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The Basics of Stem Cells and Their Utility as Platforms to Model Teratogen Action and Human Developmental and Degenerative Disorders

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1.1 Stem Cell Types and Basic Function

All organisms with body plans organized into specialized organs containing tissue-specific cell types rely upon the action of stem cells in order to produce their adult body plans over the course of development. In placental mammals, the process of development commences upon fertilization of the egg to yield the zygote, a single cell with a fixed genome from which all subsequent cells arise over the course of development. The zygote is imbued with the property of totipotency; i.e. it has the potential to give rise to all cells of the conceptus, including cells of the developing embryo proper as well as cells of the embryonic component of the placenta. After a short series of rapid cleavage-stage cell divisions, the morula forms, in which all cells are still totipotent. Morula cells then execute the first asymmetric cell division, resulting in daughter cells that are a component of either the inner cell mass (ICM), which subsequently contribute to the embryo proper, and trophectodermal cells, which give rise to the embryonic component of the placenta. Thus, even in the initial stages of mammalian embryogenesis leading to the blastocyst (3.5 days after fertilization in mice and 5.5 days in humans), cells begin with maximal developmental potential (totipotency), which is then restricted during a single-key asymmetric cell division culminating in the production of pluripotent cells of the ICM and multipotent cells of the trophectoderm. This most basic example illustrates two key features of stem cells *in vivo*: (i) the capacity for self-renewal without loss of developmental potentiality and (ii) the capacity to execute carefully

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regulated asymmetric cell divisions resulting in two differing types of cells each with distinct developmental trajectories.

These two features (self-renewal and asymmetric cell division) are the defining features of all stem cells, in both the developing embryo and adult tissues. However, in postimplantation development asymmetric cell divisions of stem cells are of two types: (i) Asymmetric cell divisions that yield two new cell types, both of which have lineage-restricted developmental potential and (ii) asymmetric cell divisions that yield a replacement daughter stem cell similar to the parental cell and a differentiated cell that is committed to differentiation. This second type of stem cell division is common in adult tissues and organs and is responsible for the establishment and maintenance of a pool of resident adult stem cells that serve to renew and replenish the organ with new cells that replace those lost to aging, degeneration, and injury.

Embryonic stem cells (ESCs) are pluripotent cells that are derived from the ICM of blastocyst-stage embryos and are becoming increasingly employed in cell culture systems that are designed to assess compounds and conditions that affect development and cellular function. ESCs were first developed in 1981 from isolated mouse blastocysts (Evans and Kaufman, 1981). However, comparable human ESCs were not derived until 1998 (Thomson *et al.*, 1998). The cells of the ICM are pluripotent, having the capacity to differentiate into the approximately 200 distinct cell types present in the adult mammal body plan, and this level of pluripotency is retained by ESCs. The ICM persists in the mammalian embryo for little more than a day *in vivo*, but culture conditions for ESC maintenance have been optimized such that this pluripotent state can be maintained indefinitely during ESC culture. This is because key signaling factors such as leukemia inhibitory factor (LIF, for mouse ESCs) and basic fibroblast growth factor (bFGF, for human ESCs) have been discovered, and these maintain the pluripotent state of ESCs indefinitely during culture *in vitro*. In addition, ESCs are conditionally immortal in culture since they can divide indefinitely without loss of pluripotency. Thus, ESCs exist in a state of suspended stasis with regard to their pluripotentiality, which is only a transient state *in vivo*. Upon removal of LIF or bFGF, ESCs spontaneously differentiate *in vitro* to form cells of endodermal, ectodermal, and mesodermal lineages, reminiscent of the process of gastrulation in their *in vivo* counterparts. Upon differentiation, conditional immortality is lost and the differentiated cells eventually senesce. In the last decade, widespread advances have been realized that allow the directed differentiation of ESCs to specific terminal cells types derived from each of the three principal germ layers, but the initial steps of each of these individual directed differentiation procedures commence with the removal of factors that support pluripotency, leading to the formation of definitive endodermal, ectodermal, or mesodermal cells.

In addition to ESCs, it is now possible to produce induced pluripotent stem cells (iPSCs) from terminally differentiated somatic cells. Similarly to ESCs,

iPSCs have the ability to differentiate into numerous distinct cell types but can be cultured indefinitely under appropriate conditions. In the iPS process, terminally differentiated cells are reprogrammed to a pluripotent state by the forced expression a key set of powerful transcription factors that normally function in the ICM. In 2006, Takahashi and Yamanaka successfully reprogrammed mouse fibroblasts to generate embryonic-like state by virally expressing four core pluripotency reprogramming factors: Oct3/4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006). The resulting iPSCs are functionally equivalent to ESCs. In the following year, they showed that the same four factors could be used to produce iPSCs by the reprogramming of adult human fibroblasts (Takahashi *et al.*, 2007). Two of these transcription factors (OCT4 and SOX2), together with NANOG, are especially important as they regulate the expression of hundreds of genes in pluripotent cells. When OCT4 and SOX2 are expressed in fibroblasts or other differentiated cells, they "boot up" the expression of a large set of embryonic genes, leading to the reprogramming of somatic cells to pluripotent cells. Since the same transcription factors can successfully reprogram both mouse and human fibroblasts to pluripotency, this finding shows that two divergent mammalian species (human and mouse) contain conserved sets of transcription factors that govern the pluripotent state. Subsequently, iPS approaches have been used to produce iPSCs from other species including rhesus monkey (Liu *et al.*, 2008), pig (Esteban *et al.*, 2009), and rat (Coppiello *et al.*, 2017, Hamanaka *et al.*, 2011, Li *et al.*, 2009, Zhou *et al.*, 2011). In addition, many types of somatic cells can be reprogrammed including nucleated cord blood cells (Haase *et al.*, 2009) epithelial cells from the urinary tract (Zhou *et al.*, 2011), and many others. iPS technology allows the facile production of pluripotent cells from fibroblasts or nucleated peripheral blood cells, which bypasses the need for embryos for the derivation of pluripotent cells. Importantly, iPS technology works well on human cells, and human iPSCs retain the entire genome of the human somatic cell donor, thus allowing the creation of patient-specific iPSC lines. This approach opens the door to personalized medicine approaches in which individual patient genomes (with an idiosyncratic set of patient-specific genetic variations) can be used to test for individualized responses to drug activation and susceptibility to toxicological effects caused by genetically altered drug metabolism.

