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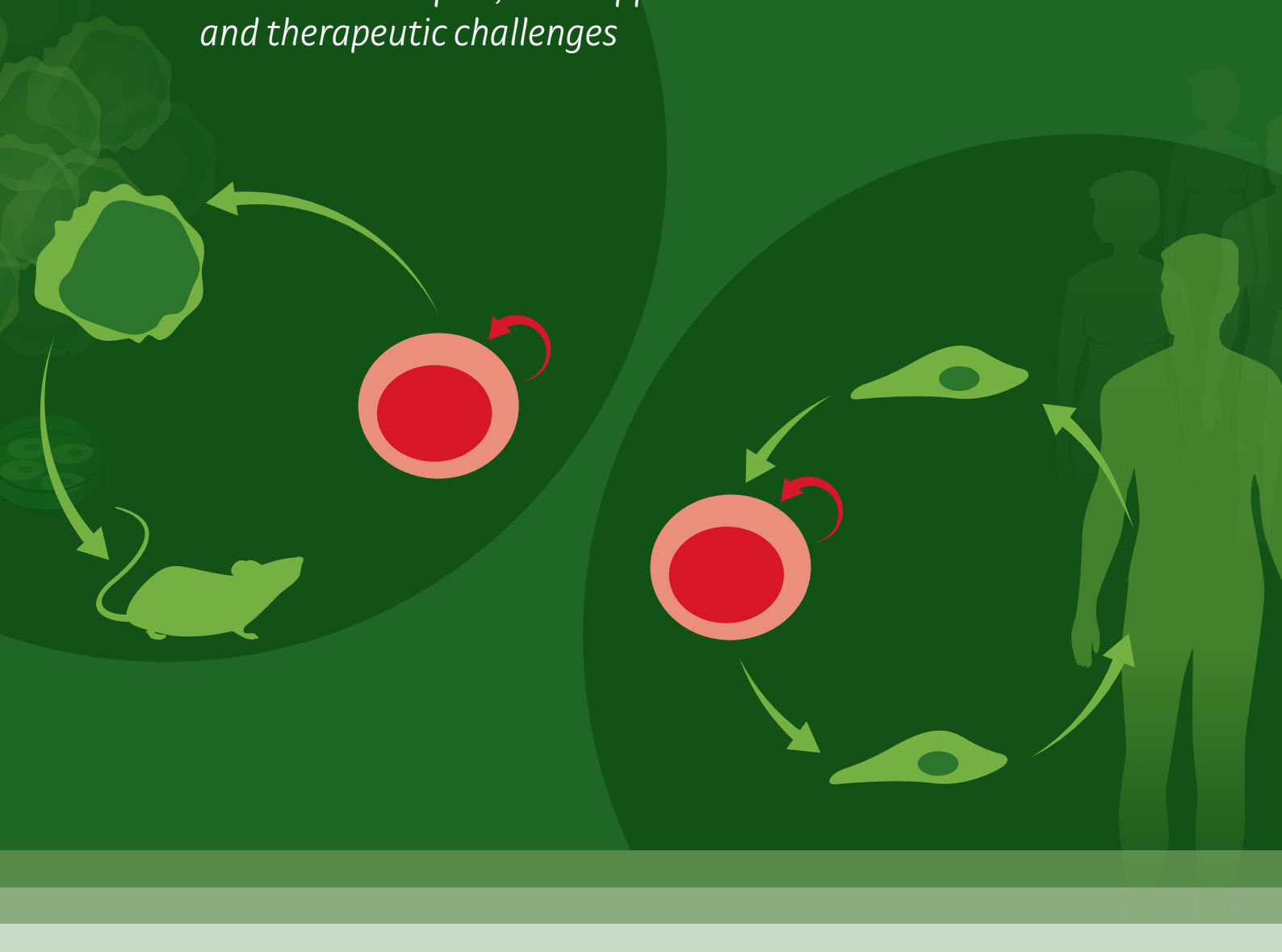
Focus

Induced Pluripotency & Cellular Reprogramming

Current techniques, basic applications and therapeutic challenges



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Induced Pluripotency & Cellular Reprogramming

Current techniques, basic applications and therapeutic challenges

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THE EMBO JOURNAL

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EDITORIAL NOTE

The technologies of induced pluripotency and cellular reprogramming have transformed basic research and sparked hope for therapeutic applications. With this collection of nine reviews by leaders in the field, we aim both to distill the rapid progress made in many laboratories during the nearly ten years of iPSC-based research, and to provide a comprehensive update about state of the art research across the field. This series of reviews focuses on clinically relevant tissues, organs and systems. We present four of them here, and the others are available online at: http://emboj.embopress.org/2015StemCellFocusIssue

We are indebted to the authors and their laboratories who volunteered their expertise, time, energy and resources.

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UPCOMING MEETINGS

7 October, 2015 **CSHL Stem Cell Biology, Cold Spring Harbor, USA**
Céline Carret (EMBO Molecular Medicine)

18 October, 2015 **EMBO Workshop on Stem cell mechanobiology in development and disease, Capri, Italy**
Céline Carret (EMBO Molecular Medicine)

19 October, 2015 **CSHA/ISSCR Joint Meeting – Stem Cells: from Basic Biology to Disease Therapy, Suzhou, China**
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Review

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Focus: Induced Pluripotency & Cellular Reprogramming

Transcription factor-mediated reprogramming toward hematopoietic stem cells

Wataru Ebina[1,2] & Derrick J Rossi[1,2,3,4,*]

Abstract

De novo **generation of human hematopoietic stem cells (HSCs) from renewable cell types has been a long sought-after but elusive goal in regenerative medicine. Paralleling efforts to guide pluripotent stem cell differentiation by manipulating developmental cues, substantial progress has been made recently toward HSC generation via combinatorial transcription factor (TF)-mediated fate conversion, a paradigm established by Yamanaka's induction of pluripotency in somatic cells by mere four TFs. This review will integrate the recently reported strategies to directly convert a variety of starting cell types toward HSCs in the context of hematopoietic transcriptional regulation and discuss how these findings could be further developed toward the ultimate generation of therapeutic human HSCs.**

Keywords cell fate conversion; hematopoietic stem cells; induced reprogramming; transcription factors
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Introduction

The process by which differentiated cell types arise from more primitive stem and progenitor cells generally proceeds down a strict lineal hierarchy defined by progressive functional specialization concomitant with restriction of lineage potential. From embryogenesis initiated by a single totipotent zygote to the lifelong homeostasis of organ parenchyma by tissue-specific stem cells, physiological differentiation of progenitor cells largely proceeds both unidirectionally and irreversibly, with differentiated cell types and even intermediate progenitors being remarkably fixed with respect to their cellular identity and functional potential. This paradigm, however, was challenged by the seminal works of Gurdon and others that demonstrated the sufficiency of factors present in oocyte cytoplasm to reverse differentiation of somatic nuclei and allow cloning of whole animals (Briggs & King, 1952; Gurdon, 1962; Wilmut *et al*, 1997). Subsequent efforts to identify the trans-acting factors capable of altering cell fate revealed the central role of transcription factors (TFs) in determining cellular identity. This was first demonstrated by the ability of a single TF MyoD to imbue myogenic identity on fibroblasts (Davis *et al*, 1987), which then established the foundation for the landmark discovery by Yamanaka that mere four TFs are sufficient to induce pluripotency in somatic cells (Takahashi & Yamanaka, 2006; Takahashi *et al*, 2007). These and other studies galvanized numerous investigators to harness the power of TFs in directly respecifying multiple cell fates: hepatocytes (Huang *et al*, 2011; Sekiya & Suzuki, 2011) cardiomyocytes (Ieda *et al*, 2010), cardiac pacemaker cells (Kapoor *et al*, 2013), oligodendrocytes (Najm *et al*, 2013; Yang *et al*, 2013), various types of neurons (Vierbuchen *et al*, 2010; Son *et al*, 2011; Liu *et al*, 2013), neural stem cells (Han *et al*, 2012), pancreatic beta cells (Zhou *et al*, 2008), sertoli cells (Buganim *et al*, 2012), thymic epithelium (Bredenkamp *et al*, 2014), endothelial cells (Han *et al*, 2014), and intestinal progenitors (Morris *et al*, 2014).

Studies of the hematopoietic system have borne profound insights into the transcriptional regulation of cellular identity. The ability to prospectively isolate hematopoietic stem, progenitor, and effector cells with defined lineage potentials has allowed dissection of molecular mechanisms underlying blood differentiation as well as identification of characteristic TFs governing diverse hematopoietic lineages (Orkin, 1995). However, the wave of breakthroughs from cell fate conversion studies transformed the hematopoietic system from a subject of scrutiny to a destination to be reached from alternative cell types. Initial studies focused on manipulating lineage potential by means of oncogenic transformation (Beug *et al*, 1979; Graf *et al*, 1992), lineage switching of hematopoietic progenitors (Heyworth *et al*, 2002), and direct cell fate conversion toward non-progenitor blood cells such as macrophages (Xie *et al*, 2004; Laiosa *et al*, 2006; Feng *et al*, 2008); however, substantial progress has been made recently toward *de novo* generation of hematopoietic stem cells (HSCs) (Szabo *et al*, 2010; Doulatov *et al*, 2013; Pereira *et al*, 2013; Batta *et al*, 2014; Pulecio *et al*, 2014; Riddell *et al*, 2014; Sandler *et al*, 2014). HSCs reside at the apex of the hematopoietic hierarchy and serve as the lifelong reservoir for all downstream blood cells. Their remarkable regenerative capacity to durably restore the entire hematopoietic system in transplant recipients has been harnessed as the standard of care for treatment of a number of

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morbid conditions including hematological malignancies, bone marrow failure syndromes, and immunodeficiency syndromes with ~50,000 allogeneic or autologous transplants performed each year (Gratwohl *et al*, 2010). Many factors contribute to transplantation outcomes including relapse of primary disease, graft failure, and opportunistic infection. Moreover, in addition to the challenge of identifying histocompatible donors, allogeneic transplantation is further complicated by graft-versus-host disease (GVHD), which remains a significant cause of morbidity and mortality for a large number of patients who undergo allogeneic transplantation despite the use of prophylactic immunosuppressants (Petersdorf, 2013). Another major factor contributing to transplant success is the number of stem cells transplanted, with an increased number of CD34+ HSPCs being the strongest predictor of transplantation success as measured by rapid and durable hematopoietic recovery (Siena *et al*, 2000). Transplants that contain too few HSCs either fail to engraft altogether or result in delayed blood reconstitution post-transplantation that is associated increased morbidity and mortality. This is particularly clinically challenging for the 10–30% of patients from whom sufficient numbers of autologous stem cells cannot be harvested due to poor responsiveness to mobilizing agents such as GCSF (Bakanay & Demirer, 2012). Therefore, an ability to produce an inexhaustible supply of autologous HSCs from relatively dispensable and potentially expandable cell types via cell fate conversion represents an attractive solution to these challenges. This review will discuss the critical roles played by TFs in hematopoietic cell fate regulation and how this knowledge has propelled efforts to convert alternative cell types toward fully functional HSCs.

Transcriptional regulation of cellular identity

TFs dictate the specific gene expression pattern necessary for a cell to perform its unique functions. Mechanistically, TFs directly impact chromatin state by recruiting epigenetic modifiers to specific DNA sequence motifs present in gene regulatory regions such as promoters and enhancers (Rosenfeld *et al*, 2006). Depending on whether a TF recruits transcriptional coactivators or corepressors, it may either promote or suppress gene expression, respectively. Consequently, a TF may contribute to enforcing a particular cell fate by simultaneously activating genes required for maintaining the function and identity of that cell while antagonizing lineage inappropriate genes (Cantor *et al*, 2008; Pongubala *et al*, 2008; Schaffer *et al*, 2010; Qi *et al*, 2013). In addition to recruiting cofactors, TFs often bind cooperatively to DNA as components of multiprotein complexes (Huang *et al*, 2009; Ravasi *et al*, 2010; Kazemian *et al*, 2013). Thus, the same TF may exhibit completely different genome-wide binding patterns and regulate non-overlapping sets of target genes in different cell types (Hoffman *et al*, 2010; Pimkin *et al*, 2014). Indeed, cell fate and function is invariably the result of the combinatorial action of TF complexes that form interdependent nodes that comprise larger regulatory networks. The wiring of cell-type-specific TFs as self-reinforcing circuits allows robust, stable sustenance of specific transcriptional landscapes (Rao *et al*, 2002; Chew *et al*, 2005; Bonzanni *et al*, 2013). On the other hand, antagonistic relationships between TF sets governing alternate cell fates serve as barriers to cellular plasticity and thus provide a basis for their mutual exclusivity (Graf & Enver, 2009).

TFs exert differential spheres of influence over cell fate depending on their connectivity within gene regulatory networks. Some TFs function as critical hubs, and their loss may lead to the collapse of network integrity (Albert *et al*, 2000). The potency of TFs may also derive from their ability to act as pioneering factors that can directly trigger nucleosomal remodeling to grant chromatin access to additional TFs (Zaret & Carroll, 2011). Some of these factors can be so potent that they can single handedly convert cell fate as is the case for MyoD in establishing the myogenic transcriptome, Cebpa in activating the myeloid program (Xie *et al*, 2004), and Runx1 in coordinating the endothelial-to-hematopoietic transition during development (Feng *et al*, 2008). More often, however, multiple TFs must act in concert to access and activate cell-type-specific gene regulatory networks (Wilson *et al*, 2010a).

Reprogramming and cell fate conversion take advantage of the connectedness and interdependencies of TFs in orchestrating cell fate programs (Buganim *et al*, 2013). Ectopic expression of a subset of TFs enriched in a destination cell type can be sufficient to actuate the destination gene regulatory network in an alternate starting cell type. The destination cell gene regulatory network may then predominate over that of the starting cell type, thus altering its identity. This process depends on extensive chromatin reconfiguration including reversal of chromatin inaccessibility, installation of destination cell specific enhancers, and shutdown of regulatory elements specific to the starting cell type, the carryover of which could lead to retention of what has been termed 'epigenetic memory' (Hu *et al*, 2010; Kim *et al*, 2010; Apostolou & Hochedlinger, 2013; Vaskova *et al*, 2013).

The self-reinforcing nature of gene regulatory networks implies that a small number of TFs may be sufficient to trigger their establishment and maintenance of destination cell regulatory networks. Moreover, recent evidence has demonstrated that regulatory networks governing cell identity could potentially be seeded by multiple distinct combinations of TFs. Indeed, extensive studies of TFs regulating embryonic stem cells have yielded multiple TF combinations capable of iPS cell generation that are different and even completely distinct from that originally reported by Yamanaka (Montserrat *et al*, 2013; Shu *et al*, 2013; Buganim *et al*, 2014; Takashima *et al*, 2014).

Transcriptional regulation of hematopoietic cell fates

The hematopoietic system has been studied extensively as a model tissue hierarchy for dissecting transcriptional regulation of cellular identity and cell fate transitions. As cells descend from HSCs, they are subjected to tiers of decisions that successively commit them to their final effector function. The molecular events underlying developmental cell fate decisions have been attributed in part to cross-antagonism between lineage-specific TFs, perhaps best illustrated by the activities of Gata1 and Pu.1 in promoting erythroid and myeloid differentiation programs, respectively (Arinobu *et al*, 2007). Physical interaction between Gata1 and Pu.1 leads to mutual extinction of transcriptional activity (Nerlov *et al*, 2000; Zhang *et al*, 2000). While they are both expressed in multipotent progenitors, offset in their relative levels, modulated by parameters such as cell cycle length (Kueh *et al*, 2013) and instructive cytokine signaling (Sarrazin *et al*, 2009), allows the higher expressed factor to

dominate cell fate decisions and hence tip the balance toward the respective lineage (Huang *et al*, 2007). Multiple such mutually antagonistic interactions could be assembled to form a greater landscape of attractors and transitional states that models multilineage differentiation (Krumsiek *et al*, 2011).

Pax5 is another TF whose role in hematopoietic lineage commitment has been studied extensively (Urbanek *et al*, 1994). Pax5 is essential in B-cell commitment, the lack of which arrests differentiation at the early pro-B stage (Nutt *et al*, 1999). Mechanistically, Pax5 locks progenitors into B-cell fate by both activating B cell promoting signaling pathways while silencing those important for the development of alternative lineages. Intriguingly, Pax5 null pro-B cells retain not only self-renewal potential but also multilineage differentiation potential spanning myeloid lineages and T cells (Mikkola *et al*, 2002) though Pax5-deficient progenitors do not give rise to B cells (Urbanek *et al*, 1994) and have very limited potential to give rise to erythrocytes or platelets (Nutt *et al*, 1999; Schaniel *et al*, 2002). The importance of Pax5 for limiting self-renewal and locking in B-cell fate is further illustrated by experiments in which deletion in CD19-positive B cells results in an aggressive and highly penetrant lymphoma *in vivo* (Cobaleda *et al*, 2007).

In addition to regulating developmental lineage commitment, TFs may impact subtype specification within hematopoietic lineages. For example, peripheral CD4 T-helper cells can develop into induced regulatory T cells (iTregs) with appropriate immunosuppressive functions upon induction of Foxp3, a TF critical for all regulatory T-cell development (Rudensky, 2011). However, inflammatory signals can extinguish Foxp3 expression in iTregs and convert them back to effector CD4 T-helper cells (Zhou *et al*, 2009). Furthermore, diverse subsets of macrophages are specified by the integration of Pu.1, a macrophage lineage determining TF, with transcriptional regulators downstream of tissue-specific environmental signals (Gosselin *et al*, 2014). These examples suggest that cellular identity can be a composite of multiple transcriptional modules each presided over by unique TFs or their combinations.

Artificial hematopoietic lineage conversions toward therapeutic application

Artificial manipulation of TFs has yielded important insights into the molecular underpinnings of lineage choice (Iwasaki *et al*, 2006). Overexpression together with the loss of function experiments has been used to confirm the nature of interaction between TFs and led to surprising discoveries on cellular plasticity. In addition to obtaining valuable mechanistic information, excitement in cell fate conversion research has been fueled by its enormous clinical potential. Cell fate conversion has the potential to generate patient-specific cells that are rare, inaccessible, or clinically useful from relatively dispensable autologous cells. If realized, such procedures could be applied to supplying cells for human disease modeling, therapeutic screening, and cell replacement therapy (Robinton & Daley, 2012; Cherry & Daley, 2013; Kamao *et al*, 2014; Nakamura *et al*, 2014; Stewart, 2014; Wainger *et al*, 2014). Thus, this strategy represents an attractive means to address both patient specificity and overcoming the rarity, and lack of means for expansion that currently limits the therapeutic use of HSCs.

A parallel strategy toward generating HSCs has been via stepwise differentiation of pluripotent stem cells such as embryonic stem cells or induced pluripotent stem cells (Sturgeon *et al*, 2013). However, attempts to direct differentiation of pluripotent stem cells toward HSCs by recapitulating the embryonic developmental trajectory *in vitro* has seen limited success. Simulating the temporal (Tober *et al*, 2013), spatial (Peeters *et al*, 2009; Wilkinson *et al*, 2009), mechanical (North *et al*, 2009), and cellular (Clements *et al*, 2011; Espin-Palazon *et al*, 2014) complexity of the embryonic milieu has proved technically challenging. Moreover, since the precise developmental intermediates en route to HSCs are only recently becoming elucidated (Rybtsov *et al*, 2011, 2014), the directed differentiation approach has thus far suffered from paucity of reliable developmental guideposts. For example, induction of T lymphoid potential has been used to guide directed differentiation as it correlates with definitive hematopoiesis, a temporal wave of embryonic hematopoiesis during which HSCs are specified (Kennedy *et al*, 2012). However, it is still unknown whether the *in vitro* derivatives with T lymphoid potential indeed possess the eventual capacity to produce HSCs or whether they might represent a developmental intermediate similar to embryonic T-cell progenitors that arise independently of HSCs (Yoshimoto *et al*, 2012). Due to such state of the field, little is known about the functional and molecular correlation between the developmental intermediates obtained *in vitro* and *in vivo*, and the knowledge is particularly lacking in the context of human HSC ontogeny (Ivanovs *et al*, 2014). Furthermore, the necessity to transit through multiple distinct intermediate cell types to reach HSCs means that culture conditions may need to be optimized for every intermediate and that deviation in any one step may extinguish the eventual potential to develop HSCs. Therefore, in spite of 'forceful' nature of TF manipulations, TF-mediated cell fate conversion may be a relatively simpler, direct, and even more tractable of a strategy for deriving HSCs as it only requires knowledge of the properties of the destination cell type.

Generation of hematopoietic stem cells via direct cell fate conversion

HSCs are functionally defined as cells capable of engrafting conditioned recipients and giving rise to all blood lineages (i.e., myeloid, thrombo-erythroid, and lymphoid), for an extended period (at least 4 months in mice). These functional hallmarks of HSCs, namely multilineage differentiation potential and extensive self-renewal capacity, are embodied at the clonal level such that only few HSCs are required to durably sustain the entire hematopoietic system (Holstege *et al*, 2014). Although fully functional human HSCs meeting the aforementioned criteria have yet to be produced *in vitro*, several strategies have recently been described that bring the goal of deriving fully function HSCs from alternative cell types within reach (Table 1).

Szabo *et al* (2010) and Pulecio *et al* (2014) converted human fibroblasts to hematopoietic cells possessing multilineage myeloid potential aided by pluripotency-associated TFs, namely OCT4 and SOX2, respectively. The latter study also showed improved hematopoietic conversion with the addition of mir125b, a microRNA enriched in human hematopoietic progenitors. Since transient expression of pluripotency factors or OCT4 is sufficient to confer

Table 1. Summary of studies using transcription factor-mediated reprogramming to derive primitive blood progenitors.

	Szabo *et al* (2010)	**Pulecio *et al* (2014)**	**Pereira *et al* (2013)**	**Batta *et al* (2014)**	**Doulatov *et al* (2013)**	**Riddell *et al* (2014)**	**Sandler *et al* (2014)**
Species	Human	Human	Mouse	Mouse	Human	Mouse	Human
Starting cell type	Fibroblast	Fibroblast	Fibroblast	Fibroblast	ES cell-derived myeloid restricted progenitor	B-cell progenitor, myeloid progenitor, bone marrow myeloid effector	Umbilical vein endothelial cells, microvascular endothelial cells
Transcription factors	OCT4	SOX2, mir125b	Gata2, Gfi1b, cFos, Etv6	Erg, Gata2, Runx1c, Scl, Lmo2	ERG, HOXA9, RORA, SOX4, MYB	Runx1t1, Hlf, Lmo2, Pbx1, Zfp37, Prdm5, Mycn, Meis1	FOSB, GFI1, RUNX1, SPI1
Factor inducibility	Constitutive	Constitutive	Inducible	Constitutive	Inducible	Inducible	Constitutive
Medium	*In vitro*	*In vitro*	*In vitro*	*In vitro*	*In vitro*	*In vivo*	*In vitro* with endothelial stroma
In vitro colony formation	+	+	+	+	+	+	+
Erythroid	+	+	Not shown	+	+	+	+
Myeloid	+	+	+	+	+	+	+
B	–	–	–	–[a]	–	+	+
T	–	–	–	–[a]	+[b]	+	+[c]
Engraftment	+[d]	+[d]	–	+[e]	+	+	+
Serial Transplantation	–	–	–	–	–	+	+
HSC?	No	No	No	No	No	Yes	No[f]

[a]Lymphoid differentiation potential acquired with p53 deletion.
[b]Although modest T-cell differentiation potential was confirmed *in vitro*, no T cells were detected *in vivo*.
[c]Minimal *in vitro* T-cell differentiation possible when TFs are expressed using inducible system.
[d]Engrafted cells express low levels of CD45, a pan-lympho-myeloid hematopoietic marker.
[e]Very short-term (2 week), primarily erythroid engraftment.
[f]*In vivo* function not assayed with cells derived using inducible system.

tri-germ layer differentiation potential on fibroblasts, fate conversion specifically to the blood lineage with OCT4 or SOX2 was likely mediated by the inductive effects of hematopoietic cytokines (Mitchell *et al*, 2014), which has previously been shown to be able to reprogram blood cell identity (Kondo *et al*, 2000). Although the resulting cells were able to engraft *in vivo*, the majority of them expressed low levels of the pan-hematopoietic antigen, CD45, and did not peripheralize, possibly suggesting incomplete hematopoietic conversion.

Instead of transitioning through a developmentally plastic state, a more direct, autonomous fate transition could be achieved by overexpressing lineage-specifying TFs enriched in destination cells. Toward this, Pereira *et al* (2013) screened 18 candidate TFs enriched in quiescent mouse HSCs that could activate exogenous human CD34 promoter inserted into mouse fibroblasts. The screen identified transient expression of Gata2, Gfi1b, cFos, and Etv6 to be sufficient for generating hematopoietic cells from fibroblasts via an intermediate cell type that coexpressed both endothelial and hematopoietic markers. Although the converted hematopoietic cells were similar to mouse hematopoietic stem/progenitor cells with respect to gene expression, they were devoid of *in vitro* clonogenic potential unless cocultured with placental stroma, suggesting that maturation into progenitor-like blood cells required additional signals. Clonal multilineage potential or *in vivo* functionality was not assayed. A similar fate conversion strategy from fibroblasts was employed by Batta *et al* (2014) who screened a curated set of 19 hematopoietic TFs for morphological change of murine fibroblasts to round hematopoietic cells. Five TFs, Erg, Gata2, Lmo2, Runx1c, and Scl, were found to robustly induce hematopoietic colonies from both embryonic and adult fibroblasts, and the reprogrammed cells were shown to possess erythroid, megakaryocytic, granulocytic, and macrophage differentiation potentials. Similar to Pereira *et al*, Batta *et al* also observed that fibroblasts converted to hematopoietic cells via an endothelial intermediate. *In vitro* clonogenic assays confirmed the presence of cells possessing multilineage potential; upon transplantation, however, these cells only gave rise to very short-term (2 weeks) erythroid chimerism. Interestingly, p53 nullizygosity not only enhanced the efficiency of reprogramming but also increased erythroid differentiation potential in addition to permitting production of receptor rearranged B and T lineage cells.

