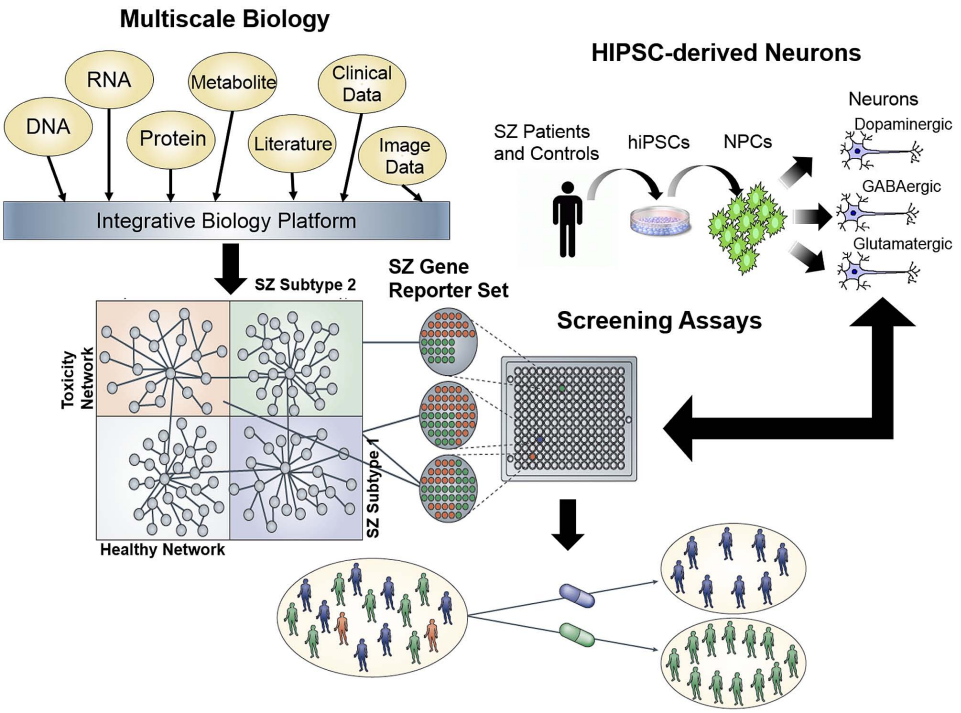

FIGURE 2 | Schematic for a network screening approach to drug discovery. First, integration of multiscale data sets can be reduced to a minimal gene reporter set specific to a disease of interest, in this case, schizophrenia. Second, expression of this gene reporter set can be used as a platform for high throughput screening of hiPSC neurons generated from schizophrenia patients, in order to identify compounds capable of ameliorating the gene expression signature in patient-derived neurons.

interaction and differential connectivity networks, which characterize the connectivity patterns of the molecular networks in disease relevant cell types between those with and without disease. Interaction networks and differential connectivity measures, such as the module-centric differential co-regulation (MDC) measure, provide for deeper insights into the molecular processes involved in disease. For example, by applying MDC to the molecular interaction networks generated from late-onset AD brain regions compared to these same brain regions in non-demented controls (Zhang et al., 2013a), we determine that one module with a significant gain of connectivity in AD patients was enriched for immune function and microglia. This type of analysis, comparing disease cases to controls, can now be carried out on hiPSC-derived cell types in order to generate sets of genes from co-expression modules that are differentially connected.

The network constructs discussed above provide a convenient framework for understanding the core biological processes involved in a given disease of interest, as well as for elucidating the master regulators of disease. Individual signatures of disease, or a given perturbation, can be projected onto the network models, in order to identify the sub-networks that best organize the signatures according to biological processes. For example, gene expression traits monitored in hiPSC-derived neurons can be identified as changed or not, in response to treatment with a given small molecule compound. This signature would represent a complex mixture of changes that reflect the proteins specifically targeted by the compound, the primary response of genes to those specific targets, downstream changes that result from changes in these molecular states, changes induced by unintended targets, and so on. By projecting this complex signature onto a more comprehensive multiscale network model, the signature can be broken up into different coherent components that reflect different biological processes and molecular functions associated with the action of the drug on the cell system under study (**Figure 3**). The identified components represent sub-networks that can in turn elucidate potential disease mechanisms defined by them. The master regulators of these sub-networks can in turn be identified using key driver analysis methods (Zhu et al., 2008, 2012; Tran et al., 2011; Zhang et al., 2013a) that involve finding the largest connected graphs containing the sub-networks, and then perturbing each node (or combination of nodes) in this expanded sub-network *in silico* to predict the network response. Those nodes that significantly alter the state of the network are declared as key drivers or master regulators of the sub-network. We have previously demonstrated this type of key driver analysis to identify networks and their corresponding key drivers associated with inflammatory bowel disease (IBD), AD, and other such common human diseases (Jostins et al., 2012; Wang et al., 2012; Zhang et al., 2013a).

Application of multiscale networks to high throughput screening

Multiscale models defining networks for a given neurological disease can be used to construct gene expression assays for high throughput screening (**Figure 2**). The effect of any given

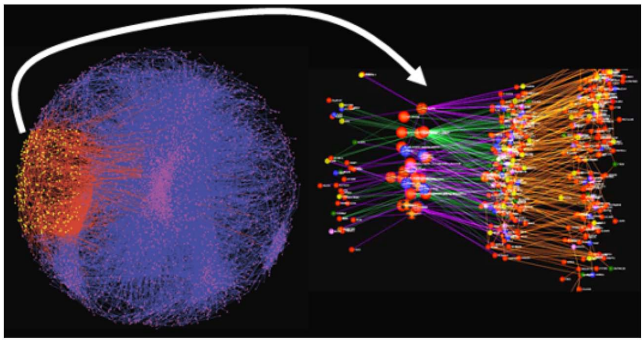

FIGURE 3 | Inflammatory bowel disease-associated gene set projected onto a predictive network model. The yellow nodes and red edges indicate the sub-network most significantly enriched for the signature gene set. The right panel is an expanded view of the impacted sub-network. The larger nodes represent key drivers that are >10-fold enriched for genes associated with IBD, whereas the sub-network itself is roughly 4-fold enriched.

perturbagen, whether a small molecule compound, natural product, RNAi-based construct and so on, on a specific network of interest can be assayed directly in cell-based systems (such as hiPSC-derived neurons derived from SZ or AD patients), which more accurately reflect the states of networks underlying disease. Complementing the network-based screens that use molecular network state as a readout, are cellular phenotyping assays that also aid in the linking of molecular states of disease to pathophysiological states. Screening carried out in this way can lead to the rapid identification of compounds that affect disease networks in favorable ways, while simultaneously identifying compounds that hit networks associated with toxicity or other adverse events (**Figure 2**). In this way, compounds can be identified that target specific subtypes of disease without targeting networks that can lead to toxicity or adverse events.

Network constructs can be used to inform on molecular responses to perturbations with small molecules or other perturbagens, where the networks enable a direct link between molecular biology and pathophysiology. In a high throughput screening context, where transcription or other molecular features are the readout, in addition to cell-based phenotypes, the aim is to identify molecular responses to the perturbagens that are predicted to associate with physiological changes in favorable directions, while simultaneously being predicted to have a minimal adverse event profile. Networks can be integrated with molecular screening data to identify those perturbagens from the screen that have similar mechanisms of action, that impact key disease related processes, or that impact key driver genes of diseases of interest. We and others have previously made use of network models to inform on perturbagen-induced molecular signatures as a means of predicting and validating the impact a given gene or genes had on molecular states and the pathophysiology of disease-associated with those states (Mehrabian et al., 2005; Schadt et al., 2005; Chen et al., 2008; Zhu et al., 2008, 2012).

This type of approach has been more generally applied to repurpose existing drugs for novel indications. For example, IBD signatures were derived from surgical specimens and intersected with Connectivity Map data representing transcriptional readouts across a number of cell lines in response to treatment with many 100s of drugs using a novel pattern-matching algorithm (Dudley et al., 2011). From this search the anticonvulsant drug topiramate was identified and experimentally validated as a novel treatment for IBD (Dudley et al., 2011). Topiramate has primary indications for seizure disorders and no history of efficacious use for IBD or other inflammatory diseases. Using a chemically induced (2,4,6-trinitrobenzenesulfonic acid) rodent model of IBD to evaluate the activity of topiramate administered in the presence of an IBD phenotype, a statistically significant reduction in gross pathophysiological and histopathological measures of severity of the induced IBD phenotype in the population of animals receiving topiramate compared to untreated vehicle controls was observed.

