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Novel in vitro models based on undifferentiated or selectively differentiated human stem cells will be instrumental for increasing the R&D productivity in the pharmaceutical industries.

The application of human embryonic stem cell technologies to drug discovery

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The isolation of human embryonic stem cells about a decade ago marked the birth of a new era in biomedical research. These pluripotent stem cells possess unique properties that make them exceptionally useful in a range of applications. Discussions about human stem cells are most often focused around the area of regenerative medicine and indeed, the possibility to apply these cells in cell replacement therapies is highly attractive. More imminent, however, is the employment of stem cell technologies for drug discovery and development. Novel improved *in vitro* models based on physiologically relevant human cells will result in better precision and more cost-effective assays ultimately leading to lower attrition rates and safe new drugs.

Introduction

Drug discovery and development are dynamic processes and the challenges are ever changing. There are many external forces that affect the pharmaceutical industry today and the complexity of designing a winning R&D strategy include several elements such as focus on diseases with the highest potential return on investment, increase of R&D productivity, achievement of unquestionable benefit versus risk, and the drive to be ''best-in-class''. During recent years, the pharmaceutical industry has been struggling with declining R&D productivity. Some of the factors causing the lower productivity are long R&D cycles and approval times, drug attrition and large clinical trial sizes. From a regulatory perspective, it appears difficult to speed up the approval times and to decrease the clinical trial sizes. However, the long R&D cycles and high attrition rates are factors that could be addressed by the pharmaceutical companies using novel or improved technologies for drug discovery. The drug development costs for 2005 have been calculated to be at least in the order of US$40 Billion. In 2004, 36 new drugs were approved by the US FDA while only 20 new drugs were approved during 2005. The success rate of self-originating new chemical entities has historically been between 10% and 30%, depending on therapeutic class [1]. One major issue today is the high failure rate in phase III clinical trials. More than 40% of the trials are unsuccessful, which contributes substantially to the high costs for drug development [2].

In order to increase R&D productivity, one possibility is that the pharmaceutical industry evaluates and implements novel technologies that could positively affect the efficiency of drug

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discovery. For instance, the impact of high-throughput technologies, such as high-throughput screening, high-throughput organic synthesis, high-throughput crystallography, and high-throughput metabolism, needs to be maximized. Other opportunities for increasing R&D productivity are to use technologies to expedite clinical proof-of-concept such as biomarker discovery (e.g. proteomics and microarrays), imaging for rapid identification of efficacy, and pharmacogenetics. Furthermore, in order to achieve unquestionable benefit versus risk and to reduce late stage attrition it is imperative to assess potential toxicity or other adverse effects of a compound as early as possible in the drug development project [3]. In many cases, the toxicity is not discovered before clinical trials are conducted. Animal models have an important role for detecting adverse effects of compounds, but these are costly and, besides the issue of clinical relevance of these models, there are ethical and political concerns associated with the use of experimental animals. Many of the needs in drug development could make use of human stem cell technologies and the discovery process could be made more efficient and ultimately reduce the need for *in vivo* experimentation.

The common denominator in most *in vitro* drug discovery applications is the biological component (i.e. the cell) whose functionalities and responses are being assayed. There is a substantial need for physiological cell models and, in particular, for efficacy and safety studies. The properties of stem cells that make them so attractive to use for *in vitro* testing are that they have the capacity of self-renewal and differentiation into virtually any cell type. Stem cells can be genetically modified using reporter gene construct to improve the throughput of the assays and they can provide specific disease models [4]. In addition, they are important for understanding differentiation pathways and for identifying factors needed to manipulate cell lineages, as well as for identifying and validating disease targets using, for example, siRNA-library or compound-library screens. In the adult organism, stem cells mediate tissue homeostasis and repair. There are several tissue sources of adult stem cells including brain, bone marrow, and skin [5]. It is currently being speculated if every tissue has its own stem cell population. When a stem cell is removed from its niche (i.e. site of self-renewal), it usually starts to differentiate along a certain pathway. The niche provides various stimuli such as extracellular matrix, growth factors, and cell–cell interactions [6]. One area of extensive exploration is the identification of low molecular weight compounds that control stem cell fate [7]. Potentially, such compounds could be able to activate resident tissue stem cells and thus could lead to the development of drugs for treatment of degenerative diseases provided they are selective and sufficiently potent.

There are a number of different types of human stem cells and these cell populations present varying degrees of developmental potency, but they also share similarities related to molecular mechanisms involved in maintenance of the stem cell state [8]. Multipotent adult stem cells can be derived from specific organs or bone marrow [9], whereas populations of multipotent fetal stem cells can be obtained from fetal tissues or umbilical cord blood [10]. In the following sections, we will discuss the current and future prospects of human embryonic stem (hES) cells, and illustrate some of the possibilities these cells provide for the development of novel and improved tools for drug discovery. The use of mouse ES cells in several areas of the drug discovery process was recently reviewed and supports the efforts undertaken with the human analogues [11].

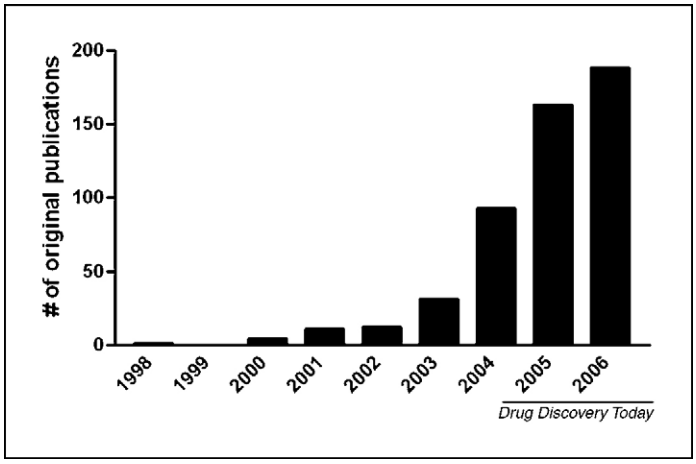


FIGURE 1

Number of published original papers describing experimental work using hES cells. The figure is redrawn and updated from [14]. Notably, scientific reviews, commentaries, and papers on ethical or legal aspects of hES cell research are excluded from the figure.

