Review

Embryonic stem cell application in drug discovery

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Embryonic stem (ES) cells and their differentiated progeny offer tremendous potential for regenerative medicine, even in the field of drug discovery. There is an urgent need for clinically relevant assays that make use of ES cells because of their rich biological utility. Attention has been focused on small molecules that allow the precise manipulation of cells *in vitro*, which could allow researchers to obtain homogeneous cell types for cell-based therapies and discover drugs for stimulating the regeneration of endogenous cells. Such therapeutics can act on target cells or their niches *in vivo* to promote cell survival, proliferation, differentiation, and homing. In the present paper, we reviewed the use of ES cell models for high-throughput/content drug screening and toxicity assessment. In addition, we examined the role of stem cells in large pharmaceutical companies' R&D and discussed a novel subject, nicheology, in stem cell-related research fields.

Keywords: embryonic stem cells; induced pluripotent stem (iPS) cells; differentiation; small molecules; high-throughput/content screening; drug discovery; toxicity assessment; nicheology

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Introduction

Stem cell research promises to make significant progress in regenerative medicine to replace damaged tissues and organs. An outstanding landmark in the past decade has been the development between stem cell biology and chemistry^[1–6]. The combination of the two fields will likely provide an impetus for revealing intricate molecular interactions and functions under complex cellular differentiation, which offers systems for drug discovery and sources for potential cell therapy^[7–10].

Embryonic stem (ES) cells can mimic embryogenesis and differentiate into cells of the three embryonic germ layers (endoderm, mesoderm, and ectoderm) from which various specialized cell types of the entire organism are produced^[11]. Human ES (hES) cells can also differentiate into all terminal cell types of the human body. Small molecules with inducing ability *in vitro* are expected to possess their own mechanism of promoting differentiation *in vivo*. This makes them potentially valuable for pharmaceutical development and safety assessment. Indeed, small molecules can be screened using hES cell models to find new chemical entities that modulate the fate of adult stem cells, or they could be used directly in cell therapies^[4, 8, 10, 11].

Mouse ES (mES) cells have been in use for several years in drug discovery to develop genetically modified mice for target

validation, target selectivity, model development and toxicity evaluation^[12–17]. Combined with advances in hES cell technology, the drug discovery community may soon have a physiologically relevant screening tool (with limitless availability) that shows normal growth and genetic structure.

Understanding molecular mechanisms that determine stem cell fate (*ie*, self-renewal or differentiation) will significantly promote the realization of the therapeutic potential of stem cells^[16, 18–22]. To date, much attention has been paid to signal transduction and the related molecular events during ES cell differentiation. ES cells probably had some novel drug targets, especially in a definite differentiation medium containing low-molecular-weight compound(s). Studies have suggested that a stem cell niche is dynamic, not static, and can be modified or even created^[23–27]. Therefore, the stem cell niches regulated by small molecules can control the cell fate, which offers a suitable microenvironment to screen drugs.

A chemical approach of differentiation by small molecules has currently been developed. This review will address ES cell lines that could potentially be used to discover low-molecular-weight agents for the control of differentiation and drugs for the treatment of degenerative diseases. In addition, small molecules could participate in producing induced pluripotent stem (iPS) cells for lower oncogenic and higher reprogramming efficiency by small-molecule hitting.

ES cell models for drug screening

The development of new drugs is costly and time-consuming.

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In particular, the initial stages of research and development (R&D) require *in vitro* models to screen the activity and toxicity of a large number of compounds. Thus, a suitable model that can be used for both effects and safety assessment is extremely important. Cell-based *in vitro* assays with high human relevance are urgently needed for pre-clinical activities. Previous studies have suggested that ES cells could serve as a screening platform to identify low-molecular-weight compounds that affect endogenous stem cell populations and repair damaged tissue^[4, 8, 10, 11, 28, 29], and a set of screening protocols is available (*ie*, the primary screen and the secondary assay). In addition, several cell lines, such as D3 mES cells and H1, H7, and H9 hES cells, are available for drug screening^[12, 30, 31]. Table 1 lists some characteristics of ES cell and other cell types and their respective advantages and disadvantages.