Returning one last time to novel successes in cancer therapeutics, in a separate study, this same computational drug repurposing strategy was applied to transcriptional profiles of small cell lung cancer (SCLC; Jahchan et al., 2013). The system identified imipramine (a tricyclic antidepressant), bepridil (a calcium channel blocker), and promethazine (a phenothiazine antihistamine) as having anti-SCLC activity. These predictions were experimentally validated, demonstrating anti-SCLC activity across a number of *in vitro* and *in vivo* experiments using human and animal model systems. The same approach was used to identify and validate anti-neoplastic activity of the anti-ulcer drug cimetidine against NSCLC (Sirota et al., 2011).

While low cost sequencing assays have provided an unprecedented amount of data on genetic loci and variants associated with common syndromic disorders, these loci by themselves are not sufficient to garner the most informative insights into disease mechanisms upon which new drug discovery efforts may be founded. Again, analogous to the oncology field, multiscale predictive models now provide a computational platform that has the potential to significantly improve the success rate of neurological drug discovery if integrated appropriately.

CONCLUSION

After considering the frequent clinical failures for novel drugs together with the novel cancer research paradigms that have led to improved drug discover, here we have discussed two transformational technologies that have and will continue to provide unprecedented insights into molecular mechanisms associated with complex diseases of the brain, and thereby de-risk drug discovery. First is the ability to generate on a large-scale hiPSCs derived neurons; this cellular model recapitulates disease mechanisms *in vitro*, thereby enabling studies of: disease mechanisms, genotype-phenotype relationships, and causative or risk factors. Furthermore, hiPSC derived neurons offer the ability to engineer assays that have direct relevance to disease biology, analogous to proliferation assays on cancer cell lines. Second, advances in biotechnology have enabled very low cost sequencing of nucleic acids, leading to big data and identification of large ensembles of gene loci and variants, which necessitated a revolution in computing and big data analytics to more comprehensively integrate very large-scale data and infer predictive models from it. Using these methods, we have already identified novel insights into

disease mechanisms in AD, which are now the focus of drug discovery in small and large pharmaceutical companies; we expect that a similar approach will yield a better understanding into the mechanisms and treatment of psychiatric disorders, such as SZ. Together, these technologies can spawn a new generation of drug screening paradigms where screening assays (either *in vitro* or *in silico*) that capture far more of the relevant biology for common human diseases may be performed. The prediction is that such screens will significantly improve discovery of targets and drugs, as well as diagnostic tests, to make personalized therapies a reality for CNS disorders.

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Label-free drug discovery

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Current drug discovery is dominated by label-dependent molecular approaches, which screen drugs in the context of a predefined and target-based hypothesis *in vitro*. Given that target-based discovery has not transformed the industry, phenotypic screen that identifies drugs based on a specific phenotype of cells, tissues, or animals has gained renewed interest. However, owing to the intrinsic complexity in drug–target interactions, there is often a significant gap between the phenotype screened and the ultimate molecular mechanism of action sought. This paper presents a label-free strategy for early drug discovery. This strategy combines label-free cell phenotypic profiling with computational approaches, and holds promise to bridge the gap by offering a kinetic and holistic representation of the functional consequences of drugs in disease relevant cells that is amenable to mechanistic deconvolution.

Keywords: cell phenotypic screen, drug safety/toxicity, label-free drug discovery, lead selection, molecular mechanism of action, phenotypic screen, polypharmacology, target identification

INTRODUCTION

Early drug discovery is achieved mainly through two strategies, target-based and phenotypic approaches (Hart, 2005; Swinney and Anthony, 2011). Target-based screens use high-throughput and label-dependent molecular assays to measure the effect of compounds on a specific target protein *in vitro*, while phenotypic screen use unbiased phenotypic assays to examine the effect of compounds on a specific phenotype of cells, tissues or animals. Target-based approaches have been dominating early drug discovery in the past quarter of century, which is coincident with the continuous decline in productivity of pharmaceutical research and development (Paul et al., 2010; Pammolli et al., 2011; Scannell, 2012). Several factors contribute to this productivity crisis. First, there have been increasing efforts in high-risk projects for unmet therapeutic needs and associated with unexploited biological mechanisms in the past decades (Kamb et al., 2007; Hopkins, 2008; Rask-Andersen et al., 2011). Second, the target chosen in a screen may be not essential to disease pathogenesis or induce undesired toxicity, and the molecular mechanism of action (MMOA) investigated may be unable to produce therapeutic benefits (Hopkins, 2008; Swinney and Anthony, 2011). The MMOA describes the interaction between a drug and its target (or targets) that creates a specific response. Third, many, if not all, drugs display clinically relevant polypharmacology – the specific binding of a drug to more than one target (Roth et al., 2004; Yildirim et al., 2007; Hopkins, 2008; Rask-Andersen et al., 2011), suggesting that single target-based screen may *de facto* be ineffective. Fourth, molecular assays for target-based screens generally rely on the use of labels, which may cause artifacts in results (Beher et al., 2009; Pacholec et al., 2010; Hu et al., 2012). Lastly, traditional phenotypic approaches suffer disadvantages associated with low-to-moderate throughput, and difficulty in target deconvolution and in governing medicinal chemistry optimization (Kenakin, 2009; Swinney and Anthony, 2011).

In the past years, phenotypic screens have gained renewed interest in discovering first-in-class or best-in-class medicines (Lee et al., 2012; Eggert, 2013). Comparing to traditional phenotypic approaches, label-free cell phenotypic profiling techniques afforded by optical or electric biosensors offer clear advantages in rich information content, real-time kinetics, highly flexible assay formats, and high-throughput, beside wide pathway coverage and ability in multi-target profiling and screening that are common to all phenotypic assays (Fang, 2013). Optical biosensors such as resonant waveguide grating (RWG) measure drug-induced dynamic mass redistribution (DMR) signals, while electric biosensors measure drug-induced impedance signals (Fang, 2010). In parallel, similarity analysis based on two-dimensional structures of compounds has been used to predict drug–target interactions (Keiser et al., 2007, 2009; Lounkine et al., 2012), while molecular docking using ever increasing numbers of three-dimensional protein structures are also productive (Carlsson et al., 2011; Koutsoukas et al., 2011; Shoichet and Kobilka, 2012; Stevens et al., 2012).

Herein, I propose a label-free strategy combining label-free cell phenotypic profiling techniques with computational approaches for early drug discovery (**Figure 1**). Essential to this strategy is that label-free cell phenotypic profiling techniques are used for multi-target screening, target identification, MMOA determination, and lead selection. Bioinformatics analysis of the label-free profiles of compounds is used to provide analytical support for target identification, and chemical similarity analysis is used to expand compound library for lead optimization and selection. Of note, the principles and applications of label-free biosensors for cell analysis have been widely reviewed in literature (Fang, 2006, 2011b; McGuinness, 2007), and thus not included in the present review.

LABEL-FREE CELL PHENOTYPIC SCREENING

CHOICE OF CELLS

As the basic unit of life cells have been widely used for drug discovery, mostly because the functional responses of drugs in

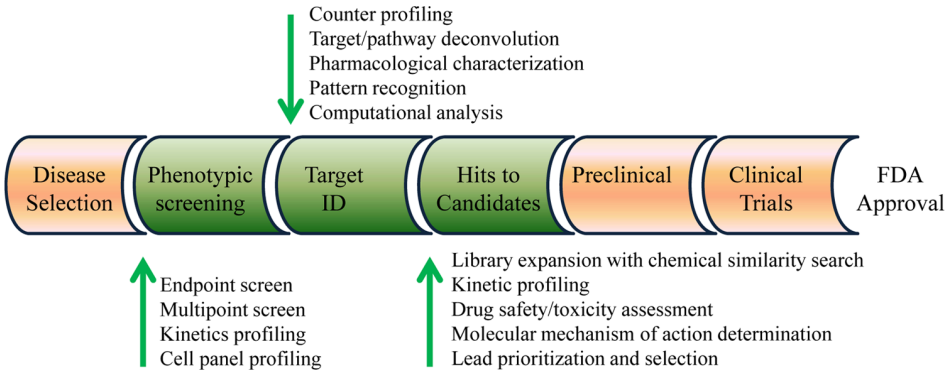

FIGURE 1 | Label-free drug discovery strategy. Combining computational approaches with label-free cell phenotypic profiling and screening techniques can be used for high-throughput screening, target engagement determination, compound library expansion, lead optimization, molecular mechanism of action determination, drug safety/toxicity assessment, and lead prioritization and selection.