Human pluripotent stem cells

Pluripotent hES cells are isolated from early stage human embryos (i.e. blastocysts) and have the potential for unlimited self-renewal. Human ES cell lines were originally derived about a decade ago from supernumerary fertilized eggs created for the purpose of assisted reproduction [12]. Before this scientific breakthrough, researchers had been employing mouse ES cells for various purposes. Most dramatic is the impact of genetically engineered mice in which gain-of-function and loss-of-function of specific genes can be studied in an *in vivo* setting, and transgenic mice are now used routinely for pharmaceutical target validation [13]. In recent years, these animal models have been further developed and have become more sophisticated, providing researchers with tools for switching on and off the expression of certain genes in specific tissues at selected time points. This has become especially valuable, since genetic engineering can lead to unpredictable developmental complications or lethal consequences. Furthermore, studies using mouse ES cells have resulted in a considerable wealth of data regarding the molecular mechanisms dictating lineage specification. However, there are noteworthy differences between humans and rodents and this has left a gap in our understanding of human embryonic development and tissue specification. The expanding interest in hES cells and the use of these cells in research is illustrated well by the number of scientific original papers published annually since the initial derivation of hES cells in 1998 (Figure 1) [14]. Notably, in 2003 the number of papers started to increase substantially, probably reflecting the fact that a few years were required to successfully implement hES cell research in different laboratories, and the hES cell research field has been growing rapidly over recent years.

Ethical and legal issues

The procurement of hES cell lines has been surrounded by ethical and legal considerations which, in most parts of the world, have led to the establishment of guidelines and regulations concerning

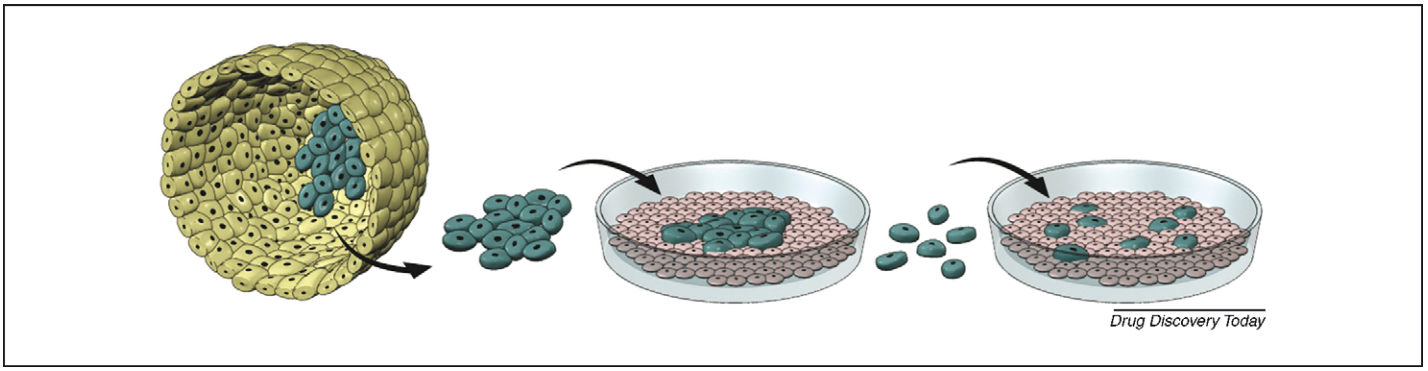


FIGURE 2

Schematic of the establishment and propagation of hES cells. The inner cell mass cells are isolated from the human blastocysts at day 5–7 post-fertilization. The pluripotent cell population is cultured on top of a growth inhibited feeder layer that provides the necessary signals that sustain undifferentiated proliferation of the stem cells. The cultures are passaged regularly using either mechanical or enzymatic dissociation, and the cells are transferred to fresh culture dishes.

hES cell research. For instance, in the US, federal funding is only available for hES cell research using cell lines established before 9 August 2001, but in the private sector there are no such limitations. In addition, individual states have allocated specific state funding for hES cell research, whereas others have introduced local restrictions. In Europe, the situation is complex and certain countries have a very permissive legislation concerning hES cell research (e.g. UK, Sweden, and Belgium), whereas others have a complete prohibition (e.g. Ireland and Austria). Based on the diversity in regulations between member states, the European Union currently only funds hES cell research programs performed on existing cell lines. The complicated situation in the US and Europe is also mirrored in the rest of the world. In order to promote uniform practices worldwide, there are several organizations that have published guidelines for the conduct of hES cell research which specify rigorous ethical standards for scientists. These organizations include The International Society for Stem Cell Research (http://www.isscr.org/), The National Academy of Sciences (http://www.nasonline.org/), the National Institutes of Health (http://www.nih.gov/), and the UK Stem Cell Bank (http://www.ukstemcellbank.org.uk/).

Isolation, characterization, and propagation of hES cells

The experimental approach for the derivation of hES cell lines was initially adapted from the previously developed methods for mouse and primate ES cells, as well as the early attempts to culture inner cell mass cells from human blastocyts [12,15–18]. The procedure involves proteolytic digestion of the zona pellucida of an expanded blastocyst followed by immunosurgery to lyse the trophectoderm by an antibody/complement reaction. The isolated inner cell mass cells are subsequently placed on a layer of growth-inhibited mouse embryonic fibroblasts feeder cells in tissue culture dishes. After 1–2 weeks, the initial outgrowth from the inner cell mass is dissected manually and transferred to new culture dishes as schematically illustrated in Figure 2. Successful propagation of the inner cell mass is associated with the appearance of cells with undifferentiated hES cell morphology (Figure 3) [19]. Till date, hES cell lines have been derived in a number of independent laboratories worldwide using this traditional derivation method [20–22], but alternative approaches such as whole embryo culture or partial embryo culture have also been applied [23,24]. In total, more than 400 hES lines have been reported by various investigators, though the level of characterization of these lines varies substantially [14]. Under appropriate culture conditions, hES cell lines can be maintained in culture indefinitely and exhibit a stable developmental potential to differentiate into all the cells of the human body. In contrast to mouse ES cells, hES cells can also give rise to trophectoderm-like cells *in vitro* [25].