Although iPS cells have the developmental potential to be differentiated toward potentially transplantable autologous tissues, their hematopoietic differentiation has yielded progenitors with greatly restricted self-renewal and differentiation potentials quite unlike those of true HSCs. Doulatov *et al* (2013) sought to respecify iPS cell-derived myeloid restricted progenitors toward HSCs using TFs enriched in both human and mouse HSCs that appeared underexpressed in the blood progenitors cells derived from pluripotent cells.

Screening nine candidate TFs and using serial plating as a readout, ectopic expressions of ERG, HOXA9, and RORA were found to instill robust *in vitro* clonogenic potential but not multilineage potential or engraftment capacity. However, additional ectopic expression of SOX4 and MYB enabled the acquisition of myelo-erythroid differentiation potential as well as short-term myeloid engraftment capacity in immunocompromised mice. Although modest T lineage potential was confirmed *in vitro*, the grafts failed to produce lymphoid lineage *in vivo*. Long-term engraftment was not achieved.

As opposed to respecifying embryonic-like hematopoietic cells derived from pluripotent stem cells, Riddell *et al* (2014) undertook reprogramming of primary adult lineage committed murine hematopoietic progenitors and effectors using gene regulatory factors exhibiting restricted expression in mouse HSCs relative to the majority of their differentiated progeny. An unbiased screen of 36 factors, which included 33 TFs and three translational regulators, was performed in the transplantation setting to take advantage of the sensitivity of the assay in reading out HSC activity at the single-cell level, and potentially co-opt signals present in the *in vivo* environment that might facilitate cell conversion. The screen identified six genes *Hlf, Runx1t1, Pbx1, Lmo2, Prdm5*, and *Zfp37* whose transient ectopic expression was sufficient for instilling multilineage reconstituting potential on otherwise lineage committed hematopoietic cells. Inclusion of Meis1 and Mycn was found to improve reprogramming efficiency. Long-term multilineage reconstitution, serial transplantability, reconstitution of bone marrow progenitor compartments and secondary hematopoietic organs, and single-cell gene expression profiling confirmed that the reprogrammed cells possessed the functional and molecular properties of endogenous HSCs and thus were termed 'induced HSCs' (iHSCs).

HSCs and endothelial cells share an intimate ontological relationship as HSCs are specified from hemogenic endothelial-like intermediates during embryogenesis via endothelial-to-hematopoietic transition (EHT). Sandler *et al* hypothesized that EHT may be recapitulated in mature, non-hemogenic endothelial cells by ectopic expression of key TFs and provision of an inductive environment (Zovein *et al*, 2008; Bertrand *et al*, 2010; Boisset *et al*, 2010; Gordon-Keylock & Medvinsky, 2011; Sandler *et al*, 2014). Screening 25 TFs expressed at a higher level in human cord blood HSPCs relative to human umbilical vein endothelial cells (HUVECs) identified the minimal set of FOSB, GFI1, RUNX1, and SPI1 to be sufficient and necessary for robustly generating hematopoietic colonies from HUVECs and human adult dermal microvascular endothelial cells. The reprogramming was found to strictly depend on an endothelial stroma previously developed by the authors' group for maintaining human cord blood HSPCs (Butler *et al*, 2012). The reprogrammed cells possessed both *in vitro* and *in vivo* multilineage differentiation potential, long-term reconstitution potential, *in vivo* homing/engraftment capacity, and serial transplantability, only with the caveat of defective T-cell differentiation potential, thus earning the label of multipotent progenitors (MPPs).

Despite sharing the same destination identity, the studies summarized above show striking diversity with respect to experimental design and the TF combinations identified (Fig 1). However, in totality, the reported TFs are highly enriched for both developmental genes involved in embryonic specification of hematopoiesis and those implicated in leukemogenesis. Classical developmental hematopoiesis genes such as *Scl, Gata2, Gfi1, Runx1, and Spi1 (Pu.1)* (Wilson *et al*, 2010b) appear to be important in fate conversions that involve lineage switching, such as from fibroblasts or endothelial cells, whereas they do not appear to be important in reprogramming or respecification within the blood lineage. This could reflect that even post-embryogenesis, the same small set of TFs that specified hematopoiesis earlier in life can pioneer the establishment of hematopoietic program in non-hematopoietic adult cell types, though such activity may be redundant in conversions within hematopoietic lineage. The requirement for TFs such as HOXA9, MYB, Lmo2, Pbx1, Mycn, and Meis1 in reprogramming of committed blood cells is consistent with their classical roles in tumor development (Thorsteinsdottir *et al*, 2001; Kawagoe *et al*, 2007; Jin *et al*, 2010; McCormack *et al*, 2010). In particular, components of Hox effectors, namely *HOXA9*, *Pbx1*, and *Meis1*, are found in TF sets reported by both Doulatov *et al* and Riddell *et al*. This particular set of TFs has been shown to function as a heterotrimeric complex (Shen *et al*, 1997) and is frequently represented in regenerative processes (Mercader *et al*, 2005; Capellini *et al*, 2006; Chen *et al*, 2013; Roensch *et al*, 2013) and across multiple types of cancers (Morgan *et al*, 2007; Shears *et al*, 2008; Plowright *et al*, 2009; Sun *et al*, 2013), suggesting that it may regulate generic properties of stem/progenitors such as self-renewal, anti-apoptosis, and differentiation arrest. In spite of the apparently central role played by the Hox complex in regulating stemness, it is intriguing that ectopic stimulation of this pathway was not required to generate progenitor cells as highly functional as the MPPs directly from endothelial cells (Sandler *et al*, 2014). However, this could be explained by the fact that endothelial cells exhibit intrinsic HoxA cluster activity, which is integral to vascular development and function (Rössig *et al*, 2005; Bandyopadhyay *et al*, 2012).

Species-specific differences may also contribute to the identification of distinct HSPC–inducing TF combinations. It is possible, though not rigorously studied, that divergent gene regulatory networks may govern mouse and human HSCs. For example, although constitutive ectopic expression of Hoxb4 allows the generation of engraftable mouse HSPCs from mouse embryonic stem cells (Wang *et al*, 2005b; Matsumoto *et al*, 2009), the same robust effect was not seen using human cells (Wang *et al*, 2005a). In the erythroid lineage, noticeable transcriptional divergence was found between mouse and human cells isolated from comparative stages of differentiation (Pishesha *et al*, 2014). Furthermore, overexpression of a dominant-negative isoform of IKAROS (IKZF1) was recently found to impart diametric effects on mouse and human HSPCs (Beer *et al*, 2014). Whereas IKZF1 overexpression in mouse HSPCs suppressed B but enhanced T lineage outputs, the same manipulation in human cord blood HSPCs significantly increased B lineage cell production without affecting T lineage. Therefore, although the majority of TFs discovered in the studies using murine cells (Pereira *et al*, 2013; Riddell *et al*, 2014) are highly homologous between mouse and human, it is uncertain whether the same set of factors capable of inducing mouse HSPCs would also be sufficient for inducing human HSCs.

Another major variable among the studies is the system used for ectopic expression of TFs. An important criterion for complete cell fate conversion is transgene-independent sustenance of destination cell gene regulatory networks. Although transduction with viruses encoding TFs under constitutive promoters may become passively silenced over time, the use of an inducible transgene

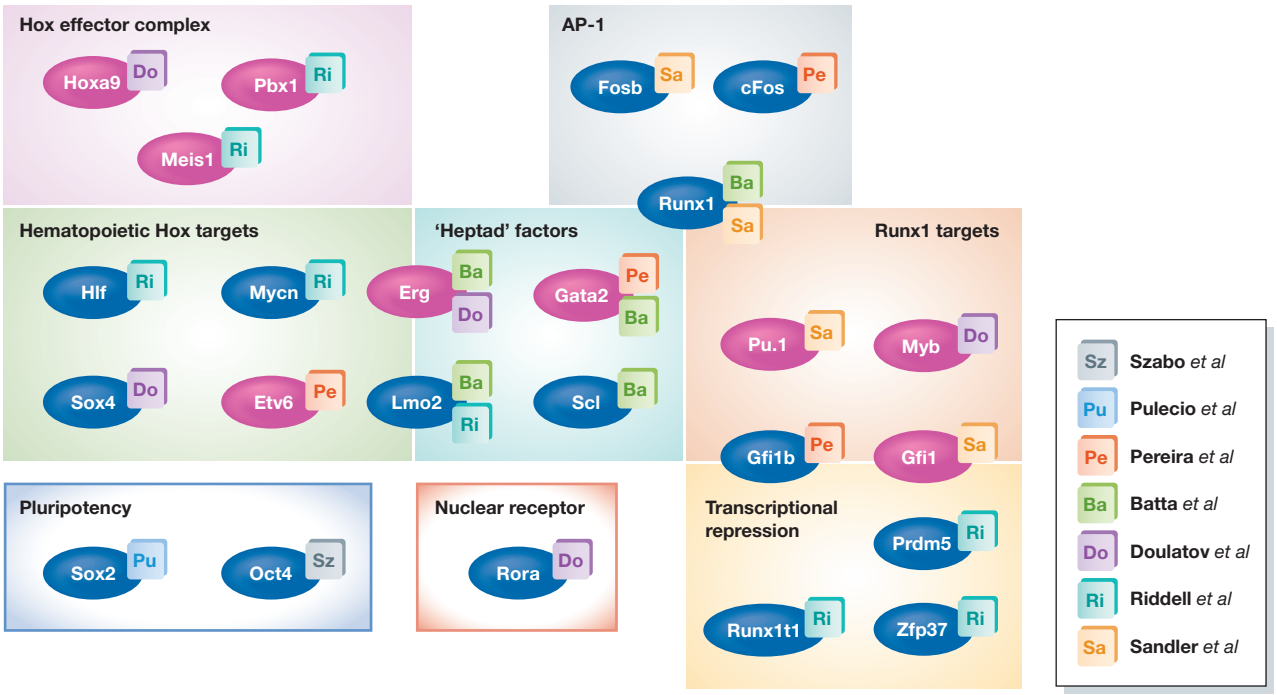

Figure 1. Categorization of transcription factors employed toward *de novo* HSC generation.
TFs employed by Szabo *et al* (Sz), Pulecio *et al* (Pu), Pereira *et al* (Pe), Batta *et al* (Ba), Doulatov *et al* (Do), Riddell *et al* (Ri), and Sandler *et al* (Sa) are classified into categories based on previously described activities. 'Heptad' factors refer to classical hematopoietic TFs known for their physical interactions (Wilson *et al*, 2010a). Individual loss of function of TFs highlighted in pink has been shown to impair HSC activity.

system (i.e., doxycycline inducible) can give better temporal control over TF expression as well as ensure that the ectopic genes are turned off upon completion of cell fate conversion. Importantly, continued ectopic expression of TFs involved in 'respecification/reprogramming' may also interfere with the function of the destination cells. For example, continued ectopic expression of SPI1 in endothelial cell-derived multipotent progenitors (MPPs) was shown to block T-cell differentiation potential (Sandler *et al*, 2014). In the same study, as only MPPs obtained using constitutive expression vectors were assayed *in vivo*, it remains to be shown definitively whether the MPPs can stably maintain their identity independent of residual transgene expression.

Since environmental responsiveness and developmental plasticity represent cardinal properties of HSCs, it is conceivable that environmental cues may strongly influence cell fate conversion toward HSCs. Reasoning that reprogramming in the context of the native HSC niche (Morrison & Scadden, 2014) may facilitate the acquisition of HSC identity, Riddell *et al* (2014) conducted reprogramming experiments *in vivo*. Similarly, Sandler *et al* took advantage of an endothelial stromal coculture system developed for *ex vivo* maintenance of human HSCs to provide an inductive environment for *de novo* HSC generation (Butler *et al*, 2012; Sandler *et al*, 2014). Intriguingly, both of these studies gave rise to serially transplantable cells possessing lympho-myeloid multilineage potential, a feat unattained by any of the other cell fate conversion strategies, which were all conducted in the absence of HSC supportive milieu. Although Riddell *et al* did not show the absolute requirement of the *in vivo* environment for HSC induction, an HSC supportive environment was shown to be a necessity in the conversion of endothelial cells to MPPs by Sandler *et al* Insights from other cell fate conversion systems also point to the importance of the environment. In particular, STAT3, a transcription factor directly activated by growth factor signaling, has been shown to play an central role in embryonic stem cells as well as in induction of pluripotency (Niwa *et al*, 1998; Raz *et al*, 1999; Yang *et al*, 2010; van Oosten *et al*, 2012), thus emphasizing that cell extrinsic cues can be as important as the intrinsic ones.

Toward understanding the transcriptional regulation of HSC identity

In spite of enormous progress, the era of HSC induction has only dawned. With little consensus on the optimal combination of TFs and/or environmental cues for inducing HSCs, it may be necessary to perform comparative studies or even 'mix and match' findings from hitherto studies for even better results. With respect to reevaluating TF combinations for human HSC induction, it may be worth noting the heterogeneity of primary human HSCs. Although immunophenotypically defined single human HSC has been successfully isolated (Notta *et al*, 2011), a number of studies show that the human HSC compartment can still be fractionated into subpopulations with distinct functional potentials (Anjos-Afonso *et al*, 2013; Chitteti *et al*, 2014) similar to the HSC subfractionation that has been demonstrated in the mouse (Dykstra *et al*, 2007; Beerman *et al*, 2010; Morita *et al*, 2010; Babovic & Eaves, 2014). Therefore,

TFs enriched in each of these subpopulations may differentially impact HSC induction.

Understanding the function of individual TFs in HSCs constitutes an important step toward elucidating the mechanism of HSC induction. Consistent with the ability to access the HSC gene regulatory program, many of the TFs identified by the studies highlighted in this review have been previously implicated in HSC and progenitor biology. Individual loss of function of *Hoxa9* (Lawrence *et al*, 2005), *Gata2* (Lim *et al*, 2012), *Pbx1* (Ficara *et al*, 2008), *Meis1* (Unnisa *et al*, 2012), *Gfi1* (Hock *et al*, 2004; Zeng *et al*, 2004), *Etv6* (Wang *et al*, 1998), *SPI1* (Pu.1) (Staber *et al*, 2013), *Myb* (Lieu & Reddy, 2009), or *Erg* (Ng *et al*, 2011) in HSCs has each been shown to compromise their homeostatic maintenance and/or competitive repopulation potential. Although direct loss of function of *Lmo2* in HSCs has not been conducted, its critical role in hematopoiesis is underscored by the inability of *Lmo2* null ES cells to contribute to postnatal hematopoiesis in chimeric blastocysts (Yamada *et al*, 1998). Similarly, *Runx1* null ES cells cannot contribute to adult hematopoiesis (Okuda *et al*, 1996) due to impairment of EHT (Chen *et al*, 2009). Intriguingly, however, conditional loss of *Runx1* in the adult hematopoietic system only impairs megakaryocytic and lymphoid differentiation with minimal impact on HSC activity (Ichikawa *et al*, 2004; Cai *et al*, 2011). Although the consequence of loss of function of *Sox4, Rora, Runx1t1, Zfp37, Prdm5*, or *Hlf* in HSCs has yet to be reported, overexpression and leukemia studies have implicated some of these factors in HSC function. For example, *Sox4* has been implicated in self-renewal of leukemic cells (Zhang *et al*, 2013) and ectopic expression of *Hlf*, a leukemia-associated TF (Hunger *et al*, 1992), has been shown to enhance repopulation potential of human HSCs (Shojaei *et al*, 2005) and allows maintenance of *in vitro* multilineage differentiation potential and serial plating capacity of murine HSCs and progenitors (Gazit *et al*, 2013). Also of note, many of these understudied factors, namely *Runx1t1* (Lindberg *et al*, 2003), *Zfp37* (Dreyer *et al*, 1998), and *Prdm5* (Duan *et al*, 2007), identified by Riddell *et al* (Riddell *et al*, 2014) function as transcriptional repressors, suggesting that active repression of differentiation-associated genes may be an important component of reprogramming differentiated blood cells back to HSCs. Finally, loss of function of some TFs may not result in HSC defects due to functional redundancies with other TFs. For example, *Mycn* deletion in HSCs is compensated by the presence of *c-Myc* (Laurenti *et al*, 2008). However, combined deletion of *Mycn* and *c-Myc* demonstrated the requirement of the Myc genes in the exit from stem cell state as well as preventing apoptosis of HSCs. Similarly, *Scl* nullizygosity has no effect on HSCs due to its functional redundancy with *Lyl1*, but their combined ablation leads to loss of HSCs via apoptosis (Souroullas *et al*, 2009).

Detailing the interactions between the TFs used in HSC induction may provide deeper insights into the gene regulatory network governing HSC identity, which could lend to discoveries of more efficient or alternative TF combinations for inducing HSCs. Many of the reported TFs have already been shown to interact physically and/or at the transcriptional level. As mentioned previously, Hoxa9, Meis1, and Pbx1 directly interact to coregulate transcription (Shen *et al*, 1997), although they may also function independently as their individual deletions result in differential HSC phenotypes (Lawrence *et al*, 2005; Ficara *et al*, 2008; Unnisa *et al*, 2012). The Hox complex cannot only autoregulate Hox gene expression (Trivedi *et al*, 2008; Horman *et al*, 2009) but also enhance the expression of a number of other reprogramming TFs such as Erg, Myb, Sox4, Lmo2, Etv6, Mycn, and Hlf in blood cells (Palmqvist *et al*, 2007; Nagel *et al*, 2011; Huang *et al*, 2012) although there also appears to be redundancies among these targets such as the cross-regulation between Lmo2 and Erg (Oram *et al*, 2010) and induction of *Lmo2* by Hlf (de Boer *et al*, 2011). However, the diverse and wide-ranging targets of the Hox complex combined with the fact that it regulates distinct processes in non-hematopoietic tissues (i.e., body segmentation, blastema formation, and non-hematopoietic cancers, mentioned previously) suggests that the complex may require interaction with lineage-restricted TFs to exert tissue-specific functions. In support of this, Hoxa9/Meis1 has been shown to specifically bind and activate myeloid enhancers in cooperation with hematopoietic TFs such as SPI1(Pu.1), and Runx1 (Huang *et al*, 2012). This model implies that HSC specification by the Hox complex requires either *a priori* patterning of relevant enhancers or coexpression of hematopoietic-specific TFs that can pioneer enhancer establishment. Following this logic, under certain circumstances, overexpression of the Hox complex may impede cell fate conversion by promoting maintenance of enhancers specific to the starting cell type, akin to the antagonism of EHT by Hoxa3 during embryogenesis (Iacovino *et al*, 2011).

Extensive interactions have also been reported between TFs classically associated with hematopoiesis such as Scl, Runx1, Gata2, Gfi1, and SPI1(Pu.1) that appear in the reprogramming TF cocktails. For example, a heptad of TFs regulating HSPCs, SCL, LYL1, LMO2, GATA2, RUNX1, ERG, and FLI1 have been shown to directly bind each other and cooperatively regulate hematopoietic genes (Wilson *et al*, 2010a) as well as autoregulate their own expression (Grass *et al*, 2003; Pimanda *et al*, 2007; Diffner *et al*, 2013). While the majority of these TFs is implicated in both hematopoietic and endothelial development (De Val & Black, 2009), Runx1 has received much attention for its specific and critical role in embryonic HSC specification (North *et al*, 2002). During mid-gestation, Runx1 specifies HSCs by acting as a pioneering TF that initiates expression of hematopoietic genes such as SPI1(Pu.1) in an endothelial-like cell (Huang *et al*, 2008; Chen *et al*, 2009; Lichtinger *et al*, 2012; Tanaka *et al*, 2012). The activity of Runx1 is modulated not only by its obligate binding partner Cbfb and other members of the heptad TFs but also by the AP1 complex, whose motifs are highly enriched at genomic Runx1 binding peaks (Pencovich *et al*, 2011; Lie *et al*, 2014). AP1 complex has been shown to physically interact with Runx1 (Hess *et al*, 2001; D'Alonzo *et al*, 2002) and is itself a heterodimer of JUN and FOS family proteins, the latter of which were found to be necessary to induce hematopoiesis from non-hematopoietic cells in the studies by Pereira *et al* (2013) and Sandler *et al* (2014). The same studies also identified Gfi1/Gfi1b and SPI1 (Pu.1), direct targets of Runx1 that promote the loss of endothelial identity associated with EHT (Lancrin *et al*, 2012; Pereira *et al*, 2013; Sandler *et al*, 2014; Wilkinson *et al*, 2014). Interestingly, Gfi1 has been shown to directly repress Hoxa9, Pbx1, and Meis1 (Horman *et al*, 2009), which could support the aforementioned notion that suppression of Hox complex may be important to facilitate lineage switching toward blood. Ultimately, the kinetics and expression levels of TFs may need to be regulated for optimal HSC induction.

TFs mediate cell fate conversion by rewriting epigenetic information, which has been shown to be facilitated by directly modulating nuclear enzymes responsible for chromatin modifications. A number of small molecules that increase chromatin accessibility have been shown to enhance the efficiency of iPSC generation (Federation *et al*, 2014). One study even demonstrated that a G9a histone methyltransferase inhibitor was able to replace Oct4 in inducing pluripotency from mouse fetal neural progenitors (Shi *et al*, 2008). Although the mechanisms governing induction of pluripotency or HSCs may differ, barriers to accessing the HSC program may be similarly lowered by the use of such small molecules. Epigenetics has also been shown to underlie functional heterogeneity within the HSC compartment, especially with respect to aging-induced increase in self-renewal and myeloid lineage bias (Beerman *et al*, 2013; Sun *et al*, 2014a). Although reprogramming to pluripotency has been demonstrated to be sufficient in reversing epigenetic components of HSC aging (Wahlestedt *et al*, 2013), other studies have shown functional and molecular heterogeneity of iPSCs that originate from retention of epigenetic information associated with the starting cell type (Hu *et al*, 2010; Kim *et al*, 2010; Vaskova *et al*, 2013). Therefore, an intriguing question would be to determine whether the starting cell age is reset upon direct cell fate conversion to HSCs and whether HSCs induced from committed blood cell lineages exhibit differentiation bias toward the respective lineages. This latter point was not however evident in iHSCs generated in the Riddell *et al* (2014) study as iHSCs derived from either B-cell progenitors or myeloid progenitor or effector cells appeared comparable in their capacity to give rise to lymphoid and myeloid effector cells *in vivo*.

Complementary to overexpressing HSC-specific TFs, direct extinction of gene regulatory networks governing starting cell types may further augment cell fate transition. Since deletion of Pax5 has already been shown to be sufficient for liberation from B lineage fate (Nutt *et al*, 1999), such manipulation may enhance HSC induction from B-cell progenitors. The use of RUNX1 and GFI1 by Sandler *et al* in inducing hematopoiesis from endothelial cells indeed follows the logic of their developmental role in extinguishing the endothelial program (Iacovino *et al*, 2011; Lancrin *et al*, 2012).

Toward human HSCs

Given the rapid progress in the field, derivation of the first human HSCs via cell fate conversion appears increasingly attainable. The diverse strategies presented thus far provide lessons that may help refine the approach to generating human HSCs as well as gain deeper insights into the mechanism of HSC induction.

Though already mentioned earlier, the importance of using inducible expression vectors should be reemphasized. Inducibility is critical to ensure transient ectopic expression of TFs in order to demonstrate that resulting HSCs possess self-sustaining and stable identity. Another advantage of this system is the re-inducibility of the TFs, which can be exploited for secondary reprogramming experiments (Wernig *et al*, 2008; Riddell *et al*, 2014).

To accelerate the study of HSC induction, it would be critical to perform experiments in defined media. Although the endothelial stromal system used by Sandler *et al* appears to provide a powerful inductive milieu, it remains to be seen whether it is generally applicable. Nonetheless, it is also possible that a special environment may not be necessary for certain starting cell types and/or TF combinations. In either case, with an ability to perform the entirety of cell fate conversion *in vitro*, it should be possible to gain finer control over experimental parameters as well as to obtain kinetic information on the induction of HSC identity.

The crux of HSC induction is the functional evaluation of test cells. Rigorous assays should be employed to confirm the *in vivo* potential of induced HSCs to self-renew and generate multilineage progeny at the clonal level. Long-term reconstitution and serial transplantation are commonly employed to assess the *in vivo* self-renewal potential of HSCs. These assays also indirectly provide indications on the ability of test cells to properly home to and engraft in the niches. *In vivo* functionality of HSCs is contingent upon their successful engraftment. To arrive at bone marrow niches, HSCs must home to the correct vasculature, extravasate, and then migrate to gain contact with the niche components (Lapidot *et al*, 2005). The surface molecules and signal transduction components necessary for orchestrating this process are thus integral to HSC function. Although it is possible that this facet of HSC identity is within the domains governed by HSC-specific TFs, it could represent a generic functional module shared with other bone marrow resident cell types. In the latter scenario, orthogonally acting TFs or specific environmental signals may be required in addition to ectopic expression of HSC-enriched TFs to induce engraftable HSCs. For instance, it has been shown that expression of Cxcr4, an essential chemokine receptor for HSC migration, is regulated by Hif1a, suggesting that Cxcr4 induction may dependent on hypoxia rather than HSC-specific TFs (Speth *et al*, 2014).

The prerequisite for homing and engraftment complicates functional assessment of human HSCs in the setting of xenotransplantation experiments. To circumvent graft rejection, immunodeficient mice that lack B, T, and NK cells are used as hosts for human HSCs (Shultz *et al*, 2012). Even then, the engraftment potential of human HSC in murine hosts is inferior to that of mouse HSCs, likely due to cross-species differences between cytokines and signaling/homing receptors or even due to the incompatibility of human CD47, a 'don't eat me' signal, with host phagocytes (Jaiswal *et al*, 2009; Kwong *et al*, 2014). To obtain robust chimerism, human HSCs may need to be injected directly into the bone marrow cavity via intrafemoral injection, thus bypassing the complex orchestration of cellular maneuvers leading to engraftment. The caveat, however, is that lowering the hurdle to engraftment or differences in transplantation procedures may result in incongruous definitions for human HSCs. Although intrafemoral injection of induced HSCs could be justified if one were to claim the necessity of the *in vivo* environment in inducing proper homing/engraftment capacity, this needs to be confirmed with secondary transplantation via intravenous route of injection. The difference in the rigor of *in vivo* assays used to define HSCs as well as cross-species mismatches in signaling may also underlie molecular differences between mouse and human HSCs, and it is unknown whether experimentally derived cells tested in xenograft models will ultimately function effectively in human patients. An important step, therefore, is to develop better models for native engraftment of human HSCs, such as better humanized mice or reconstituted human bone marrow, so that their functional definition could be refined (Drake *et al*, 2012; Scotti *et al*, 2013; Cosgun *et al*, 2014; Torisawa *et al*, 2014).