cells provide better understanding of receptor physiology and drug pharmacology than *in vitro* binding studies. Target-based approaches often use recombinant cell lines expressing a specific target implicated in a disease, while cell phenotypic approaches often use native cells including immortalized cell lines, primary cells, and stem cells. As surface sensitive and non-invasive techniques label-free biosensors can examine drug-induced minute changes in a confluent layer of eventually all types of cells (Fang, 2010, 2011a), including primary (Hennen et al., 2013) or stem cells (Bagnaninchi and Drummond, 2011; Abassi et al., 2012; Pai et al., 2012). Compared to recombinant cell lines, primary or stem cells retain many functions seen *in vivo* and express endogenous targets of interest in their native signaling circuitry, thus permitting drug profiling using more physiologically and clinically relevant cell phenotypes (Kenakin, 2009; Eglen and Reisine, 2011). Owing to its spatially resolved capability the recently developed RWG imager enables drug profiling using partially confluent cells (Ferrie et al., 2010) or even single cells (Ferrie et al., 2012), and thus opens an unique opportunity to screen drugs using primary or stem cells when homogeneous cell populations are difficult to obtain (Pai et al., 2012).

CHOICE OF CELLULAR PHENOTYPES

Disease relevant cellular phenotypes can be structural, morphological, or physiological abnormalities involving cells or cell components. Structural abnormalities can be classified based on cellular component hierarchy, whereas abnormal morphology phenotypes is either the (abnormal) absence of required cellular parts, the (abnormal) presence of additional cellular parts, or abnormal qualities of cellular parts, and abnormal physiology of a cell component refers to abnormal functionality of a cell component (Hoehndorf et al., 2012). Thus, drug profiling and screening can be performed using a great number of cellular phenotypes such as angiogenesis, cell death, cell division, and inflammation; depending on the MMOA of interest one or more specific cellular phenotype may be examined (Welsh et al., 2009; Kepp et al., 2011). For label-free cell phenotypic screening, two common approaches developed are endpoint and kinetic based screens (see below). Given that label-free biosensors are sensitive to cell numbers, cell signaling and morphological changes, these biosensors permit screening and profiling compounds in the context of a great number of cellular phenotypes ranging from cell adhesion to cell life cycle (cell cycle progression, division, and growth), receptor signaling, cell death, viral infection, cell migration and invasion, and cell-cell communication (**Figure 2**).

ENDPOINT HIGH-THROUGHPUT SCREENS

The endpoint screens leverage the ability of a biosensor to record and encode the signaling events of a specific receptor in a population of cells (typically confluent cells) into an integrated and kinetic biosensor response for identifying active molecules specific to the receptor (Fang, 2010). Here, once the biosensor profile of a receptor cognate agonist in the cells is obtained, its response at a specific time point is monitored and used as the readout to fish out ligands for the receptor of interest from a compound library. In order to identify distinct classes of ligands screening can be performed using different formats. For instance, one-step assay may be useful for discovering agonists, wherein the cells are stimulated with compounds, each individually. Considering the wide presence of compensatory pathways in cell signaling, the one-step agonist screen may result in relatively high false positives for the receptor of interest. Such false positives can be minimized using two-step endpoint screens, wherein the cells are stimulated with compounds first, followed by stimulation with a cognate agonist specific to the receptor. Compounds that are active in the first step and also desensitize the second agonist stimulation would be agonists for the receptor, while compounds that are inactive in the first step but block the second agonist stimulation would be antagonists for the receptor (Tran and Fang, 2008; Deng et al., 2011a). In addition, a three-step assay wherein a compound washout step is introduced between compound and receptor cognate agonist stimulation steps can be useful for identifying long-acting antagonists or agonists (Goral et al., 2011; Deng et al., 2012b).

MULTI-PARAMETER SCREENS

Receptor signaling is encoded by the coupling of temporal dynamics with spatial gradients of signaling activities (Kholodenko,

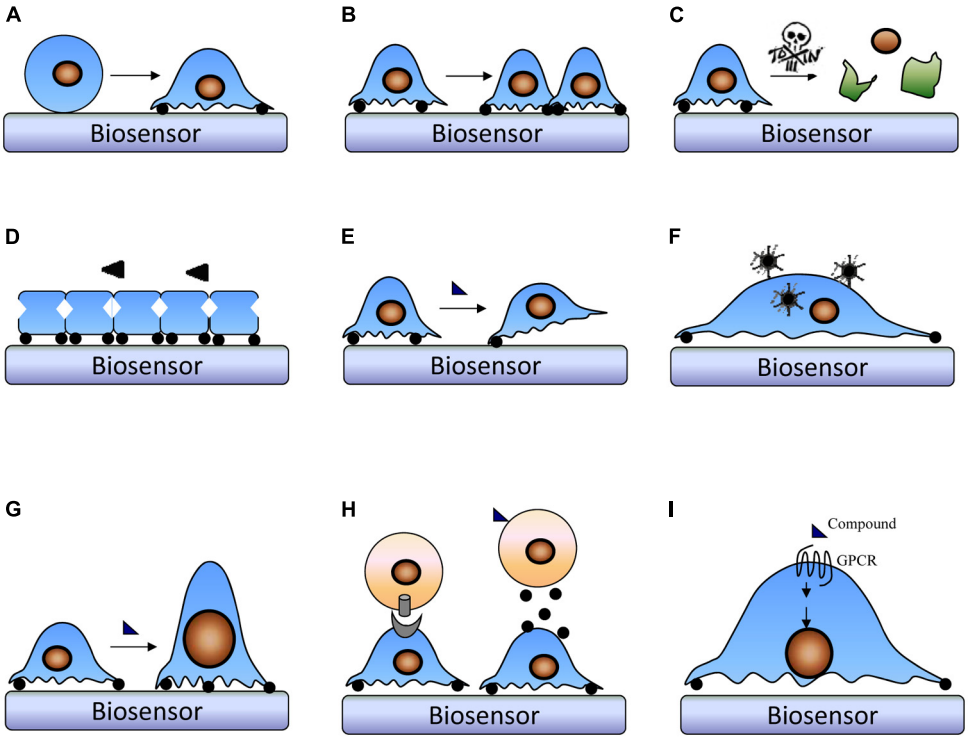

FIGURE 2 | Representative label-free cellular phenotypes examined with label-free techniques. Label-free biosensors can be used to monitor in real-time a great number of cellular process ranging from cell adhesion **(A)** to cell proliferation **(B)**, cell death **(C)**, cell barrier function **(D)**, cell migration **(E)**, viral infection **(F)**, cell morphology **(G)**, cell–cell communication **(H)**, and cell signaling **(I)**. To monitor different cellular phenotypes, different assay conditions may be applied.

2006), and may come in multiple pathways and waves (Ferrie et al., 2013; Lohse and Calebiro, 2013). Consequently, label-free biosensors as a non-invasive recorder mirror the dynamics of receptor signaling, and the biosensor signature arising from the activation of a receptor could contain multiple phases. Therefore, multi-parameter profiling and screening may be feasible and offer additional information regarding to the specificity and mechanisms of action of hits to a receptor, a signaling protein, or a pathway.

REAL-TIME KINETIC PROFILING

For label-free cell phenotypic screens, real-time kinetic measurements of drug action would be more informative but with lower throughput than end-point screens. The kinetic profiling of compounds may be performed in the context of a specific cellular process such as cell adhesion, cell growth and death, or cell signaling. Label-free biosensors allow for interrogating drug molecules with wide coverage in targets and pathways of native cells (**Figure 3**; Fang, 2011b, 2013). The modulation of many classes of targets including G protein-coupled receptors (GPCRs), receptor tyrosine Kinases (RTKs), transporters, Toll-like receptors (TLRs), immune receptors, enzymes, cell structural proteins, and kinases can directly lead to rapid biosensor responses. The most popular is to profile compound-induced cell signaling in confluent cells, given that the cells once reach confluency start to enter a new growth cycle or a quiescent state and the compound-induced response is almost exclusively due to cell signaling (Fang, 2010, 2011a). Alternatively, the long-term impacts of compounds on cell growth can also be used to screen compound library (Abassi et al., 2009; Fu et al., 2011). Of note, this approach may be able to identify ligands for other classes of targets such as nuclear receptors whose activation by themselves may not result in rapid signaling-related biosensor responses.