Adult stem cell populations exist in most if not all adult organs and serve to replenish cells lost to aging and damage. However, adult stem cells have in general proven to be much more difficult to isolate and culture *in vitro* (with the notable exceptions of hematopoietic and mesenchymal stem cells). In addition, adult stem cells are tissue specific and typically are limited in their differentiation abilities, as they can usually only produce the terminally differentiated cell types present within a specific organ. Thus, adult stem cells are considered multipotent. In summary, developmental potentiality *in vivo* becomes progressively restricted, transiting from totipotent (the zygote and

morula) to pluripotent (the ICM) to multipotent (adult stem cells), and finally unipotent (terminally differentiated cells), which often become postmitotic as a final stage.

1.2 Pluripotency

Pluripotency is a developmental biology term that describes the breadth of developmental potential of cells of the blastocyst inner cells mass. ESCs and iPSCs have a highly similar level of pluripotency. This is exemplified by the finding that individual mouse ESCs and iPSCs can completely contribute to embryogenesis *in vivo* after transfer into tetraploid host blastocysts, which are then implanted into pseudopregnant surrogate female mice (Kang *et al.*, 2009). In this embryological method, the host tetraploid blastocyst (which is made artificially tetraploid) supports the engrafted ESCs or iPSCs resulting in the fetal development and live birth of pups entirely derived from ESCs or iPSCs. To date, this achievement provides the best proof that ESCs and iPSCs have pluripotency comparable to that of normal embryonic ICM cells. In addition, a vast body of work from the field of stem cell research has shown that directed differentiation approaches can be devised that guide pluripotent cell differentiation *in vitro* to a wide variety of terminal cell types.

In the case of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), proof of pluripotency *in vivo* is not possible due to medical ethics, but hESCs and hiPSCs have been successfully differentiated to a wide variety of terminal cellular types, perhaps even more numerous than has been achieved with mouse ESCs. In addition, numerous "omics" reports have shown that the transcriptome of hESCs and hiPSCs are very similar to native human ICM cells that the epigenome of hESCs and hiPSCs are also very similar to native human ICM. Finally, detailed and comprehensive comparisons between human and mouse pluripotent cells have shown a high degree of similarity in terms of their relative transcriptomes and epigenomes, though notable differences exist, probably due to divergent evolution resulting in species-specific idiosyncrasies. Overall, the transcriptional and epigenomic state of mouse and human ICM cells, ESCs, and iPSCs share a high degree of concordance, and key pathways that collude to maintain the pluripotent state are shared by all these cells.

1.2.1 Poised Chromatin of the Pluripotent Epigenome

The configuration of the epigenomes is key for the maintenance of the pluripotent state and serves as a framework to establish transcriptional states. Pluripotent cells contain developmentally poised chromatin, which is exquisitely assembled upon the genome with a level of precision that marks key

genes for later developmental expression or silencing in appropriate cellular lineages. This is achieved in large measure by the presence of bivalent domains that are positioned throughout the genome on a gene-by-gene basis (Bernstein *et al.*, 2006). Bivalent chromatin is unusual in that it contains marks in the histone code that designate specific genes for later expression or silencing, which unfolds in a tissue specific manner over the course of development. Bivalent marks occur on the core nucleosomal histone H3 and consist of the dual trimethylation of lysine residues H3 at positions 4 and 27 within H3 N-terminal tails (H3K4me3 and H3K27me3). Individually, H3K4me3 specifies active transcription, while H3K27me3 specifies transcriptional silence. However, in pluripotent chromatin, these histone marks co-occur, resulting in an epigenetic signature that is later resolved during ensuing development, as bivalently marked genes become monovalently marked by the maintenance of either H3K4me3 or H3K24me3 alone, resulting in gene activation or silencing, respectively. Bivalent domain resolution occurs on a tissue-specific basis. For example, a neurally expressed gene is bivalently marked in the ICM, and in ESCs and iPSCs, but in a terminally differentiated neuron, the resolved mark consists of H3K4me3 while H3K27 becomes unmethylated. The same gene in a nonneuronal differentiated cell (a liver cell for instance) becomes unmethylated at H3K4 and retains H3K27me3, resulting in gene silencing.