The question of *in vivo* clonal multilineage differentiation potential, though challenging, can potentially be addressed using two molecular approaches. First, if HSCs can be induced from B or T lineage cells that have undergone receptor rearrangement, it would be possible to use the unique sequences of the recombined loci as a bar code to track the clonal progenitor cell origin of effector cell progeny as was done by Riddell *et al* (2014). Receptor rearrangement also provides direct evidence for the cell of origin of the reprogrammed cells, an important consideration given that even a single contaminating HSC inadvertently introduced during transplantation experiments has the potential to confound interpretations. The second approach is viral integration analysis that takes advantage of viral insertion sites that can serve as cellular barcodes. Finally, single-cell transplantation experiments can be used to assure clonal multilineage potential *in vivo*—though this is a very high bar to surmount, especially if cell fate conversion efficacy is low.

Toward clinical translation

Despite the progress, a number of challenges must be met before *de novo* generated human HSCs can reach clinical translation (Fig 2). Despite the diversity of TF combinations hitherto used for hematopoietic induction, a commonality among most of them is their proto-oncogenicity. Although no tumors have thus far been reported by hematopoietic cell fate conversion studies, the potential dangers of these potent TFs combined with the risks of insertional mutagenesis by lentiviral vectors cannot be tolerated for use in generating clinical-grade human HSCs. Therefore, the current lentiviral methods for HSC induction may be most applicable for such uses as disease modeling.

Given the risks associated with lentiviral transduction, non-integrating approaches will need to be considered toward clinical translation. A number of such methods developed for generating integration-free iPSCs including protein transduction, non-integrating viruses, and mRNA based transient protein expression systems could potentially be applied to HSC induction (Fusaki *et al*, 2009; Warren *et al*, 2010; Zhang *et al*, 2012; Mandal & Rossi, 2013; Yoshioka *et al*, 2013; Elcheva *et al*, 2014). However, as the non-integrating approaches are highly variable with respect to the duration of ectopic factor expression, the kinetics of respecification/reprogramming will need to be investigated in parallel to optimize the generation of integration-free HSCs. Furthermore, the level of TF induction may also need to be controlled since the stoichiometry of TFs may impact reprogramming efficiency as well as the functionality of reprogrammed cells as has been reported in other systems (Carey *et al*, 2011).

In addition to guaranteeing safety, it will be necessary to increase the efficiency of HSC induction to obtain sufficient numbers for transplantation. Although the efficiency of generating iPSCs has increased dramatically, their utility is augmented by their limitless self-renewal potential and well-defined culture conditions that support it. Since it is unclear whether HSCs could ever be expanded to the same extent as iPSCs while avoiding functional decline as seen with aging (Rossi *et al*, 2005; Beerman *et al*, 2013; Beerman & Rossi, 2014; Sun *et al*, 2014a), an ideal solution would entail near deterministic induction of HSCs from readily available somatic cells such as peripheral blood or fibroblasts. Until such efficiency can be reached, robust, defined means for *ex vivo* HSC expansion should be pursued.

Finally, the assays used to confirm HSC identity should be reevaluated. Currently, the only reliable, accepted method for assessing HSC function is competitive transplantation, a resource and time draining procedure incompatible with routine quality control of patient-derived HSCs. Therefore, a bioinformatic metric, like those developed for iPSC quality control (Nestor & Noggle, 2013), predictive of HSC functionality will not only benefit clinical translation but also accelerate the pace of HSC induction research.

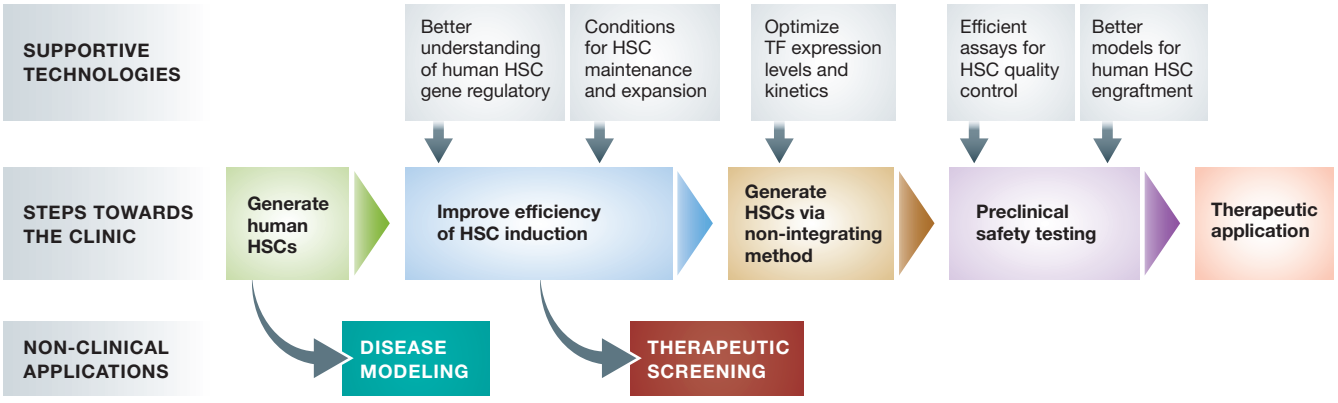

Figure 2. Road map to clinical translation of human induced HSCs.
Upon establishment of a method for generating human HSCs from patient-derived cells, a number of critical steps must be taken *en route* to clinical translation. Although the initial, suboptimal method may be sufficient for initiating patient-specific disease modeling research, substantial improvements in the efficiency of HSC generation must be made to obtain sufficient numbers of HSCs toward therapeutic screening and for use in reconstituting adult patients. Prior to preclinical testing, a non-integrating approach to generating HSCs must be established in order to eliminate the risks associated with insertional mutagenesis and accidental re-induction of reprogramming TFs, many of which are potently oncogenic. During the preclinical phase, quality control and safety testing should be performed at molecular and functional levels. Functional testing should involve the best methods for modeling human HSC engraftment such as humanized immunodeficient animal recipients.

Conclusion

"*What I cannot create, I do not understand.*"
Richard Feynman

HSCs have captivated generations of researchers not only because of their clinical importance but also for their intriguing yet elusive biological properties. Arguably, a major contributor to this allure has been the challenges associated with the seemingly simple task of obtaining more HSCs. Even while the biology of HSCs continues to be elucidated in staggering resolution with the advent of increasingly sensitive and high throughput methods (Lu *et al*, 2011; Gazit *et al*, 2013; Cabezas-Wallscheid *et al*, 2014; Lara-Astiaso *et al*, 2014; Sun *et al*, 2014a,b; Wu *et al*, 2014) as well as more accurate description of the HSC niche (Morrison & Scadden, 2014), robust *ex vivo* expansion or *de novo* generation of human HSCs still remains elusive. However, major strides made by the recent cohort of fate conversion studies have introduced new hope and perspectives to a field historically reigned by attempts to mimic the natural processes that regulate and specify HSCs. Despite having to induce non-physiologic cell fate transitions, fate conversion toward HSC has begun to yield relevant insights into HSC biology that may synergize with preexisting paradigms to better understand the ontogeny, maintenance, dysregulation, and therapeutic potential of HSCs.

Conflict of interest

The authors declare that they have no conflict of interest.

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Review

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Focus: Induced Pluripotency & Cellular Reprogramming

Probing disorders of the nervous system using reprogramming approaches

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Abstract

The groundbreaking technologies of induced pluripotency and lineage conversion have generated a genuine opportunity to address fundamental aspects of the diseases that affect the nervous system. These approaches have granted us unrestricted access to the brain and spinal cord of patients and have allowed for the study of disease in the context of human cells, expressing physiological levels of proteins and under each patient's unique genetic constellation. Along with this unprecedented opportunity have come significant challenges, particularly in relation to patient variability, experimental design and data interpretation. Nevertheless, significant progress has been achieved over the past few years both in our ability to create the various neural subtypes that comprise the nervous system and in our efforts to develop cellular models of disease that recapitulate clinical findings identified in patients. In this Review, we present tables listing the various human neural cell types that can be generated and the neurological disease modeling studies that have been reported, describe the current state of the field, highlight important breakthroughs and discuss the next steps and future challenges.

Keywords directed differentiation; disease modeling; neurologic disorder; neuronal development

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Introduction

Diseases of the nervous system represent an enormous burden for society in terms of human suffering and financial cost. While significant advancements have been achieved over the last few decades particularly in terms of genetic linkage, clinical classification and patient care, effective treatments are lacking. The inaccessibility of the relevant tissues and cell types in the central nervous system (CNS) and the complex multifactorial nature of most neurological disorders have hampered research progress. While animal models have been crucial in the investigation of disease mechanisms, fundamental developmental, biochemical and physiological differences exist between animals and humans. The importance of utilizing human cells for these purposes is evident by the large number of drugs that show efficacy and safety in rodent models of diseases but subsequently fail in human clinical trials, which are partly attributed to these species differences (Rubin, 2008). Furthermore, the overwhelming majority of neurological disease is of a sporadic nature, rendering animal modeling ineffective, while it is unclear whether the relatively rare monogenic forms of disease truly represent the vast majority of sporadic cases.

The simultaneous development of methods for reprogramming adult cells into induced pluripotent stem cells (iPSCs; Takahashi *et al*, 2007; Yu *et al*, 2007; Park *et al*, 2008) and the directed differentiation of pluripotent stem cells into distinct neuronal subtypes (Williams *et al*, 2012) suggested an attractive route to a novel model system for the study of neurological disorders. Patient-specific iPSCs can be generated by epigenetic reprogramming of various adult cell types such as skin fibroblasts and blood mononuclear cells and just like embryonic stem cells (ESCs), self-renew indefinitely and retain the potential to give rise to all cell types in the human body (Takahashi *et al*, 2007). More recently, sophisticated lineage conversion approaches have allowed for the direct generation of neurons and neural cell types from adult cells by means of overexpressing key transcription factors (for a detailed description see Tsunemoto *et al*, 2014). These methods have overcome some of the limitations of directed differentiation and have enabled for the generation of cell types that in many cases were previously unattainable.

The overwhelming advantages of using iPSCs and lineage conversion to develop models of diseases of the nervous system are that they allow one to study disease mechanisms in the context of human neurons and in the context of each patient's own unique genetic constellation. In many cases, established differentiation protocols allow for the generation of the particular neuronal subtype that is most vulnerable to the particular disease, such as spinal motor neurons (Davis-Dusenbery *et al*, 2014) and dopaminergic neurons (Kriks *et al*, 2011). These neurons can be produced in

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abundance from variable genetic backgrounds and could provide useful platforms for drug discovery.

The concept of using iPSCs and lineage conversion to study neurological disease appears straightforward: Both of these approaches allow for the generation of patient-specific neurons, which are relevant to the disease of interest, and when these are compared to neurons generated from healthy controls, any differences identified could be related to the disease. In practice, this approach has been proven to be more challenging than initially believed. What is the right cell type to make and study? How should quality control of neurons be performed? What are the right controls to use when assessing a disease-related phenotype? How do phenotypes identified *in vitro* relate to the clinical presentation of patients? These are just some of the questions that the community has struggled with, since the initial description of iPSCs and the onset of the development of *in vitro* patient-specific disease models. Perhaps the seemingly biggest advantage of this approach —the ability to study disease in the genetic background of the patient—has created the biggest challenge, as genetic background contributes to high variability in the properties of the patient-derived cells. This variability is a reality that neurologists have been facing for years, as often, two patients diagnosed with the same condition might present with very different clinical profiles. The technology of cellular reprogramming has brought this reality of clinical heterogeneity seen in patients from the bedside to the lab bench.

Since the initial description of reprogramming technologies, neuroscientists, neurologists and stem cell researchers have generated and characterized hundreds of patient-specific stem cell lines as well as neuronal cells derived from them (Table 1). The first "wave" of disease modeling studies focused on generating patient-specific human neurons and confirming previously described pathologies (Dimos *et al*, 2008; Ebert *et al*, 2009; Marchetto *et al*, 2010; Brennand *et al*, 2011; Seibler *et al*, 2011; Bilican *et al*, 2012; Israel *et al*, 2012). More recent studies have revealed novel insights into disease mechanisms and employed gene editing approaches to clearly demonstrate the association of identified phenotypes with known genetic variants that contribute to disease (An *et al*, 2012; Corti *et al*, 2012; Fong *et al*, 2013; Reinhardt *et al*, 2013; Kiskinis *et al*, 2014; Wainger *et al*, 2014; Wen *et al*, 2014b). At the same time, there has been tremendous progress in our ability to generate neuronal subtypes both via directed differentiation and through the exogenous expression of transcription factors. Here, we review the current state of disease modeling and neuronal differentiation approaches, highlight breakthrough studies and discuss the shift in focus that is expected over the next few years.

You can study only what you can make

With an eye on modeling neurological disease, stem cell scientists have steadily developed protocols for generating relevant human neural subtypes *in vitro* (Fig 1 and Table 2). Many directed differentiation and lineage conversion studies have focused on cell types that are selectively vulnerable in neurodegenerative or neurological diseases such as spinal motor neurons (amyotrophic lateral sclerosis, ALS), midbrain dopaminergic neurons (Parkinson's disease, PD) and striatal medium spiny neurons (Huntington's disease, HD). Their selective vulnerability in patients provides confidence that the phenotypes identified in iPSC-derived or lineage-converted cells *in vitro* represent relevant disease processes. In addition, it provides the opportunity to sift out phenotypes that may be disease non-relevant by using neuronal subtypes that are not affected *in vivo* as negative controls.

One important area requiring further development of *in vitro* protocols is region-specific cortical differentiation. Many diseases affect specific regions of the cortex, such as frontotemporal dementia (FTD), which affects the anterior cingulate, orbitofrontal cortex, and temporal lobes, or ALS, which affects layer V neurons in the motor cortex. Thus, region-specific attributes play a large role in the disease vulnerability of neuronal subtypes. While protocols exist to generate neurons from both deep and upper layers of the cortex (Shi *et al*, 2012b; Kadoshima *et al*, 2013), they have not shown to be specific for a given region of the cortex. The identification of marker genes and neuronal projection patterns specific to neurons in different cortical regions will greatly facilitate the development and validation of region-specific cortical neuron protocols.

Adult neural stem cells of the dentate gyrus play a key role in memory formation and pattern separation tasks and could be an important therapeutic target for Alzheimer's disease (AD). Although it is possible to generate human neural stem or progenitor cells *in vitro*, these are likely more embryonic and it is not clear how closely these mimic adult stem cells of the dentate gyrus (Chambers *et al*, 2009, 2011; Shi *et al*, 2012a,b). One reason is that until recently, rigorous molecular characterization of these cells was missing. We are now starting to get a clearer picture. There seem to be several adult neural stem cell populations or states that can be distinguished by markers such as Ascl1 or Gli1, and single-cell RNA sequencing data have been generated (Bonaguidi *et al*, 2011). This new information will serve as a template for generating adult neural stem cells *in vitro*.

A third cell type that has not been produced on a patient-specific level *in vitro* is microglia. Microglia perform inflammatory and non-inflammatory tasks that enable normal neuronal function. Through these roles, they are known to regulate the progression of ALS and AD (Zhong *et al*, 2009; de Boer *et al*, 2014; Johansson *et al*, 2015), and potentially other neurodegenerative diseases. Mouse studies showed that microglia from *SOD1*G93A ALS mice express higher levels of the prostaglandin E2 receptor (Di Giorgio *et al*, 2008; de Boer *et al*, 2014). Similarly, microglia from an AD model sharply upregulate the prostaglandin E2 receptor in response to amyloid-β (Ab) exposure in an age-dependent manner (Johansson *et al*, 2015). Higher prostaglandin E2 signaling in microglia caused reduced microglial cytokine generation, chemotaxis, clearance of Aboligomers, resolution of inflammatory responses to Ab_{42} and trophic factor release (Johansson *et al*, 2015). In both the ALS and AD models, deletion of the prostaglandin E2 receptor significantly slowed disease progression (de Boer *et al*, 2014; Johansson *et al*, 2015). Under normal conditions, microglia are derived from the embryonic yolk sac and go through a maturation process after they enter the nervous system (Nayak *et al*, 2014). The signaling and gene expression changes that occur during this process are not well understood and will need to be characterized further to enable the production of patient-specific microglia.

Table 1. List of published studies modeling human neurological diseases with iPSCs.

Disease	References	Patient genotype	Cell type analyzed	Identified phenotype	Notable
Alzheimer's Disease	Yagi *et al* (2011)	*PSEN1, PSEN2* mutations	Neurons	Increased amyloid β42 secretion	
Alzheimer's Disease	Israel *et al* (2012)	*APP* mutations, sporadic cases	Neurons	Increased amyloid β40, Tau and GSK3β phosphorylation, accumulation of endosomes	One of two sporadic patients exhibited phenotypes
Alzheimer's Disease	Kondo *et al* (2013)	*APP* mutations, sporadic cases	Cortical neurons, astrocytes	Accumulated Aβ oligomers, ER & oxidative stress	One of two sporadic patients exhibited phenotypes
Alzheimer's Disease	Muratore *et al* (2014)	*APP* mutation	Forebrain neuron	Increase in Aβ42, Aβ38, pTAU	Aβ-antibodies reduce pTAU
Alzheimer's Disease	Sproul *et al* (2014)	*PSEN1* mutation	Neural progenitors	Higher Aβ42/Aβ40 ratio, gene expression differences	Verification of gene expression differences in human AD brains
Alzheimer's Disease	Duan *et al* (2014)	Sporadic *ApoE3/E4*	Basal forebrain cholinergic neurons	Higher Aβ42/Aβ40 ratio, increased vulnerability to glutamate-stress	
Alzheimer's Disease	Hossini *et al* (2015)	Sporadic	Neurons	Gene expression analysis	
Amyotrophic Lateral Sclerosis (ALS)	Dimos *et al* (2008)	*SOD1* mutations	Motor neurons	N.D.	First report of patient-specific neurons
Amyotrophic Lateral Sclerosis (ALS)	Mitne-Neto *et al* (2011)	*VAPB* mutations	Fibroblasts, iPSCs, motor neurons	Reduced VAPB protein levels	Although VAPB levels were highest in neurons, the reduction was not specific to neurons
Amyotrophic Lateral Sclerosis (ALS)	Bilican *et al* (2012)	*TDP43* mutations	Motor neurons	Cell death	Real-time survival analysis of $HB9^{+}$ neurons
Amyotrophic Lateral Sclerosis (ALS)	Egawa *et al* (2012)	*TDP43* mutations	Motor neurons	Expression differences, TDP43 pathology, shorter neurites	Rescue by anacardic acid, multiple clones per patient used
Amyotrophic Lateral Sclerosis (ALS)	Sareen *et al* (2013)	*C9orf72* expansion	Motor neurons	RNA foci, hypoexcitability, gene expression differences	Repeat-containing RNA foci colocalized with hnRNPA1 and Pur-α, rescue of gene expression by ASO treatment
Amyotrophic Lateral Sclerosis (ALS)	Donnelly *et al* (2013)	*C9orf72* expansion	Neurons	RNA foci, irregular interaction with ADARB2, susceptibility to glutamate excitotoxicity	Colocalization of repeat with ADARB2 validated in patient motor cortex. Rescue of gene expression by ASO treatment
Amyotrophic Lateral Sclerosis (ALS)	Yang *et al* (2013b)	*SOD1, TDP43* mutations	Motor neurons	Sensitivity to growth factor withdrawal	Rescue by kenpaullone
Amyotrophic Lateral Sclerosis (ALS)	Serio *et al* (2013)	*TDP43* mutations	Astrocytes	Cell death, TDP43 mislocalization	
Amyotrophic Lateral Sclerosis (ALS)	Wainger *et al* (2014)	*SOD1, C9orf72, FUS* mutations	Motor neurons	Hyperexcitability	Phenotype rescued by gene correction in *SOD1*, and by treatment with a Kv7 agonist
Amyotrophic Lateral Sclerosis (ALS)	Kiskinis *et al* (2014)	*SOD1, C9orf72* mutations	Motor neurons	Cell death, reduced soma size, ER stress, mitochondrial abnormalities, gene expression changes	Phenotypes rescued by gene correction in *SOD1*

Table 1 (continued)

Disease	References	Patient genotype	Cell type analyzed	Identified phenotype	Notable
Amyotrophic Lateral Sclerosis (ALS)	Chen *et al* (2014)	*SOD1* mutations	Motor neurons	Neurofilament aggregation, cell death	Phenotype rescued by gene correction
Amyotrophic Lateral Sclerosis (ALS)	Barmada *et al* (2014)	*TDP43* mutations	Neurons, astrocytes	Sensitivity to TDP43 accumulation	Autophagy stimulation increases survival
Amyotrophic Lateral Sclerosis (ALS)	Devlin *et al* (2015)	*TDP43* and *C9orf72* mutants	Neurons	Electrophysiological dysfunction	Hyperexcitability followed by loss of action potential output
Angelman & Prader–Willi Syndrome	Chamberlain *et al* (2010)	*15q11-q13* deletions	Neurons	*UBE3A* expression	Genomic imprint is maintained in iPSC neurons
Ataxia Telangiectasia	Lee *et al* (2013)	*ATM* mutations	NPCs & neurons	Defective DNA damage response	SMRT compounds rescue phenotype
Best Disease	Singh *et al* (2013)	*BEST1* mutations	RPE cells	Delayed RHODOPSIN degradation, defective Ca^{2+} responses, oxidative stress	
Dravet Syndrome	Higurashi *et al* (2013)	*SCN1A* mutation	Neurons (mostly $GABA^{+}$)	Reduced AP firing	
Dravet Syndrome	Liu *et al* (2013b)	*SCN1A* mutation	Neurons (GABA & $Glutamate^{+}$)	Increase Na^{+} current density, altered excitability	
Dravet Syndrome	Jiao *et al* (2013)	*SCN1A* mutation	Neurons	Abnormal Na^{+} currents, increased firing	
Familial Dysautonomia	Lee *et al* (2009)	*IKBKAP* mutation	Peripheral neurons, neural crest precursors	Mis-splicing & *IKBKAP* expression, neurogenesis & migration defects	Phenotypes are tissue specific
Familial Dysautonomia	Lee *et al* (2012)	*IKBKAP* mutation	Neural crest precursors	*IKBKAP* expression levels	First large-scale drug screening approach, first follow-up study
Fragile X Syndrome	Sheridan *et al* (2011)	*FMR1* expansion	NPCs & neurons	*FMR1* promoter methylation & reduced expression, reduced length of processes	
Fragile X Syndrome	Liu *et al* (2012b)	*FMR1* expansion	Neurons	Decreased PSD95 expression & density, neurite length, electrophysiological defects	
Fragile X Syndrome	Doers *et al* (2014)	*FMR1* expansion	Neurons	Neurite extension & initiation defects	
Friedreich's Ataxia	Liu *et al* (2011)	*FXN* expansion	Peripheral neurons, cardiomyocytes	*FXN* expression, repeat instability	
Friedreich's Ataxia	Hick *et al* (2013)	*FXN* expansion	Neurons, cardiomyocytes	*FXN* expression, mitochondrial dysfunction	
Friedreich's Ataxia	Eigentler *et al* (2013)	*FXN* expansion	Peripheral neurons	*FXN* expression	
Frontotemporal Dementia	Almeida *et al* (2013)	*C9orf72* expansion	Neurons	RNA foci, RAN products, sensitivity to autophagy inhibitors	
Frontotemporal Dementia (Bv)	Gascon *et al* (2014)	Sporadic patients	Neurons	Alterations in miR-124 & AMPAR levels	Confirmation of mouse model findings in iPSC neurons & patients
Frontotemporal Dementia	Raitano *et al* (2015)	PGRN mutation	Cortical & motor neurons	Cortical differentiation defects	Rescue by PGRN expression

Table 1 (continued)

Disease	References	Patient genotype	Cell type analyzed	Identified phenotype	Notable
Gaucher's Disease	Mazzulli *et al* (2011)	*GBA1* mutations	Dopaminergic neurons	Declined proteolysis, increased α-synuclein	Provides links between GD & PD
Gaucher's Disease	Tiscornia *et al* (2013)	*GBA1* mutations	Neurons & macrophages	Reduction in acid-β-glucosidase activity	Identification of two small molecules
Gyrate Atrophy	Meyer *et al* (2011)	*OAT* mutation	RPE cells	Decreased OAT activity	Rescued by BAC-mediated introduction of *OAT*
Hereditary Spastic Paraplegia	Denton *et al* (2014)	*SPAST* mutation	Glutamatergic neurons	Axonal swelling, increased levels of acetylated tubulin	
Hereditary Spastic Paraplegia	Zhu *et al* (2014)	*ATL1* mutation	Forebrain neurons	Impaired axonal growth, defects in mitochondrial motility	
Huntington's Disease	Camnasio *et al* (2012)	*HTT* expansion	Neurons	Altered lysosomal activity	
Huntington's Disease	Juopperi *et al* (2012)	*HTT* expansion	Astrocytes	Cytoplasmic vacuolization	
Huntington's Disease	HD Consortium (2012)	*HTT* expansion	NPCs & $GABA^+$ neurons	Altered gene expression, morphological alterations, survival deficit, sensitivity to stressors	Correlation between repeat length & vulnerability to cell stress
Huntington's Disease	An *et al* (2012)	*HTT* expansion	NPCs, neurons	Cell death, gene expression, mitochondrial dysfunction	Genetic correction rescued phenotypes
Huntington's Disease	Guo *et al* (2013)	*HTT* expansion	Neurons ($GABA^+$)	Mitochondrial damage	
Huntington's Disease	Yao *et al* (2015)	*HTT* expansion	Striatal neurons	Cell death, caspase-3 activation	Identified Gpr52 as a stabilizer of HTT
Lesch–Nyhan Syndrome	Mekhoubad *et al* (2012)	*HPRT1* mutation	Neurons	Neuronal differentiation efficiency and neurite number defects	Demonstrate that X-inactivation erodes in culture & could affects modeling of X-linked disease
Microcephaly	Lancaster *et al* (2013)	*CDK5RAP2* mutation	Cerebral organoids	Smaller neuroepithelial regions & RGs, premature neurogenesis, RG spindle disarray	Generated 3-dimensional brain structures
Neuronal ceroid lipofuscinosis	Lojewski *et al* (2014)	*CNL2, CNL3* mutations	NPCs, neurons	Morphological abnormalities in ER, Golgi, mitochondria & lysosomes	Rescue by expression of NCL proteins
Niemann–Pick type C1 disease	Trilck *et al* (2013)	*NPC1* mutation	NPCs & neurons	Accumulation of cholesterol	
Parkinson's Disease	Byers *et al* (2011)	*SCNA* triplication	Dopaminergic neurons	Oxidative stress, α-synuclein accumulation	
Parkinson's Disease	Nguyen *et al* (2011)	*LRRK2* mutations	Dopaminergic neurons	Oxidative stress, α-synuclein accumulation, sensitivity to stress reagents	
Parkinson's Disease	Seibler *et al* (2011)	*PINK1* mutations	Dopaminergic neurons	Increased mitochondrial copy number, PGC1a upregulation	Rescue by *PINK1* overexpression
Parkinson's Disease	Devine *et al* (2011)	*SNCA* triplication	Dopaminergic neurons	Upregulation of α-synuclein	