CELL PANEL PROFILING AND SCREENING

Large panels of disease relevant cell lines annotated with both genetic and pharmacological data are powerful tools for drug discovery (Barretina et al., 2012; Garnett et al., 2012). For instance, NCI60 consists of 60 (now 59) human cancer cell lines from nine different tissues introduced in 1990 by the US National Cancer Institute (NCI) in Bethesda, Maryland, and has been widely used for discovering new anticancer drugs (Shoemaker, 2006). For label-free profiling, cell panels may consist of multiple cell lines for a disease, a parental cell line and its recombinant counterparts, or a stem cell and its differentiated cells. Each cell line has unique expression pattern of functional receptors and signaling circuitry. Thus, the use of cell panels not only expands the number of addressable targets/pathways, but also offers confirmative information regarding to the potential mechanism of action of active compounds identified in label-free cell phenotypic screens (Verdonk et al., 2006; Morse et al., 2011, 2013; Pai et al., 2012; Ferrie et al., 2014). For instance, using the DMR assay we profiled

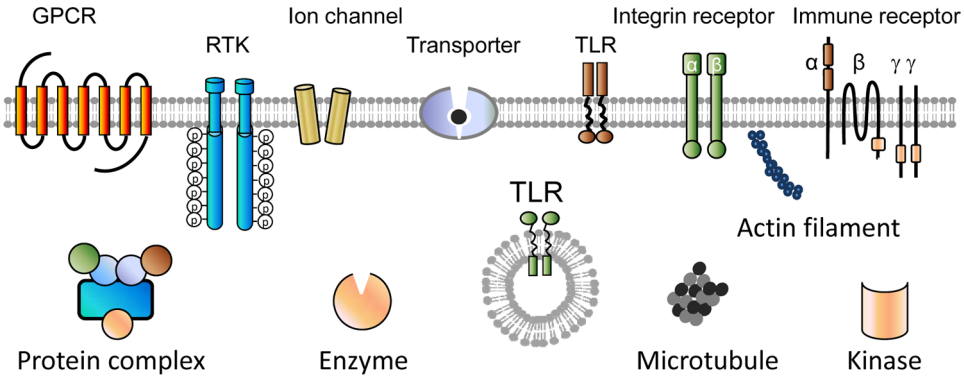

FIGURE 3 | Target receptor classes whose activation or modulation has been shown to trigger characteristic biosensor responses in living cells. Label-free receptor signaling profiling has wide coverage in targets and pathways. GPCR, G protein-coupled receptor; RTK, receptor tyrosine kinase; TLR, Toll-like receptor.

a library of sixty-nine ligands of adrenergic receptor (AR) with a cell panel consisting of the parental HEK-293 and four β_2-AR-stably expressed cell lines, and found that HEK-293 endogenously expresses functional G_i-coupled α_2-AR and G_s-coupled β_2-AR, and these ligands displayed divergent label-free cell phenotypic pharmacology (Ferrie et al., 2014).

TARGET IDENTIFICATION AND VALIDATION

Identifying the target for a specific phenotype is vital to guide lead optimization and to understand the potential toxicity for the target (Hart, 2005). Common to classic phenotypic approaches for determining target engagement is to first generate target hypotheses using pattern recognition to compare small molecule phenotypic profiles to those of known reference molecules, followed by confirmation using direct proteomic approaches (Young et al., 2008; Schenone et al., 2013). However, classical phenotypic approaches mostly rely on descriptive, empirical, and end-point measurements, which, by themselves, generally offer little insights about the biological mechanisms of action of drugs (Feng et al., 2009). In contrast, label-free cell phenotypic approaches all measure real-time kinetic responses of compounds in cells, which contain target- and pathway-specific information (Fang, 2011a). For label-free endpoint and multi-parameter screens, target hypothesis is predefined by the reference agonist cognate to the receptor of interest, so target engagement can be confirmed using direct binding assays or counter profiling using another cell line that does not express the target receptor. For instance, using the DMR signal at 25 min post-stimulation with methacholine in CHO-M_3 cells as the readout, screening a library of 83,000 compounds led to identification of 49 novel muscarinic M_3 receptor ligands that had pIC_{50} values between 4.8 and 6.3 and were further confirmed using radiobinding assays (Dodgson et al., 2009). Here, methacholine is used as the reference agonist for the M_3 receptor. Of note, these novel ligands were found to be false negatives in a calcium flux assay.

For label-free kinetic profiling, target hypothesis can be generated using several approaches. First, target/pathway deconvolution may be directly achieved through investigating the impact of chemical probes and/or genetic manipulations (e.g., RNAi) on the kinetic response of a compound itself (Fang et al., 2005; Deng et al., 2011b; Verrier et al., 2011). Second, counter profiling between a recombinant cell line expressing the receptor of interest and its parental cell line without the receptor is also effective to confirm the target specificity (Ferrie et al., 2014). Third, multiple assays including agonist, antagonist, desensitization, and antagonist reversal assays when respective pharmacological tools are available can be used to ascertain the specificity of a compound to the receptor of interest (Ferrie et al., 2013). Fourth, pattern recognition based on label-free profiles of compounds can be used to generate target hypothesis through comparison of their profiles with databases of the activity profiles of other reference molecules with known targets (Abassi et al., 2009; Fu et al., 2011). Traditional approaches including proteomics-, genetics-, and bioinformatics-based approaches can then be used for determining target engagement (Ziegler et al., 2013). Fifth, computational approaches based on similarity analysis of known probe molecules (Keiser et al., 2007, 2009; Lounkine et al., 2012) or molecular docking (Carlsson et al., 2011; Shoichet and Kobilka, 2012) can be used to predict the probability of the binding of small molecules to a specific target.

HIT IDENTIFICATION

For label-free endpoint screens, hits are selected based on the label-free profile arising from the activation of the receptor of interest, similar to classical target-based screens (Dodgson et al., 2009). For label-free kinetic profiles, hits are selected based on a specific phenotypic response in the context of a specific cellular process, such as the increase in label-free profile of cell adhesion, alteration of the label-free profile of cell growth, or a specific label-free profile of cell signaling. For instance, screening a library of 660 compounds led to identification a characteristic DMR signal in HT-29 cells for a subset of compounds (Deng et al., 2011b). Combining DMR antagonist/desensitization assays with GPR35 knockdown with interference RNA, receptor internalization, and Tango β-arrestin translocation assays revealed that two novel series of chemical compounds, 2-(4-methylfuran-2(5H)-ylidene)malononitrile and

thieno[3,2-b]-thiophene-2-carboxylic acid derivatives, are GPR35 agonists.

LEAD OPTIMIZATION

Once hits are identified and confirmed, searching similar compounds from commercial and public databases can quickly expand compound library for generating structure-activity relationship (SAR) analysis. These databases include PubChem (Wang et al., 2009), ChemBank (Seiler et al., 2008), DrugBank (Knox et al., 2011), ChemBL (Gaulton et al., 2012), and ZINC (Irwin and Shoichet, 2005). With the ever-increasing number of compounds annotated with biological and pharmacological activities in these databases, it is highly possible to quickly identify lead-like compounds with high specificity and potency to the target receptor. For instance, according to the similarity of tyrphostins to 2-(4-methylfuran-2(5H)-ylidene)malononitrile compounds, we hypothesized and confirmed that a group of tyrphostins such as tyrphostin-51 are GPR35 agonists with moderate potency (Deng et al., 2011a). Given that tyrphostins, the first generation of tyrosine kinase inhibitors, are tyrosine analogs (Levitzki and Mishani, 2006), we hypothesized and confirmed that multiple tyrosine metabolites are GPR35 agonists (Deng and Fang, 2012b; Deng et al., 2012a). From these SAR studies, we further expanded the chemical library by searching public databases and identified a group of nitrophenols as GPR35 agonists, among which 4,4'-(2,2-dichloroethene-1,1-diyl)bis(2,6-dinitrophenol) displays high potency with an EC_{50} of 6nM (Deng and Fang, 2012a).