Once pluripotent cells are coaxed to differentiate *in vitro*, they exit the pluripotent state and undergo a process analogous to gastrulation where they proceed along endodermal, ectodermal, and mesodermal lineages. In practice, differentiation methods have been developed that employ two general approaches: (i) undirected differentiation, which is induced by the removal of pluripotency-signaling factors (LIF or bFGF) leading to the stochastic production on mixtures of endodermal ectodermal and mesodermal cells and (ii) the removal of pluripotency-supporting factors combined with the addition of specific germ layer inducing signaling molecules. An example of the latter is the removal of bFGF from hESC or hiPSC culture, combined with the addition of Activin A, which induces differentiation to a relatively uniform population of endodermal progenitor cells (D'amour *et al.*, 2005). These can subsequently be exposed to custom series of growth factors, media, and conditions to yield a desired type of endodermal cell, such as hepatocytes for example.

1.2.2 Undirected Differentiation of Pluripotent Cells to Embryoid Bodies

Undirected (spontaneous) differentiation of pluripotent cells is a poor choice if the goal is to produce a uniform population of differentiated cells of a single cellular identity. However, undirected differentiation is highly useful for the production of embryoid bodies (EBs), which are useful for the detection of potential teratogens (compounds that can cause birth defects). This is because

EBs serve as a reasonable model for early postimplantation embryogenesis. EBs are initiated from starter cultures consisting of cohesive aggregates of undifferentiated pluripotent cells. Especially in the case of hESCs and hiPSCs, aggregates are crucial since human pluripotent cells form junctions with neighboring cells mediated by E-cadherins and other cell adhesion proteins. These contacts are preserved in aggregates. Aggregates consisting of dozens to hundreds of cells are transferred to medium lacking pluripotency-signaling growth factors (i.e. LIF of bFGF is removed). Cells within the aggregate then begin to spontaneously differentiate into cell of all three germ layers, and after several days, a variety of cell types of endodermal, ectodermal, and mesodermal origin form. Many of these cells differentiate quite far in developmental terms, and mature EBs typically contain abundant neurons and cardiomyocytes, which begin to spontaneously contract leading to observable rhythmic contractions in EBs (often called "beating heart" EBs at this stage). Classic methods of EB production start with aggregates of varying cell number made simply by the partial disaggregation of ESC cultures. These are then plated in nonadherent cell culture wells or flasks and as differentiation proceeds, a collection of variously sized EBs form. Individual aggregates have also been cultured in hanging drop cultures, which allows the individual culture of EBs of random size. Recently, it has become possible to mass-produce human EBs of uniform from pluripotent cells. This is achieved by disaggregating hESC colonies to single cells (which is now possible with ROCK inhibitor, which can compensate for the disruption of E-cadherins cell–cell interactions), followed by aggregation of a chosen number of cells into EBs. This allows cohorts of EBs of similar size to differentiate synchronously, and these can be cultured individually in multiwell formats (Flamier *et al.*, 2017). This improvement now makes human EB systems suitable for the testing of a large number of suspected environmental and pharmaceutical teratogens.

1.2.3 Directed Differentiation of Pluripotent Cells

A great deal of research effort has been devoted to the directed differentiation of pluripotent cells to specific final cell types. This effort has been driven in large measure by the desire to eventually utilize such cells for cell replacement (stem cell) therapies. However, a useful outcome of this work is that it is now possible to use these cells as platforms for toxicological assays. Primary human cell culture in which cultures are derived from postmortem human organs and biopsies are difficult at best, and directed stem cell differentiation procedures can reproducibly produce such cells. Thus, it is now possible to test compounds for toxicity on relevant cell types from the appropriate species. For instance, hepatotoxicity, neural toxicity, and cardiotoxicity can now be tested on human hepatocytes, neurons, and cardiomyocytes of stem cell origin – a vast improvement over the methods in use only a decade ago (and even now) in which

compounds were routinely tested on abnormal immortalized human cell lines and often on cells of irrelevant identity and/or species origin.

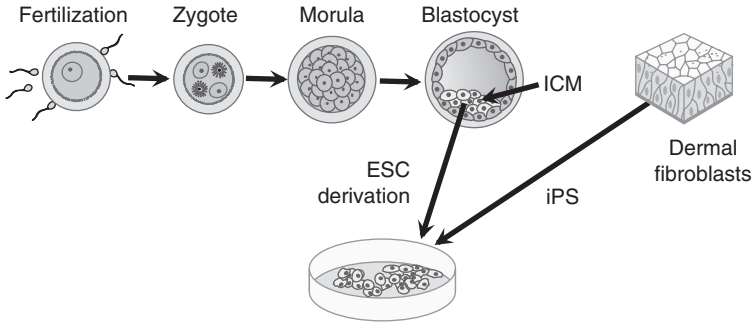
1.3 *In vitro* Uses of Pluripotent Cells

Classic approaches to toxicological and teratological assays have made heavy use of rodents (mice and rats) as well as rabbits. Though animal testing has the advantage of being an *in vivo* system, species-specific differences between animal models and humans are often large, resulting in frequent false-positive and false-negative errors. Furthermore, animal testing of compounds is laborious, costly, and time-consuming and requires the use of a large number of animals. Now, it is possible to use pluripotent cells (ESCs and iPSCs) as platforms to investigate both toxicological effects and teratogenic effects as an alternative to animal approaches (Figure 1.1). These approaches supplement existing animal approaches and provide a platform that utilizes relevant cells, and in the case of human ESCs and iPSCs, from the appropriate species. Furthermore, the advent of human iPS platforms now allows the testing of pharmaceuticals for their impact upon specific human populations with genetically altered drug metabolism.