Table 1 (continued)

Disease	References	Patient genotype	Cell type analyzed	Identified phenotype	Notable
Parkinson's Disease	Sanchez-Danes *et al* (2012)	Sporadic & *LRRK2* mutations	Dopaminergic neurons	Reduction in neurite number & density, vacuolization, sensitivity to lysosomal inhibition	A total of 15 patients examined, long-term culture ~75 DIV
Parkinson's Disease	Cooper *et al* (2012)	*PINK1* & *LRRK2* mutations	Dopaminergic neurons	Mitochondrial dysfunction in response to stressors	Pharmacological rescue of phenotypes
Parkinson's Disease	Imaizumi *et al* (2012)	*PARK2* mutations	Dopaminergic neurons	Oxidative stress, mitochondrial dysfunction, Nrf2 induction, α-synuclein accumulation	
Parkinson's Disease	Liu *et al* (2012a)	*LRRK2* mutation	Neural stem cells	Susceptibility to proteosomal stress, differentiation & clonal expansion deficiencies	Genetic correction rescued phenotypes
Parkinson's Disease	Reinhardt *et al* (2013)	*LRRK2* mutation	Dopaminergic neurons	Gene expression differences, ERK phosphorylation & activity	Genetic correction rescued phenotypes
Parkinson's Disease	Su and Qi (2013)	*LRRK2* mutation	Dopaminergic neurons	Mitochondrial damage, shorter neuritis, lysosomal hyperactivity	Pharmacological rescue
Parkinson's Disease	Chung *et al* (2013)	*SNCA* mutation	Cortical neurons	Nitrosative & ER stress	Pharmacological rescue, combination between a yeast and an iPSC platform
Parkinson's Disease	Miller *et al* (2013)	*PINK1* & *PARKIN* mutations	Dopaminergic neurons	TH reduction, dendritic degeneration	Phenotypes induced only after overexpressing progerin
Parkinson's Disease	Ryan *et al* (2013)	*SNCA* mutation	Dopaminergic neurons	Nitrosative stress, gene expression alterations, mitochondrial stress	Genetic & pharmacological rescue of phenotypes
Parkinson's Disease	Flierl *et al* (2014)	*SNCA* triplication	NPCs	Viability, metabolism & stress resistance defects	Rescue by *SNCA* knockdown
Parkinson's Disease	Sanders *et al* (2014)	*LRRK2* mutations	NPCs & neurons	Mitochondrial DNA damage	Genetic correction rescued phenotypes
Phelan–McDermid Syndrome	Shcheglovitov *et al* (2013)	22q13.3 deletion	Forebrain neurons	Defective excitatory synaptic transmission	Rescue by *SHANK3* expression or IGF1 treatment
Retinitis Pigmentosa	Jin *et al* (2011)	*RP1, RP9, PRPH2, RHO* mutations	Rod photoreceptors	Cell death, oxidative & ER stress	Differential response to treatment with α-Tocopherol
Retinitis Pigmentosa	Tucker *et al* (2011)	MAK mutations	Retinal precursors	Defective *MAK* mRNA splicing	
Retinitis Pigmentosa	Jin *et al* (2012)	*RHO* mutations	RPE cells	Cell death & ER stress	
Retinitis Pigmentosa	Tucker *et al* (2013)	*USH2A* mutations	Retinal precursors	*USH2A* transcript defects, ER stress	
Rett Syndrome	Marchetto *et al* (2010)	*MeCP2* mutations	Neurons	*MeCP2* expression, reduced synapses, spine density, soma size, altered calcium signaling	
Rett Syndrome	Ananiev *et al* (2011)	*MeCP2* mutations	Neurons	Reduced nuclear size	
Rett Syndrome	Cheung *et al* (2011)	*MeCP2* deletion	Neurons	*MeCP2* expression, reduced soma size	

Table 1 (continued)

Disease	References	Patient genotype	Cell type analyzed	Identified phenotype	Notable
Rett Syndrome	Kim *et al* (2011c)	*MeCP2* mutations	Neurons	Lower TUJ1 & Na^+ channel expression	
Rett Syndrome	Amenduni *et al* (2011)	*CDKL5* mutations	Neurons	No phenotype described	
Rett Syndrome	Ricciardi *et al* (2012)	*CDKL5* mutations	Neurons	Aberrant dendritic spines	
Rett Syndrome	Larimore *et al* (2013)	*MeCP2* mutations	Neurons	Reduced expression of *PLDN*	
Rett Syndrome	Griesi-Oliveira *et al* (2014)	*TRPC6* mutation	NPCs & cortical neurons	Gene expression differences, Ca^{2+} influx defects, decreased axonal length & arborization	Overlap in molecular pathways between *TRPC6 & MeCPT2*
Rett Syndrome	Williams *et al* (2014)	*MeCP2* mutations	Astrocytes	Mutant astrocytes cause morphological and firing defects in healthy neurons	Demonstrates non-cell autonomous contribution of astrocytes in Rett Syndrome
Rett Syndrome	Djuric *et al* (2015)	*MeCP2e1* mutation	Cortical neurons	Reduced soma size, dendritic density, capacitance & firing defects	Rescue of phenotypes by overexpression of *MeCP2e1*
Rett Syndrome	Livide *et al* (2015)	*MeCP2* & *CDKL5* mutations	NPCs & neurons	Gene expression differences	Identified *GRID1* as a common target in two distinct genetic classes of RTT
Schizophrenia	Brennand *et al* (2011)	Familial & sporadic SCZD patients	NPCs & neurons	Decreased connectivity, neurite number, PSD95 protein, gene expression changes	Recovery after treatment with loxapine
Schizophrenia	Pedrosa *et al* (2011)	22q11.2 deletion & sporadic SCZD	Glutamatergic neurons	No phenotype described	
Schizophrenia	Paulsen Bda *et al* (2012)	SCZD patient	NPCs	Elevated ROS, extramitochondrial consumption	Treatment with valproic acid reduced ROS
Schizophrenia	Robicsek *et al* (2013)	SCZD patients	NPCs, dopaminergic, glutamatergic neurons	Differentiation & maturation deficiencies, mitochondrial defects	
Schizophrenia	Yoon *et al* (2014)	*15q11.2* microdeletion	NPCs	Deficits in adherent junctions & apical polarity	Identified haploinsufficiency of *CYFIP1* as a potential contributor to neuropsychiatric disorders
Schizophrenia	Hook *et al* (2014)	SCZD patients	Neurons	Increased secretion of catecholamines, higher numbers of TH^+ neurons	
Schizophrenia	Wen *et al* (2014b)	*DISC1* mutations	Forebrain neurons	Synaptic vesicle release deficits, gene expression changes	Isogenic controls included in this study
Schizophrenia	Brennand *et al* (2015)	Familial & sporadic SCZD patients	NPCs & neurons	RNA & protein-level differences related to cytoskeleton & oxidative stress, aberrant migration	
Spinal Muscular Atrophy	Ebert *et al* (2009)	Type 1 *SMA*	Motor neurons	Cell death, soma size, reduced SMN levels	First study of iPSC-based approach to report a disease-associated phenotype

Table 1 (continued)

Disease	References	Patient genotype	Cell type analyzed	Identified phenotype	Notable
Spinal Muscular Atrophy	Sareen *et al* (2012)	Type 1 *SMA*	Motor neurons	Cell death, increased caspase-8 & 3 activation	Rescue by apoptotic inhibitors
Spinal Muscular Atrophy	Corti *et al* (2012)	Type 1 *SMA*	Motor neurons	Cell death, smaller soma size, reduced axonal length, gene expression and RNA splicing defects	Gene correction, transplantation of iPSC motor neurons extends lifespan of SMA mouse model
Tauopathy	Fong *et al* (2013)	*TAU* mutation	Neurons	TAU fragmentation & phosphorylation, axonal degeneration	Gene editing to correct the mutation & generate a homozygous mutant used as controls
Timothy Syndrome	Pasca *et al* (2011)	*CACNA1C* mutations	NPCs & cortical neurons	Ca^{2+} signaling, activity-dependent gene expression	Rescue by roscovitine treatment
Timothy Syndrome	Krey *et al* (2013)	*CACNA1C* mutations	Cortical neurons	Activity-dependent dendrite retraction	Rescue by GTPase Gem

NPCs, neural progenitor cells; RPE, retinal pigment epithelium; ND, not determined; ASO, allele-specific oligonucleotide; GD, Gaucher's disease; PD, Parkinson's disease; AP, action potential.
The table includes neurodevelopmental and neurodegenerative diseases for which patient-specific iPSCs have been generated and neuronal cells differentiated to develop a cell-based model of disease.

Specificity of phenotypes: the importance of controls

Significant technical advancements achieved over that last few years currently allow for the generation of patient-specific iPSCs that are free from genomic integration of the reprogramming factors (Malik & Rao, 2013). The essential quality of any newly derived iPSC can be easily assessed by (i) immunocytochemistry for pluripotency markers (e.g. NANOG/SSEA3), (ii) a quantitative pluripotency assay such as the *Scorecard* or the *Pluritest* and (iii) analysis of genomic integrity (karyotype, array CGH).

Disease modeling studies based on iPSC technology have relied on the use of diseased cells derived from patients as a model for disease, and cells derived from healthy individuals as controls. However, genetic and potentially epigenetic heterogeneity of iPSC lines contributes to functional variability of differentiated somatic cells, confounding evaluation of disease modeling experiments (Sandoe & Eggan, 2013). Such variability can be introduced at multiple different levels including generation of stem cell lines, continuous *in vitro* culture, variation in cell culture reagents, differential efficiencies of neural generation and genetic background. There are different approaches to overcoming this variation. One approach is through the use of targeted gene editing that results in the generation of a control stem cell line that is isogenic to the patient one, except for the disease-causing mutation. Such an approach effectively minimizes line-to-line differences and is a very important tool for iPSC-based disease modeling.

CRISPR/Cas9, a recent technology that has emerged, allows for the efficient generation of such isogenic stem cell lines (Hsu *et al*, 2014). The system contains two essential components, an enzyme that can cleave DNA such that a double-strand break or a single nick is generated and a guide RNA that targets the enzyme to a specific genomic location. By simultaneously introducing either a single-stranded oligodeoxynucleotide (ssODN) containing the desired edit or a targeting plasmid with larger desired sequence alterations, the genomic sequence can be precisely edited via the cells' own endogenous repair mechanism, homologous recombination. Given the incredible versatility of the CRISPR/Cas9 system and the continuous evolvement of the technical aspects of this approach, it should be expected that every iPSC study that focuses on genetic forms of disease should include an isogenic control cell line. The rescue of a phenotype by genetic correction can lead to the conclusion that the genetic lesion is *necessary* for the onset of the phenotype. The same technique can be used to introduce a disease-associated mutation in a healthy iPSC line in order to assess whether the mutation in itself is *sufficient* for the onset of particular phenotypes.

An alternative approach to the concern of variation would be to utilize multiple stem cell clones from each individual patient and compare the desired measurement against multiple healthy individuals. The use of multiple patient clones would ensure that the phenotype is not an artifact of a defective clonal cell line, while the use of multiple healthy controls should encapsulate sufficient technical and genetic variation, so that the measured cellular properties neuronal firing, dendritic density, etc. will represent a true average. This approach will be important in studies of sporadic disease.

Additionally, approaches that are complementary to the iPSC method should also be considered for the verification of identified phenotypes. These could include the generation of neurons via direct conversion as well as the investigation of human patient material such as postmortem CNS tissue and cerebrospinal fluid (CSF). Other non-invasive techniques such as transcranial magnetic stimulation (TMS) (Fox *et al*, 2014), which allows *in vivo* neurostimulation and neuromodulation, and an electroencephalogram (EGG), can also be used to examine changes associated with electrical excitability of neurons.

An important point to consider when assessing the specificity of an identified phenotype is whether it is only apparent in the cell type known to be most vulnerable to the disease being modeled. In

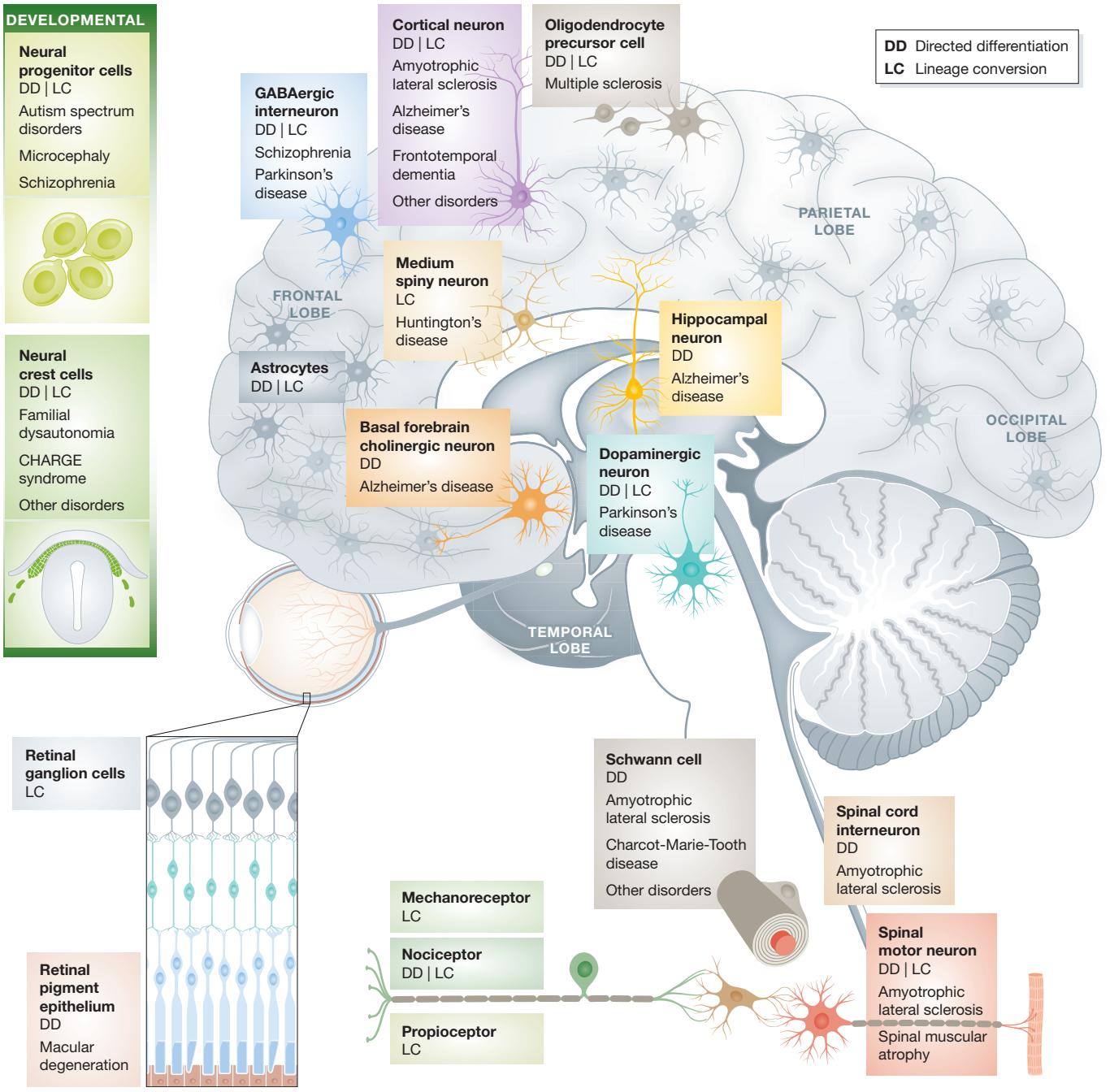

Figure 1. You can model only what you can make.
A number of different human neural cells can be efficiently generated by directed differentiation (DD) from pluripotent stem cells, or by lineage conversion (LC) from somatic cell types.

ALS patients for example, it is the upper and lower motor neurons that are initially targeted by disease mechanisms and gradually lost, while sensory neurons remain relatively unaffected. It would therefore be predicted that a phenotype that is truly relevant to disease would not be evident in a sensory neuron generated from the same individual. Although this could be a valuable approach, it should be taken with caution for two reasons: firstly because a sensory neuron might simply be resistant to a phenotype, and therefore, it is the effect of the phenotype on the sensory cell that should be considered and not simply the presence of the phenotype in itself, and secondly because it might be the *in vivo* microenvironment of a sensory neuron that confers resistance and not a cell autonomous trait. Nevertheless, studies have demonstrated neuronal-type specificity of a phenotype including the sensitivity of mutant PD tyrosine hydroxylase (TH)-positive neurons but not TH-negative neurons to H_2O_2-induced toxicity (Nguyen *et al*, 2011), and morphometric

Table 2. List of neural cells generated by directed differentiation of stem cells and lineage conversion of somatic cells.

Initial cell population	Target cell type	Morphogens/Small molecules	Reprogramming factors	References
Lineage conversion				
Fibroblasts	Neural crest cells		SOX10	Kim *et al* (2011a)
Fibroblasts	Neural stem cells		SOX2	Ring *et al* (2012)
Fibroblasts	Neurons	CHIR99021, SB431542	ASCL1, NGN2	Ladewig *et al* (2012)
Fibroblasts	Neurons		ASCL1	Chanda *et al* (2014)
Pericyte-derived cells	Neurons		SOX2, ASCL1	Karow *et al* (2012)
Fibroblasts	Dopaminergic neurons		ASCL1, BRN2, MYT1L, LMX1A, FOXA2	Pfisterer *et al* (2011)
Fibroblasts	Dopaminergic neurons		ASCL1, LMX1A, NURRL	Caiazzo *et al* (2011)
Fibroblasts	Dopaminergic neurons		Lmx1a, Foxa2, Ascl1, Brn2	Sheng *et al* (2012a)
Fibroblasts	Dopaminergic neurons		Ascl1, Pitx3, Lmx1a, Nurr1, Foxa2, EN1	Kim *et al* (2011b)
Fibroblasts	Dopaminergic neurons		ASCL1, NGN2, SOX2, NURR1, PITX3	Liu *et al* (2012c)
Fibroblasts	Glutamatergic Neurons		ASCL1, BRN2, MYT1L, NEUROD1	Pang *et al* (2011)
Fibroblasts	Glutamatergic neurons		BRN2, MYT1L, miR-124	Ambasudhan *et al* (2011)
Fibroblasts	Glutamatergic neurons	Forskolin, Dorsomorphin	NGN2	Liu *et al* (2013a)
Fibroblasts	Glutamatergic and GABAergic neurons		ASCL1, MYT1L, NEUROD2, miR-9/9*, miR-124	Yoo *et al* (2011)
Fibroblasts	Medium spiny neurons		DLX1, DLX2, MYT1L, CTIP2, miR-9/9*, miR-124	Victor *et al* (2014)
Fibroblasts	Nociceptor, mechanoreceptor, proprioceptor neurons	Brn3a, Ngn1/2		Blanchard *et al* (2015)
Fibroblasts	Nociceptor Neurons	ASCL1, MYT1L, ISL2, KLF7, NGN1		Wainger *et al* (2015)
Fibroblasts	Oligodendrocyte progenitor cells		Sox10, Olig2, Zfp536	Yang *et al* (2013a)
Fibroblasts	Oligodendrocyte progenitor cells		Olig1, Olig2, Nkx2.2, Nkx6.2, Sox10, ST18, Gm98, Myt1	Najm *et al* (2013)
Fibroblasts	Spinal motor neurons		ASCL1, BRN2, MYT1L, NGN2, ISL1, LHX3, NEUROD1	Son *et al* (2011)
Fibroblasts	Astrocytes		Nfia, Nfib, Sox9	Caiazzo *et al* (2015)
Fibroblasts	Neural precursor cells		Brn2, Sox2, FoxG1	Lujan *et al* (2012)
Fibroblasts	Neural progenitor cells	VPA, CHIR99021, RepSox		Cheng *et al* (2014)
Fibroblasts	Neural stem cells		Brn4, Sox2, Klf4, c-Myc, E47	Han *et al* (2012)
Fibroblasts	Neural stem cells		Sox2, Klf4,c-Myc, Oct4	Thier *et al* (2012)
Sertoli cells	Neural stem cells		Ascl1, Ngn2, Hes1, Id1, Pax6, Brn2, Sox2, c-Myc, Klf4	Sheng *et al* (2012b)
Astrocytes	Neuroblasts		Sox2	Niu *et al* (2013)
Hepatocytes	Neurons		Ascl1, Brn2, Myt1l	Marro *et al* (2011)
Fibroblasts	Neurons		PTB repression	Xue *et al* (2013)
Astrocytes	GABAergic neurons		Ascl1, Dlx2	Heinrich *et al* (2010)
Directed differentiation				
Pluripotent stem cells	Forebrain neuronal precursors	SB431542, LDN189193, N2, B27		Chambers *et al* (2009)

Table 2 (continued)

Initial cell population	Target cell type	Morphogens/Small molecules	Reprogramming factors	References
Pluripotent stem cells	Forebrain neurons	SB431542, LDN189193, N2, B27		Chambers *et al* (2009)
Pluripotent stem cells	Telencephalic neurons	N2, B27, IGF1, Heparin, SHH, DKK1, WNT3A, BDNF, GDNF		Li *et al* (2009)
Pluripotent stem cells	Forebrain neural cells	Heparin, N2, B27, BDNF, GDNF, IGF1		Zeng *et al* (2010)
Pluripotent stem cells	Cortical neurons	B27, N2, BSA, sodium pyruvate, 2-mercaptoethanol, Noggin, Y27632		Espuny-Camacho *et al* (2013)
Pluripotent stem cells	Granule cerebellar neurons	FGF2, heparin, N2, Glutamax, FGF8, retinoic acid, ITS, FGF4, WNT1, WNT3A, B27, BMP7, BMP6, GDF7, SHH, NT3, JAG1		Erceg *et al* (2012)
Pluripotent stem cells	Hypothalamic neurons	Neurobasal-A, Glutamax, N2, B27, sodium bicarbonate, dibutyryl cyclic AMP, GDNF, BDNF, CNTF		Merkle *et al* (2015)
Pluripotent stem cells	Dopaminergic neurons	Heparin, N2, serum replacer, cAMP, ascorbic acid, BDNF, GDNF, SHH, FGF8		Yan *et al* (2005)
Pluripotent stem cells	Dopaminergic neurons	LDN193189, SB431542, SHH C25II, purmorphamine, FGF8, CHIR99021, N2, B27, L-Glut, BDNF, ascorbic acid, GDNF, TGFβ3, dibutyryl cAMP, DAPT		Kriks *et al* (2011)
Pluripotent stem cells	Spinal motor neurons	SB431542, LDN189193, N2, B27, retinoic acid, smoothened agonist		Amoroso *et al* (2013)
Pluripotent stem cells	Astrocytes	B27, BMP2, BMP4, LIF		Gupta *et al* (2012)
Pluripotent stem cells	Astrocytes	EGF, FGF, Glutamax, N2, CNTF		Krencik and Zhang (2011)
Pluripotent stem cells	Oligodendrocytes	N2, N1, cAMP, biotin, heparin, retinoic acid, SHH, purmorphamine, FGF2, B27, PDGF, IGF, NT3		Hu *et al* (2009)
Pluripotent stem cells	Hippocampal neurons	DKK1, SB431542, Noggin, cyclopamine, N2, B27, Wnt3a, BDNF, FGF2, ascorbic acid, cyclic AMP, fetal bovine serum		Yu *et al* (2014)
Pluripotent stem cells	Astrocytes (ventralized)	SB431542, LDN189193, RA, SHH, N2, B27, FGF1, FGF2		Roybon *et al* (2013)
Pluripotent stem cells	Basal forebrain cholinergic neurons	RA/SSH/FGF8/BMP9	*Lhx8* & *Gbx1*	Bissonnette *et al* (2011)
Pluripotent stem cells	Cortical interneurons	SB431542, LDN189193, XAV939, SHH, purmorphamine, N2, B27		Maroof *et al* (2013)

deficiencies of mutant ALS, ISL-positive motor neurons but not ISL-negative neurons grown in the same culture dishes (Kiskinis *et al*, 2014).

A major advantage of using reprogramming approaches to study a neurological disease is the ability to assess the biological variation associated with a specific neuronal defect. Consider that a

phenotype, for example, defective lysosomal function, has been identified in neurons derived from a patient cell line and that this phenotype is mutation dependent (i.e. it is corrected in an isogenic control line). The first level of biological variation can be addressed by examining neurons derived from a different individual that harbors the exact same mutation in the same gene. If the phenotype is not present, then additional genetic or epigenetic factors might be necessary for the onset of the defect. The next level of biological variability can be addressed by examining neurons from a patient with a different mutation in the same gene. Lastly, the broader relevance of the identified phenotype for the disease can be assessed by examining the lysosomal function of neurons from patients with mutations in different disease-causing genes as well as in a large number of sporadic cases.

A more direct route to the CNS?