High-throughput/content screening (primary screen)

Small-molecule libraries can contain millions of compounds. Thus, screens must be employed to effectively eliminate molecules that are toxic or do not have any biological activity. High-throughput screening (HTS) technologies already allow the rapid testing of thousands of compounds. Studies have suggested, however, that screening against a defined molecular target usually exploits a protein's function and results in expression or transcription of differentiation-related genes^[29, 32, 33]. For instance, HTS assays usually use 96- or 384-well plates, and the assays can be performed on chemical compounds in various formats.

Generally, enzymatic activity, signal transduction, or molecular interactions with partners that contain reporter systems have been used to assess whether compounds activate par-

ticular signaling pathways of interest^[34]. Recently, a notable report demonstrated an image-based, high-content assay for detecting compounds that affect hES cell survival or pluripotency^[35]. In that study, 1040 compounds were screened using an hES cell colony-based assay. The assay was designed to detect changes in the phenotype of hES cell colonies by quantifying multiple parameters, including the number of cells in a colony, colony area and shape, intensity of nuclear staining, the percentage of cells in the colony that expressed a marker of pluripotency, and the number of colonies per well. As a result, several steroids that promoted hES cell differentiation were identified, and the antihypertensive drug pinacidil was shown to affect hES cell survival. Phenotypic screens can be advantageous because they can be carried out in cells by examining multiple markers and functional changes (for example, cell morphology and behavior) using automated high-content imaging technologies in a high-throughput manner^[1, 4, 36, 37].

HTS based on receptors in ES cell-derived neurons have also been reported^[38]. That study described an mES cell-based screen of a library of 2.4×10^6 compounds, as well as the identification of novel chemical "hits" for alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate-subtype glutamate receptors, structure-function relationships of compounds and receptors, and validation of lead compounds. The emergence of automated cellular systems has allowed rapid visualization of large groups of cells and phenotypic analysis in a quantitative manner.

Primary screens can also be directly evaluated under a contrast-phase microscope to examine cell differentiation into terminal cells. One protocol for hES cell differentiation into neuroepithelial cells has been recommended, and evaluation

Table 1. Advantages and disadvantages of different cells available for drug screening.

Cell types	Advantages	Disadvantages
Immortalized cell lines	<ul style="list-style-type: none">• Homogeneous cell population• Growth and maintenance cheap• Time- and labor-saving	<ul style="list-style-type: none">• Lack important aspects of native function• Genetic mutations that cause permanent and heritable change in the phenotype
Primary cells	<ul style="list-style-type: none">• Close approximation of native function• Reflect <i>in vivo</i> physiological or pathological condition	<ul style="list-style-type: none">• Require fresh preparation• Difficult to procure in sufficient quantity• prone to batch-to-batch variability in quality
Stem cells	<ul style="list-style-type: none">• Continuously propagated <i>in vitro</i> and retain the potential to generate all the cell types of the body (ES cell).• Compounds can modulate more than one target to achieve a desired biological effect.• High quantity• Readily available source of all cell types• Both develop-dependent and fully differentiated cell types	<ul style="list-style-type: none">• Limited potential for expansion and are lineage restricted (adult stem cells),• Growth and maintenance expensive• A lot of time needed to obtain fully differentiated cell types• Require more effort to achieve purified populations
iPS cells	<ul style="list-style-type: none">• With genetic information of the patients• Reflect <i>in vivo</i> physiological or pathological condition• Mimic both develop-dependent and fully differentiated cell types	<ul style="list-style-type: none">• Very low efficacy for harvest, ($\leq 1\%$), expensive for preparations,• Growth and maintenance expensive• A lot of time needed to obtain fully differentiated cell types• Require more effort to achieve purified populations