1.3.1 Pluripotent Cells for Toxicology

Animal research to assess toxicological effects of compounds *in vivo* comes with a substantial logistical load, but in general, toxicological assays can be conducted on adult mice followed by sacrifice to assess consequences of compound exposure for tissue histology combined with assessment of biomarkers of toxicity such as release of liver enzymes into peripheral blood. The U.S. Environmental Protection Agency (EPA) ToxCast program has identified thousands of compounds that have been deemed of importance for toxicological assessment (Dix *et al.*, 2007), but the low throughput nature of animal testing makes the complete assessment of ToxCast compounds in animals a huge endeavor, which will require decades of testing and the use of a large, nearly prohibitive number of rodents to complete this task. In addition, rodents developmental biology differs from that of humans, and false-positive and false-negative results due to species differences are frequent. Though the use of animals will likely not be supplanted, testing in cell culture platforms is of obvious interest for reasons of expediency and the ability to use human cells of relevant identity.

In pharmaceutical drug development, the most common types of toxicity that result in failure of candidate drugs are cardiotoxicity and hepatotoxicity. Cardiotoxicity is a common pharmaceutical side effect, and cardiomyocytes derived from stem cells serve as a platform for predictive toxicology. For



Cell type	Culture methods	Applications
ESCs, iPSCs	Embryoid bodies	Teratology developmental toxicology
	Directed differentiation	Toxicology
iPSCs (patient-specific)	Embryoid bodies	Genetic birth defects
	Directed differentiation	Toxicogenomics

Figure 1.1 Derivation and use of pluripotent cells for *in vitro* teratology and toxicology applications. ESCs are derived from the ICM of blastocyst-stage embryos (mouse or human) and a pluripotent. Alternatively, iPSCs can be produced by factor-mediated reprogramming of terminally differentiated somatic cells such as fibroblasts. iPSCs are also pluripotent. Pluripotent cells (ESCs or iPSCs) can be differentiated into EBs for modeling of early development and used to detect teratogens or study human genetically specified birth defect mechanisms (provided that iPSCs containing relevant genetic developmental mutations are used). In addition, both ESCs and iPSCs can be subjected to directed differentiation to detect chemically induced impacts on specific cell types. Finally, patient-specific iPSCs can be used to detect and study individualized pharmacogenomic responses to pharmaceuticals (in terms of efficacy or toxicity).

example, doxorubicin can induce cardiotoxic effects in human subjects. The cardiotoxicity of doxorubicin is evident in *in vitro* stem cell systems in which stem-cell-derived cardiomyocytes are used (Farokhpour *et al.*, 2009; Singla, 2015; Maillet *et al.*, 2016). In fact, patients exhibit idiosyncratic cardiotoxicity in response to doxorubicin and patient-specific susceptibility to doxorubicin can be detected in cardiomyocytes derived from patient-specific iPSCs (Burridge *et al.*, 2016). Hepatotoxicity is also a common pharmacological side effect, and similar stem cell systems to detect drug-induced hepatotoxicity have also been developed (Greenhough *et al.*, 2010; Takayama and Mizuguchi, 2017).

1.3.2 Pluripotent Cells for Teratology

In the United States, approximately 3% of live-born infants are affected by recognizable birth defects according to the Centers for Disease Control and Prevention (CDC). In addition, spontaneous abortion is frequent, and many of these may be caused by teratogenic exposure. Potentially teratogenic compounds exist in the environment, in food and drinking water, and in pharmaceuticals, and these may contribute to birth defects of nongenetic origin. Often, teratogenic pharmaceuticals have become known only after release of a drug to the public. In the late 1950s and early 1960s, over 10 000 birth defects occurred after thalidomide use by pregnant women (Franks *et al.*, 2004; Gaffield *et al.*, 1999; Holmes, 2002; Matthews and Mccoy, 2003). Thalidomide-induced defects included limb and neural tube closure defects (Holmes, 2002). Sodium valproate is a mood-stabilizing antiepileptic drug that also impacts chromatin acetylation, which is also teratogenic (Lammer *et al.*, 1987; Nau, 1994; Nau *et al.*, 1991; Ornoy, 2009; Smith and Whitehall, 2009). Unintentional exposure can occur during the first few days of pregnancy, especially if the pregnant state is unknown to the mother. Unfortunately, preimplantation and early postimplantation development stages are highly susceptible to teratogen action.

The use of animals for testing of suspected teratogens is even more fraught with logistical impediments than standard toxicology because exposure to compounds must occur *in utero*. Thus, pregnant animals must be dosed with compounds under test. In addition, there are complexities for animal teratology testing having to do with fetal support of dosed pregnant female animals and developmental biology of the germ line. In animal teratology testing, the pregnant female must be dosed with a suspected teratogen, and if altered offspring or fetal defects are observed, it is not immediately clear if this is due to *bona fide* teratogenicity, or simply toxicological effects on the pregnant female that cause compromised fetal support. The situation is further complicated if it is suspected that effects might impact the fertility of the exposed fetus. In placental mammals (both mice and human), gonadogenesis and the development of germ line cells occur *in utero*. This means that when dosing a pregnant female with a suspected developmental reproductive toxin, the developing fetal gonad is also exposed. Thus, effects on reproductive toxicity must be assessed in mice in the F2 generation, as these are the first generation in which the developing fetal gonads are not directly exposed to a chemically impacted *in utero* environment. Thus, comprehensive teratology and reproductive toxicity tests require multiple generations of animals, rendering this approach exceedingly low throughput and highly animal intensive.