Lineage conversion provides a progenitor-free approach for generating various neural types. Lineage conversion relies on the overexpression of transcription factors to internally drive differentiation programs. The forced expression of these factors replaces external developmental morphogens utilized in iPSC differentiation by directly activating downstream genes. Additionally, either purified external cues or other cell types normally present *in vivo* are sometimes added to further guide the developmental trajectory and maturation of various cell types (Son *et al*, 2011; Meyer *et al*, 2014).

An advantage of this approach is that it simplifies the identification of protocols for generating new neural subtypes because it only requires knowledge of transcription factor expression during the terminal stages of development, as opposed to requiring a deep understanding of morphogen signaling dynamics starting from the pluripotent state through the terminally differentiated state. In the same way that identifying the signals that produce the target cell type is simpler for lineage conversion, optimizing the efficiency of their production is more complicated for iPSC-directed differentiation because one must optimize the efficiency of each progenitor step as opposed to one step as in lineage conversion. Due to these advantages, reprogramming biologists have rapidly developed lineage conversion protocols for almost all neural subtypes attainable by directed differentiation just a few years after the initial demonstration of iPSC reprogramming (Takahashi *et al*, 2007), which showed that dramatic changes in cell fate are possible (see Fig 1).

Several groups have taken advantage of the modular nature of transcriptional networks to generate distinct neuronal subtypes. Genetic neuralization through introduction of *BRN2*, *ASCL1* and *MYT1L* (BAM) to fibroblasts generates induced neurons (iN; Pang *et al*, 2011). The transcription factor *ASCL1* is sufficient in generating iNeurons alone, indicating that it is the key driver in this reprogramming approach (Chanda *et al*, 2014). The addition of *NEUROD1* further enhances this conversion (Pang *et al*, 2011). From iNs, a secondary layer of transcription factors guide cells to particular neurons. Spinal motor neurons have been generated by adding *ISL1*, *LHX3*, *NGN2* and *HB9* to the BAM factors (Son *et al*, 2011). Addition of *LMX1A* and *FOXA2* to the BAM cocktail results in dopaminergic neurons (Pfisterer *et al*, 2011). Striatal medium spiny neurons can be generated using a microRNA-based neuralization platform (Yoo *et al*, 2011) supplemented with *CTIP2, DLX1, DLX3* and *MYT1L* (Victor *et al*, 2014). Neuronal induction has also been achieved through the repression of polypyrimidine tract binding (PTB), a single RNA binding protein (Xue *et al*, 2013). Oligodendrocyte precursor cells follow a separate glial lineage that is independent of BAM-mediated neuralization. Induced oligodendrocyte precursor cells can be made by overexpressing either *SOX10*, *OLIG2* and *ZFP536* (Yang *et al*, 2013a) or *OLIG1*, *OLIG2*, *NKX2.2*, *NKX6.2*, *SOX10*, *ST18*, *GM98* and *MYT1* (Najm *et al*, 2013). Just like in iPSC differentiation, inductive signals are added during lineage conversion protocols to further guide cells to mature fates.

During development, early neural progenitors produce neurons whereas late progenitors differentiate into astrocytes (Stiles & Jernigan, 2010). iPSC-directed differentiation recapitulates this developmental process. As a result, while the production of neurons from human iPSCs occurs within 30 days, astrocytes only emerge after 3 months (Krencik & Zhang, 2011). Recently, Broccoli and colleagues reported that three transcription factors, *NFIA*, *NFIB* and *SOX9,* convert fibroblasts into astrocytes (Caiazzo *et al*, 2015). A major advantage of this approach is that it requires < 3 weeks to generate functional astrocytes (Caiazzo *et al*, 2015).

The key consideration in evaluating the utility of lineage-converted cells is how similar they are to their primary counterparts and whether they reliably recapitulate disease phenotypes. We and others have shown that lineage-converted cells such as motor neurons (Son *et al*, 2011), dopaminergic neurons (Kim *et al*, 2011b) and pancreatic beta cells (Zhou *et al*, 2008; Li *et al*, 2014) express transcriptional profiles and DNA methylation patterns (Li *et al*, 2014) very similar to their primary targets. Although bulk analysis of lineage-converted cultures suggested that these cells retained more residual gene expression from the starting somatic cells than iPSC-derived cells (Cahan *et al*, 2014), single-cell studies suggest that this reflects heterogeneous cultures of converted and non-converted cells rather than "confused" or mixed-property cells (Li *et al*, 2014). Detailed epigenetic and single-cell analysis for more lineage-converted cell types will be required to rigorously assess the quality of these cells.

Recent studies have shown that lineage-converted cells are able to recapitulate disease phenotypes and provide insight into pathogenic mechanisms. Induced motor neurons derived from patients with *C9orf72* ALS degenerated rapidly in cell culture relative to control neurons (Wen *et al*, 2014a). In addition, they expressed dipeptide repeat proteins specific to the *C9orf72* form of the disease, indicating that they reproduce the phenotypes observed *in vivo* (Wen *et al*, 2014a). The authors used this model to determine that dipeptide repeat proteins induce toxicity in *C9orf72* ALS (Wen *et al*, 2014a). Meyer and colleagues used lineage conversion to generate astrocytes from sporadic ALS patients (Meyer *et al*, 2014). Astrocytes from the familial *SOD1* form of the disease induce the degeneration of motor neurons (Di Giorgio *et al*, 2008; Marchetto *et al*, 2008; Meyer *et al*, 2014), and the authors used this approach to assess the neurotoxicity of sporadic ALS patient astrocytes. They found that lineage-converted astrocytes from sporadic ALS patients consistently induced neurodegeneration, suggesting that an inherent disease mechanism is maintained in most sporadic patients. These studies demonstrate that lineage-converted cells are effective tools for studying CNS diseases.

Which approach would be better for disease modeling experiments—lineage conversion or iPSC-directed differentiation? It depends on several considerations. Has the disease affected cell type been generated *in vitro* previously? If not, how much is known about their developmental signaling or transcriptional profile? This would dictate which approach would be more effective to pursue. If both developmental signaling and transcriptional profiling are known, then lineage conversion might be a faster route to disease studies.

How many cells are required for the designed study? If a large number of cells are needed, for example for biochemical or epigenetic studies, iPSC-directed differentiation would be more suitable because the number of differentiated cells gets amplified at each progenitor step, whereas lineage conversion does not amplify the number of differentiated cells.

Are lineage-related cell types desirable or undesirable for the specific model? For example, non-cell autonomous neurotoxic stimuli from astrocytes are a key aspect of ALS (Di Giorgio *et al*, 2008; Marchetto *et al*, 2008; Meyer *et al*, 2014) and perhaps AD disease processes. It therefore would be informative to have patient-derived astrocytes included in these disease models. Most iPSC-directed differentiation protocols result in the production of multiple cell types within the same developmental lineage. In addition, several groups have started to develop three-dimensional protocols that produce several cell types from the same tissue that self-organize into structures that mimic the primary tissue (Eiraku *et al*, 2011; Nakano *et al*, 2012; Koehler *et al*, 2013; Koehler & Hashino, 2014). For certain tissues, such as the inner ear, this may enable more relevant disease models.

In contrast, lineage conversion strategies would not be expected to produce developmentally related cell types at a substantial rate nor 3D structures (unless a progenitor was formed that gives rise to self-organizing structures). But this would be desirable for screening applications where pure cultures of one neuronal subtype simplify high-throughput scaling and assay interpretation.

Overall, there are advantages and disadvantages to both approaches for the production of *in vitro* patient-derived cells depending on the disease and application. However, the emergence of the same phenotype in cells derived by both methods would certainly enhance confidence in the results.

A shift in focus: from developing neurons to maturing and aging them

A critical area that deserves further investigation is the maturity and aging of *in vitro* derived cells (Fig 2). We like to think that there are three stages we need to consider when setting up *in vitro* models of disease: the development, the maturation and the natural aging process of a neural cell type. While significant advancements have been achieved in generating and maturing neural cell types—either by directed differentiation or lineage conversion—little has been done in terms of affecting the aging of cells. For late onset diseases such as ALS, FTD, HD, PD and AD, it is possible that changes elicited by aging are required to induce the disease process. Age is the strongest risk factor for neurodegenerative diseases, and although there are rare cases with early onset presentation, the overwhelming majority of patients develop clinical symptoms in the later stages of their lives. The nature of the age-related risk remains largely unknown, and whether it arises from cell autonomous mechanisms or as a result of a systemic dysfunction remains to be determined. A number of studies support the notion that cellular epigenetic

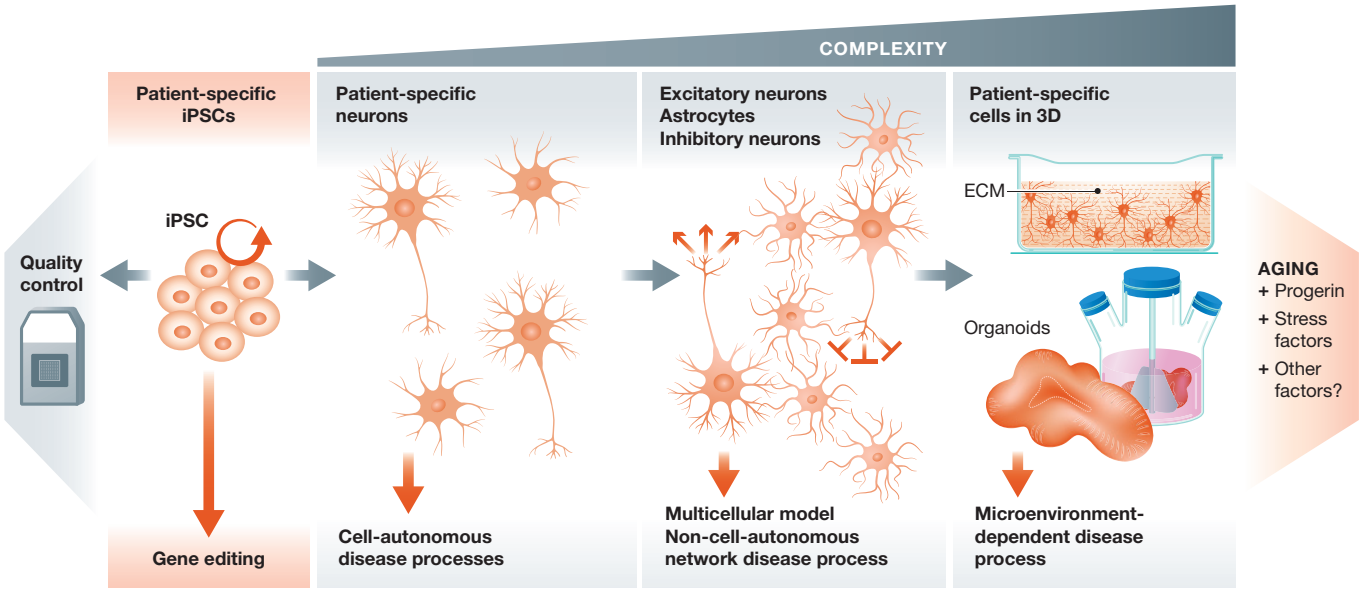

Figure 2. Developing stem cell-based models of neurological disorders.
Patient-specific iPSCs should be properly quality controlled for genomic integrity and pluripotent potential, while gene editing techniques allow for the generation of isogenic controls in cases where the disease-causing allele is known. Simple cell autonomous or more sophisticated multi-cellular and 3D disease models can be developed depending on the hypothesis being addressed. Neuronal maturity increases with the complexity of the cellular system, while methods for effectively aging neurons are lacking.

changes in the CNS correlate with aging. For example, recent work has demonstrated that profound changes in DNA methylation levels occur in the brains of mice with age (Lister *et al*, 2013), while aging oligodendrocytes lose their ability to effectively remyelinate damaged nerves (Ruckh *et al*, 2012). Importantly, under conditions of heterochronic parabiosis in mice, the effects on oligodendrocytes were reversible, implicating some aspect of epigenetic regulation.

Current studies suggest that the transcriptional and electrophysiological properties of both iPSC-derived and lineage-converted neurons are more similar to fetal neurons than adult (Son *et al*, 2011; Takazawa *et al*, 2012). It is likely that extrinsic factors present during normal development or aging are required to activate the maturation process. We and others have shown, for example, that the addition of primary astrocytes to lineage conversion cultures significantly improves the maturation of induced neurons (Son *et al*, 2011; Chanda *et al*, 2013; Wainger *et al*, 2015). Additional progress in generating more mature and aged cells will require a better understanding of the gene expression and functional changes associated with maturation and aging. This has been difficult to obtain for specific neuronal subtypes because of the scarcity of available human tissue. Efforts such as those of the Allen Brain Institute have shed some light on these markers, but future studies will need to analyze specific neuronal subtypes in order to be sure that differences between aged neurons and young neurons are truly due to aging and not different neuronal subtypes.

In addition to glial-derived factors, Rubin and colleagues recently showed that circulatory factors also contribute to the aging process in the CNS (Katsimpardi *et al*, 2014). They were able to identify a single factor, GDF11, which normally declines in expression with age. Interestingly, restoring GDF11 levels in old mice rejuvenated the proliferative and neurogenic properties of neural stem cells in the mouse (Katsimpardi *et al*, 2014). This raises the notion that there may be other factors that control the aging of neurons and could be exploited to regulate this process *in vitro*.

Studer and colleagues took a more intrinsic approach to inducing aging in iPSC-derived neurons by expressing *Progerin*, which is a mutant form of the Lamin A protein that causes accelerated aging phenotypes in humans (Miller *et al*, 2013). Expression of Progerin induced higher levels of DNA damage and mitochondrial reactive oxygen species in dopaminergic neurons derived from PD patients, which enabled the detection of PD-associated disease phenotypes such as dendrite degeneration, mitochondrial enlargement, Lewy body precursor inclusions and suppression of tyrosine hydroxylase expression (Miller *et al*, 2013). It remains unclear whether this approach induces the recapitulation of *bona fide* disease processes, but it represents a new line of targeted aging procedures.

From cell autonomy to more sophisticated systems

Neurons do not exist in isolation in the human nervous system. They form elaborate and functional networks with other neurons and also rely on a sophisticated microenvironment that is created by the interactions with other neural and non-neural cell types, which provide structural, metabolic and functional support as well as effective communication (Abbott *et al*, 2006). Glial cells, astrocytes, oligodendrocytes, microglia and endothelial cells exist in abundance in the nervous system and play vital functional roles. Glial cells buffer harmful ions, astrocytes provide nutrients and circulate neurotransmitters around synapses, oligodendrocytes form myelin sheaths around axons, microglia scavenge and degrade dead cells, and endothelial cells are important in maintaining the blood–brain barrier. Cell–cell interactions and the microenvironment as a whole might mediate important neuroprotective or neurotoxic activities in response to disease or injury. In fact, a number of studies over the last few years have clearly demonstrated that non-cell autonomous processes involving astrocytes, oligodendrocytes and microglia play a critical role in mediating disease progression and potentially onset in neurodegeneration including in ALS, HD, PD, prion disease, the spinal cerebellar ataxias (SCAs) and AD *in vivo* (Ilieva *et al*, 2009). The strength of utilizing iPSCs to study neurological disease is in their ability to generate a range of different cell types from the same genetic background (Fig 2). This allows for the assessment of how a specific genetic lesion, for example, might differentially impact neuronal subtypes. It also allows for a rational step-by step approach to assess how cellular interactions might contribute toward the evolvement of a disease-associated phenotype or a cellular response to stress.

The co-culture of spinal motor neurons with cortical astrocytes has previously been utilized in one of the first stem cell-based models of ALS to demonstrate how mutant or healthy astrocytes significantly compromised or maintained, respectively, the health of a pure population of motor neurons (Di Giorgio *et al*, 2008; Marchetto *et al*, 2008). The co-culture of cortical excitatory with cortical inhibitory neurons and the establishment of functional circuitry might be beneficial when studying epileptic syndromes. The clinical presentation of epileptic patients is the result of the functional control—or lack thereof—of a network of neurons, and recapitulating such a network could be an essential step toward the development of a cellular disease model. The importance of the local microenvironment in neuronal function and potentially dysfunction during disease is also relevant in the context of the three dimensionality that it creates. Neither the brain nor the spinal cord hosts isolated neurons surrounded by an entirely liquid trophic support (akin to culture media) in which nutrients, molecules and proteins can freely diffuse and float around. Recently, Kim, Tanzi and colleagues were able to successfully recapitulate amyloid-β plaques, and tau neurofibrillary tangles—the two pathological hallmarks of AD—in a single 3D human neural cell culture system (Choi *et al*, 2014). Although this system is not based on iPSCs and their cell lines expressed slightly elevated protein levels of *PSEN1* and *APP*, they designed a simple but innovative cell culture system with neurons grown embedded within a 0.3-mm layer of an extracellular matrix composed of BD Matrigel. This viscous layer reduced the diffusion of secreted amyloid-β and led to the accumulation of aggregated plaques. This is the first time this has been achieved in a cell-based *in vitro* system and demonstrates the importance of a 3D environment for disease modeling assays.

The recent description of cerebral organoids generated from human pluripotent stem cells and resembling the three-dimensional regional organization of a developing brain has created an exciting opportunity for iPSC-based disease modeling approaches (Lancaster *et al*, 2013). These brain-like structures, formed by the combination of external growth factor patterning and intrinsic and environmental cues, exhibit distinct regional identities that functionally interact and importantly recapitulate human cortical organization. The

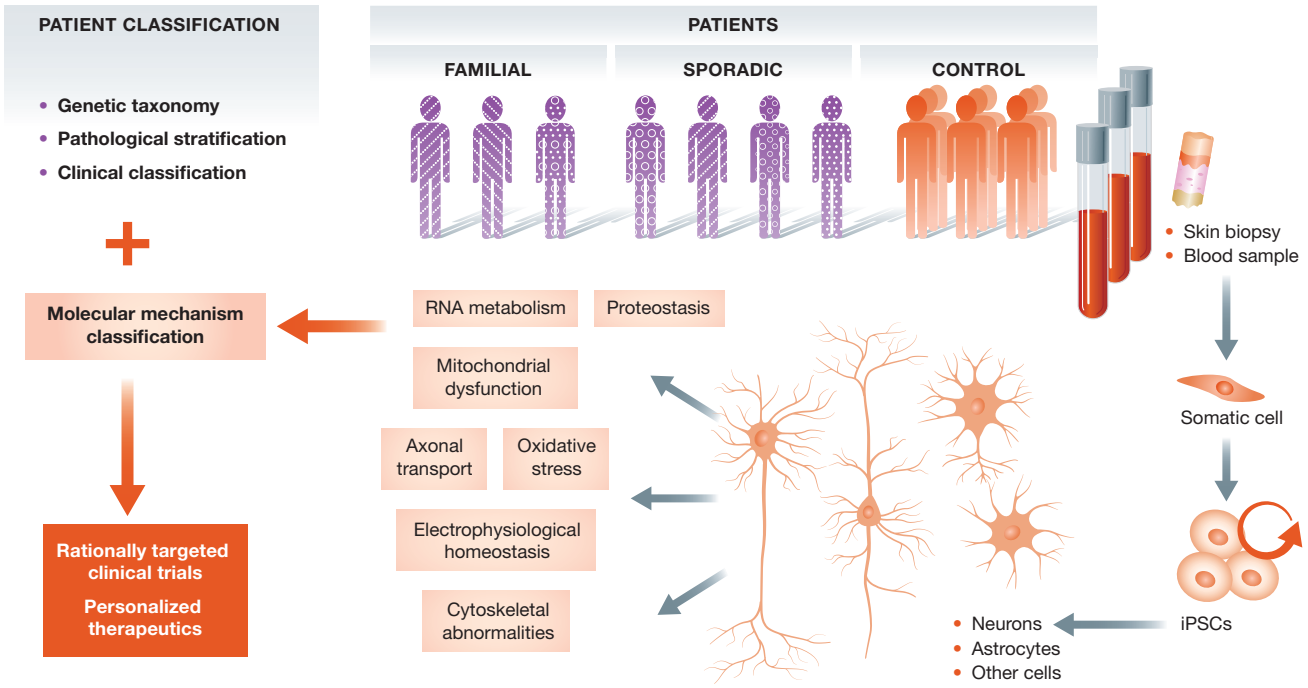

Figure 3. Patient stratification based on the molecular pathways that are affected.
Reprogramming and stem cell-based disease modeling can be utilized to address the level of heterogeneity by defining the molecular mechanisms that lead to disease in different patients. This novel classification of patients could lead to rationally targeted clinical trials and personalized therapeutic approaches.

authors utilized this method to study microcephaly and demonstrate that patient-specific organoids show premature neuronal differentiation and are only capable of developing to a smaller size. Importantly, mouse models have failed to effectively recapitulate these disease phenotypes for microcephaly, probably due to the dramatic differences in the development and regional organization of the brain as mice do not have an outer subventricular zone (SVZ). This system may be suitable for the study of other neurodevelopmental and neuropsychiatric syndromes in which there are moderate but crucial defects in cortical organization and function. This approach may also be useful in recapitulating human neurodegenerative models that primarily affect brain function as it may allow for the establishment of neuronal circuitry as well as biochemical networks.

Patient stratification based on molecular pathways affected

Neurological disorders including schizophrenia, ALS, PD, FTD and epilepsy are often characterized by a profound clinical and genetic heterogeneity, suggesting that they might represent a syndrome rather than a single nosological entity (Fanous & Kendler, 2005; Tremblay *et al*, 2013; Jeste & Geschwind, 2014). The variable combination of positive and negative symptoms in schizophrenia, the variable degree of upper and lower motor neuron dysfunction in ALS, the heterogeneity of cognitive symptoms in PD, the variable rate of progression in FTD and the differential response to anti-epileptic treatments in epileptic syndromes are some examples of the clinical diversity in neurological disorders. In addition, genetic studies in ALS, for example, have demonstrated that the disease can be caused by mutations in genes that encode proteins involved in diverse cellular functions ranging from RNA metabolism, vesicle transport, cytoskeletal homeostasis and the processing of unfolded proteins (Cleveland & Rothstein, 2001; Pasinelli & Brown, 2006; Sreedharan & Brown, 2013). While progress has been achieved in terms of genetic taxonomy, pathological stratification and the classification of patients based on their clinical presentation, little is known about how similar or different patients are, in terms of the molecular pathways that mediate their disease processes. Reprogramming technologies can be used to develop *in vitro* models of genetic and sporadic disease cases and effectively stratify patients, based on (i) the neuronal subtype that exhibits a disease-associated phenotype and (ii) the pathway that leads to this phenotype in each case (Fig 3). This approach may lead to the identification of overlapping disease mechanisms that will be broadly relevant and represent the best therapeutic opportunities, or toward a personalized approach to clinical trials and therapeutic treatments.

Concluding remarks

Tremendous progress has been achieved in our efforts to develop cellular models of neurological disease since 2007 and the initial description of induced pluripotency and the concept of cellular reprogramming (Takahashi *et al*, 2007). We are now able to generate a wealth of different neural subtypes, have created and characterized hundreds of patient-specific iPSCs and their neural derivatives, have developed efficient gene editing approaches and

are continuously establishing elaborate methods for the functional analysis of neurons. During the next phase in the field, it is imperative that the research community offers unrestricted access to cell lines, human samples and differentiation protocols, maintains close communication and attempts to further establish standards for the quality control of pluripotent stem cells and the neuronal subtypes that are utilized for disease modeling and drug screening experiments. It is also worth pointing out that broad collaborative efforts with substantial financial support need to take center stage in order to address important outstanding questions. What is the variation in the properties of neurons generated from a significant number of healthy individuals? Can we assess the broader relevance of phenotypes identified in genetic types of disease by monitoring hundreds of sporadic cases? Can we predict how patients will respond to a potential therapeutic treatment by studying their stem cell-derived neurons? Can we match an *in vivo* clinical trial with an *in vitro* iPSC-based clinical trial to monitor the correlation of outcome measures? The answers to these questions will help us conclude what are the capabilities and limitations of this promising technological tool. Despite the challenges that have arisen over the last few years, the community has responded with sustained effort and is steadily moving forward toward the development of systems that will have an impact in our efforts to understand and treat diseases that affect the nervous system.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Review

THE EMBO JOURNAL

Focus: Induced Pluripotency & Cellular Reprogramming

Generating human intestinal tissues from pluripotent stem cells to study development and disease

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Abstract

As one of the largest and most functionally complex organs of the human body, the intestines are primarily responsible for the breakdown and uptake of macromolecules from the lumen and the subsequent excretion of waste from the body. However, the intestine is also an endocrine organ, regulating digestion, metabolism, and feeding behavior. Intricate neuronal, lymphatic, immune, and vascular systems are integrated into the intestine and are required for its digestive and endocrine functions. In addition, the gut houses an extensive population of microbes that play roles in digestion, global metabolism, barrier function, and host–parasite interactions. With such an extensive array of cell types working and performing in one essential organ, derivation of functional intestinal tissues from human pluripotent stem cells (PSCs) represents a significant challenge. Here we will discuss the intricate developmental processes and cell types that are required for assembly of this highly complex organ and how embryonic processes, particularly morphogenesis, have been harnessed to direct differentiation of PSCs into 3-dimensional human intestinal organoids (HIOs) *in vitro*. We will further describe current uses of HIOs in development and disease research and how additional tissue complexity might be engineered into HIOs for better functionality and disease modeling.

Keywords endoderm; intestinal development; organoids; stem cells; tissue engineering

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Introduction

The human intestine averages 8 m in length (Hounnou *et al*, 2002) with an epithelial surface area of 250 m^2, roughly equivalent to the area of a tennis court. Epithelial surface area is amplified by the presence of villi, which are finger-like projections that protrude into the lumen of the intestine and microvilli, which are hair-like structures on the surface of absorptive epithelial cells (enterocytes). The epithelial morphogenesis that occurs in late embryonic and early postnatal stages also creates the crypts of Lieberkühn at the base of the villi. Crypts contain a specialized subset of intestinal cells including stem and progenitor cells that constantly renew the intestinal epithelium. In the mouse, the entire intestinal epithelium is turned over once every 5 days. There are two major cell types in the intestinal epithelium: the secretory cells and absorptive enterocytes. Comprising 90% of the epithelium, the enterocytes are responsible for the uptake and subsequent distribution of nutrients to the blood vasculature. The three secretory lineages are the hormone-secreting enteroendocrine cells (EECs), the mucus-producing goblet cells, and the Paneth cells in the crypt, which secrete antimicrobial peptides and help maintain the stem cell niche (Fig 3).