under a contrast-phase microscope has been described^[39]. Recent studies in hES cells have identified a novel neural stem cell stage (*ie*, the rosette stage). In this stage, cells exhibit plasticity by generating a broad range of neuronal cell types in response to appropriate developmental signals. Such rosette-stage neural stem cells can also be distinguished from other neural stem cell populations with a contrast-phase microscope because of their specific rosette cytoarchitecture ("morphology")^[40]. The striking cytoarchitecture of rosettes could become a powerful tool for translational medicine and applications in HTC assays that require large numbers of homogeneous cell populations. Recently, a study reported that light microscopy could be used to evaluate embryoid body (EB) structure and size, and the number of wells containing contracting cardiomyocytes was determined^[41]. In addition, hepatocytes containing two morphologically distinct populations, a mononuclear population and a binuclear population, were clearly identified under light microscopy. Another study used immunohistochemical analysis to confirm that albumin-positive cells (*ie*, hepatocytes) were present in the outgrowths of EBs with binuclear cells^[42]. Examining differentiation into terminal cells under a contrast-phase microscope can save both time and labor during a primary screen. Indeed, this approach has been shown to be helpful for successful screening^[14].

More recently, a screen for chemical mediators of reprogramming has been reported^[39]. So far, most studies of direct reprogramming have been performed with lentiviruses/retroviruses, which encode the reprogramming factors. This represents a major limitation to therapeutic applications because viral integration in the host genome increases the risk of tumorigenicity. Low-level residual expression of reprogramming factors may alter the differentiation potential of human induced pluripotent stem (iPS) cells. Indeed, both the viral vectors used for gene transfer and the encoded reprogramming factors are probably oncogenic and possess low transduction efficiency^[43]. Some small molecules can replace transcription factors, and the combined activity of transcription factors can reprogram adult cells into iPS cells. For example, one study using a high-throughput/content screen showed that a TGF- β inhibitor replaced Sox2 in reprogramming and produced unmodified iPS cell lines^[44]. Therefore, small-molecule replacement of transcription factors may be one potential solution to lower the oncogenic potential and increase the reprogramming efficiency^[39, 43, 44].

Another valuable report demonstrated a strategy to adapt hES cells to HTS conditions, which resulted in an assay that is suitable for the discovery of small molecules that drive the self-renewal or differentiation of hES cells^[45]. Use of this new assay (*ie*, global gene expression analysis) has led to the identification of several marketed drugs and natural compounds that promote short-term hES cell maintenance and direct early lineage choice during differentiation. Desbordes *et al* demonstrated the feasibility of hES cell-based HTS and enhanced the repertoire of chemical compounds that can manipulate hES

cell fate.

A series of secondary assays

It has also been recommended that, in general, hit compounds identified from a primary screen be further confirmed using a series of more functional secondary assays and analyzed using informatics tools^[29, 45, 46]. Before a compound enters mechanistic studies, it is normally optimized through structure-activity relationship studies to improve its properties, such as increasing its potency and specificity and enhancing its pharmacokinetic properties. All these data can be expected to be obtained via secondary assays.

To identify the molecular targets and pathways of an unknown compound, biochemical and cellular or sub-cellular experiments are commonly used. One study reported that phosphoserine (P-Ser) increased neurogenesis in hES cell-derived neural progenitors^[1]. It also confirmed that the effects of P-Ser are mediated by the group III metabotropic glutamate receptor 4 (mGluR4). Saxe and colleagues highlighted the tremendous potential of developing effective small-molecule drugs for use in regenerative medicine or transplantation therapy.

Similarly, protein interactions can be mapped in ES cells to enable systematic discovery of regulatory pathways^[47, 48]. Indeed, a method for defining the proteome of stem cell populations using isobaric tags for relative and absolute quantitation has been developed. This allows simultaneous analysis of samples to be analyzed simultaneously, which can give relative quantification for hundreds of proteins from a relatively small (1–5 million) cell number. It is a rapid secondary assay system that precedes more-focused, hypothesis-driven research. The available techniques to study translational regulation of protein levels have decreased the reliance on proteomic confirmation of mRNA-based analyses. In addition, these techniques offer opportunities to develop new quantitative biological approaches to investigate stem cell self-renewal or differentiation, which increase the probability of discovering proteins that regulate development.