EBs and ESC differentiation systems provide workable platforms for the identification of potentially teratogenic compounds. EB systems have been successful in the detection of the teratogenic action of thalidomide, where thalidomide-exposed EBs exhibited impaired angiogenesis and increased hydroxyl

radical levels (Sauer *et al.*, 2000). In addition, human iPSC undergo higher rates of apoptosis even in their undifferentiated state (Tachikawa *et al.*, 2017). EBs and ESCs have also been successfully used to detect teratogenic activities of valproic acid. Differentiation of ESCs to cardiomyocytes is inhibited after exposure to valproic acid (Murabe *et al.*, 2007). In addition, valproic acid caused dramatic defects in differentiating EBs (Flamier *et al.*, 2017). Finally, EBs provide a medium-throughput approach to screen multiple potential teratogens, and altered EB morphology serves as a useable endpoint (Mayshar *et al.*, 2011; Warkus *et al.*, 2016).

1.3.3 Limitations of Pluripotent Stem Cells

In vitro stem cell culture platforms have obvious advantages for teratology, developmental toxicology, and general toxicology research, but there are limitations to such systems. Perhaps the most significant of these limitations is the lack of metabolic activities compared to animal systems. Many compounds (be they environmental toxins or pharmaceuticals of concern) must be metabolized in the liver to yield active or toxic metabolites, and the liver is also the major organ that is responsible for the metabolism of drugs, which allows the drugs to be absorbed, distributed, metabolized, and excreted from the body. Crucial drug-metabolizing enzymes include phase I and phase II enzymes and transporters that are highly expressed in liver. Phase I enzymes mainly catalyze oxidation, reduction, and hydrolysis reactions of parent compounds. Most notable among phase I enzymes are cytochromes P450s (CYPs), which are abundantly expressed in the liver. Phase II enzymes primarily catalyze conjugation reactions including glucuronidation, sulfation, and acetylation. Lastly, hepatic transporters allow drugs to be absorbed and eliminated from the liver.

When parent compounds are added directly to stem cell cultures, metabolic activities normally found in the liver are absent from the system. Hepatocytes derived from pluripotent stem cells have been of interest because these might provide a solution to this problem, but unfortunately, most hepatocyte-like cells (HLCs) derived from stem cells are significantly deficient in their metabolic activities. This problem is compounded by the fact that primary hepatocytes from both mouse and human liver rapidly lose their metabolic activities upon culture *in vitro*. Most drug-metabolizing enzymes and transporters are regulated by xenobiotic-sensing nuclear receptors, CAR (constitutive androstane receptor) and PXR (pregnane X receptor) that are poorly expressed in HLCs (Kim *et al.*, 2016). For example, CYP3A4 is a major phase I enzyme that is abundantly expressed in the liver. However, in HLCs, the expression of CYP3A4 is not present until the fourth stage of differentiation and is still not equivalent to native hepatocytes (Yu *et al.*, 2012). Advances are being made in both of these areas (improved hepatic directed differentiation from stem cells

and improved methods for the culture of primary hepatocytes), but the state of the art in both of these areas is still problematic at present.

In the case of birth defects research, ESC-based systems have become useful models for early stages of teratogenesis, and these systems can also detect teratogenic compounds. In short, EBs are of keen interest because human cells with embryonic identities with developmentally relevant potency can be used. However, limitations also exist for the use of stem-cell-based teratology platforms. Differentiating EBs most closely model very early stages of development, but certainly do not model later stages including organogenesis. Thus, EB models are best for investigations of effects on early postimplantation development. This is clearly a limitation, but it must also be noted that early postimplantation development is a developmental stage of great susceptibility for exposure to teratogenic compounds and is also a stage in which pregnancy is often not known, thus increasing the risk of unintentional exposure to hazardous compounds.

1.4 Adult Stem Cells *In vivo*

The preceding sections describe the use of pluripotent cells (ESCs and iPSCs) for *in vitro* platforms to detect and study teratogens and cytotoxins. However, adult stem cells are crux cells during organogenesis of fetal development and are also the key cells that contribute to tissue homeostasis and organ repair in postnatal and adult life. Thus, another emerging field of medical study is the impact of environmental and pharmaceutical compounds on resident adult stem cells (Figure 1.2). When adult stem cells are impacted, "stem cellopathies" can result, which are diseases and medical syndromes that are caused by loss or dysfunction of adult stem cells. In addition, dysfunctions of adult stem cells *in vivo* that are unrelated to compound exposure can also have significant consequences. To this end, an entire section in this book is largely devoted to this topic (see Part III). An emerging theme in cancer is that most cancers (whether they be solid tumors or leukemic) have cancer stem cell populations that differentiate to yield the bulk mass of neoplastic cells and tumors. Thus, cancers may easily be viewed as "stem cellopathies." However, the field of cancer stem cell research is vast and a book devoted entirely to this subject would still not be adequate. For that reason, this volume is confined to noncancer stem cellopathies.