Undifferentiated hES cells are characterized by their expression of a number of molecular markers, largely consisting of markers previously used to distinguish human embryonic carcinoma cells, mouse ES cells, and hematopoietic stem cells [26]. The markers include the cell surface molecules SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. In addition, expression of POU5F1/Oct3/4 and Nanog is tightly associated with the undifferentiated state of the cells and these transcription factors are commonly used as identifiers of hES cells [27,28]. Additional markers linked to pluripotent human stem cells have also been suggested, although no functional connection between pluripotency and the expression level of

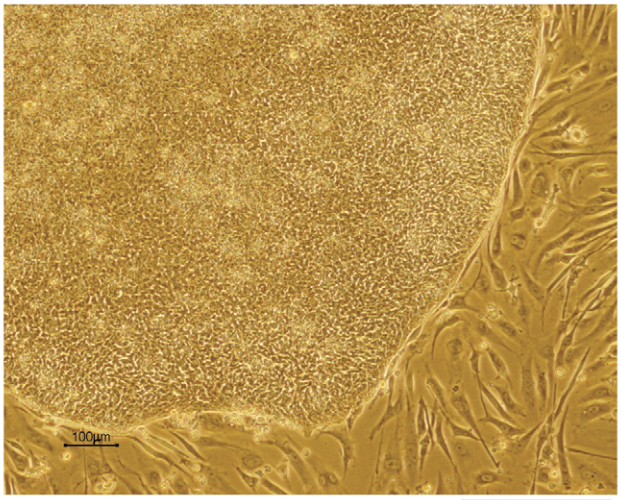


FIGURE 3

Micrograph showing undifferentiated hES cells (cell line SA002, Cellartis AB) on a mouse embryonic fibroblast feeder layer. Within the colony, the tightly packed, small hES cells with a high nucleus-to-cytoplasma ratio can be observed. The colony has a sharply delineated border towards the surrounding elongated feeder cells.

the individual markers has been reported [29]. The availability of appropriate molecular markers of undifferentiated hES cells is instrumental for the rapid detection of these cells in various experimental settings. In particular, translational research of hES cell-based therapeutic applications relies on the ability to detect, with high sensitivity, the presence of any undifferentiated hES cells in a population of differentiated progenies.

Pluripotent hES cells have the capacity for extensive, or possibly even indefinite, self-renewal. Expansion of hES cells up to several hundred population doublings has been reported by several investigators and we have similar experiences from our own laboratories and cell lines [30]. However, the most important feature of hES cells is their ability to differentiate into virtually any cell type present in the adult (reviewed in: [31]). This pluripotency can be assayed *in vitro* by removing the hES cells from the culture conditions that are designed to inhibit their differentiation and subsequently maintain the cells under differentiation promoting culture conditions. In the resulting heterogeneous cell populations, differentiated progenies representing all three embryonic germ layers (endoderm, ectoderm, and mesoderm) can be identified using standard laboratory techniques. Currently, the gold standard to demonstrate the pluripotency of hES cells is by xeno-grafting undifferentiated hES cells to immuno-deficient mice where the human cells give rise to teratomas [12,19,21]. The teratomas contain various types of tissues representing all three embryonic germ layers. Striated muscle, cartilage, bone, gut epithelium, and neural rosettes are commonly observed in the teratomas [19]. These tissues show a sufficiently high degree of differentiation to allow histological evaluation and can thus provide experimental proof of pluripotency *in vivo*. In order specifically to derive functionally differentiated cells from hES cells, investigators are working intensively to improve protocols beyond the spontaneous differentiation of hES cells obtained *in vitro* or *in vivo*. This topic will be discussed in more detail below and Table 1 summarizes various cell types that have been successfully derived from hES cells till date.

TABLE 1

Summary of specialized cell types derived from hES cells

Cell type	Reference[a]
Trophoblast	[25]
Endothelial cell	[94]
Cardiomyocyte	[79]
Smooth muscle cell	[95]
Hepatocyte	[62]
Insulin producing endocrine cell	[96]
Keratinocyte	[97]
Oligodendrocyte	[98]
Neuron and astrocyte	[99]
Glia	[100]
Germ cells	[101]
Adipocyte	[102]
Chondrocyte	[103]
Osteoblast	[104]
Natural killer cell	[105]
T cells	[106]
Dendritic cell	[107]
Megakaryocyte	[108]
Erythrocyte	[109]
Macrophage	[110]
Melanocyte	[111]
Retinal neurons	[112]
Motor neurons	[113]
Type II pneumocytes	[114]
Prostate tissue	[115]
Lung alveolar epithelial type II cells	[116]

[a] The reference list includes examples of key studies related to each cell type and should not be considered a comprehensive list of all related published studies.

One of the most important issues at the very basis of hES cell research today is the proper maintenance and expansion of the undifferentiated cells. Pluripotent hES cell lines are traditionally propagated in co-culture with a mitotically inactivated feeder layer. This feeder layer provides certain currently unknown factors, which support undifferentiated growth of hES cells [32]. There are substantial differences between the culture systems for mouse – and hES cells. In particular, unlike in feeder-free mouse ES cell cultivation, leukemia inhibitory factor (LIF) does not prevent hES cells from differentiating [12,21]. The most widely used method to propagate hES cells has been manual microdissection. In this delicate process, manually drawn or commercially available micropipettes are used for dissection of individual hES cell colonies into small clumps. These clusters of hES cells are subsequently transferred to fresh culture dishes. The main advantage of the mechanical transfer method is the possibility to perform a positive selection at every passage by isolating undifferentiated hES cells from differentiated cells. This method, however, is labor-intensive and time-consuming, making it very difficult to process many cells simultaneously. This has incited the development of alternative methods for hES cell expansion. The use of enzymes for cell dissociation during passage is obviously considerably faster and simpler than microdissection, and different enzymes such as collagenase IV [33], trypsin [34], dispase [35], and Tryple™ Select [36] have been employed for the expansion of hES cells. Interestingly, in a recent study, enzymatic passaging was combined with a synthetic ROCK-inhibitor resulting in increased hES cell survival during propagation of the cells [37]. One disadvantage with the use of enzymes for hES cell passaging is the increased risk of introducing genomic aberrations during propagation *in vitro* [38]. It is still not clear, however, in which way culture conditions and the occurrence of chromosomal abnormalities relate to each other. In order to expand hES cells in the absence of feeder cells, the commonly used feeder-free culture systems take advantage of the fact that soluble factors, which are necessary for maintenance of undifferentiated hES cells, are secreted into the culture medium by the feeder cells. Thus, the hES cells can be grown in the absence of feeder cells on a suitable growth substrate such as Matrigel™ using feeder cell conditioned medium [33]. Other reports indicate that culture additives which activate the canonical Wnt pathway [39], a combination of growth factors such as LIF, transforming growth factor-β1, and basic fibroblast growth factor (bFGF) [40], a combination of noggin and bFGF [41] or high levels of bFGF alone [42], may be sufficient to sustain undifferentiated hES cells in the absence of supporting feeders.