The intestine is comprised of two major segments, the small and large intestines. Proximally located, the small intestine is further subdivided into the duodenum, jejunum, and ileum, while the distal large intestine includes the cecum and colon. Morphologically, the proximal intestine can be distinguished by long, leaf-like villi, which become shorter and flatter in the colonic epithelium. These morphological changes reflect the unique functions that different segments of the intestine have in the breakdown of food and the uptake of nutrients. For example, the duodenum is responsible for acid neutralization and enzymatic breakdown of chyme from the antrum, while the jejunum is the main sight of total nutrient absorption. More distal, the ileum is responsible for bile reabsorption and the colon is the main sight of short-chain fatty acid (SCFA) production. These examples are only a few of the many roles each region plays in digestion and absorption of nutrients. For a more comprehensive look at the digestive physiology of the intestine, refer to Gastrointestinal Anatomy and Physiology: The Essentials (Reinus & Simon, 2014). The unique structure and function of each region of the intestine only highlights the complex underlying cellular differences. These include region-specific EEC subtypes as well as nutrient transporters and sub-epithelial populations that aid in the function of each region.

Infection, cancer, inflammation, intestinal motility disorders, and malabsorptive diarrhea are among the most prevalent human maladies. As a result, there have been many studies in model organisms aimed at understanding the development, homeostasis, regeneration,

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and pathologies of the intestinal tract. However, until recently there were no *in vitro* systems available that adequately modeled the development and pathophysiology of the gut. In the past few years, several approaches have been developed to generate human intestinal tissues *in vitro*. One approach used intestinal biopsies from patients, from which are derived from primary cultures of intestinal epithelium (Sato *et al*, 2011), which has been extensively reviewed elsewhere (Sato & Clevers, 2013). This review focuses on another approach using directed differentiation of human pluripotent stem cells (PSCs) into three-dimensional, human intestinal organoids (HIOs) (Spence *et al*, 2011b). HIOs, generated either from embryonic stem cells or from induced pluripotent stem cell lines (iPSCs), are being used to study many aspects of human intestinal development, including cell–cell interactions, the stem cell niche, patterning of the intestine, the differentiation of multiple cell types from intestinal stem cells (ISCs), and intestinal diseases. Below we will outline our current knowledge of intestinal development, function, and disease and discuss how HIO-based models are currently being used to understand these processes in a human context.

Development of the intestines *in vivo* and in pluripotent stem cell cultures

Endoderm formation

The directed differentiation of PSCs into intestinal tissue *in vitro* was achieved by temporally manipulating the signaling pathways that are involved in normal intestinal development *in vivo*. This begins with formation of the endoderm germ layer during gastrulation (see reviews Zorn & Wells, 2007, 2009; Spence *et al*, 2011a). At the molecular level, endoderm formation is a highly conserved process in mouse, fish, frog, and humans, requiring the TGF-beta family member Nodal. Nodal signaling launches a cascade of gene expression to both initiate gastrulation and direct cells into a mesoderm or endodermal fate. Adopting an endoderm fate involves downstream transcription factors including Sox17, Foxa2, Mix, Gata4/6, and Eomes (Sinner *et al*, 2006; Zorn & Wells, 2007, 2009). Activation of the Nodal pathway, with either Nodal protein or the Nodal mimetic Activin, can direct differentiation of mouse and human ES cells into definitive endoderm *in vitro* (Kubo *et al*, 2004; D'Amour *et al*, 2005) (Fig 1A). Cultures of definitive endoderm are multipotent and can give rise to numerous lineages including thyroid, lung, stomach, pancreas, liver, and intestine (Lumelsky *et al*, 2001; D'Amour *et al*, 2006; Cai *et al*, 2007; Cao *et al*, 2011; Spence *et al*, 2011b; Antonica *et al*, 2012; Longmire *et al*, 2012; Mou *et al*, 2012; Takebe *et al*, 2013; McCracken *et al*, 2014).

Anterior–posterior patterning of the endoderm and the intestine

At the end of gastrulation in chicks, mice, and humans, DE is a two-dimensional sheet of cells. DE is subsequently patterned along the anterior–posterior axis and undergoes morphogenesis into a primitive gut tube, from which all of the endodermal organs will arise. The anterior section of the tube is called the foregut and the posterior sections are the mid- and hindgut, which give rise to the small and large intestines. A number of signaling pathways are involved in posterior endoderm patterning, including Fgf, Wnt, retinoic acid (RA) (Huang *et al*, 1998; Stafford & Prince, 2002; Niederreither *et al*, 2003; Wang *et al*, 2006b; Bayha *et al*, 2009), and BMP. Multiple Wnt ligands are expressed in the posterior of gastrula stage vertebrate embryos and the role of Wnt/β-catenin signaling at this stage of development is to promote a posterior fate, while inhibiting an anterior fate in the endoderm (McLin *et al*, 2007; Sherwood, 2009). Likewise, FGF and BMP ligands that are expressed in the posterior act on the endoderm to promote a posterior fate in chick and mouse embryos (Roberts *et al*, 1995; Wells & Melton, 2000; Tiso *et al*, 2002; Kumar *et al*, 2003; Dessimoz *et al*, 2006; Rankin *et al*, 2011). WNT and FGF act in part by regulating the transcription of the Caudal homeobox (Cdx) family of transcription factors that play an evolutionarily conserved role to specify embryonic posterior fate. The Cdx family of genes in vertebrates (*Cdx1, Cdx2, Cdx4*) work in tandem with FGF signaling to promote the expression of posterior Hox genes *Hoxa7* and *Hoxb9* (Pownall *et al*, 1998; Ho *et al*, 1999; Zorn *et al*, 1999; Ehrman & Yutzey, 2001; Bel-Vialar *et al*, 2002; Spence *et al*, 2011b; Chen *et al*, 2013). These data indicate that Wnt/β-catenin and FGF signaling regulate the posteriorization of endoderm through activation of the *Cdx* genes.

The pathways that govern posterior fate of endoderm in embryos can also direct human pluripotent stem cell-derived definitive endoderm into a posterior fate (McCracken *et al*, 2011; Spence *et al*, 2011b; Chen *et al*, 2013). In one example, the combined activation of WNT and FGF was sufficient to direct human definitive endoderm (DE) cultures to adopt a hindgut identity as evidenced by the broad expression of *CDX2* (McCracken *et al*, 2011; Spence *et al*, 2011b). This work further identified that maximal and sustained *CDX2* expression required the activity of both FGF and WNT signaling and that posterior specification required 4 days of exposure to FGF/WNT (Spence *et al*, 2011b). Together, these findings demonstrate that embryonic studies inform our approaches to direct differentiation of PSCs into posterior cell types and highlight how PSC cultures can be used to identify new developmental mechanisms regulating endoderm organogenesis.

In addition to their involvement in establishing early posterior endoderm fate, FGF, WNT, BMP, and Cdx factors play multiple roles at later stages of intestinal development including intestinal elongation, segment identity, and growth. In mice, disruption of Wnt/β-catenin signaling by deletion of the transcriptional cofactors Tcf1 and Tcf4 ($Tcf1^{-/-};Tcf4^{-/-}$) resulted in a loss of *Cdx2* expression and ectopic *Sox2* expression at e13.5 (Gregorieff *et al*, 2004), a phenotype that is similar to deletion of *Cdx2* in the mid-gestation embryo (Grainger *et al*, 2010). Deletion of Wnt5a caused shortening of both the small and large intestines as well as disruption of apical–basal polarity of the epithelium (Yamaguchi *et al*, 1999; Cervantes *et al*, 2009). BMP ligands that are expressed in the posterior mesoderm act in a signaling loop with Sonic Hedgehog from the endoderm to regulate posterior *Hox13* gene expression in the hindgut of chick embryos (Roberts *et al*, 1995). Lastly, FGF10 signaling, mediated through FGFR2IIIb, is involved in the development of several regions of the intestine and embryos with defective FGF10 signaling have cecal defects and colonic atresia (Burns *et al*, 2004; Sala *et al*, 2006). It should be possible to manipulate these pathways to regulate regional identity of HIOs, although this has not yet been reported.

Intestinal morphogenesis in vivo *and in HIO cultures: the importance of 3-dimensional structure for function*

The initial transition from a two-dimensional sheet of DE to a three-dimensional tube occurs shortly after gastrulation. While BMP,

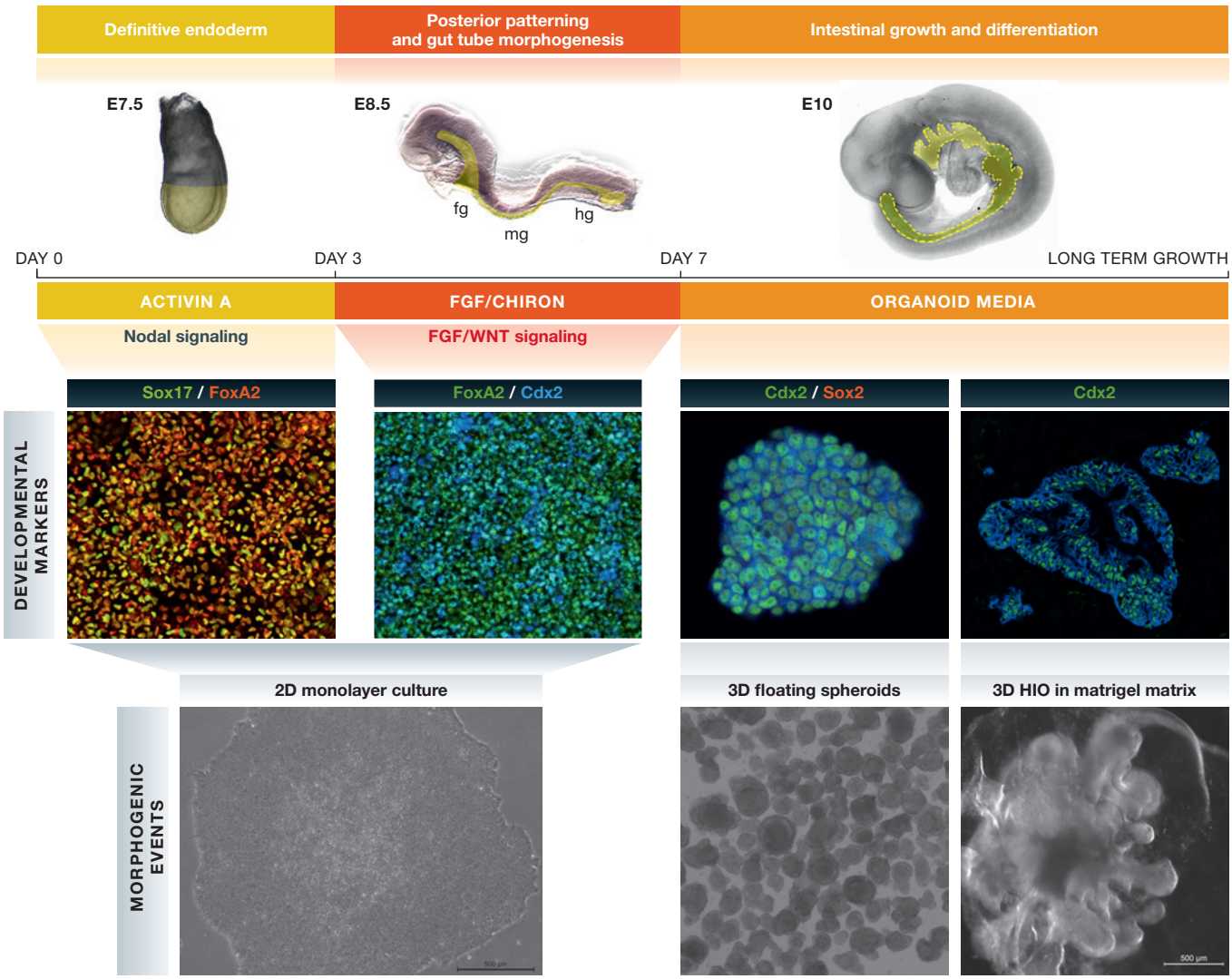

Figure 1. Generation of HIOs from PSCs.
Early stages of embryonic development guide the generation of HIOs from pluripotent stem cells into intestine. Definitive endoderm is established by the end of gastrulation (e7.5 in the mouse) and requires TGFβ molecule Nodal signaling. Differentiation of PSCs into definitive endoderm is achieved using the Nodal mimetic Activin A, which promotes differentiation into definitive endoderm as marked by Foxa2 (green) and Sox17 (red). The 2-dimensional sheet of DE folds to form a 3-dimensional primitive gut tube that is functionally subdivided along the anterior–posterior axis into a foregut, mid-gut, and hindgut (e8.5 in the mouse). Morphogenesis and posteriorization are induced in DE cultures via activation of FGF/WNT signaling. This causes the 2-D endoderm monolayers to undergo morphogenesis to form 3-D spheroids that additionally express the posterior marker CDX2. Spheroids that are grown in 3-D culture conditions for 4 weeks form HIOs that express CDX2 and have all major secretory and absorptive lineages of the fetal intestine. HIOs can be routinely split and re-plated in fresh 3-D matrigel every 2 weeks and have been passaged in this manner for up to a year.

WNT, and FGF pathways were known to function in endoderm posteriorization, how they act to regulate gut tube morphogenesis is poorly understood. It was known that non-canonical Wnt signaling may play a role in posterior development and intestinal morphogenesis, promoting elongation of the endodermal hindgut during development (Yamaguchi *et al*, 1999; Cervantes *et al*, 2009; Zhang *et al*, 2013). However, the first evidence that FGF and canonical WNT may cooperate during gut tube morphogenesis came from HIO studies. In this case, the combined activities of FGF4 and canonical WNT signaling promoted the morphogenesis of a 2-D sheet of DE into 3-D gut tube-like structures, termed gut tube spheroids (McCracken *et al*, 2011; Spence *et al*, 2011b). While the molecular basis for how FGF and WNT act to promote gut tube morphogenesis is not known, the ease of manipulating and imaging HIO cultures should allow for a better understanding of this process.

Another key event that occurs during hindgut tube formation is the assembly of a primitive mesenchyme around the gut tube epithelium. In HIO cultures, a mesenchymal layer assembles next to the gut tube epithelium during morphogenesis, although it is not known which cell types drive morphogenetic processes. The source of the mesenchyme in these cultures is a small population of mesoderm that expands in response to FGF4. It is also possible that FGF drives morphogenesis through directed cell migration of mesoderm, as has been observed in chick, where FGF4 and 8 act as

chemoattractants and repellants to direct the posterior migration of hindgut mesoderm (Lickert & Kemler, 2002; Yang *et al*, 2002; Kinkel *et al*, 2008; Bayha *et al*, 2009).

After gut tube formation *in vivo*, there are a number of morphogenetic events that result in the formation of intestinal villi with differentiated columnar epithelium (Spence *et al*, 2011a). The simple cuboidal epithelium of the e8.5 gut tube expands and transitions into a pseudostratified epithelium by e12.5 (Grosse *et al*, 2011). The epithelium transitions into a columnar type of epithelium with folds that extend into the lumen, the first stage in villus morphogenesis. Mesenchyme has been implicated in villus morphogenesis in mice, where PDGFRA-positive mesenchymal clusters influence the formation of villi through Hedgehog (Hh)-mediated signaling (Karlsson *et al*, 2000; Walton *et al*, 2012). In addition, the formation of smooth muscle layers and the resulting tension on the epithelium has been shown to be required for both early and later stages of villus morphogenesis in chickens (Shyer *et al*, 2013).

When grown in a three-dimensional matrix, PSC derived hindgut spheroids transition through very similar stages of intestinal morphogenesis *in vitro* resulting in the formation of human intestinal organoids (HIOs) (Fig 1). Similar to an e9 gut tube, day 0 spheroids start as a cuboidal epithelium surrounded by a primitive mesenchyme. However, after 14 days of culture, HIOs form a pseudostratified epithelium that resembles a e12.5 mouse intestine. By 28 days, HIOs contain columnar epithelium with protrusions into the organoid lumen, similar to early villus-like structures. As the epithelium transitions through these stages, the mesenchyme also differentiates into layers containing smooth muscle cells, subepithelial fibroblasts, and fibroblasts (Spence *et al*, 2011b). As with other organoid systems (reviewed in Lancaster and Knoblich, 2014), there appears to be some level of 'self assembly' involved in the formation of HIOs. This suggests that communication between the epithelium and the mesenchyme during HIO development may resemble the signaling that occurs during gut development *in vivo*. Additionally, these signals are still needed in adult intestinal organoid systems and the communication has been shown to influence the epithelial ISCs and their niche in these culture conditions (Ootani *et al*, 2009). Therefore, these organoids represent a new model to study human intestinal epithelial morphogenesis, stem cell differentiation, and cell–cell interactions.

Differentiation and function of intestinal subtypes

The major differentiated cell types in the intestine can be detected by e16.5 in the mouse, just after villus morphogenesis. These include the absorptive enterocytes, and two secretory cell types: goblet cells and enteroendocrine cells (Fig 3). Paneth and tuft cells arise after birth along with the formation of the crypts of Lieberkuhn and the establishment of adult ISCs. Absorptive enterocytes (small intestine) and colonocytes (large intestine) (Cheng & Leblond, 1974) function to transport peptides, ions, water, sugars, lipids, B12, and reabsorb bile acids. These cells contain microvilli on their apical surface to increase absorptive surface area and act as a protective brush border from the external environment (Mooseker, 1985). Though Notch signaling has been known to be the regulator between absorptive and secretory fates (Fre *et al*, 2005; Stanger *et al*, 2005; Zecchini *et al*, 2005), little is actually known about the specific genes required for enterocyte differentiation. Rather, the presence of Notch allows inhibition of Atoh1, a pro-secretory transcription factor, and loss of the Notch effector Hes1 promotes an enterocyte fate (Jensen *et al*, 2000; Yang *et al*, 2001; Shroyer *et al*, 2007; Noah *et al*, 2011).

In the adult, Atoh1 is required for all secretory lineages: goblet cells, enteroendocrine cells (EECs), Paneth cells, and tuft cells. Downstream of Atoh1, it has been suggested that goblet and Paneth may share a common progenitor given their dependence on Gfi1, Spdef, and LkB1/STK11 (reviewed in Noah *et al*, 2011). Goblet cells function to secrete mucins in order to protect the epithelium from damaging agents such as acidity and microbes in the lumen. Though these cells are present in both the embryonic and adult intestine, goblet cells may not differentiate the same way, as Klf4 has been shown to be required for embryonic goblet cell fate but not for those differentiated postnatally (Katz *et al*, 2002; Pellegrinet *et al*, 2011). Paneth cells develop postnatally in the mouse following crypt formation, but are present in humans by the first trimester (Underwood, 2012). Secreting antimicrobial peptides, Paneth cells regulate innate intestinal immunity, protecting the ISCs from pathogens, and shaping the gut microbiome. Wnt/beta-catenin signaling and its downstream target Sox9 are essential for the specification of Paneth cells (Bastide *et al*, 2007; Mori-Akiyama *et al*, 2007). Paneth cell dysfunction is associated with progression of necrotizing enterocolitis (NEC) in premature infants, though our knowledge is limited due to a lack of proper disease models (reviewed in Underwood, 2012). Lastly, markers such as Gfi1b have identified tuft cells; however, the development and function of these cells are poorly understood. There has been some speculation that tuft cells may help neutralize the acidic chyme in the duodenum and function in chemoreception and inflammation (reviewed in Noah *et al*, 2011; Gerbe *et al*, 2012).

Enteroendocrine cells (EECs) occupy approximately 1% of the total epithelium in the intestine but secrete upwards of 15 different types of hormones. These hormones have a variety of roles that affect metabolism, the digestive process, satiety, hunger, and glucose homeostasis. The endocrine role of hormones in regulating glucose metabolism and satiety is well studied (reviewed in Strader & Woods, 2005; Deacon & Ahrén, 2011; Crespo *et al*, 2009). In both mice and humans, specification of all EECs requires the basic helix-loop-helix transcription factor Neurogenin3 (NEUROG3) (Jenny *et al*, 2002; Wang *et al*, 2006a; López-Díaz *et al*, 2007; Pinney *et al*, 2011; Rubio-Cabezas *et al*, 2011). Loss of *NEUROG3* leads to a loss of all endocrine cells in the intestine and a subsequent failure in the absorption of nutrients. Mouse studies have demonstrated that the transcription factor Neurod1 is downstream of Neurog3 and is required for the development of CCK and secretin cells (Naya *et al*, 1997; Rindi *et al*, 1999) and Arx is required for proximal subtypes CCK, GIP, and secretin, and distal subtypes PYY, GLP-1, and neurotensin (Beucher *et al*, 2012). Other TFs such as Pdx1, Nkx2.2, Pax4, and Pax6 have been shown to influence the differentiation of EECs (Larsson *et al*, 1998; Desai *et al*, 2008; Chen *et al*, 2009; Wang *et al*, 2009). Interestingly, deletion of *Foxo1* from the small intestinal epithelium caused EECs to express and secrete insulin (Talchai *et al*, 2012), suggesting that intestinal and pancreatic endocrine hormones utilize similar transcriptional machinery. Moreover, ectopic expression of *MafA*, *Pdx1*, and *Ngn3* within intestinal cell crypts led to the formation of 'neo-islets' in the intestine (Chen *et al*, 2014). These papers also demonstrate that insulin-expressing cells in the

gut restore normal glucose levels in diabetic mice, suggesting that converted EECs may be used as a diabetes therapy in the future.

Using HIOs to study development of intestinal cell types

The columnar epithelium in HIOs contain all the main cell types of the human fetal small intestine including enterocytes, goblet cells, Paneth cells, enteroendocrine cells, and a proliferating progenitor zone (Spence *et al*, 2011b). HIO enterocytes are polarized, have a brush boarder, and have dipeptide transport activity resulting in absorption of dipeptides that are injected into the organoid lumen. Goblet cells actively secrete mucins into the lumen of the organoid, and EECs are capable of basal secretion of hormones (unpublished observation). HIOs contain proliferating progenitor cells that express *SOX9, KLF5, ASCL2,* and *LGR5*, which are localized at the base of villus-like structures. Moreover, the differentiated cell types in HIOs are present in similar proportions as their *in vivo* counterparts (Spence *et al*, 2011b). The fact that HIOs are functionally and architecturally similar to human fetal intestine, in combination with their ability to be passaged long term (Spence *et al*, 2011b; Talchai *et al*, 2012), makes them an excellent model to study human intestinal development, as well as neonatal physiology and disease. One example is seen in Spence *et al*, where the authors modeled loss of EECs that is caused by mutations in *NEUROG3* (Wang *et al*, 2006a; Pinney *et al*, 2011; Rubio-Cabezas *et al*, 2011). Knocking down of *NEUROG3* in HIOs similarly resulted in loss of EECs; moreover, ectopic expression of *NEUROG3* caused precocious differentiation of EECs. Because rodents lack some of the hormone subtypes, such as motilin that are found in humans, HIOs are an ideal model to study specification of endocrine cell lineages. Two recent examples used genetic manipulation of HIOs to convert human EECs into insulin-expressing cells (Talchai *et al*, 2012; Chen *et al*, 2014). Additionally, using CRISPR/Cas9 system, McGrath *et al*, have mutated the *NEUROG3* locus in PSCs, a tool that could provide means to model or correct human mutations causing rare intestinal diseases. Moreover, induced pluripotent stem cell lines from patients with genetic forms of intestinal diseases such as cystic fibrosis and intractable diarrhea in infancy (enteric anendocrinosis) could be used to generate HIOs to model the diseased phenotype, providing a means to understand the biology behind EECs and nutrient absorption as well as how to go about treating these patients.

Much is known about adult ISC maintenance and their ability to differentiate into all of the cell types of the adult intestinal epithelium. For example, Wnt/β-catenin signaling components are essential for maintenance of the ISCs in the crypt, whereas BMP signaling is involved in differentiation of progenitors. Wnt signaling has also been implicated in the formation of ISCs from a very early intestinal progenitor population (Fordham *et al*, 2013; Mustata *et al*, 2013); however, establishment of the niche and the exact mechanisms of action have yet to be elucidated. As the first *in vitro* model of human intestinal development, HIOs are a potentially valuable tool to identify mechanisms by which fetal intestinal progenitors give rise to differentiated cells types as well as intestinal stem cells. Manipulation of signaling pathways with small molecules, genetic manipulation of stem cell factors, and real-time imaging using cell-specific reporter lines makes HIOs a powerful new tool to study ISC development.

Maturation of PSC-derived HIOs *in vivo*

Immaturity is a common feature of human PSC-derived cells and tissues and has been reported for liver hepatocytes, pancreatic beta cells, and cardiomyocytes (Mummery *et al*, 2002; Shirahashi *et al*, 2004; D'Amour *et al*, 2006). Despite the remarkable architectural and cellular similarities to fetal intestine, HIOs do not have molecular and structural features of the adult intestine. For example, the villus-like structures that form are short and fetal-like, there are no crypts and there is no evidence for the presence of functional ISCs. Possible reasons for an immature phenotype could be that 4-week-old HIOs have not had time to mature. HIOs can be cut into smaller pieces and passaged *in vitro* for up to a year, and older HIOs acquire a somewhat more mature phenotype (Spence *et al*, 2011b; Talchai *et al*, 2012). Another cause of immaturity could be missing cell types such as vascular cells or systemic factors that could only be found *in vivo*. *In vitro*, the maximal size of an HIO is about 2 mm, which is likely due to limitations of oxygen and nutrient diffusion.

To overcome the absence of a functional vasculature system *in vitro*, a recent study described the growth of HIOs following engraftment under the kidney capsule in mice (Watson *et al*, 2014). Engrafted HIOs became vascularized by the host endothelial cells and after 6 weeks grew into intestinal tissue ranging from 1 to 2 cm in size. The resulting tissue had mature villi with a full complement of highly differentiated adult cell types and maintained regional intestinal identity in the host environment (Watson *et al*, 2014). HIOs matured *in vivo* also had *bona fide* crypts that contained Paneth cells and cells expressing ISC markers LGR5 and ASCL2. Moreover, isolated crypts had ISC activity in that they gave rise to expandable enteroids in ISC growth conditions (Sato *et al*, 2009). Lastly, HIOs grown under the kidney capsule were able to proliferate in response to small bowel resection (SBR), resulting in expansion of crypts and lengthening of villi, establishing this as a model to identify humoral factors regulating intestinal adaptation following SBR. These data suggest that HIOs engrafted *in vivo* can be used to study intestinal development and maturation, for example, the processes involved in specification of ISCs and the niche (Fig 2).