The term "adult stem cells" is somewhat of a misnomer, since adult stem cells become established during organogenesis of fetal development. They then persist in specialized niches unique to each organ during childhood, adolescence, and into adulthood. In general, these multipotent cells have the capacity to contribute to the organ in which they reside. The human brain contains adult neural stem cells that participate in the later development, maturation, and

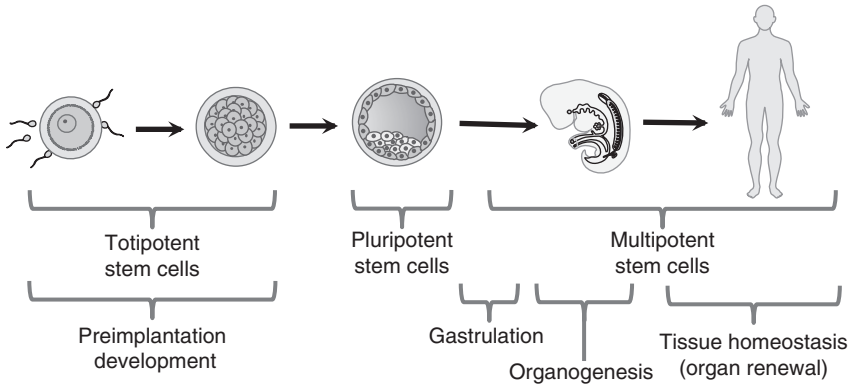


Figure 1.2 Adult (endogenous) stem cells and developmental toxicity. An emerging theme is that some human disorders occur due to impacts upon endogenous adult stem cells ("stem cellopathies"). Adult stem cells arise during organogenesis, and if they are compromised during gestation, birth defects and developmental disorders that play out later in life can occur. In addition, adult stem cells are required for tissue homeostasis and organ regeneration, and insults to adult stem cell populations in the adult can also lead to human disorders, impacting a variety of organs. Successive restriction in developmental potency (from totipotency to multipotency) of endogenous stem cells and major landmarks in human development and maturation are shown.

maintenance of the brain and are responsible for some degree of neurogenesis that occurs throughout adulthood. The adult brain is an organ that develops slowly compared to other organs, with significant growth and specialization occurring during early childhood, with further maturation that persists into early adulthood. Given this lengthy period, it is not surprising that the course of neurodevelopmental and neurodegenerative disorders may be impacted by insults to adult neural stem cells.

Certain tissues within the adult are highly proliferative throughout life and rely heavily upon adult stem cell differentiation to replace lost cells. Examples include the blood where hematopoietic stem cells (HSCs) support a prodigious turnover of cells throughout life, the skin, and also intestinal epithelia. In fact, most organs have a substantial amount of cellular replacement that occurs throughout life. Indeed, aging itself has features in common with stem cellopathies, in that organ degeneration and senescence occur in large measure due to the eventual depletion of resident populations of adult stem cells.

1.5 Emerging Trends in Stem Cell Culture

Tissue engineering is a multidisciplinary field that combines biomaterials, cells, biologically active factors, and engineering technology to reconstruct biological tissues and organs. Adult stem cells, ESCs, and iPSCs and are often

used in engineering tissues along with a biologically inert scaffold materials that serve in the capacity of extracellular matrix (ECM). When such engineered tissues are implanted into tissues, these matrix materials degrade in a controlled manner and are replaced by ECM secreted by the cells. There are several methods for engineering tissues and organs, some of which are discussed in the following sections.

1.5.1 Use of Coculture

Coculture is a multicell system comprised of a target cell and assisting cells. Stem cells play a pivotal role in a coculture as target cells, due to their multilineage differentiating potential. Assisting cells provide functions including cellular metabolism, immune suppression, inhibition of fibrosis and apoptosis, enhancement of angiogenesis, mitogen, and morphogen activities. Together these activities provide "trophic effects" through secretion of cytokines and growth factors to target cells (Caplan and Dennis, 2006). Stem cells can be used as an alternative to primary cells and donor tissues in bioengineering. Often, coculture systems provide stem cells with a scaffold for their physical attachment and an *in vitro* stem cell "niche" for signaling factor regulation, self-renewal, and differentiation into specific cell types (Scadden, 2006).

Coculture systems can be either direct or indirect. Direct coculture consists of a mixed population of cells in two-dimensional (2D) or three-dimensional (3D) culture. 2D direct coculture consists of a monolayer of admixed cells that are grown in flasks or dishes. 3D coculture systems more closely recapitulate the *in vivo* physiological environment. In a 3D direct coculture, multiple cell layers are cultured on a basal substrate scaffold layer. Scaffolds are often made up of natural materials such as collagen, fibrin, hyaluronic acid, and alginate beads, or synthetic materials such as Arg-Gly-Asp (RGD). Matrigel™ is made of ECM proteins including laminin and collagen IV and is used as a substrate for stem cells proliferation and differentiation in feeder cell-free culture (Dawson *et al.*, 2008). Cell signaling in direct coculture is mediated through direct cell–cell interaction, cell-ECM adhesion, or by paracrine signaling. In indirect coculture platforms, the various cell types are separated by a physical but permeable barrier such as a trans-well insert chamber. Such permeable barriers permit the passive diffusion of growth factors but not cells and can be used for both 2D and 3D cultures. Encapsulation of vascular endothelial growth factor (VEGF) in dextran sulfate nanoparticles was shown to increase angiogenesis within 3D implants (Des Rieux *et al.*, 2011). ESCs were shown to undergo cardiomyocyte differentiation when indirectly cocultured in a 3D environment with cardiac fibroblasts (Ou *et al.*, 2011). Mouse ESCs encapsulated in alginate have been shown to respond to added chemicals and can be used for *in vitro* cytotoxicity screens (Oberai *et al.*, 2015). Soluble factors such as cytokines and growth factors are released upon the establishment cell–cell interactions, or

from scaffolds themselves, and thus such systems bear resemblance to *in vivo* niches. Fibroblast growth factor signaling regulates self-renewal and proliferation of human ESCs and regulates migration, proliferation, and lineage commitment in differentiated cells (Coutu and Galipeau, 2011). Bone morphogenetic proteins (BMPs) regulate ESC pluripotency, block neural differentiation, and regulate HSC Proliferation by controlling the HSC niche size (Zhang and Li, 2005).