Ultimately, the development of chemically defined media is preferable [43,44]. Despite the large body of reports on various culture methods for hES cells, no universal protocol has been adapted though attempts along this line are currently being undertaken, for example in a large international consortium [45].

Much progress has been made over recent years concerning establishment, expansion, and characterization of hES cells. Therefore, it is now realistic to believe that the scientific community can soon generate hES cell lines in a standardized manner for various applications, including future clinical use. Major challenges remain concerning the scale-up of hES cell production, but these issues are being addressed seriously by the launch of several focused programs. We consider the use of bioreactor technology, as well as the automation of processes, as promising paths toward hES cell production to an industrial level [46,47]. In parallel, it is equally important to develop adequate robust and efficient characterization methods to verify the quality of the cells that are being manufactured. These issues are currently being addressed as well [48].

Human pluripotent stem cells: applications for drug discovery

Discovering one new drug and bringing it to the market typically takes 10–15 years and costs around US$900 million [3]. The complex process of drug discovery and development is designed to make certain that only safe and effective new medicines are brought to the public. Unmet medical need is the constant driver for the development of new therapies, and the evolution of the drug discovery process is driven by the need to add medical value, but, at the same time, limit the costs for the pharmaceutical and biotechnological companies. The industry is now taking a more disease-based approach, in which the understanding and treatment of the underlying human pathology is emphasized instead of simply focusing only on symptomatic relief. This approach requires model systems based on humans rather than animals. For obvious reasons, the use of animal-derived cells or tissues to develop selective drugs has its pitfalls, since activity in animals or animal models does not always translate into efficacy in humans. Technical advancement in, for example, non-invasive imaging, genomics, and clinical genetics, together with the sequence of the human genome will certainly be instrumental for employing the biological systems approach in drug discovery. In addition, the development of human cell-based models will also be important for evaluating novel targets and compounds in a close to physiological environment. Presumably, attrition, because of lack of efficacy, when new mechanisms are applied to humans will be diminished if better validation could be performed in human models and subjects as early as possible. Besides failure from lack of efficacy, other major reasons for attrition are toxicity, poor biopharmaceutical properties, and market reasons [3]. Identification of the toxicity potential of novel drug candidates at an early stage of development is essential in order to determine any unacceptable safety profile. The provision of predictive high-throughput cell-based *in vitro* toxicity screens is likely to be highly beneficial in order to address this issue. Technical advances in instrumentation have contributed substantially to improve the throughput of these assays. However, these models are still restricted by the lack of relevant and validated cell types. The optimal cell types would obviously be human that display the appropriate organ phenotype. Specialized cell types can be derived by differentiation of pluripotent human stem cells. One advantage of human stem cell technologies is that the use of cells derived from one single hES cell line would minimize the donor and preparation variability. On the other hand, pharmacogenetic information can be obtained by applying cell lines representing different genotypes. In addition, disease-specific cell lines could be isolated from patients carrying specific genetic traits or by genetic manipulation of normal cell lines. Such models could be critical for understanding the pathogenic progression of the disease as well as testing drug efficacy. Here we highlight some hES cell-based approaches for drug discovery and toxicity testing. To exemplify the possibilities we use hepatocytes and cardiomyocytes, since these two cell types are central in the drug development process and have implications in specific disease areas in addition to wide general applicability. Recent results also indicate the usefulness of neural derivatives of hES cells for toxicity testing [49]. It should be pointed out that the possibilities are far from limited to these cell types and in principle any application requiring normal human cells can be envisioned. Furthermore, undifferentiated hES cells also provide novel opportunities for embryotoxicity testing [50].

Hepatocytes – derivation and characteristics

Stem cell differentiation into hepatocytes is of great interest, since access to large numbers of these cells would enable their use in place of whole organ transplantation as a potential treatment for severe liver diseases [51]. Of specific interest in this review is, however, the idea that a convenient source of hepatocytes could also substantially facilitate the development of new drug discovery strategies and provide possibilities to perform *in vitro* metabolism studies and toxicity assessment. Notably, the complexity and function of the liver is not mirrored by any cell type available today. Although primary human liver cells are available, they rapidly lose functional properties when cultured *in vitro*, and therefore the usefulness of these cells relies on repeated sourcing, which is a major limitation [52]. Available hepatic cell lines contain very low levels of metabolizing enzymes and they have a distribution of other important proteins that is substantially different from the native hepatocyte [53].

Similar to many other aspects of human development, the molecular program that initiates and sustains human liver development remains elusive, though many molecular details have been discovered in rodents [54]. Human hepatocytes could potentially be derived from either adult or EC cells. A natural source of mature hepatic cells is the intrahepatic stem cells [55], but also extrahepatic stem cells can differentiate to the hepatic lineage [56]. Hepatocyte-like cells have been derived from mouse ES cells using a variety of different culture conditions resulting in heterogeneous cultures containing several other cell types besides the hepatic lineage [57,58]. In a recent study, the efficiency of mouse ES cell differentiation and maturation was improved using a combination of growth factors (activin A, BMP-4, and bFGF) and a high proportion of cells positive for α-fetoprotein and albumin was generated [59].

Interestingly, cells with hepatocyte-like morphology and function have been derived from hES cells and reported in a few studies. The initial studies reported spontaneous differentiation of hES cells without specific efforts to enrich for hepatocyte-like cells [60].