DRUG SAFETY/TOXICITY ASSESSMENT

Drug toxicity/safety assessment is essential to drug discovery and development, and may be studied using several label-free cell phenotypic profiling approaches. First, the recently developed high frequency electric impedance biosensor system can be used to monitor the impact of drugs on the beating patterns of primary or stem cell-derived cardiomyocytes; and this system can recapitulate known effects of various known modulators of cardiac function (Abassi et al., 2012). Cardiac toxicity is one of the major concerns in drug development, and accounts for one-third of all drug withdrawals from the market (Wilke et al., 2007). Second, potential adverse drug reactions (ADRs) of compounds can be assessed using a panel of cells consisting a parental cell line and a number of recombinant cell lines, each expressing a specific target receptor that is known to be associated with and prone to cause ADRs. ADRs are the second leading cause for attrition of drug candidates in clinical trials, behind lack of efficacy (Arrowsmith, 2011). Factors that cause ADRs include the primary target of the drug itself, non-specific interactions of reactive metabolites of the drug, or unintended activity at off-targets. The number of off-targets that is known to be associated with ADRs is relatively small (∼75; Bender et al., 2007; Campillos et al., 2008; Lounkine et al., 2012), almost all of which can be directly examined using label-free profiling. Practically, these recombinant cell lines can be made readily to be profiled as cell bank (e.g., frozen cell batches), or transitly transfected *in situ* using classical viral or plasmid DNA-based approaches. Third, computational approaches based on chemical structures or molecular docking can be used to calculate the probability of drug candidate molecules binding to these ADR-related targets (Lounkine et al., 2012).

MMOA DETERMINATION

Elucidating the MMOA of drug candidate molecules is a critical step in drug discovery. Label-free biosensor such as surface plasmon resonance and RWG is well-known for its ability to determine the affinity and kinetics of drugs binding to their primary target (Schuck, 1997; Fang, 2012). Label-free cell phenotypic profiling also can provide information regarding to the MMOA of compounds. This is done through leveraging the sensitivity of the label-free profiles of compounds to their polypharmacology (Wermuth, 2004), functional selectivity (or biased agonism; Kenakin, 2012), binding kinetics (Deng et al., 2013a), binding orientation (Bock et al., 2014), cell membrane permeability (Ferrie et al., 2013), and transport mechanisms (Deng et al., 2013b; reviewed in Fang, 2013).

The biased agonism describes ligand-dependent selectivity for a specific signal transduction pathway over others downstream the same receptor, and is common to ligands for GPCRs and potentially other classes of receptors (Fang, 2013). Owing to its integrative nature in measurement (Fang et al., 2006), label-free cell phenotypic profiling, by *de facto*, is not ideal for directly assessing biased agonism (Morse et al., 2013). However, several label-free approaches may be useful to manifest biased agonism. First, multi-parameter kinetic analysis may sort ligands for a specific target into different clusters. For instance, profiling of a set of β_2-adrenergic receptor (β_2-AR) ligands in A431 cells using DMR assays revealed that multiple kinetic parameters extracted from their responses allow fine classification of these ligands based on their efficacy and biased agonism (Fang and Ferrie, 2008; Fang, 2010). Second, the recent developed integrative pharmacology ontarget (iPOT) approach can classify ligands based on their specificity, pathway selectivity, and efficacy for the target receptor of interest (Ferrie et al., 2011; Morse et al., 2011, 2013). The iPOT approach leverages distinct sensitivity of the label-free profiles of different drugs acting through the same receptor in different cell lines, or the same cell line but with different preconditioning. The cell preconditioning can be achieved using specific probe molecules to impair or alter specific pathways, or genetic tools to alter the expression of a specific signaling protein. Using this approach, we had obtained a pharmacological heatmap of all adrenergic receptor drugs approved by the US Food and Drug Administration that correlates well with their *in vivo* indications (Ferrie et al., 2011).

The functional consequence of different binding kinetics of a family of ligands for a specific receptor can also be assessed using label-free biosensor ligand washout assay. The onboard microfluidics is the most effective means to control the duration of a ligand exposed to the cells, so it is possible to determine whether the effect of the ligand is short or long acting. For instance, using microfluidics to control the agonist stimulation duration we found that the activation of the β_2-AR in A431 with a pulse of its agonist as short as 1 min is sufficient to trigger a sustained response, whose sustainability is dependent on the type and concentration of agonists, and the stimulation duration (Goral et al., 2011; Ferrie et al., 2013).

In another study, combining radiobinding results with electric biosensor profiling results revealed that the efficacy of adenosine A_{2A} receptor agonists is positively correlated to their receptor residence time (the reciprocal of off rate; Guo et al., 2012). Almost identical trend was found for a family of agonists for endogenous muscarinic M_3 receptors in six different cell lines (Deng et al., 2013a).

The cell membrane permeability and transport mechanism are important mostly for the efficacy of drugs acting at intracellular targets. A recent DMR study of three inhibitors for epidermal growth factor receptor (EGFR) in A431 and HT-29 showed that the recovery of EGFR signaling after inhibitor removal from the extracellular buffer was faster in HT-29 than in A431, and also dependent on the duration of inhibitor removal (Deng et al., 2013b). Furthermore, the potency of three inhibitors including gefitinib, erlotinib, and AG1478 was generally higher in A431 than HT-29 cells. The most possible mechanism for this is that the drug uptake and retention paly a dominating role in determining the whole cell efficacy of these kinase inhibitors. The cellular retention of these inhibitors is a function of cell uptake and effluxing via efflux transporter such as breast cancer resistance protein (BCRP/ABCG2). All three inhibitors tested are ABCG2 substrates; A431 cells express little ABCG2, while HT-29 expresses high amount of ABCG2.

LEAD SELECTION AND PRIORITIZATION

Effective lead selection and prioritization is essential for getting the cost of early drug discovery under control. In a typical screening campaign, tens of thousands of hits are often identified. After optimization, about one hundred lead-like molecules are selected for animal testing. The iPOT approach, label-free profiling techniques in general, are useful to classify these lead-like molecules into distinct clusters, each of which may share a common MMOA (Ferrie et al., 2011). Representative lead-like molecules from each cluster can then be selected for *in vivo* testing (Morse et al., 2013). Computational approaches, in particular chemical similarity analysis against the ADR-related receptor panel, would also be beneficial to lead selection process.

CONCLUSION

In the recent years, there has been a renaissance in phenotypic approaches for drug discovery. Label-free cell phenotypic profiling and screening holds great promise in discovering disease-modifying activities of drug molecules via validated or previously undescribed targets, or by acting simultaneously on more than one target. Combining computational approaches, in particular similarity analysis based on chemical structures and molecular docking based on three-dimensional structures of target proteins, with label-free approaches would greatly facilitate early drug discovery by permitting target engagement determination, compound library expansion, MMOA deconvolution, safety and toxicity assessment, and lead optimization and selection.

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Quantitative phenotypic and pathway profiling guides rational drug combination strategies

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Advances in target-based drug discovery strategies have enabled drug discovery groups in academia and industry to become very effective at generating molecules that are potent and selective against single targets. However, it has become apparent from disappointing results in recent clinical trials that a major challenge to the development of successful targeted therapies for treating complex multifactorial diseases is overcoming heterogeneity in target mechanism among patients and inherent or acquired drug resistance. Consequently, reductionist target directed drug-discovery approaches are not appropriately tailored toward identifying and optimizing multi-targeted therapeutics or rational drug combinations for complex disease. In this article, we describe the application of emerging high-content phenotypic profiling and analysis tools to support robust evaluation of drug combination performance following dose-ratio matrix screening. We further describe how the incorporation of high-throughput reverse phase protein microarrays with phenotypic screening can provide rational drug combination hypotheses but also confirm the mechanism-of-action of novel drug combinations, to facilitate future preclinical and clinical development strategies.

Keywords: phenotypic screening, drug combinations, high-content, proteomics, network pharmacology

INTRODUCTION

The evolution of many complex human diseases has generated multiple biological redundancies in the genetics, pathway signaling networks and pathophysiology of disease thus counteracting the efficacy of new therapeutics. In such complex diseases exemplified by cancer, neurodegeneration, cardiovascular, bacterial, and viral infections, combination therapies represent the standard of care (Zimmermann et al., 2007; Al-Lazikani et al., 2012; Yap et al., 2013). Examples of fixed dose and co-administration drug combination therapies approved across multiple disease indications are described in **Table 1**.