During the early stage of development, differentiation-related gene expression or transcriptomics can be regulated, and a genome-wide transcriptional analysis has been used as a powerful assay^[32, 33, 49]. For example, genome-wide transcriptional analysis has shown more than just the effects of *Mesp1* dramatically accelerates and enhances multipotent cardiovascular progenitor specification through an intrinsic and cell-autonomous mechanism. In addition, analysis at various time points identified 1355 genes that were significantly regulated, and the differentiation track was described in a principal component analysis^[33]. Differentiation cultures exposed to monobutyl phthalate or 6-aminonicotinamide (from the EB stage onward) for 24 or 96 h showed RNA expression patterns that deviated from the differentiation track. Studies like these demonstrate that secondary assays can be used as informatics tools and are involved in further functional evaluation.

ES cell models for drug toxicity assessment

Stem cells have been used to evaluate cardiotoxicity for more than 10 years. ES cells are an important *in vitro* model to test drugs, and they have the potential to predict toxicity in human^[32, 33, 50]. For example, a stem cell-based reporter assay was developed to detect drug-induced alterations in the canonical Wnt/ β -catenin signaling pathway, which is involved in the regulation of early embryonic development. The so-called ReProGlo assay allows simultaneous determination of cell viability and luciferase reporter activity in a high throughput 96-well microtiter format^[50]. Several test chemicals were analyzed in the new assay system, and known embryo toxicants like retinoic acid and lithium chloride induced concentration-dependent increases in reporter activity. The potency of valproic acid and a series of structural analogs that activate the Wnt pathway correlated well with their reported teratogenic activity in the mouse. The new test may help to predict embryotoxic potential of chemicals in drug discovery.

Studies have also utilized differentiated fibroblastic progenies of hES cells for *in vitro* toxicological screening^[51]. These cells were generated through random spontaneous differentiation within standard culture media over several passages. The cytotoxic response of the differentiated hES cell fibroblastic progenies to mitomycin C was observed to be more sensitive than the L929 cell line. Therefore, ES cell tests represent reliable, scientifically validated *in vitro* systems for the detection and classification of compounds according to their teratogenic potency^[41, 51–57].

Using contrast-phase microscope is a simple way to examine cell differentiation into terminal cells. One study used light microscopy to determine the number of wells containing contracting cardiomyocytes and evaluate EB structure and size^[41]. The results indicated that a decrease in the pH may be the mechanism by which the alkoxyacetic acid metabolites of glycol ethers cause embryotoxicity. Interestingly, scientists have developed a new molecular approach based on an analysis of the expression of certain marker proteins specific for developing heart tissue^[52]. The approach involves a combination of quantitative flow cytometry, to measure marker proteins (*ie*, sarcomeric myosin heavy chain and α -actinin) in mES cells on day 7, and concurrent cell viability analysis. This approach was subsequently referred to as a molecular fluorescence-activated cell sorting (FACS)-ES cell test, which offered the same sensitivity as the validated protocol, but did not require as much time. This new molecular method has the potential to be a sensitive, rapid and reproducible screening tool, which is highly suited to predict developmental toxicity *in vivo* from *in vitro* data. This protocol was also suitable for *in vitro* embryotoxicity assays by measuring the disturbance of the differentiation of ES cells into endothelial cells (*ie*, the reduction in the expression of platelet/endothelial cell adhesion molecule-1, *ie* PECAM-1 and vascular endothelial cadherin)^[53]. High-content image analysis was used to assess the utility of dissociated human N2TM (HN2TM) cultures as an *in vitro* model for neurite outgrowth^[54]. In addition, the molecular phenotype of these cells was examined using immunocytochemical staining. The hn2TM cultures are amenable for examining