These observations were later followed by more directed differentiation strategies and modified culture conditions that supported hepatic differentiation of hES cells [61–65]. The cells obtained displayed appropriate morphology and expressed some hepatocyte-associated markers, for example albumin, α-1-antitrypsin, and cytokeratin 8 and 18. In addition, hepatic transcription factors, such as Fox A2, HNF-1, and GATA-4 were expressed by the hepatocyte-like cells. Functional analysis of the cells indicated glycogen accumulation, inducible cytochrome P450 activity, production of urea and albumin, and uptake of indocyanine green. The first study to report on actual drug-metabolizing effects in hES cell-derived hepatic cells was recently published and the authors showed that lidocaine was significantly metabolized by the cells [66]. We have obtained similar results in our laboratories and the capacity of hES cells to differentiate into early stage hepatoblast-like cells, as well as more differentiated hepatocyte-like cells, was recently reported [67]. Besides the expression of several mature liver markers, these cells express functional glutathione transferase activity at levels comparable to human hepatocytes. For the future industrial use of stem cell-derived hepatocytes, the presence of specific biotransforming enzymes in the cells are of utmost importance. From a developmental biology stand point, it is currently unclear whether the hES cell-derived hepatocyte-like cells described above are derived from a population of cells differentiating from definitive endoderm, which is considered the origin of the liver in mammalian development. Interestingly, cells with detoxifying capacity and a phenotype resembling hepatocytes in certain aspects are also part of the extraembryonic endoderm. Because of the limited availability of specific markers capable of discriminating between extra- and definitive endoderm, the results from previous studies have been somewhat inconclusive in this respect. However, recent studies have begun to shed some light on this issue and the derivation of definitive endoderm from hES cells [68], and later the derivation of hepatocyte-like cells from definitive endoderm, was reported [69]. For hepatocyte differentiation, hES cells were induced by activin A, and further treated with FGF-4 and BMP-2. The resulting cells showed expression of hepatic genes and the presence of protein markers, in addition to exhibiting functions similar to adult liver cells. However, no metabolism, biotransformation, or transport of pharmaceutical compounds was reported. In our own laboratories, we have taken a slightly different approach for exploiting activin A induction of definitive endoderm and subsequent derivation of hepatocyte-like cells (G. Brolén, N. Heins, Manuscript in preparation). Besides general hepatocyte-specific properties, these cells also metabolize several commonly used pharmaceuticals. It will be interesting to ascertain the further differentiation of the definitive endoderm cells toward the hepatic lineage and specifically determine if these cells are functional enough to be useful for broad drug discovery and toxicology applications. Critical functions to be further investigated are metabolic competence, biotransformation capacity, and transportation of exogenous compounds.

Hepatocytes – applications

As indicated above, hepatocytes represent key cells in the drug discovery process and they are used for investigations of novel targets in, for example, metabolic and dyslipidemic diseases. In addition, these cells have broad uses in studies of liver metabolism and pharmacokinetic properties of novel compounds, as well as in hepatoxicity assessment. Notably, unexpected human metabolism and pharmacokinetic problems are major causes for removal of potential new drugs from pharmaceutical projects, and some researchers place adverse drug reactions between the fourth and sixth leading cause of death in hospitalized patients in the USA [70]. Furthermore, liver toxicity and alterations of liver function are the most frequently occurring reasons for toxicology among drug molecules. Hence, there is a great need for novel improved models for human hepatocytes.

The pharmaceutical industry has made major investments to screen for metabolic properties early in the drug discovery process [71]. The questions addressed at the early metabolism testing stages are related to metabolic degradation of the compound, mechanisms of the metabolism, and induction or inhibition of any drug metabolizing enzymes. Hepatocytes are currently used as the gold standard in drug metabolism studies, but the tools available today still lack accurate predictive power [72]. Among the important human drug metabolizing enzymes, the CYPs and the enzymes involved in drug conjugation, such as UDP glucuronosyltransferases and flavin monooxygenases are recognized [73]. In addition, transporter proteins may be important for the clearance and elimination of a drug when it passes the liver and in particular the role of transporters in the hepato-biliary disposition has been highlighted [74]. Numerous transporters are available on the sinusoidal side of the hepatocyte to mediate uptake of drugs from the blood as well as flux them back into the blood stream. Hepatic transporters may also play an important role in the excretion of drugs and their metabolites from the hepatocyte into bile.

It is very difficult to foresee hepatotoxic actions of new compounds in humans and, unfortunately, the toxicity is often observed only in the late phases of the drug discovery process. The underlying reason for this problem is mainly related to pronounced species differences and is based on the extensive use of animal models. Much effort has been spent in order to establish predictive human hepatic cell populations that could be assayed *in vitro*. The models available today are based on human cancer cell lines or human primary cells isolated from tissue biopsies, but these have significant drawbacks. For example, a commonly used human hepatoma cell line, HepG2, is limited by its poor phenotypic and functional match to *in vivo* hepatocytes. In addition, HepG2 cells have low basal and inducible drug metabolizing capacity. The best option today, with respect to functional differentiation, is human primary isolated hepatocytes, but issues related to the acquisition and variability in this material result in practical constraints that limit its usefulness. Furthermore, the purity of the primary isolations is also a point of concern and non-parenchymal cells and hepatocytes with limited viability are, to a varying degree, contaminating the final cell preparations. Importantly, a proof-of-concept test was recently reported which demonstrated the potential of mouse ES cells to differentiate into functional hepatic cell thus providing access to cell material for assessing hepatotoxicity [75]. These results lend support to the development of analog models based on human cells. Among the challenges when trying to use hES cell derived hepatic cells for toxicity testing is to apply the cells in formats for drug discovery use. We have in our laboratories been able to reseed the hepatocyte-like cells into 96-well plates and to maintain the morphology