Cells grown in 3D cocultures offer several advantages over 2D cultures. They are similar to *in vivo* cells in morphology, proliferation, cellular differentiation, viability, response to stimuli from cell–cell interaction and external factors, drug metabolism, and secretion of metabolites (Edmondson *et al.*, 2014). However, conventional 3D techniques including matrices and scaffolds have some disadvantages. Difficulty in consistently reproducing scaffolds, unwanted interactions between scaffold materials and certain drugs, and lack of vasculature are some of the issues that are addressed by other innovative techniques including fabrication of microchannels and microfluidics (Edmondson *et al.*, 2014). Though many aspects of tissue ultrastructure are modeled by coculture systems, significant advances are still needed in order to reconstruct tissues that closely resemble natural histological structure and function.

1.5.2 Organoids

Organoids are 3D multicellular structures with properties that closely resemble the microanatomy and function of normal organs (Fatehullah *et al.*, 2016). They are derived from cell lines, primary tissues, ESCs and iPSCs, and organ explants. Pluripotent stem cells, when grown on Matrigel along with suitable exogenous factors, can form complex organoids with internal histology that resembles their *in vivo* counterparts. A dynamic stem cell niche environment is created in organoids through cell–cell autocrine or paracrine signaling or through the addition of growth factors. Intestinal organoids with spatial and temporal control of signaling can be designed from leucine-rich repeat containing G-protein-coupled receptor 5 ($Lgr5^{+}$) intestinal stem cells, Paneth cells, and epithelial cells cultured in Matrigel, and these undergo self-renewal and differentiation. Lgr5 stem cells have shown the potential to self-organize into stomach, colon and liver organoids (Sun and Ding, 2017). Organoids developed from mouse or human iPSCs include stomach, intestine, liver, lung, brain, pituitary gland, inner ear, retina, mammary gland, fallopian tube, gallbladder, and kidney (Kretzschmar and Clevers, 2016; Lancaster *et al.*, 2013; Sun and Ding, 2017).

Targeted genome editing, especially by CRISPR or TALEN approaches, can be used to improve stem cell niche function. The role of niche signaling pathways in tumor progression and micrometastases was studied through genome editing in human intestinal epithelial organoids that were transplanted into

mice (Matano *et al.*, 2015). Patient-derived iPSCs have been used for disease modeling in various diseases such as Hutchinson–Gilford Progeria Syndrome and Werner Syndrome. These organoid disease models have been used to study disease mechanisms (Li *et al.*, 2016). iPSC-derived intestinal organoids have been used in bacterial–epithelia interaction studies to study host–pathogen interactions involving *Salmonella*, *Clostridium,* and *Helicobacter pylori* (Sun and Ding, 2017). Brain organoids derived from patient-specific iPSCs have been used to study microcephaly caused by a mutation in *CDK5RAP2* (Lancaster *et al.*, 2013).

1.5.3 Microfluidics

Microfluidics technologies have been devised that employ etching or soft lithography approaches to yield structures that can support the growth and maintenance of cells that are continuously perfused by media flow (Zhang and Austin, 2012). Dynamic microfluidic technologies offer certain advantages compared to static culture systems. Microfluidics provide a versatile tool in stem cell analyses since they can incorporate various surfaces that mimic ECM, chemical gradients, and microfluidic channels that resemble *in vivo* niche environments. This approach has been widely used in tissue regeneration (Li *et al.*, 2017), high-throughput microfluidics for cytotoxicity analyses, and studies that assess iPSC and ESCs by high-throughput analyses of gene and protein expression changes over time (Chin *et al.*, 2009). Microfluidics are also used to study stem cell differentiation, cocultures, and the requirements of added factors in gradients of concentration (Zhang and Austin, 2012). Gradient microfluidics devices can set up precise gradients of chemicals, temperature, and oxygen by laminar flow or diffusion and exhibit features normally found in *in vivo* environments. Proliferation and differentiation of neural stem cells were optimized through a continuous gradient of different growth factors (Chung *et al.*, 2005). Gradient microfluidics can also be used to ascertain mechanisms of adaptation of stem cells and embryos to the changing internal environment (Lucchetta *et al.*, 2005).

The role of autocrine and paracrine signaling pathways mediated by Notch signaling, fibroblast growth factor-4 (FGF4)-dependent and -independent pathways in ESC pluripotency and lineage commitment have been demonstrated through microfluidics devices (Blagovic *et al.*, 2011). Microfluidic chips including 3D hepatocyte cultures and HepG2/C3A cultured cells have been successfully used for *in vitro* hepatotoxicity and toxicogenomic analyses (Prot *et al.*, 2011; Toh *et al.*, 2009). 3D microfluidic cell culture was shown to support the differentiation of human adipose-tissue-derived stem cells into neurons (Choi *et al.*, 2011). Human organoid-like structures resembling lung, liver, intestine, blood vessels, tumor, spleen, neurons, skeletal muscle, endothelium, and cardiomyocyte networks have been developed using microfluidic chips

(Ghaemmaghami *et al.*, 2012). In short, microfluidics approaches show promise in modeling organ function and disease states and are useful in stem-cell-based cytotoxicity assays, cell sorting, and drug screening.