With regards to cancer it is apparent that further exploitation of new targeted therapies including novel combinations of unapproved agents and targeted therapy combined with established chemo- or radio-therapy remain to be fully explored in preclinical and clinical settings. Numerous clinical trials are progressing that promise to maximize the value of targeted drugs as combination therapies in cancer (Yap et al., 2013). The challenge is to select which of the many drug combination possibilities and administration schedules are most suitable for clinical development across distinct cancer patient populations.

Given the widespread use and strong track record of clinical success of combination therapy across complex diseases, it is surprising that most typical drug discovery strategies only consider drug combinations during late-stage development or as a risk mitigation strategy. The re-emergence of phenotypic drug discovery (PDD) strategies provides a new opportunity to discover and prioritize drug combination and polypharmacology strategies objectively while appropriately tailoring their use to complex disease during early stage drug discovery (Lee and Berg, 2013). In this review article we highlight how new advances in high-content screening and high throughput pathway profiling capabilities advance phenotypic screening approaches toward a more systematic discovery of the next generation of multi-targeted drugs and drug combination therapies.

PHENOTYPIC SCREENING OF DRUG COMBINATIONS

Recent advances in automated image-based high-content microscopy provide a new opportunity to perform hypothesis-free phenotypic screening of complex compound libraries and drug combination sets in more sophisticated biological assays (Bickle, 2010). To support the discovery of novel combination therapies, experimental assay systems that maintain the integrity of biological signaling networks in their most physiologically relevant states are most desirable. In their simplest form, such phenotypic assays may represent single cell-based assays, however, more elaborate co-culture or 3-dimensional (3D) organotypic models can be employed to explore multi-targeted intervention of paracrine or juxtacrine signaling between distinct cell populations (Harma et al., 2010).

A wealth of preclinical drug combination data using cell based and *in vivo* models has been published that serves as the basis for clinical proof-of-concept studies and patent claims of novel drug combination strategies. A limitation of much of the patented and published drug combination studies to date is that they are often performed or presented as isolated studies focusing on a specific combination. As such, these combinations are not placed into context of broader combination options or

Table 1 | Examples of drug combinations approved for clinical use across disease indications.

Indication	Approved drug combinations
Melanoma	Trametinib (a MEK inhibitor) + Dabrafenib (a BRAF inhibitor) approved through the FDA accelerated approval program (Flaherty et al., 2012)
Pancreatic cancer	Gemcitabine + nanoparticle albumin-bound nab-paclitaxel (Abraxane) (Sahoo and Kumar, 2014; Saltz and Bach, 2014; Von Hoff et al., 2014); FOLFIRINOX (5-Fluorauracil + leucovorin [Wellcovorin] + irinotecan [Camptosar] + oxaliplatin) (Oikonomopoulos et al., 2014)
Ovarian cancer	Taxane/platinum combination therapy (carboplatinum/paclitaxel) represents standard of care
Breast cancer	Multiple chemotherapeutic regimes include; Adriamycin + Cyclophosphamide; Adriamycin + Cyclophosphamide + Paclitaxel; Cyclophosphamide + Adriamycin + Fluorouracil; Cyclophosphamide + Methotrexate + Fluorouracil; Fluorouracil + Epirubicin Hydrochloride + cyclophosphamide; Taxotere + Adriamycin + cyclophosphamide
Diabetes	Xigduo (sodium-glucose linked transporter 2 [SGLT2] + metformin) (Jabbour et al., 2014)
Cardiovascular	Vytorin (Eztimibe + simvastatin), Caduet (Amlodipine + Atorvistatin), and Lotrel (Amlodipine + Benezapril) (Dabhadkar and Bellam, 2013)
Antiviral (HIV)	Multi-component anti-retroviral therapies include, Atripla (efavirenz + tenofovir/emtricitabine); Complera (rilpivirine + tenofovir/emtricitabine); and Stribild (elvitegravir + cobicistat + tenofovir/emtricitabine)
Antibacterial	Combinations targeted against antibacterial resistance include, Bactrim (Trimethprim + Sulfa mexathazole); B-Lactamase inhibitor *or* carbapenem + aminoglycoside (for gram negative infection); B-Lactam + streptogramin or teicoplanin + aminoglycoside (for vancomycin resistant gram negative infection) (Sacks and Behrman, 2009)

benchmarked against standard-of-care therapies. The reductionist approaches to the study of specific combination therapies limits the ability of drug development and clinical research groups to objectively prioritize and iterate the most effective drug combination strategies to move forward into late stage preclinical or clinical development. Another major limitation of many preclinical drug combination studies is the physiological relevance of the findings. For example, the identification of synergistic activity at doses that are not achievable *in vivo* or at time points incompatible with the *in vivo* pharmacokinetic properties of the individual components of each combination are highly unlikely to succeed. Thus, advances in high-throughput phenotypic screens including, increased throughput, kinetic profiling drug response in live-cell systems, analysis of multiple phenotypic endpoints across potentially more relevant 3D and co-culture models facilitate a more comprehensive and transparent approach to both hypothesis-driven and hypothesis-free exploration of drug combinations.

The application of phenotypic drug combination screening is exemplified by dose-ratio matrix testing multiple pairwise combinations across cell based assays enabling analysis of synergy, additive, and antagonistic effects across diverse chemical libraries, annotated compound libraries and approved drug sets (Zimmermann et al., 2007). Recent examples of dedicated drug combination screening campaigns using a variety of phenotypic assays and distinct endpoints have been published (Axelrod et al., 2013; Cubitt et al., 2013; Du et al., 2013; Held et al., 2013; Schmidt et al., 2013; Li et al., 2014). Such phenotypic screens have identified novel synergistic combinations such as: Lapatanib (EGFR and Her2 inhibitor) combined with the multi-targeted inhibitor Ro31-8220 (Axelrod et al., 2013); Lapatanib combined with MK2206 (Akt inhibitor) (Held et al., 2013) and Rapamycin (mTOR) combined with Sunitinib (multi-targeted kinase inhibitor) (Li et al., 2014). For pragmatic reasons such recent examples of dedicated combination screening has been mostly limited to small focussed compound libraries and 2-dimensional (2D) cell based assays.

A limitation of screening large compound libraries in complex cell based assays compared with more traditional biochemical drug screening is throughput and cost. Both throughput and cost are particularly limiting when considering the evaluation of multiple drug combinations across a factorial dose-ratio matrix where the number of individual combination dose ratios increases quadratically with the number of agents under study. For practical reasons, medium to high-throughput phenotypic screening across cancer cell lines have traditionally employed simple single endpoint analysis of tumor cell viability or cell proliferation in 2D mono-culture (Barretina et al., 2012). While such assays can provide valuable insights into phenotypic and drug combination response across annotated cell line panels their reliance on gross cell viability and proliferation endpoints tend to favor the phenotypic discovery of cytotoxic agents. Furthermore, integration of basic cell viability endpoints with gene expression profiling provide a useful source of biomarkers that predict sensitivity to cell-cycle arrest but poorly inform on optimal combination strategies or markers for other important cancer phenotypes such as apoptosis and invasion. Recent advances in fully automated brightfield and fluorescent microscopic acquisition platforms and associated image analysis algorithms have facilitated the integration of quantitative microscopic imaging of multiple endpoints upon both fixed and live-cells assays (Perlman et al., 2004; Yarrow et al., 2004; Tanaka et al., 2005; Caie et al., 2010). Screening beyond simplistic 2D monoculture assays is a necessary aim to target more relevant pathophysiological mechanisms and discover novel synergistic drug combination activity. Drug combination screening in complex 3D and co-culture assay formats is most desirable, however, throughput is limiting when using standard cell culture assay methods especially for hypothesis-free screening. The integration of high-content microscopy platforms with sophisticated laboratory automation and optimized data-handling pipelines provide increased throughput and overcome many of the bottlenecks associated with screening large compound libraries across complex assay formats (Bickle, 2008; Alcock et al., 2010). The application of validated mathematical approaches, based upon the median-effect principal and combination index theorem (Chou and Talalay, 1981, 1984) provides robust evaluation of additive, synergy, and antagonism of drug combination effects. Further optimization of

software tools specifically designed for the analysis and visualization of large drug combination screening data sets are based upon methods such as Lowe additivity (Zimmermann et al., 2007) and are exemplified by the *combination* high throughput screening (cHTS) platform (Zalicus®) and Compound Synergy Extension (Genedata Screener®). Such tools enable rapid drug combination screening across phenotypic assays at scale to enable a more transparent review of drug combination data placed into context of multiple drug combination sets to aid benchmarking and prioritization. Incorporation of genomically annotated patient derived cell panels into high capacity drug combination screening activity further supports pharmacogenomics and personalized healthcare approaches to drug combination strategies. High-content single cell analysis of specific phenotypic events over both dose and time enable a significantly more robust evaluation of the quality of drug combination data. Such considerations support the interpretation of whether synergistic and additive drug combination data occur at physiologically relevant doses.