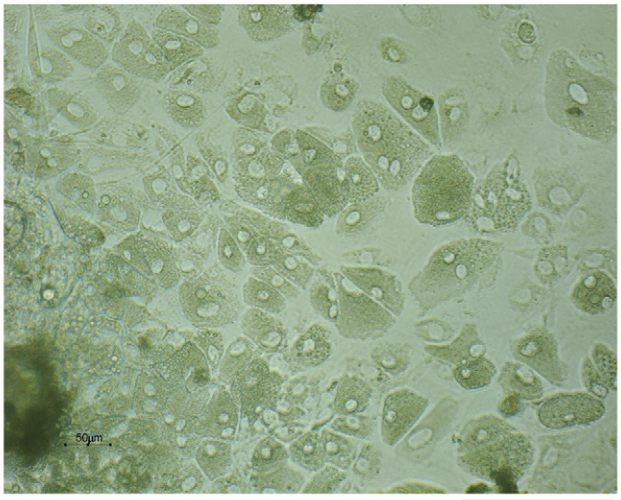


FIGURE 4

Micrograph showing hepatocyte-like cells derived from hES cell line SA002 (Cellartis AB). The hES cells were differentiated for 4 weeks on mouse embryonic fibroblasts as previously described [67] and subsequently transferred to a 96-well plate, coated with collagen I, for further culture. The cells are large, rhombic, multi-nucleated, and highly granulated.

of the cells for several weeks (Figure 4). The 96-well format is expected to enable a useful throughput of novel assays. Clearly, hepatocytes derived from hES cells may combine a high degree of specific differentiation with an excellent availability for *in vitro* testing of potential new drug candidates.

Finally, the human liver is an organ consisting of many cell types besides the hepatocyte. For example Kuppffer cells, stellate cells, and cholangiocytes are adding important pieces to the complex architecture of the liver. Therefore, to be able to fully understand, and thereby predict, positive and negative effects of new pharmaceutical compounds *in vitro*, more complex models must be developed. This further underscores the potential for hES cells as a source for human hepatotoxicity models, since basically any cell type can be generated from the pluripotent stem cells. Although speculative, there is a great hope that hES cell research will pave the first way to mimic simple liver tissue, thereby dramatically improve the chances to accurately predict human toxicity *in vitro*.

Cardiomyocytes – derivation and characteristics

Because of the lack of donor material as well as the somewhat problematic procedure of cell isolation, human primary cardiomyocytes are not currently available for preclinical drug discovery. Thus, much hope is placed on the use of pluripotent human stem cells to derive functional cardiomyocytes for *in vitro* applications in drug development.

During embryonic development, the formation of the heart, and the initiation of its functions are among the earliest events, but knowledge about the molecular program that governs cardiogenesis in humans is still in its infancy. Although a complete understanding of these events remains to be determined, some crucial factors, including members of the GATA family of transcription factors, Nkx2.5, Mef2C, Tbx5/20, and Hand1/2, have been identified together with important signaling pathways involving Wnts, BMPs, and FGFs [76]. The initial observations that mouse ES cells readily differentiate into cells with cardiomyocyte-like properties was reported about two decades ago [77]. Although the mouse system has proven very useful in certain aspects, it is clear that there are substantial disparities in cardiogenesis between mouse and human that are most probably attributed to the fundamental species differences. Following the first isolations of hES cells, several studies reported the establishment and characterization of spontaneously contracting cells derived from hES cells (for a review see [78]).

The most common method for obtaining cardiomyocyte-like cells from hES cultures is by inducing cell differentiation through embryoid body formation [79]. This technique is however laborious and the yield is relatively low, making it necessary to develop improved protocols for directed differentiation of hES cells to cardiomyocytes. Several studies have been performed toward this goal and some progress has been made [80,81]. However, one of the major obstacles, today, for the utilization of hES cell-derived cardiomyocytes is the insufficient number of cells achieved by the currently described differentiation protocols.

Supporting the initiatives to increase the yield and cell number are the mounting data on the characteristics of the cardiomyocyte-like cells that can be derived from hES cells. During recent years, a number of papers have described, in various ways, the basic characteristics of hES cell-derived cardiomyocytes. In these reports, cell analysis has been based on the expression of molecular markers for cardiomyocytes, structural architecture, and functionality. The morphology and ultrastructure of hES cell-derived cardiomyocytes share similarities with adult cardiomyocytes although the myofibrillar and sarcomeric organization indicate an immature phenotype in the stem cell-derived population [79,81–83]. This is, however, not unexpected since *in vitro* differentiation differs substantially from the *in vivo* situation in many aspects. Interestingly, there are indications that suggest the possibility to modulate the maturation of hES cell-derived cardiomyocytes *in vitro* [82]. On a molecular level, several markers expressed by cardiomyocytes are also expressed by hES cell-derived cardiomyocytes, including transcription factors, structural proteins, hormones, ion-channels, and tight junction proteins [79–81,83–86].

Taken together, the molecular and structural properties of the hES cell-derived cardiomyocytes suggest that these cells share similarities with their adult counterparts. More importantly, however, are the functional characteristics of the cells, and different pharmacological and electrophysiological approaches have been used to examine these properties. One major advantage of cardiomyocytes derived from hES cells is that they can be maintained in culture for extended time periods without losing their spontaneous contractile capacity. This allows for repeated non-invasive examination of the same cell preparations. Several studies have demonstrated that hES cell-derived cardiomyocytes respond to α/β-adrenergic and muscarinic stimuli suggesting that the cells express specific surface membrane receptors coupled to a signaling pathway that activate ion channels, membrane transporters, and myofilament proteins [79,83,84,87]. In addition, action potentials indicative of nodal-like, atrial-like and ventricular-like origin have been identified in hES cell-derived cardiomyocytes using intracellular

electrophysiological measurements [84–86]. Taken together, these results indicate that *in vitro* developed stem cell-derived cardiomyocytes have a basal functionality that makes them attractive for further evaluation in terms of applicability in drug discovery.

Cardiomyocytes – applications

The use of cardiomyocytes derived from hES cells in drug development can generally be divided into two segments. The first segment represents cardiac drug discovery where the heart is the diseased organ and pharmaceutical modulation of cardiomyocyte function is a potential intervention. The other segment represents cardiac safety assessment of novel compounds in development. Importantly, according to recommendations from the regulatory agencies all compounds in development should be tested for cardiac safety. In either of these segments, the key cell type is the cardiomyocyte but unfortunately the pharmaceutical industries currently lack human material for preclinical drug discovery. As described above, hES cells have the capacity to differentiate into spontaneously contracting cells with cardiomyocyte-like properties and, as such, they represent a potential unlimited source for human cardiomyocytes that can be utilized for *in vitro* testing.