1.5.4 Other Cell Types with Stem-Cell-Like Properties

Certain immortalized cell lines behave much like stem cells in that they retain the capacity to differentiate into other cell types. HepaRG cells are undifferentiated hepatic progenitors derived from hepatocellular carcinoma with morphological and functional characteristics similar to early hepatoblasts. HepaRG cells can be differentiated into either biliary (cholangiocyte) or hepatocyte lineages. HepaRG cells differentiate into mature hepatocytes and express phase I and II drug-metabolizing enzymes at levels comparable to primary hepatocytes. In addition, transporter proteins and nuclear receptors are highly expressed in HepaRG cells (Andersson *et al.*, 2012). Upon differentiation, HepaRG cells form polarized structures with bile canaliculi and possess efflux properties**.** These features make them an ideal candidate for drug screening, cell toxicity, and cytochrome P450 induction studies. HepaRG cells have also been differentiated into mature HLCs in 3D bioreactors (Darnell *et al.*, 2011). Thus, the scope of tissue engineering extends beyond the use of adult and pluripotent stem cells, and certain immortalized cell lines have good applications in toxicology and tissue modeling.

1.6 Future Directions

The use of stem cell strategies for teratology and toxicology research is still a very active area of research that is only now becoming established. However, this field has matured enough that it is possible to grasp the future directions and opportunities that exist. This section considers a few such forward-looking areas that lie at the intersection of stem cell research, human developmental biology and teratology, and the ways in which exogenous compounds may affect these biomedical research areas.

1.6.1 iPSCs, Pharmacogenomics, and Predictive Teratology

Recently, the field of pharmacogenomics has moved from the research realm to active medical practice. Pharmaceuticals are actively metabolized in the human body, primarily by phase I and phase II drug metabolic enzymes. There is a remarkable degree of genetic diversity in human populations within drug-metabolizing genes, and significant proportions of individuals fail to respond to specific medications because they contain polymorphisms that affect drug metabolism. In pharmacogenomics terms, individuals can be classified into

normal metabolizers, ultrarapid metabolizers, and poor or nonmetabolizers for each drug, and the genetic basis of this lies in whether or not individual patients have genetic variants that affect the expression of genes that encode drug-metabolizing enzymes. Thus, the bulk of patients typically respond to a given medication, but many are nonresponders or poor responders. Drug efficacy is one consideration in pharmacogenomics, but an equally important consideration is drug toxicity and the occurrence of unwanted side-effects. This too is mediated by human genetic variations in drug-metabolizing genes. Previously, adverse drug events have been detected in subpopulations of patients during large clinical trials, or unfortunately, once a drug has been FDA approved and marketed. In either case, whether there is variation in drug efficacy or toxic side-effects, the underlying cause is frequently genetic variation from patient to patient, but this can be ascertained through genotyping, and whole exome and genome sequencing of individual patients. Thus, the genome is predictive of drug action, and this has formed the basis of the biomedical fields of pharmacogenomics and personalized (precision) medicine. However, to date, the genomic information is typically obtained from cohorts of patients who have already failed to respond to drug treatment or already suffered adverse outcomes.

Looking forward, iPS technology may alleviate morbidity associated with pharmacologically induced adverse events since cells derived from patient-specific iPSCs representing major human haplotype groups can be used for screening for side-effects of new drugs in development. Indeed, it is now feasible to produce panels of human iPSCs that harbor the genomes and representative haplotype configurations of all major human ethnicities. In the future, it is very likely that human genetic diversity panels of iPSCs can be differentiated *in vitro* to selected cell types (especially liver and cardiac cells) and used to assess whether or not new drugs pose risk to specific human populations.

In terms of birth defects research, it has been difficult to determine *a priori* whether or not pharmaceuticals pose risk to the developing fetus. The reasons for this have already been discussed, but briefly, this is due to the tediousness of animal teratology assays, and the fact that many drugs do not affect rodent development, but turn out to have impacts on human fetal development. Though most women opt to avoid prescribed pharmaceuticals during pregnancy, many women become pregnant unintentionally while taking a variety of prescribed and over-the-counter drugs, and preimplantation and early postimplantation embryos are exposed. Therefore, there is a need to determine the teratogenicity of all drugs, if possible, and EB technology may contribute significantly to this endeavor in the near future.

1.6.2 Stem Cell Systems for Environmental Toxicology

During the human lifetime, there are many chances to become exposed to xenobiotics (foreign chemical substances) that cause toxicity directly or that

leave altered epigenetic marks on genes, which may manifest as future dysfunction. Therefore, it is important to understand environmental compounds that may cause damage to human tissues. For the most part, animal assays and *in vitro* experiments using animal cell have been employed to detect potentially toxic environmental insults. In practice, humans are exposed to complex chemical mixtures (in drinking water, in the food supply, contact with skin, or airborne), and stem cell systems can be employed to detect overt effects on cellular toxicity or fetal development. In fact, stem cells seem ideal for such studies because they (and their differentiated derivatives) can be exposed to complex mixtures (drinking water, solubilized food materials, etc.) and then monitored for positive results indicative of toxic or teratogenic potential. The expediency of stem cell systems make them an excellent first-pass assay for testing complex environmental mixtures, which can then be followed up with animal studies as needed.

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