Tissue complexity of the intestine

In addition to the diversity of epithelial cell types that formed in HIOs *in vivo*, the HIO mesenchyme differentiated into three stratified layers of smooth muscle, a smooth muscle adjacent to the mucosa, and two additional layers of smooth muscle, as well as myofibroblasts and stromal fibroblasts (Watson *et al*, 2014). Moreover, HIOs grown *in vivo* develop a vascular system that is largely derived from the host. However, there are other cell and tissue types missing from HIOs that are normally found in the intestine, including mesenteric lymph nodes called Peyer's patches, an enteric nervous system, epithelial M cells, and Brunner's glands. In addition, HIOs lack microbes that are normally found in the intestine (microbiome), which play essential roles in digestion and metabolism. Therefore, for fully functional intestinal tissues to be derived from PSCs, it may be necessary to incorporate these missing components (Fig 3). Below we will discuss the embryonic development of some of these cell types and how HIOs could be engineered to contain additional complexity.

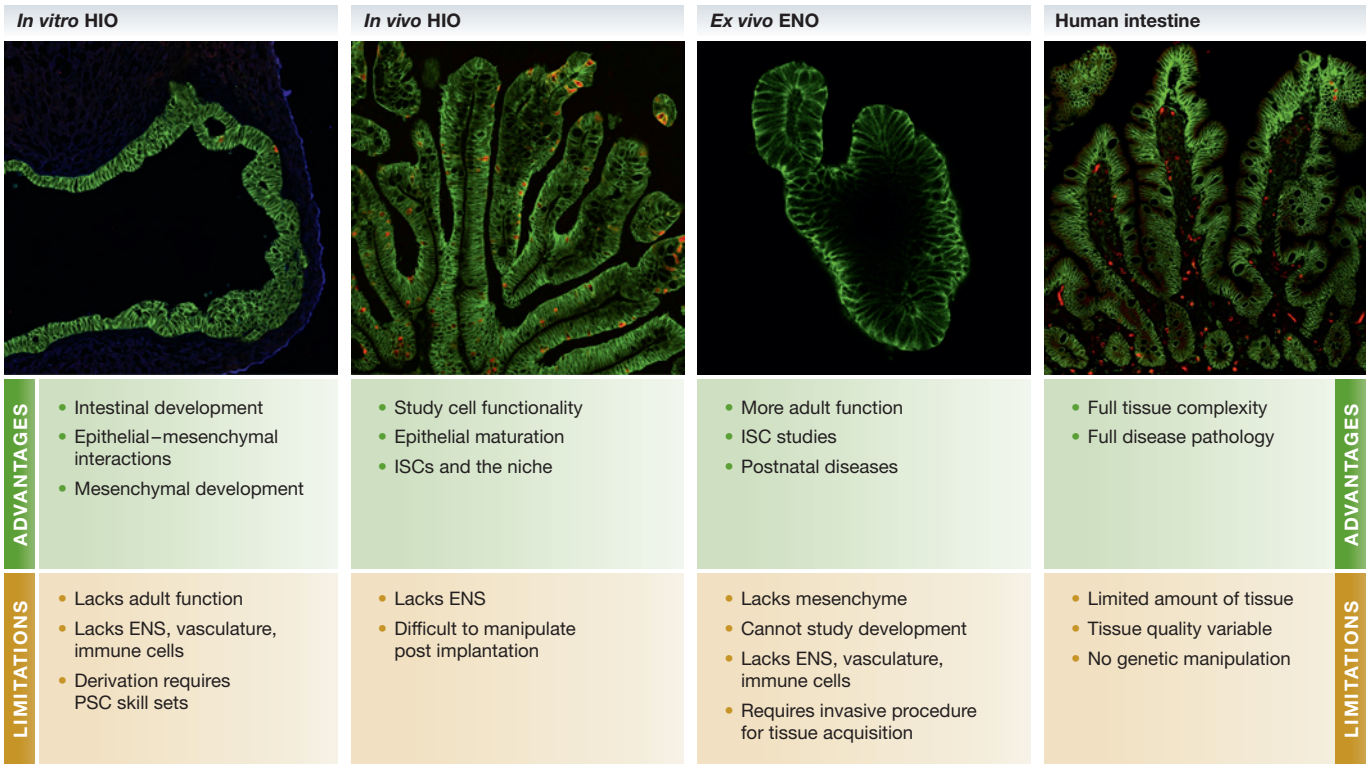

Figure 2. Unique advantages and limitations of current intestinal organoid systems.
HIOs grown *in vitro* are similar to fetal intestine and thus are uniquely suited to study human intestinal development using both genetic and pharmacologic manipulation of genes and signaling pathways. HIOs also contain mesenchyme allowing for studies of epithelial–mesenchymal interactions. HIOs grown *in vivo* contain intestinal stem cells, crypts, villi, and differentiated smooth muscle layers and can be used for functional studies of human intestine. Primary cultures of human intestinal crypts (called enteroids (ENOs) or adult intestinal organoids) have more mature characteristics and can be used to study intestinal stem cell biology. Lastly, direct analysis of patient tissues provides an important snap shot of normal and pathological states of patient tissue; there is no opportunity for experimental manipulation. Both *in vitro* systems share many advantages in studying intestinal biology and disease modeling.

The main role of the enteric nervous system (ENS) of the intestine is to coordinate peristalsis for unidirectional movement of luminal contents. There are more than 100 million neurons in the human ENS (for review, see Goldstein *et al*, 2013), which includes motor, sensory, and interneurons. Developmentally, the enteric nervous system arises from a multi-potent population of cells called neural crest cells (NCCs) that migrate into the mesenchyme of the foregut (Lake & Heuckeroth, 2013). From here, they migrate rostrocaudally and proliferate to fully colonize the gut by e14.5 in the mouse and week seven of human gestation. Migration and proliferation is driven in part by signals from the mesenchyme, including GDNF, Netrin, and BMP signaling that act via receptors on NCCs (Cacalano *et al*, 1998; Wu *et al*, 1999; Manié *et al*, 2001; Zhu *et al*, 2004). Enteric NCCs (ENCCs) will give rise to up to 18 different subtypes of neurons and glia that will form the outer myenteric plexus and the inner submucosal plexus. These plexuses are embedded within submucosal smooth muscle layer and the circular and longitudinal layers and coordinate with the interstitial cells of Cajal (ICCs) that serve as a pacemaker for peristalsis (for review on ICCs see Huizinga & Chen, 2014). In addition, the ENS and the gut epithelium are in constant communication to maintain gut barrier function, control endocrine secretions, and regulate blood flow. For example, gut hormones such as 5'HT directly stimulate ENS neurons to stimulate peristaltic movements, secretion, and vasodilation of the gut (reviewed in Mawe & Hoffman, 2013). Secretin and CCK have been shown to stimulate sensory neurons, which then act on the pancreas to release HCO^- into the duodenum as well (Nathan & Liddle, 2002; Li, 2007). Ghrelin also acts through a neuronal pathway, in order to stimulate the hunger response in basal conditions (Andrews, 2011) via a brain–gut axis.

The ENS also regulates the barrier function of the epithelium. The intestinal epithelial barrier (IEB) is composed of apically located tight junctions, as well as adherent junctions that confer mechanical strength, regulate permeability, and anchor the epithelium. Through regulation of protein subunits of epithelial junctions, the ENS controls intestinal paracellular and transcellular permeability changes that occur during postnatal development and injury (reviewed in Neunlist *et al*, 2013). Neuromediators such as acetylcholine and substance P increase permeability and cell proliferation, whereas VIP decreases it (Goode *et al*, 2003; Toumi *et al*, 2003; Cheng *et al*, 2008). In addition, glial cells secret S-nitrosoglutathione (GSNO) in response to bacterial infection, resulting in down-regulation of CDC42 and a decrease in cytoskeletal recruitment (Flamant *et al*, 2011). This increases the barrier resistance to bacteria such as *Shigella flexneri*. Lastly, in cases of intestinal injury, enteric glial cells secrete proEGF, which acts on enterocytes to increase

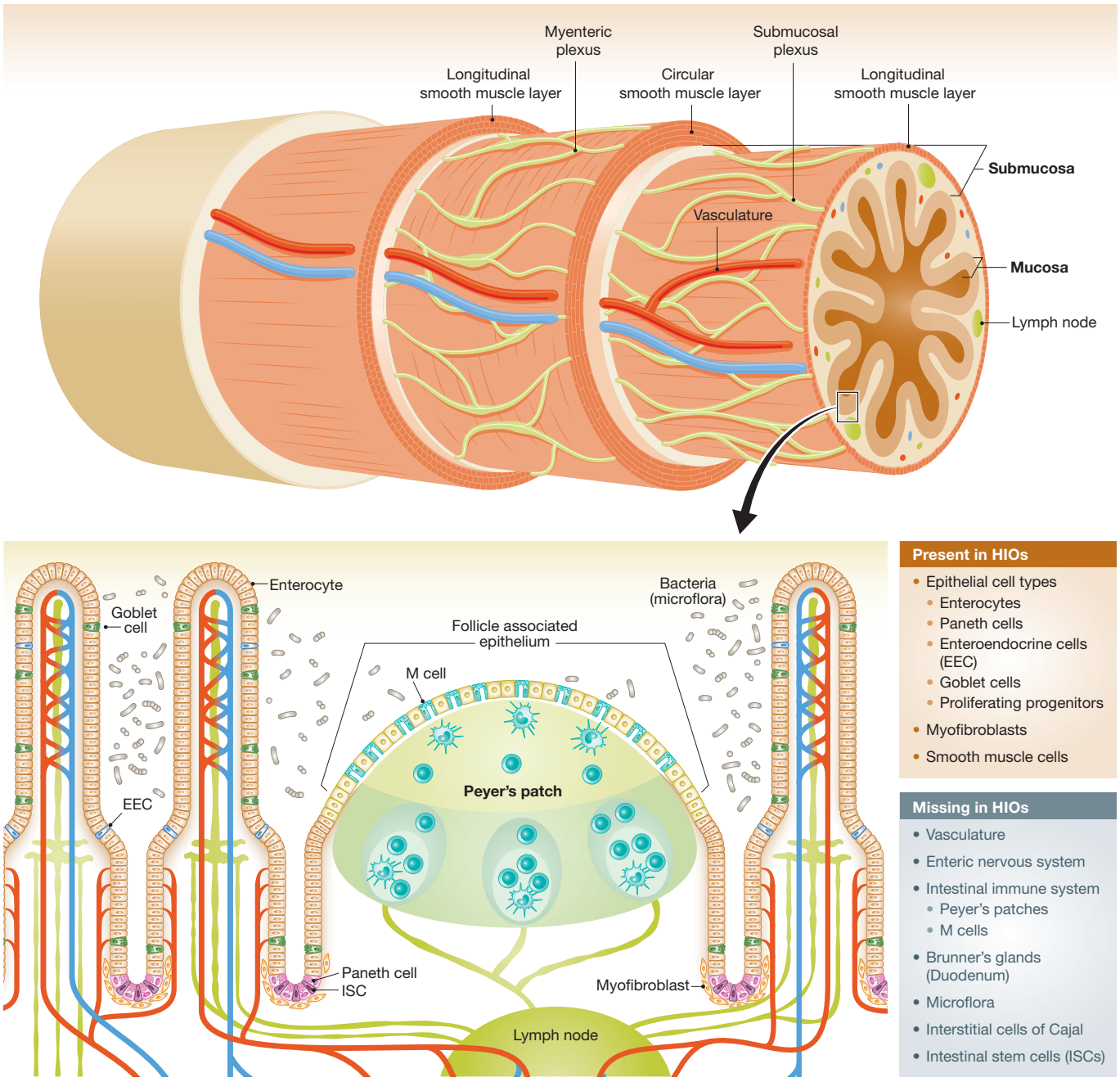

Figure 3. Complexity of the intestine.
The intestine is an assembly of multiple cell types from all 3 germ layers. The endoderm gives rise to the epithelium of the intestine (insert). This region is known as the mucosa. This includes cell subtypes such as enterocytes (yellow), enteroendocrine cells (blue), Paneth cells (pink), and goblet cells (green). Capillaries and blood vasculature as well as myofibroblasts and Peyer's patches/M cells control transport of nutrients, epithelial integrity, and immune responses, respectively. The epithelium is circled by three smooth muscle layers that are arranged in alternating longitudinal–circular–longitudinal orientations. Embedded within the muscle is the enteric neural system (ENS), the submucosal plexus and the myenteric plexus. While HIOs contain most of these epithelial and mesodermal cell types, other cell types such as neurons will need to be incorporated in order to more accurately mimic *in vivo* development and adult function. In addition, microbial components can be injected into HIOs to study immunity, gut barrier functions, and metabolism by the microbiome.

focal adhesion kinase (FAK) (Van Landeghem *et al*, 2011). FAK has major roles in intestinal cell motility, which allows cell spreading over the damaged area and increased mucosal healing.

A close association of the vasculature with the gut epithelium is essential for proper absorption and systemic transport of nutrients and hormones. The intestinal vasculature consists of 2 large mesenteric arteries that branch into arterioles, which span from the muscularis layer down to single villi. In addition, a separate arteriole branch exits the villi and submucosal layer in the opposite direction, to transport absorbed nutrient and intestinal hormones throughout

the body (reviewed in Nankervis *et al*, 2008). The temporospatial development of the vascular and enteric nervous systems suggests that their formation may be coordinated. Imaging studies of the ENS and vasculature during murine development (Hatch & Mukouyama, 2014) indicate that formation of the primary capillary plexus occurs in a similar window of time of initial neural crest colonization of the anterior gut tube, at e9.5. As the gut lengthens and grows, the capillary plexus is remodeled and gives rise to vessels that penetrate through the developing muscle layers to establish association with the epithelium, giving rise to stereotypical branching patterns and distinct arteries and veins by e15.5. During these stages, neural precursors also divide, differentiate, and remodel into the two neuronal plexuses of the ENS (Hatch & Mukouyama, 2014). The similarity between where and when the vascular and neural plexuses develop suggest they may rely on signals from each other in order to properly invade the gut. We will discuss below how endothelial and ENS progenitors might be incorporated into HIOs to study the development of these tissue types.

Additional important structures in the intestine include Brunner's glands, gut-associated lymphoid tissue (GALT), and Peyer's patches (PP). Brunner's glands are located in the submucosa of the proximal duodenum and are primarily composed of mucus-producing cells, which secrete type III mucin glycoproteins (Krause, 2000). They also secrete limited amounts of EGF, trefoil factors, surface-active lipids, and bactericidal factors that provide additional support and protection to the duodenum. Specialized gut-associated lymphoid tissue (GALT) is made up of mesenteric lymph nodes (mLN) as well as specialized lymphoid patches, called Peyer's patches (PP), which in combination function to sense antigens within the intestinal lumen and mediate immune responses. PPs are located in the lamina propria of the ileum and are comprised of follicle areas containing B and T lymphocytes as well as dendritic cells, macrophages, and antigen-sensing M cells (reviewed in Eberl & Lochner, 2009; Gibbons & Spencer, 2011). After birth, colonization of the gut by microbes is believed to further mature M cells and PPs (Bauer *et al*, 2006). However, how the immune system of the gut distinguishes between the elimination of potential pathogens and maintenance of normal gut flora remains a vastly open area of research. It is not known whether HIOs *in vitro* contain any of these structures but growth of HIOs *in vivo*, with the introduction of the hosts vasculature and immune cells, may allow for formation of GALT and Peyer's patches.

The intestinal microflora

Intestinal function depends on an extensive array of commensal and symbiotic bacteria that mediate a number of positive symbiotic host–bacterial interactions. At birth, the intestine is sterile but over time becomes populated with over 1,000 different species of microbes (Lozupone *et al*, 2012) that have very important roles in digestion, stimulating the immune system, acting in various metabolic processes, and providing a barrier against disease-causing pathogens. It is estimated that the number of bacteria in the gut outnumbers the cells of our body by 10:1 (Sekirov *et al*, 2010). The types of microbes that colonize the gut in postnatal stages of development are highly variable (reviewed in Putignani *et al*, 2010) and are influenced by antibiotic exposure during pregnancy, bacteria in the amniotic fluid, hygiene, weight gain during pregnancy, and route of birth (vaginal versus cesarean). Dysbiosis of bacterial/epithelial interactions can correlate with the development of intestinal disorders such as irritable bowel syndrome (IBS) (Li *et al*, 2014a; Wang, 2014), and obesity and diabetes (Turnbaugh *et al*, 2006; De La Serre *et al*, 2010; Han & Lin, 2014). Introduction of bacteria into the lumen of HIOs would allow for methodical screening for how patient microbiomes can impact the host epithelial maturation and function.

Engineering additional functionality into human intestinal organoids

HIOs are a highly complex organoid system containing over 10 different cell types. However, many of the cell types described above are lacking, and the generation of fully functional intestinal tissue from PSCs will ultimately require incorporation of these cells. For example, though HIOs contain mesenchymal cell types including smooth muscle, fibroblasts, and myofibroblasts, they do not have HIO-derived vascular endothelial cells and it is not clear whether they contain interstitial cells of Cajal. Moreover, sub-mucosal populations of epithelium such as Brunner's glands have not been identified in HIOs, and it is not clear whether the epithelium is competent to generate these specific cell populations. One of the most critical components lacking in HIOs is the enteric nervous system/ENS, and we will use this as an example of how one might engineer additional complexity into HIOs. The ENS is an ectodermal cell type deriving from neural crest cells/NCCs. Given that NCCs are not present in HIO cultures, the only way to generate HIOs with an ENS would be to mechanically incorporate NCCs into HIOs at some stage during their differentiation. First, one would have to generate NCCs *in vitro* and there are established protocols for the generation of cranial NCCs from PSCs (Chambers *et al*, 2009; Menendez *et al*, 2011, 2013; Kreitzer *et al*, 2013). However, vagal/sacral NCCs, which are the source of the ENS, have not yet been generated *in vitro*. Therefore, cranial NCCs would have to be posteriorized to adopt a more vagal/sacral NCC fate and then incorporated into the developing HIOs. As with all PSC differentiation protocols, the best approach has always been to follow normal developmental timing. Given that NCCs migrate into the gut tube mesenchyme shortly after gut tube formation, incorporating NCCs shortly after gut tube/spheroid formation would be the most biologically logical choice. If NCCs could be incorporated into HIOs, it would allow for *in vitro* analysis of ENS migration, proliferation, and differentiation of different neural cell types and assembly into the two neural plexuses. Little is known about how NCCs interact with the mesenchymal and endodermal cells during intestinal development and how the mature ENS modulates epithelial and immune function. Importantly, building additional functionality into HIOs would allow for studies of complex diseases like Hirschsprung's disease, irritable bowel syndrome, cancer, and infection via bacterial pathogens, as discusses below.

Current and future uses of HIOs for intestinal disease research

Intestinal diseases are caused by parasitic, genetic, environmental, and sometimes a combination of these factors and affect

60–70 million people in the USA. In the following section, we address some of the major disorders of the intestine and how organoids are being used to uncover new disease mechanisms (Fig 4).

Infectious diseases

There are two examples where HIOs have been previously used to study enteric pathogens. In one example, the microorganism *C. difficile* was injected into HIOs and resulted in epithelial barrier dysfunction, similar to *in vivo* infections (Leslie *et al*, 2014). A second study involved rotovirus, for which current cell culture models were inadequate because they did not support viral replication. In contrast, HIOs supported the rotovirus life cycle and now represent a relevant model system to study the pathology of this gastrointestinal viral infection (Finkbeiner *et al*, 2012). Moreover, the use of PSC reporter lines that mark specific subpopulations of cells, such as a recently reported LGR5-GFP line (McCracken *et al*, 2014; Watson *et al*, 2014), would allow for identification of which cell types are targeted by these various pathogenic agents. There are, however, many enteric pathogens for which no good human model exists. *Gardia duodenalis* (*G. duodenalis*) is a protozoan parasite that inhabits the upper small intestine and causes the disease human giardiasis. The parasite spreads via a fecal–oral route, most often from contaminated food or water. During infection, the parasite induces apoptosis in villus epithelial cells,

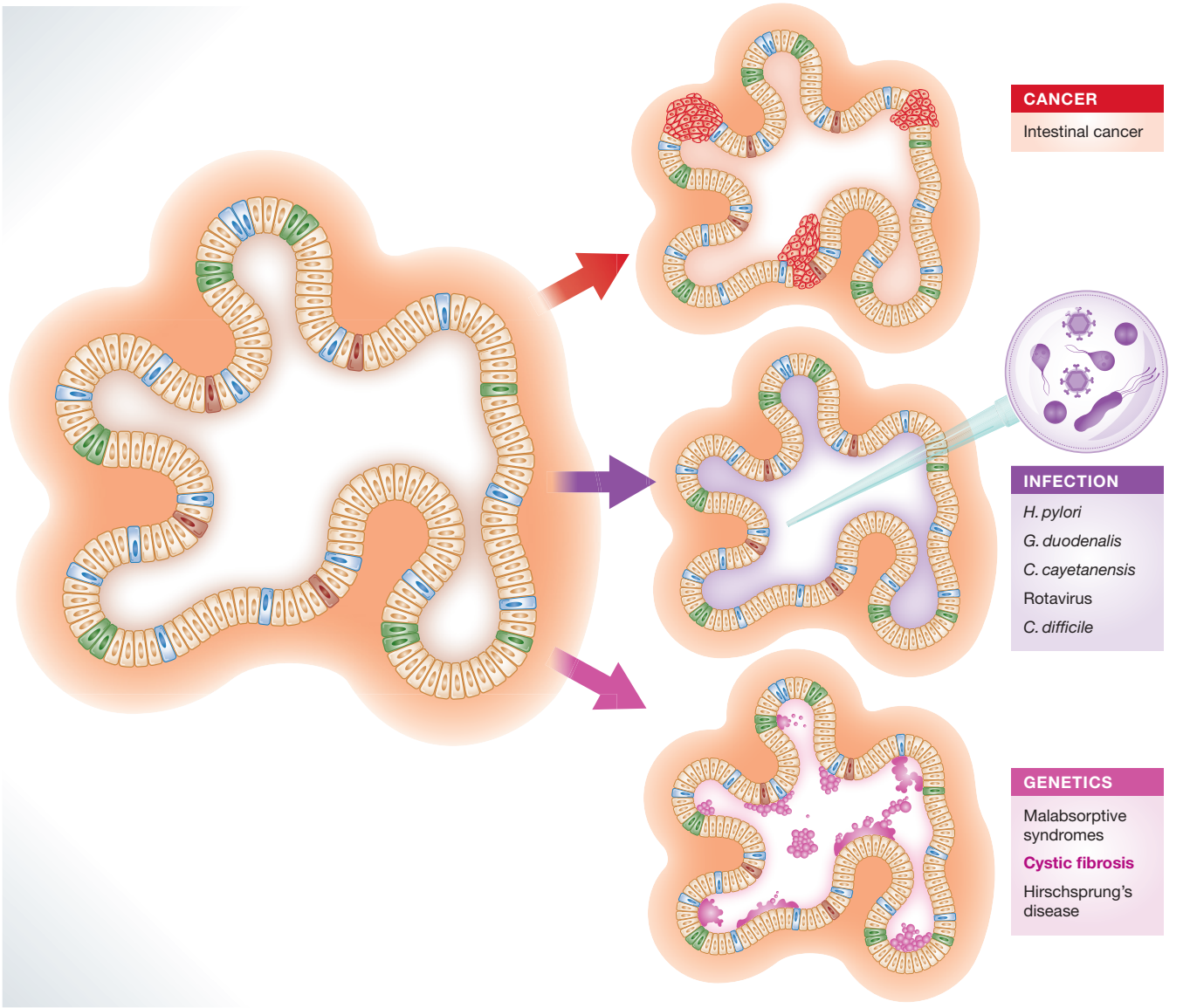

Figure 4. Using HIOs to study intestinal development and model diseases.
The *in vitro* culture of human intestinal tissues provides new opportunities to study cancer, infection, and genetic diseases. This is essential since certain human diseases, such as colon cancer, and enteric pathogens, such as *C. cayetanensis*, are not effectively studied in other model systems. This makes studying human intestinal disease very difficult. In addition, HIOs could be used as a rapid primary screen for drug absorption and GI toxicity, the most common off-target effect of new drugs.

hyperplasia of crypts, as well as intestinal barrier dysfunction (reviewed in Halliez & Buret, 2013). So little is known about the pathophysiology of this parasite that it has been recently put on the World Health Organization's Neglected Disease Initiative (World Health Organization, 2004; Savioli *et al*, 2006). *Cyclospora cayetanensis* (*C. cayetanensis*) is another food- or water-borne parasite for which the only known host is the human small intestinal epithelium. Considered an emerging pathogen, *C. cayetanensis* infection and outbreaks are quite difficult to diagnose (Eberhard *et al*, 1997; Mansfield & Gajadhar, 2004). For pathogens such as these, human intestinal organoid systems are the only known option for understanding the biology of infection.

Genetic diseases

There are several common genetic diseases that affect intestinal epithelial function. For example, autosomal recessive mutations in the cystic fibrosis trans-membrane regulator (*CFTR*) gene lead to decreased Cl^- ion movement through epithelial cells (Riordan *et al*, 1989), hyperabsorption of fluids, and subsequent dehydration of epithelial cells. While cystic fibrosis (CF) has pathology in many epithelial tissues, in the intestine it affects the motility of luminal contents, causes bacterial overgrowth, increases inflammation and ulceration, as well as leads to the accumulation of mucus. Aberrant secretion and altered mucus content often leads to intestinal blockage and malabsorptive phenotypes. Current small molecule therapies for CF are only modestly effective, and human intestinal organoids represent a new system for high-throughput, functional screens for new therapeutic compounds. For example, intestinal organoids derived from surgical biopsies of CF patients had a measurable disease phenotype that was corrected with an existing CF drug (Dekkers *et al*, 2013). Several groups have derived iPSC lines from CF patients (Mou *et al*, 2012), and HIOs from iPSC lines with specific CF mutations could uncover developmental reasons for why different CF mutations associate with more severe intestinal disease.