A significant challenge to translating effective drug combinations identified from *in vitro* screens to *in vivo* models and the clinical setting is balancing the distinct pharmacokinetic properties of each individual component of a combination to ensure drug uptake and retention times within the target tissue *in vivo* replicates optimal synergistic dose ratios identified from *in vitro* phenotypic assays. Phenotypic screening of drug combinations across live cell kinetic assays provide further information on the optimal duration of time each component of a drug combination needs to be present together to provide synergistic activity. Kinetic phenotypic analysis is enhanced by image-based reporters of functional endpoints that are compatible with live cell assays as exemplified by the cell-permeable caspase 3 biosensor probe NucView™ which provides a real time readout of caspase activation and induction of apoptosis (Smith et al., 2012). By correlating the kinetics of apoptosis induction to drug combination treatment observed *in vitro* with the *in vivo* pharmacokinetic properties of the drugs, optimal *in vivo* scheduling of drug combinations can be predicted. Kinetic profiling of phenotypic response following drug combination treatment also allows selection of the most appropriate time-points to perform analysis of synergy and antagonistic activity. Building drug combination properties into single molecules such as a multi-targeted small molecule, bi-specific antibody or formulated fixed-dose combination product (polypill) circumvents many of the challenges associated with co-dosing distinct components *in vivo*. Thus, application of more systematic approaches to evaluate the kinetics and sensitivity of drug combinations across broad dose ranges and multiple phenotypic parameters promise to enhance the quality and robustness of preclinical drug combination data and support more informed prioritization of the most appropriate combination strategies to move forward into *in vivo* and clinical settings.

Despite recent advances in automated high-content microscopy, integrating such systematic approaches to drug combination screening in the more advanced preclinical models is always going to be limited by throughput. This is particularly the case when additional considerations such as sequencing of drug combination treatments and greater than pairwise drug combination cocktails are under consideration. Application of computational biology and unbiased artificial intelligence approaches to predicting and/or guiding drug combination selection may overcome the bottlenecks associated with empirical testing of every possible drug combination dose-ratio in both hypothesis-free and hypothesis-driven screening of combinations in complex *in vitro* or *in vivo* models (Lehar et al., 2009; Azmi et al., 2010). The application of genetic algorithms from the field of computational artificial intelligence has been used to guide the selection of drug combinations for empirical testing (Zinner et al., 2009). Zinner et al. employed a genetic algorithm approach to identify novel multi-drug cocktails effective upon cancer cell line proliferation. Using a "fitness function" parameter, defined by pharmacological performance in a cell proliferation assay, the most effective combinations from first generation testing of a small combination set was used to guide algorithmic selection of subsequent generations of combinations representing a sample of a larger compound library. Iterative rounds of testing and selection of the "fittest" combinations provide a rational sampling approach of broad areas of drug combination space. The multi-drug cocktail, Feretinied, suberoylanide hydroxyamic acid, and bortezomib was determined to be the fittest in the A549 non-small cell lung carcinoma (NSCLC) proliferation assay and enhanced efficacy and synergy was subsequently validated in other NSCLC cell lines (Zinner et al., 2009). The complexity of drug combinations would not be limiting if using a genetic algorithm approach, which iteratively samples a small proportion of the best drug combinations extracted from large compound libraries. This approach enables intuitive exploration of new chemical entities, multi-drug cocktails, and alternate drug combination sequencing strategies. The application of multiparametric high content assays that inform on both the efficacy and toxic liability of preclinical drug combinations may assist in defining a multiparametric fitness function that enables a genetic algorithm to direct a guided search of drug combination space toward efficacy and away from toxicity.

FUNCTIONAL PROTEOMICS

A major challenge to the successful clinical application of targeted therapies is the existence of complex intrinsic and adaptive resistance mechanisms that have evolved to maintain the selective advantage of disease systems. While many of the underlying causes of disease occur at the genetic level, drug response and resistance are often governed by epigenetic and post-translational mechanisms. Recent studies indicate that disease pathogenesis, particularly for cancer is associated with the co-activation of multiple signaling pathways (Stommel et al., 2007; Duncan et al., 2012; Lee et al., 2012). Furthermore, targeted therapies specific for these signaling pathways reprogram signaling networks thus providing for multiple compensatory and redundancy mechanism (Duncan et al., 2012; Lee et al., 2012). Therefore, combinations of drugs will be most effective in treating such adaptive systems if we can elucidate the networks and pathway switching mechanisms that permit diseased cells to subvert single therapeutic agents. Intracellular and

paracrine signaling events are highly dynamic and drugs influence the temporal dynamics of signaling networks highlighting the importance of studying signaling events temporally following drug exposure to provide rational for simultaneous or sequential drug combination strategies (Lee et al., 2012). Recent advances in the generation and interpretation of proteomic data complements genomic analysis by providing additional information on pathway activation states providing new insight into complex biochemical pathways driving disease mechanisms and controlling therapeutic response (Kolch and Pitt, 2010). New advances in high-throughput functional proteomics combined with phenotypic screening in more relevant and informative biological models may provide the necessary rationale for selecting drug combinations and for pairing biomarkers to inform on drug combination mechanism-of-action studies and patient stratification strategies for combination therapy.

REVERSE PHASE PROTEIN ARRAYS: HIGH-THROUGHPUT CHEMICAL PROTEOMICS

Traditionally, proteomics has been dependent upon quantitative mass-spectrometry techniques that remain the standard for *de novo* identification of post-translation markers. However, limitations in speed, cost, and sensitivity of mass spectrometry approaches restrict high-throughput application across multiple samples. The evolution in antibody based array methods combined with more sophisticated automation and near infrared optical detection provides new advances in sensitivity, throughput and speed of proteomics (Weissenstein et al., 2006; Voshol et al., 2009). Reverse Phase Protein Arrays (RPPAs) have previously been used to provide quantitative analysis of multiple pathway responses at the post-translational level across large numbers of biological samples simultaneously (Tibes et al., 2006; Weissenstein et al., 2006; Carey et al., 2010; Iadevaia et al., 2010). RPPA is essentially a chip and antibody-based proteomics approach to facilitate broad multiplex analysis of protein analytes, including post-translational modifications in protein extracts isolated from small samples. In **Figure 1A** we provide a general schematic of the RPPA procedure and refer readers to the following literature for more in-depth description of the method (Mueller et al., 2010). Pragmatic benefits of using a reverse protein array approach over alternative mass spectrometric, immunoassays, and immunohistochemical proteomic methods include:

1. Increased throughput: Sample numbers are not limited by reagent costs or instrument throughput, thereby enabling proteomic analysis of multiple compounds evaluated across dynamic dose and time—series in multiple assays and distinct model systems. Such high-throughput chemical proteomics reveal the most consistent compensatory and feedback signaling mechanisms and the genomic and physiological context in which they occur.
2. Precise and sensitive quantification of multiple pathway responses at a post-translational level, including low-abundant phophorylated-epitope signatures that can be mapped directly to drug-target hypotheses including rationale combinations. High-sensitivity enables application to small samples extracted from 96-well compound screening assays and small biopsy/Fine Needle Aspirate samples.
3. Optimal antibody multiplexing format: antibodies are physically separated on the arrays. Thus, there is no potential for antibody cross-reactivity, enabling unlimited multiplexing and optimization of concentrations and incubation buffers for every antibody.
4. Application of antibody-based detection reagents that can be readily adapted to single or small multiplex diagnostic based assays using alternative immunoassay or IHC technology.