morphological changes and effects on neural outgrowth in high-to-medium-throughput developmental neurotoxicity screening because they possess cellular homogeneity, a rapid rate of neurite outgrowth, and low inter-experiment variability in automated morphological counting by FACS^[54, 55]. The hepatocyte-like cells derived by analysis of the expression of certain marker proteins specific for development method may prove to be useful as an *in vitro* model of hepatotoxicity^[14], which would provide a novel and promising alternative for obtaining large numbers of functional hepatocyte-like cells for *in vitro* drug metabolism and hepatotoxicity screening of potential drug candidates^[56].

The applications of extracellular matrix are relevant to the evaluation of drug efficacy and drug toxicity. To promote both *in vitro* and *in vivo* growth, healthy cellularized 3-D tissues are summarized as follows. Primary cells and cell lines, including ES cells constitute a new 3-D method for rapid evaluation of hepatotoxicity *in vitro*^[57]. In addition to addressing the roles and advantages of ES cells in the aforementioned toxicity models, this review also examined how genetic selection has been employed to overcome major limitations to the implementation of stem cell-based *in vitro* models for toxicology.

ES cells with novel drug targets result in “hits” by small-molecular probes

ES cell differentiation, which involves modulation of the transcription and translation of a vast number of genes and proteins, respectively, offers a desirable model for screening small-molecular probes. This system, which is modeled as a network of regulatory circuits that direct multiple steps of gene expression and mediate spatiotemporal control of a cell's proteome, can determine both cellular phenotype and plasticity^[21, 27, 39, 49, 58, 59]. Commitment to developmental lineages has been considered to be a stepwise process. To guide stem cells towards defined fates, researchers will need to know how these changes are regulated so that they can manipulate cells to change in a predictable and reproducible way^[60]. To address this, it is necessary to identify the molecular target(s) bound by the drug leads, which may be responsible for their pharmacological activity. Direct approaches, such as affinity chromatography, expression cloning and protein microarrays, have analyzed compounds bound to their targets. Indirect approaches are based on a comparison of the genome-wide activity profile of the compound with databases of the activity profiles of other compounds with known targets or activity profiles following specific genetic changes. A variety of technologies and approaches have been explored for target identification after phenotypic screening^[1, 44, 50]. Here, we would like to describe our recent work, which took advantage of chemical probing to identify signaling pathways involved in the differentiated state of ES cells (Figure 1). In the schematic drawing, we have reported at least six events initiated by small-molecule compounds (icariin, icaritin, and partly unpublished).

Differentiation is accompanied by a global increase in both transcript levels and efficiency of protein translation. Mul-

multiple vital genes have been identified where protein levels are exclusively regulated at the translational level during differentiation^[13, 16, 17]. For example^[1], high-throughput identification of small molecules revealed that the orphan ligand phosphoserine (P-Ser) was an enhancer of neurogenesis. Saxe *et al* selectively modulated molecular, cellular, and system-level properties of the mammalian brain. Phosphoserine has been shown to inhibit neural stem cell/progenitor proliferation and self-renewal, enhance neurogenic fate commitment, and improve neuronal survival. Another group^[4] described a chemical screening platform to investigate hES cell differentiation and identified a small molecule, (-)-indolactam V, which specifically works at one stage of pancreatic development to induce pancreatic progenitors from the definitive endoderm. These examples have shown that small molecules that target these regulatory processes are valuable tools for probing and

manipulating the molecular mechanisms by which stem cells self-renew, differentiate, and arise from somatic cell reprogramming.