In the area of cardiac drug discovery, there are ample opportunities to employ stem cell-derived cardiomyocytes. For target identification, validation, and evaluation studies, the access to representative human cells would certainly improve the precision of almost any assay. The possibilities to evaluate a new target in a close-to-physiologic environment are clearly advantageous to the use of animal models or transfected abnormal cell lines. Although the efficiency and costs associated with the creation of genetically modified mice have been improved during recent years [88], the issues related to species differences and extensive usage of experimental animals for research still remain problematic. The possibilities to introduce or delete genes in the stem cell-derived cardiomyocytes, either already at the undifferentiated stage of the hES cells or conditionally in the cardiomyocyte-like cells, open up novel avenues for the development of *in vitro* cell-based assays for initial target studies. Such assays could also prove very useful for lead optimization. Heart diseases with monogenetic causes (e.g. congenital long QT syndrome) can be modeled using human pluripotent stem cells and genetic engineering. In the case of more complex polygenetic disorders, new hES cell lines could potentially be created using patient specific embryos or somatic cell nuclear transfer technology, and the isolated stem cells subsequently differentiated to cardiomyocytes. The phenotype of those cells could provide new information about disease mechanisms, and screens using siRNA and compound libraries could potentially reveal new targets. In addition, there are several relevant parameters for different cardiac disease processes that could be successfully assayed using hES cell-derived cardiomyocytes. These cellular responses include, but are not limited to, contractile function, cardiac arrhythmia, response to oxidative stress, resistance to apoptosis, and protection from ischemia.

All major pharmaceutical companies have directed attention specifically toward testing drug safety and assessing a substance potential for delaying the repolarization of the cardiac ventricular action potential. One major reason for this is that the primary safety issue in development of new drugs is QT interval prolongation, which can lead to ventricular arrhythmia (Torsade de Points). Unanticipated QT interval prolongation is the primary cause of drug withdrawal from the market over the last ten years. Notably, QT prolonging drugs belong to diverse therapeutic classes including both cardiovascular and non-cardiovascular drugs [89]. Hence, there is a substantial and general need for strategies aimed at identifying the risk of drug-induced QT prolongation during early preclinical and clinical phases of drug development. One of the present problems is the lack of availability of preclinical models representative for human physiology in which a large library of substances can be screened rapidly. Various *in vitro* models are currently being utilized and examples include: cell lines heterologously expressing human cardiac ion channels, cardiac cell cultures, isolated tissue preparations, and perfused animal hearts. Because of the limited availability of human myocytes, researchers are currently restricted to using animal-derived cells or tissues. Many of these experimental models have limited predictivity for human *in vivo* response and their phenotypic and functional match to human cardiomyocytes is poor. For assessment of drug-induced QT prolongation, the optimal sub-type is the ventricular cardiomyocyte. Electrophysiological properties of these cells can be investigated in single cell preparations using standard patch-clamp techniques [85]. Aggregates of hES cell-derived cardiomyocytes can be applied in other platforms such as Micro Electrode Arrays (MEA) in which rhythm, route and origin of excitation, repolarization, and conduction can be analyzed (Figure 5) [90]. Evidence supporting this approach of drug safety testing has started to appear in the scientific literature, and, recently, the effect of D-sotalol on delayed repolarization was demonstrated in hES cell-derived cardiomyocytes using a MEA system [91]. Furthermore, incubation of hES cell-derived cardiomyocytes with the hERG-specific channel blocker E-4031 resulted in

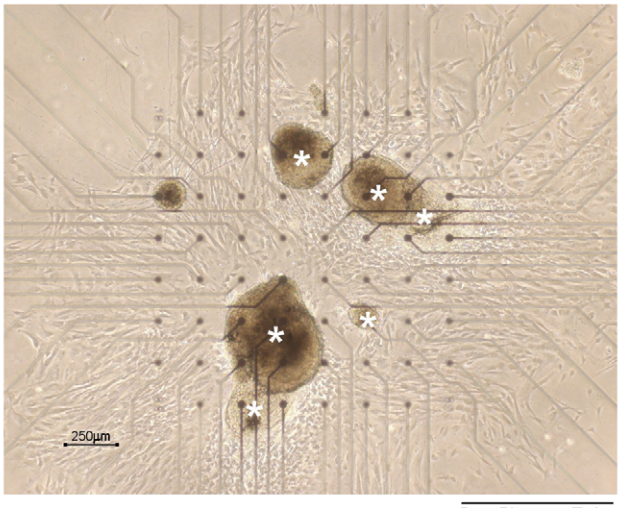
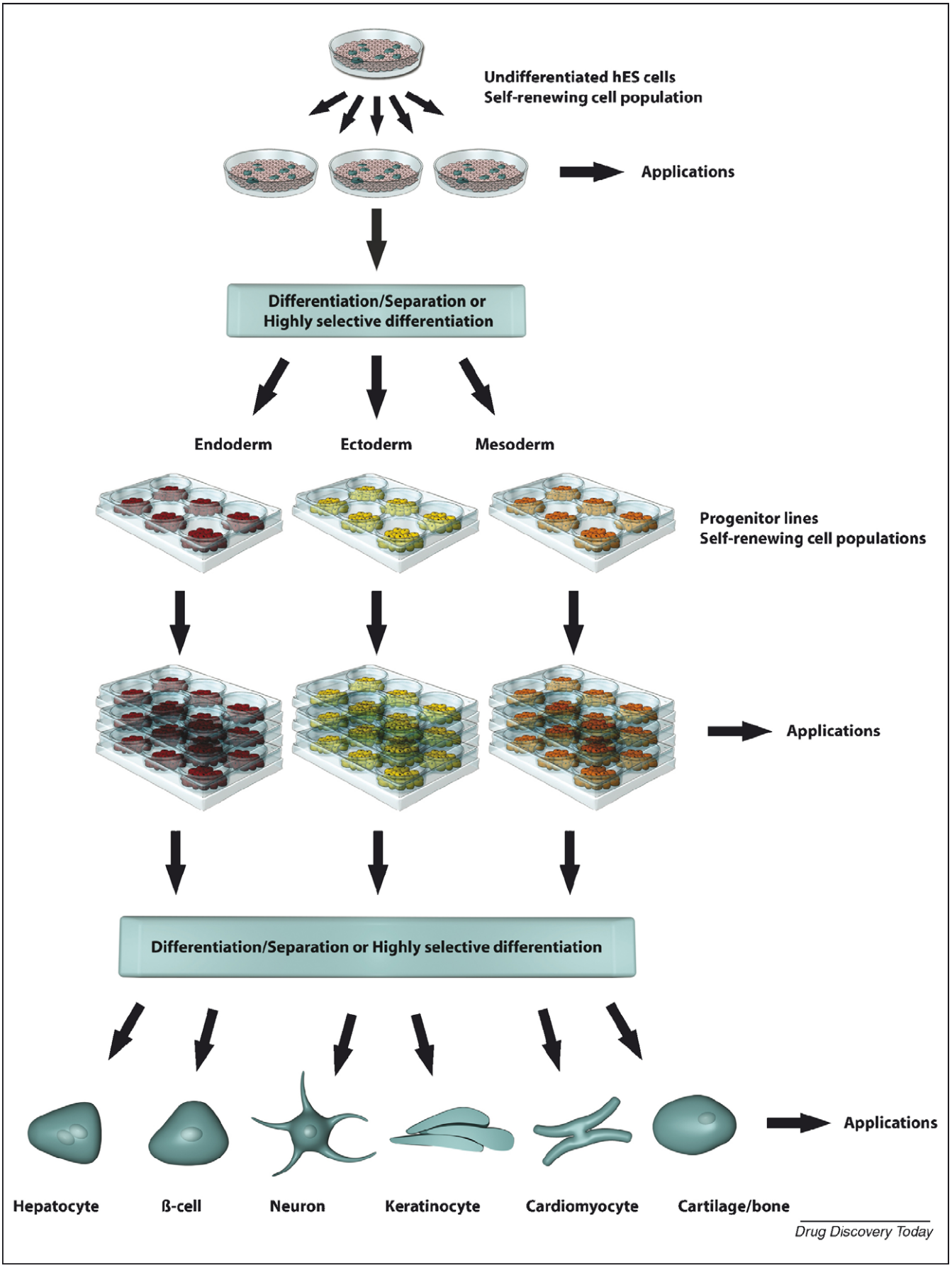