RPPA platforms can therefore be used to simultaneously profile many pathway responses across multiple samples in high-density array formats. Pathways covered include key signaling axis, EGF receptor family, PI3K, RAS-MAPK, Src/FAK, Rb/cell-cycle, and TGF and multiple DNA repair, cell-cycle, apoptosis, and epigenetic mechanisms that have all previously been implicated in drug resistant mechanisms in cancer. Ongoing international efforts such as, NCI's Antibody Characterization Program (http://antibodies.cancer.gov); the Human Antibody Initiative (http://www.immunoportal.com/); and the Human Protein Atlas Project (www.proteinatlas.org/) to derive high quality monospecific antibodies are poised to further advance antibody-based proteomics into broader areas of human pathway biology. Correlation of basal RPPA post-translational dataset with compound EC50 values from phenotypic assays performed across cell panels has identified protein level markers of drug sensitivity and resistance (Cardnell et al., 2013). Protein level markers of sensitivity may facilitate patient stratification to identify patient populations most likely to benefit from therapy. Protein level markers correlating with drug resistance can be mapped to drug target databases to identify rational combination strategies (Cardnell et al., 2013). In addition to monitoring the basal expression levels of total protein and post-translational markers, the high throughput nature of RPPA is ideally suited to profiling adaptive post-translational pathway network response across dose and time following compound exposure upon cells. While RPPA analysis is not compatible with live cell studies the throughput, accuracy, cost per sample, and automated nature of RPPA facilitates proteomic analysis of multiple experiments representing distinct time-points. Temporal profiling of pathway response by RPPA following drug treatment has revealed that many-targeted compounds have a dramatic effect upon reprogramming of dynamic signaling pathway networks (Lee et al., 2012). RPPA analysis of multiple pathways normalized to vehicle controls (e.g., DMSO) indicate that target inhibition can switch on many pathways as well as inhibiting others (**Figure 1B**). These data highlight the complexity of integrated signaling networks in living cell systems and the broad effects that targeted therapy can have upon pathway signaling. By performing RPPA studies across dose-response and time series following drug treatments, EC50 values can be calculated across multiple pathway markers over time to distinguish off-target effects from downstream signaling, feedback loop mechanisms and pathway cross talk/compensatory response. By applying RPPA to monitor the activation state of pathways, compensatory signaling response can be directly mapped to drug-target databases to build rational drug

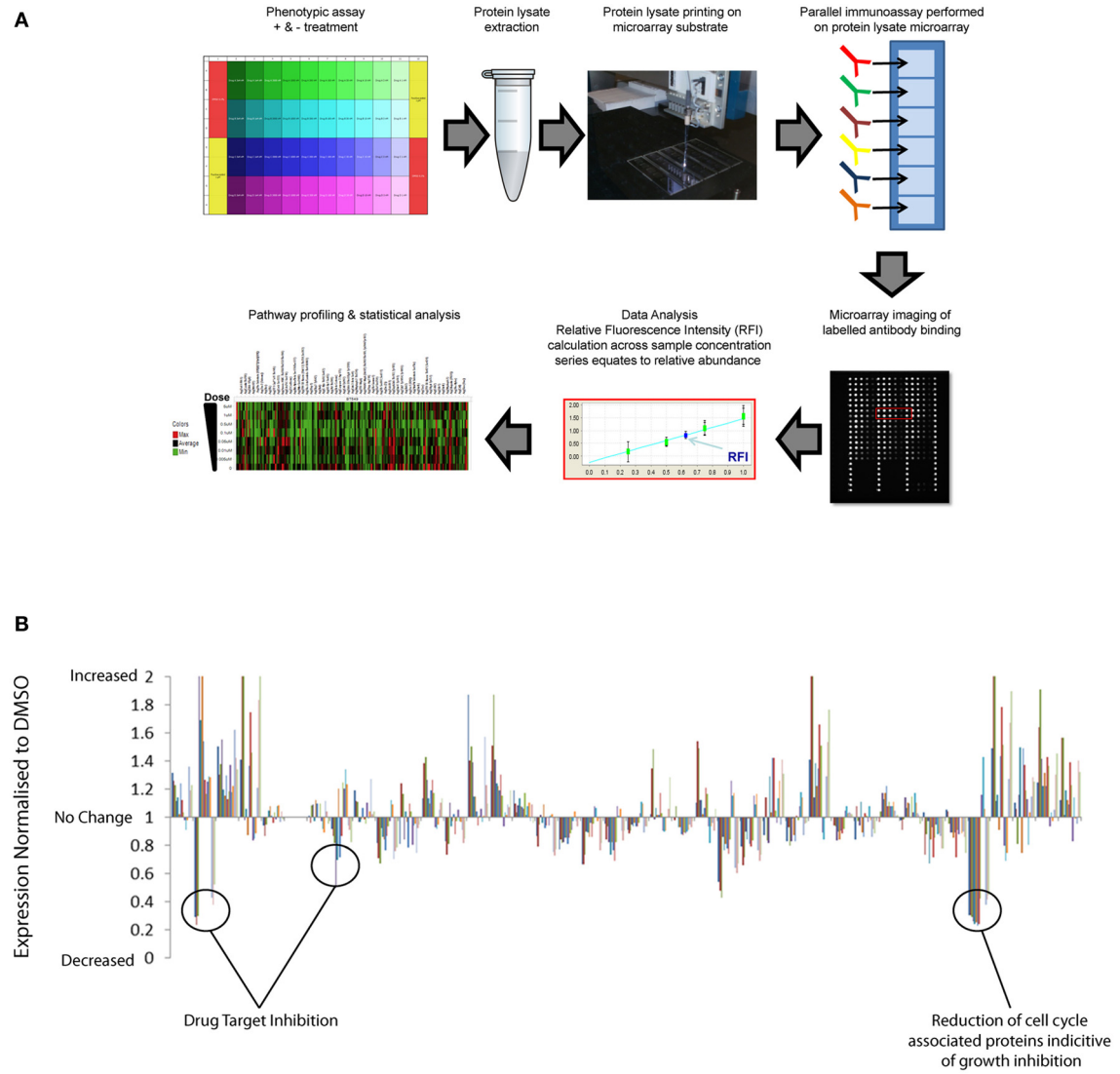

FIGURE 1 | High-throughput reverse phase protein array application to identify drug compound mechanism-of-action and compensatory response. (A) Schematic representation of RPPA workflow: Protein lysates are extracted from multiple samples and deposited across multiple sub-arrays on a microarray by high-precision printing. Validated antibodies are individually addressed to each array, followed by incubation with labeled secondary antibody detection reagent. Antibody binding to each sample is measured by an image-based microarray scanner followed by calculation of the relative abundance of each protein and post-translational modification across the sample set. **(B)** Quantification of the relative abundance of a set of 120 total protein and post-translational pathway markers following exposure to a small set of targeted therapies across dose-response. Un-annotated pathway data normalized to respective DMSO controls demonstrate significant enhancement of multiple pathway signaling events in addition to inhibition of targeted pathways.

combination strategies that can be validated across phenotypic assays or preclinical models.

CONCLUDING REMARKS

High-content imaging is leveraging functional phenotypic endpoints from more complex assay formats such as multicellular co-culture and 3D models, which are ideally suited for screening multi-targeted agents and drug combinations. An attraction of phenotypic screening across panels of human cells derived from patient's disease or human induced pluripotent stem cell (IPSC) models is correlation of phenotypic response with genomic biomarkers. Such pharmacogenomic studies support biomarker discovery and patient stratification hypothesis. However, as described in this review article, adaptive resistance mechanisms that guide combination response are often operating only at the post-translational level. Thus, RPPA analysis applied to related monotherapy and drug combination arms can be correlated with additive, synergistic, or antagonist phenotypic response across a dose matrix study to identify pharmacodynamic biomarkers that confirm the mechanism-of-action of the drug combination effect and provide biomarkers to guide future clinical development.

In 2010, the US FDA issued an updated draft guidance to support the further development of novel drug-combination therapies. Previous guidance recommended demonstrative evidence of a positive efficacy and safety response across all monotherapy and combination arms. The updated guidance now supports the proposal of clinical trial designs where single or multiple monotherapy arms can be left out on the grounds that no efficacy benefit would be expected. This new update now provides an opportunity to exploit synthetic lethality and multi-drug cocktails. Phenotypic screening campaigns are particularly suited to discovery of synthetic lethality by performing screens on matched pairs of cell models, representing suspected natural or engineered genetic vulnerabilities or distinct sensitivities to known agents. The latest advances in phenotypic screening technologies combined with high-throughput pathway profiling are now well placed to provide high quality preclinical data that provide for a more robust, transparent and objective prioritization of drug combinations.

AUTHOR CONTRIBUTIONS

Both authors contributed to the conception of this review article, drafted the paper, and approved the version for submission.

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