Niche-mediated control of stem cell fate

Complexity in the spatial organization of ES cell cultures creates heterogeneous microenvironments (niches) that influence ES cell fate. Studies have demonstrated that^[23] the rate and trajectory of hES cell differentiation can be controlled by engineering hES cell niche properties. ES cells provide an *in vitro* system that closely resembles what would occur *in vivo*^[23–26]. Significant advances in defining adult stem cell niches and understanding how they regulate stem cell function *in vivo* have provided new strategies for controlling cell fate (*ie*, by pharmacologically manipulating the niches). Typically, homogeneous and functional cell types generated

Figure 1. Schematic drawing with proposed mechanisms by which small molecules from a screening library can probe signal transduction and molecular events during stem cell differentiation. The drawing compiles the conclusions of signaling transduction and the possible molecular events. Green stars represent supposed targets for small molecular probe hits. NOX: NADPH oxidases; EGFR: epidermal growth factor receptor; MAPK: mitogen-activated protein kinase; PPAR: peroxisome proliferator-activated receptor; PI3K: phosphatidylinositol 3-kinase.

in chemically defined conditions can be used for cell-based therapy. Alternatively, conventional chemical and biological therapeutics can be developed to target patients' own cells or their niches to stimulate regeneration *in vivo*.

Therefore, the creation of "nicheology" in stem cells is a completely novel concept. Niche originally referred to the maintenance of nearby stem cells in a self-renewing state. Cells without direct contact with the niche were shown to differentiate *in vivo*^[60]. The state of stem cell differentiation can be accomplished by supplying outside signals and extracellular factors instead of genetic manipulation^[60]. The suffix "-ology" is used to describe the study of a specific science subject. Therefore, we defined nicheology as a branch of chemical biology or basic medicine that deals with heterogeneous microenvironments for stem cell fate, regenerative medicine, and drug discovery.

Chemically defined medium conditions for controlling hES cell fate will facilitate the practical application of hES cells in research and therapy. In addition, defined medium conditions will provide an excellent system for studying the molecular mechanisms underlying self-renewal and differentiation without the multiple unknown and variable factors associated with feeder cells and serum. From an *in vivo* nicheology point of view, these outside signals and the micro-environment constitute a niche in which adult stem cells are present and compete for limiting concentrations of growth factors or drugs, which maintains a balance between self-renewal and differentiation of the cells^[23].

Role of stem cells in large pharmaceutical companies

The development of new drugs is costly and requires a tremendous amount of resources. The large pharmaceutical companies are currently facing increasing developmental costs and a lower success rate of bringing new compounds to the market. Lowering costs and increasing the success rate can be accomplished by increasing the predictability of the candidate drugs in the pipeline and lowering the number of drugs that fail in later stages of testing.

The use of stem cells amongst the top 20 pharmaceutical and top 10 biotech companies was 70% (64% with hES cells) and 50% (20% with hES cells), respectively^[61, 62]. The screening of small molecules will be the predominate and most exciting approach for developing new therapeutics. The pharmaceutical industry has identified the use of stem cells for drug screening as a new and imminently necessary resource. To date, three European pharmaceutical companies, Roche Holding AG, GlaxoSmithKline and AstraZeneca, have announced that they are starting to develop ways to use stem cells for drug screening.

Prospects

Although it seems like there are extensive opportunities to use hES cells in combination with HTS systems for elucidation of differentiation pathways, there are essential complexities in these biological systems that need to be taken into consideration. For example, automated high-content analysis can

provide information about multiple properties and quantitative data of individual cells, and these analyses are well suited to study non-homogenous cell populations. From a pharmaceutical perspective, the identification of small-molecule compounds regulating cellular differentiation may provide chemical tools that can be translated into clinical applications. Small molecules can replace transcription factors and/or enhance efficiency during somatic cell reprogramming^[63]. They are also expected to be one potential solution for decreasing the oncogenic potential of iPS cells.

Endogenous adult stem cells exist in multiple tissues throughout the human body. Small molecules with inducing ability *in vitro* are expected to possess their own promoting differentiation nature *in vivo*. A novel regenerative medicine approach for tissue repair is focused on direct manipulation of these stem cell pools *in situ* using drugs from hES cell-based HTS to stimulate regeneration.

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