FIGURE 5

Micrograph showing cardiomyocyte-like cells derived from hES cell line SA002 (Cellartis AB) in a MEA. The hES cells were differentiated and spontaneously contracting clusters of cells were isolated and transferred to the MEA. The asterisks indicate contracting clusters.


Undifferentiated hES cells
Self-renewing cell population
Applications
Differentiation/Separation or
Highly selective differentiation
Endoderm
Ectoderm
Mesoderm
Progenitor lines
Self-renewing cell populations
Applications
Differentiation/Separation or Highly selective differentiation
Applications
Hepatocyte
ß-cell
Neuron
Keratinocyte
Cardiomyocyte
Cartilage/bone
Drug Discovery Today

action potential prolongation, as well as early afterdepolarization [86]. Automated platforms for high-throughput electrophysiological recordings in combination with relevant hES cell-derived cardiomyocytes and novel *in silico* modeling approaches will provide cost-effective methods for investigating potential proarrhythmic risk of novel compounds [92].

The application of hES cell-derived cardiomyocytes for drug discovery and safety assessment holds great promise. However, initial observations need to be carefully validated and cells with appropriate phenotypes need to be established using scalable culture methods. These and other challenges are being addressed by further research in this exciting new field, which has the potential to contribute with substantial improvements of existing technologies.

Concluding remarks

Research on hES cells holds great promise for the understanding and treatment of human disease. In this regard, there are huge expectations on the future application of these cells for therapeutic interventions by permitting the creation of transplantable cells to be used in regenerative medicine. In addition, hES cells provide unprecedented opportunities for basic research of human development and studies of the molecular programs that control early lineage commitment and cell differentiation. In the context of this review, we have discussed some exciting novel opportunities for hES cells and their progenies in drug discovery. The major reason for the interest in hES cell-based systems is that they offer an alternative for obtaining a large number of different specialized cell types which otherwise are difficult or impossible to acquire from other sources. Although progress has been made regarding directed differentiation of hES cells, there is still a need to improve the homogeneity and yield of the target cells by using enrichment and selection techniques. Furthermore, the generation of defined populations of specialized cells from hES cells usually takes several weeks, making it advantageous to isolate and maintain intermediate precursor cells that can be cryopreserved and still have the capacity to proliferate and differentiate upon thawing (Figure 6).

It is anticipated that there will be a widespread use of hES cell-derived hepatocytes and cardiomyocytes in predictive toxicology since two of the leading causes of preclinical failure of new compounds are hepatoxicity and cardiotoxicity [93]. Thus, novel improved models to assess adverse effects of new drugs early in the development phase are needed. However, it is important to appreciate that hES cell research represents an emerging area of investigation, and there are still many fundamental issues related to hES cell culture and differentiation that needs to be addressed. Nevertheless, the research community is now at the very early stages of putting into practice some of the new opportunities that hES cells provide. With the right resources, together with sound guidelines and regulations of stem cell research, the field of hES cells has a real potential to revolutionize many aspects of human biomedicine and the understanding of normal and abnormal human development. Specifically, the development of many cell culture tools in the pharmaceutical industry will most probably emerge over the coming years. It is likely that pharmaceutical companies that successfully integrate stem cell technologies will have a competitive advantage.

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FIGURE 6

Schematic of the generation of functional cell populations from hES cells. Undifferentiated hES cells can be propagated extensively *in vitro*, generating large quantities of cells, which subsequently are differentiated into germ layer committed progenitor cells. Depending on the efficiency of the differentiation a separation step might be required to generate pure populations of progenitor lines. The progenitor cells also have the capacity for self-renewal and can be expanded before subsequent directed differentiation takes place. The final steps generate terminally differentiated specialized cells, and a few examples of key cell types are shown in the figure. As indicated, the cells can be applied, at the various stages, for *in vitro* use in drug discovery.

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