Hirschsprung's disease (HSCR) is characterized by absence or paucity of neurons in the intestine and occurs in about 1:5,000 live births (McKeown *et al*, 2013). Depending on the severely, this can cause severe megacolon, chronic constipation, and a distended abdomen, requiring surgical resection of the aganglionic region. Several mutations have been shown to affect NCC migration, proliferation, and innervation including GDNF-GFRA1-Ret signaling (reviewed in McKeown *et al*, 2013), *Endothelin 3*, and *Sox10* (Kapur, 1999; Southard-Smith *et al*, 1998; Cantrell *et al*, 2004). Single allele mutation of *RET* in humans can cause the disease; however, this is not the case in mouse (Schuchardt *et al*, 1994; Gianino *et al*, 2003). An HIO-based approach might be an effective way to study the molecular basis of HSCR, but would require the incorporation of the neural crest-derived ENS progenitor cells. In support of this concept, adult neural progenitors from human or mouse healthy intestinal tissue have been incorporated into aganglionic fetal intestinal explants in culture and *in vivo* mouse models of HSCR (Natarajan *et al*, 1999; Bondurand *et al*, 2003; Dupin & Sommer, 2012). When incorporated, these neural progenitors differentiated and reduced the severity of the HSCR phenotype, yet formation of a complete neural plexus was not observed. A similar approach could be used with patient-specific NCC-derived neural progenitors and HIOs.

There are several additional multifactorial diseases that affect the intestine and colon. Inflammatory bowel disease (IBD), which is chronic inflammatory condition, can affect the small intestine, as with ulcerative colitis (UC), or large intestines, as with Crohn's disease (CD). Immunosuppression is the primary therapy, yet it is not known what triggers the abnormal immune response. Studies suggest that alterations in the microbiome may play a role in IBD (Li *et al*, 2014a; Wang, 2014). The ease of manipulating HIOs makes them amenable for IBD studies. For example, it has been shown that organoids can be injected with bacteria and this can be used to study early disease inductive events (McCracken *et al*, 2014). Moreover, it should be possible to study inflammatory responses by incorporating macrophages from healthy and IBD patients into HIO cultures. It may also be possible to repair damage caused by IBD using human colonic stem cells. In mice, it has been shown that engraftment of $Lgr5^+$-derived colonic epithelium improves acute colitis and resulted in repopulation of damaged colonic tissue (Yui *et al*, 2012). Organoids derived from adult mouse intestine have been used recently to model colon cancer (Li *et al*, 2014b). While it is not clear whether HIOs, which are fetal in nature, could be used to model intestinal cancers, HIO tissues that are matured *in vivo* have adult characteristics and could be used to induce oncogenic mutations as was done in mouse (Li *et al*, 2014b).

Concluding remarks

The intestine is a remarkable organ that incorporates cell and tissue types from all three embryonic germ layers to carry out its absorptive, secretory, endocrine, barrier, and mechanical functions. Disruption of any of these myriad of intestinal functions can result in disease, underlying the need for human model systems to study various pathologies. The complexity of this organ has made it difficult to derive intestine from PSCs using traditional 2-D approaches, thus necessitating the development of 3-D, organoid-based systems. While the current HIO system has provided new opportunities for developmental and disease research, tissue engineering additional complexity into HIOs will allow for increased functionality and expanded utility.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Review

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Focus: Induced Pluripotency & Cellular Reprogramming

Reprogramming of human cancer cells to pluripotency for models of cancer progression

Jungsun Kim & Kenneth S Zaret*

Abstract

The ability to study live cells as they progress through the stages of cancer provides the opportunity to discover dynamic networks underlying pathology, markers of early stages, and ways to assess therapeutics. Genetically engineered animal models of cancer, where it is possible to study the consequences of temporal-specific induction of oncogenes or deletion of tumor suppressors, have yielded major insights into cancer progression. Yet differences exist between animal and human cancers, such as in markers of progression and response to therapeutics. Thus, there is a need for human cell models of cancer progression. Most human cell models of cancer are based on tumor cell lines and xenografts of primary tumor cells that resemble the advanced tumor state, from which the cells were derived, and thus do not recapitulate disease progression. Yet a subset of cancer types have been reprogrammed to pluripotency or near-pluripotency by blastocyst injection, by somatic cell nuclear transfer and by induced pluripotent stem cell (iPS) technology. The reprogrammed cancer cells show that pluripotency can transiently dominate over the cancer phenotype. Diverse studies show that reprogrammed cancer cells can, in some cases, exhibit early-stage phenotypes reflective of only partial expression of the cancer genome. In one case, reprogrammed human pancreatic cancer cells have been shown to recapitulate stages of cancer progression, from early to late stages, thus providing a model for studying pancreatic cancer development in human cells where previously such could only be discerned from mouse models. We discuss these findings, the challenges in developing such models and their current limitations, and ways that iPS reprogramming may be enhanced to develop human cell models of cancer progression.

Keywords cancer; iPS; pluripotency; progression
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Introduction

Over the past two decades, diverse cellular models of cancer, in combination with biochemical and imaging tools, have greatly improved the early diagnosis and treatment of various cancers. Tests exist for the early detection of cervical, colon, and breast cancers and mortality has been steadily declining (Arteaga *et al*, 2014). Despite these advances, cancers such as pancreatic ductal adenocarcinoma (PDAC) retain a dismal prognosis due to a paucity of clinical symptoms and biomarkers for early-stage disease (Cantley *et al*, 2012). The failure to detect early disease prevents the application and testing of therapeutics that could be used to inhibit disease progression. Genetically engineered animal models in mouse and zebrafish have been developed for diverse cancers (Tuveson & Jacks, 2002; Liu & Leach, 2011; Ablain & Zon, 2013). Such models can provide insights into the basis of cancer development that have helped generate treatments, such as for acute promyelocytic leukemia (Wang & Chen, 2008; Nardella *et al*, 2011). Yet there are cross-species differences between animal and human cancers with regard to the size of tumors, cancer susceptibility, spectrum of age-related cancers, and telomere lengths (DePinho, 2000; Rangarajan & Weinberg, 2003). Cancer therapeutics that work in mouse models can fail in clinical human trials (Ledford, 2011; Begley & Ellis, 2012). To complement these limitations, human solid tumors, organoid cultures thereof (Gao *et al*, 2014; Li *et al*, 2014), and cell lines (Sharma *et al*, 2010) have been engrafted into immunocompromised mice either as tumor fragments (Tentler *et al*, 2012), dispersed cells, or cells sorted for tumor initiating cells/cancer stem cells (CSC) (Ishizawa *et al*, 2010; Nguyen *et al*, 2012). In these contexts, tumors inevitably arise that resemble the parental tumor state from which the cells were derived and do not undergo early-stage progression (Tentler *et al*, 2012). Primary human mammary epithelial cells can undergo cancer progression in mouse xenografts, but require prior transformation with oncogenes rather than employing endogenous genetic changes found in tumors (Wu *et al*, 2009). Currently, there are few human cell models of cancer progression that are dependent upon naturally occurring genetic mutations.

Here, we describe the use of somatic cell nuclear transfer (SCNT) on mouse cancer cells and induced pluripotent stem (iPS) cell technology to model human cancer progression. Cancer progression by the SCNT or iPS reprogrammed cells can help reveal new networks for early-stage disease, potential early-stage biomarkers, and human cell models in which therapeutics can be assessed. We first discuss the history of reprogramming of cancer cells to pluripotency, as initiated with blastocyst injection and somatic nuclear transfer. We also describe mechanisms that may underlie a temporary

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dominance of pluripotency over the cancer genome and the use of iPS technology to elicit such dominance. We then review the advances and challenges associated with iPS-based approaches, along with the future prospects for developing new human cell models of cancer progression.

Early examples of cancer cell reprogramming to pluripotency

Several lines of evidence show that cancer progression can be elicited by reversible epigenetic changes as well as by irreversible mutations in oncogenic and tumor suppressor genes (Esteller, 2007). The first experimental evidence of tumor reversibility was described in crown-gall tumors in plants over a half century ago (Braun, 1959). Braun had hypothesized that the individual pluripotent teratoma cells of crown-gall tumors might be recovered from the cancer in grafting experiments. To test his idea, he grafted the shoots from crown-gall teratoma cells serially to the cut stem ends of the healthy tobacco plants. The grafted teratoma tissue gradually developed more normal appearing shoots, which eventually flowered and developed seeds. He concluded that 'the cellular alteration in crown gall did not involve a somatic mutation at the nuclear gene level and rather some yet uncharacterized cytoplasmic entity is responsible for the cellular changes that underlie the tumorous state in the crown-gall disease.'

In mammalian development, the fertilized egg, or zygote, undergoes cell divisions to reach the blastocyst stage. The blastocyst contains the first differentiated structures, consisting of an outer layer of trophectoderm and an inner cell mass (ICM) that develops into the embryo and yolk sac. Many cells within the inner cell mass are considered pluripotent, because they give rise to all three germ layers (endoderm, ectoderm, and mesoderm) and derived tissues in the embryo. The most stringent test of whether exogenous mammalian cells are pluripotent is to inject them into a blastocyst and determine whether they contribute to the embryo and, ultimately, the animal proper (Lin, 1966). Individual malignant stem cells of murine embryonal teratomas (embryonic carcinoma, EC) injected into 280 blastocysts yielded 45 apparently normal embryos or fetuses, and such cells injected into 183 blastocysts yielded 48 apparently normal live mice (Mintz & Illmensee, 1975). All of three fetuses analyzed were mosaic, with substantial tissue contributions from teratoma cells, indicating the cells' pluripotency. Notably, many genes that had been silent or undetectable in the tumor, such as immunoglobulin, hemoglobin, MUP, agouti genes, were expressed in the appropriate tissues. The authors concluded that 'conversion to neoplasia did not involve structural changes in the genome, but rather a change in gene expression.'

The developmental pluripotency of somatic cell nucleus can be tested by implanting them into enucleated oocytes, that is, somatic cell nuclear transfer (SCNT) (Gurdon *et al*, 1958). By SCNT, a subset of cancer cells such as certain renal tumor cells, medulloblastoma cells, RAS-induced melanoma cells, and EC cells were able to be reprogrammed to pluripotency (McKinnell *et al*, 1969; Blelloch *et al*, 2004; Hochedlinger *et al*, 2004). Donor nuclei from triploid frog renal tumors were reprogrammed by injection into enucleated frog eggs, developed into blastulas with a higher efficiency than diploid nuclei (23% versus 7.7%). Yet only 21% of the triploid, tumor-derived blastulas developed into swimming embryos compared to 100% of the blastulas from normal diploid nuclei (McKinnell *et al*, 1969). The living triploid tadpoles differentiated into functional embryonic tissues of many types and the descendant tail fins regenerated appropriately, demonstrating pluripotency of the reprogrammed tumor genome (McKinnell *et al*, 1969).

Hochedlinger *et al* (2004) attempted the reprogramming by SCNT of diverse mouse cancer cells, including a $p53^{-/-}$ lymphoma, moloney murine leukemia virus-induced leukemia, PML-RAR transgene-induced leukemia, hypomethylated Chip/c lymphoma, $p53^{-/-}$ breast cancer cell line, and an ink4a/$Arf^{-/-}$, RAS-inducible melanoma cell line. All SCNT-reprogrammed cancer cell lines, but no primary tumor cells, were able to develop normal appearing blastocysts, with much greater efficiency in cancer cell lines harboring mutant tumor suppressors. SCNT-derived blastocysts whose zona pellucida was removed were placed onto irradiated murine embryonic fibroblast to derive embryonic stem (ES) cells. However, such SCNT-ES cell lines were only made from an Ink4a/$Arf^{-/-}$, RAS-inducible melanoma cell line, suggesting that only certain cancer genomes or cell types are amenable to the manipulation.

To assess their autonomous developmental potential, melanoma SCNT-ES cells were injected into tetraploid blastocysts, where transplanted wild-type ES cells can exclusively give rise to the embryo and tetraploid cells become the placenta (Wang *et al*, 1997). The resulting embryos developed up to day 9.5 of mouse gestation (E9.5) with a beating heart, closed neural tube, and developing limb and tail buds. Yet at later stages, embryos were not recovered, presumably because of the reactivation of irreversible genetic alterations of the melanoma.

However, in a chimeric blastocyst assay, where normal and donor cells can contribute to the embryo, the SCNT-ES cells, despite having major severe chromosomal changes from the original cancer, showed remarkable differentiation by contributing to skin, intestine, heart, kidney, lungs, thymus, and liver in newborn chimeric mice and to adult lineages such as the lymphoid compartment in Rag2-deficient chimeric mice (Hochedlinger *et al*, 2004). These findings show that the oocyte cytoplasm can reprogram the epigenetic state of the donor cancer cell nucleus into a pluripotent state that supports differentiation into multiple somatic cell types.

The SCNT-ES-derived adult chimeric mice developed multiple primary melanoma lesions with an average latency of 19 days, comparable to the latency required for the development of recurrent tumors or the emergence of tumors derived from transplanted melanoma cells (Chin *et al*, 1999). Interestingly, 33% of the chimeras from melanoma SCNT-ES cells developed rhabdomyosarcoma, which has an overlapping pathway with melanoma, showing the consequence of the 'melanoma genome' expressed in a different tissue (Hochedlinger *et al*, 2004) (Fig 1, top).

Li *et al* (2003) tested the epigenetic reprogramming of medulloblastoma, a pediatric brain tumor, originating from the granule neuron precursors of the developing cerebellum. The medulloblastoma cells were isolated from $Ptc^{+/-}$ mice and used for SCNT. Although transferred SCNT cells developed into blastocysts that were morphologically indistinguishable from those derived nuclei of spleen control cells, no viable embryos were identified after E8.5 in the transplanted pseudo-pregnant mice. Intriguingly, while the embryos at E7.5 days appeared grossly normal and contained all three germ layers as well as an ectoplacental cone, a chorion, an

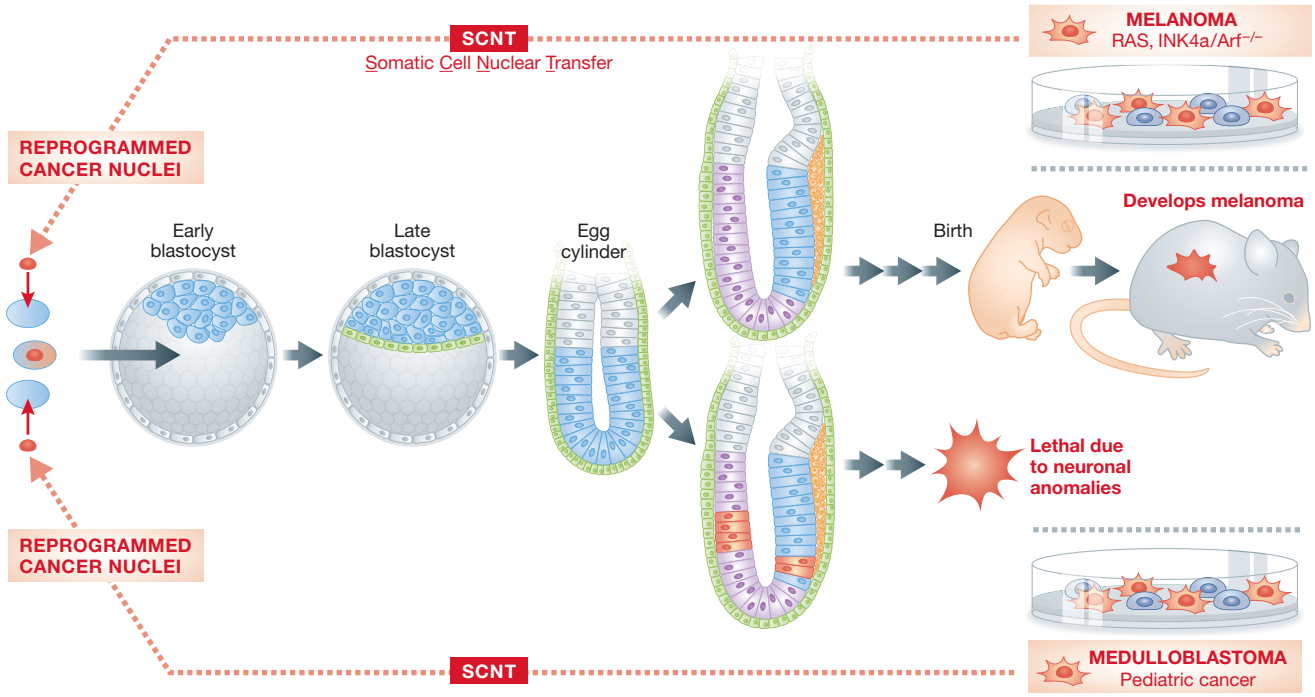

Figure 1. Cancer cell reprogramming by somatic cell nuclear transfer (SCNT).
Somatic cell nuclear transfer from medulloblastoma (left) or melanoma (right) cells leads to reprogramming of the cancer cell nucleus. When the resulting reprogrammed cells are injected into blastocysts, dominance of the early pluripotent state apparently suppresses the cancer phenotype and allows partial embryonic development. From medulloblastoma cells, lethality results shortly after gastrulation, whereas from melanoma cells in chimeric blastocysts, the cancer genome cells contribute to many tissues but melanoma ultimately ensues.

amnion, a Reichert's membrane, a yolk sac cavity, and an amniotic cavity, embryos at E8.5 showed more extensive differentiation of the cephalic vesicles and neural tubes, implying that the lack of viable embryos after E8.5 could be attributed to dysregulated neuronal lineages. Thus, this report demonstrates the mutation(s) underlying medulloblastoma was suppressed during pre-implantation and early germ layer stages, and became activated within the context of the cerebellar granule cell lineage, ultimately leading to embryonic lethality (Fig 1, bottom).

In summary, the cancer genome can be suppressed during the pre-implantation blastocyst stage when certain cancer cells are first reprogrammed to pluripotency by nuclear transfer (SCNT-ES). The resultant pluripotent cells can then differentiate into multiple early developmental cell types of the embryo. Yet, later in organogenesis, the cancer genome becomes activated, particularly in the cell lineage in which the original cancer occurred. This leads to the question of how the pluripotency network can suppress the cancer phenotype sufficiently to allow early tissue differentiation and development.

Expression of proto-oncogenes during development and suppression by pluripotency

The expression of proto-oncogenes is spatially and temporally regulated during embryogenesis, with certain proto-oncogenes being transiently activated in only certain tissues and in late lineage specification (Pfeifer-Ohlsson *et al*, 1984, 1985; Slamon & Cline, 1984; Adamson, 1987; Wilkinson *et al*, 1987; Pachnis *et al*, 1993). For examples, tyrosine kinase c-Src mRNA is transiently expressed between days E8–10 of mouse gestation and gradually decreases thereafter (Slamon & Cline, 1984). In human cancers, Src tyrosine activity is correlated with Src protein expression (Verbeek *et al*, 1996).

Proto-oncogene expression during development can be modulated by epigenetic states. For instance, the repressive histone mark H3K27me3 and active histone mark H3K4me3 both are enriched at the promoter of proto-oncogenes c-*SRC*, *AKT1*, and *MYB* in human ES cells, but only the active histone mark H3K4me3 is enriched in the K562 cancer cell line (ENCODE). (Ram *et al*, 2011). While inactivation of tumor suppressor genes such as *CDKN2A* and *TP53* is observed in many human cancers, including PDAC (Nigro *et al*, 1989; Caldas *et al*, 1994), genes encoding histone modifying enzymes are often mutated in cancer (Dawson & Kouzarides, 2012; Kadoch *et al*, 2013) and such enzymes can bind to and repress the *CDKN2A* locus (Bracken *et al*, 2007). Moreover, the conformation status of p53 can be changed in the pluripotent state. Undifferentiated ES cells express high levels of p53 in a wild-type conformation, but there is a shift in the conformational status of p53 to a mutant form upon RA-induced differentiation (Sabapathy *et al*, 1997). Mouse ES cells harboring oncogenic mutant alleles of *p53* maintain pluripotency and are benign, with normal karyotypes compared to ES cells, when the *p53* gene is knocked out (Rivlin *et al*, 2014).

The embryonic stem cell microenvironment, presumably mimicking that in the blastocyst, can contribute to the suppression of uncontrolled cell growth in the pluripotent state; this helps

to keep the balance between self-renewal and differentiation. Aggressive melanoma cells were reprogrammed into melanocyte-like cells and invasiveness was reduced, at least in part, by culturing the cells on Matrigel that was conditioned by human ES cells, suggesting suppressive, anti-invasive cues associated with the huES microenvironment (Postovit *et al*, 2006). The aggressive melanoma and breast carcinoma cells express Nodal, which is essential for human ES cell pluripotency, yet these cancers did not express Lefty, an inhibitor of Nodal, which is expressed in human ES cells. Exposure of the cancer cell lines to ES-conditioned Matrigel resulted in a decrease in tumorigenesis accompanied by a reduction in clonogenicity and an increase in apoptosis, directly associated with secretion of Lefty from huES cells (Postovit *et al*, 2008).

In conclusion, early developmental signals naturally regulate proto-oncogenes so that their expression can be suppressed until an appropriate developmental stage where the genes function. Concordantly, the changing early embryonic environment and the mimic of such environments during ES cell culture can suppress oncogenic phenotypes of cancer-derived cells.

Reprogramming of somatic cells to pluripotency by OSKM

Human and mouse somatic cells (such as fibroblasts, blood cells, etc.) can be reprogrammed into a pluripotent ES-like cells, called induced pluripotent stem (iPS) cells, by the ectopic expression of transcription factors such as Oct4, Sox2, Klf4, and c-Myc (OSKM) (Takahashi & Yamanaka, 2006). As this method does not need oocytes or blastocysts that are used for SCNT, it is a much more accessible technique and it side-steps ethical issues associated with using early human embryos. The pluripotency of human iPS cells can be validated by cell markers, genomic RNA expression profiles, epigenetic profiles, and teratoma assays; the latter being when (e.g., human) iPS cells are injected subcutaneously into mice that are genetically deficient in innate and acquired immunity (e.g., NOD-SCID (Shultz *et al*, 2005)). Details of iPS cell generation and use are discussed elsewhere in this issue.

Reprogramming of cancer cells to pluripotency or near-pluripotency by OSKM

In the first experiments on cancer reprogramming by SCNT described above, only a subset of cancer cell types could be reprogrammed to pluripotency. Despite the remarkable demonstration of the potential dominance of the pluripotent state over cancer, major questions remained. Are there particular cancer mutations that allow or block the ability of a cancer cells to be reprogrammed to pluripotency? Does such ability to be reprogrammed relate to the tissue type of the cancer? At what stage of using re-differentiation of the reprogrammed cancer cells does the cancer genome regain dominance over the cell phenotype? Can understanding the transition between pluripotency and cancer provide new insight into how to control the growth of cancer cells? Now that the relatively simple iPS technology can be applied to reprogram cancer cells, independent of oocytes and blastocysts, these questions have been revisited.

Reprogramming of chronic myeloid leukemia (CML)

The initial chronic phase of CML, which originates from hematopoietic stem cells of the bone marrow, is caused by a BCR-ABL fusion mutation that drives cell expansion, while the CML clones retain differentiation potential (Melo & Barnes, 2007). The chronic phase of CML progresses into an accelerated phase, followed by the blast crisis, terminal phase of CML upon acquisition of a second lesion. Once CML reaches the blast crisis stage, the cells lose the ability to differentiate and immature leukemia cells overgrow. Based upon the dependency of CML on BCR-ABL activated tyrosine kinase, tyrosine kinase inhibitors such as imatinib improved the long-term survival rate of CML patients. However, the inhibitor cannot completely eradicate CML cells and often lead to the recurrence of CML clones after its discontinuation (Melo & Barnes, 2007). Can CML in the terminal blast crisis stage be reprogrammed into iPS cells? If so, can CML-iPS cells recapitulate the initial chronic phase of CML, which has differentiation potential? Can be the dependency of CML on BCR-ABL signaling be altered?

Carette *et al* (2010) reprogrammed a cell line derived from blast crisis stage CML by infecting with a retrovirus expressing OSKM. Subcutaneous injection of the resulting CML-iPS cells into NOD-SCID mice revealed teratomas which contained cells of three germ layers, indicating pluripotency. During *in vitro* differentiation, the CML-iPS cells were able to differentiate into cells expressing the pan-T cell marker $CD43^+$ and the hematopoietic lineage marker $CD45^+$, as well as the stem cell marker $CD34^+$, demonstrating a restoration of differentiation potential into hematopoietic lineages. The loss of the CML phenotype in CML-iPS cells and the recovery of differentiation can be viewed as a recapitulation of the chronic phase of CML, despite starting with blast crisis stage CML-iPS cells.

Interestingly, whereas parental CML cell lines were dependent on the BCR-ABL pathway, the CML-iPS cell lines were independent of BCR-ABL signaling and showed resistance to imatinib, an inhibitor of BCR-ABL signaling (Carette *et al*, 2010). The loss of BCR-ABL dependency was also observed in cells differentiated *in vitro* into neuronal or fibroblast-like cells. Yet when the cells were differentiated *in vitro* to hematopoietic lineage cells, they became sensitive to imatinib, suggesting that the recovery of oncogenic dependency as the CML-iPS cells underwent hematopoietic differentiation. Thus, oncogenic mutations can be dynamically expressed when cancer cells are converted to pluripotency and then re-differentiated.

Similar observations were seen by another group that generated iPS cells from primary $CD34^+$ cells which were isolated from bone marrow mononuclear cells of a CML chronic phase patient, by retroviral infection with OSKM (Kumano *et al*, 2012). The CML-iPS cells underwent normal hematopoiesis during *in vitro* differentiation. The differentiated hematopoietic progenitors ($CD34^+CD45^+$) from CML-iPS cells produced colonies of mature erythroid cells, granulocyte–macrophage cells, or mix lineages with a distribution of colony size, morphologies, and kinetics of growth and maturation that was similar to non-cancer iPS cells. The CML-iPS-derived hematopoietic progenitors did not show a CML phenotype *in vivo*, when intravenously engrafted into NSG mice receiving minimal irradiation. Whereas the parental $CD34^+$ cells responded to imatinib, the CML-iPS derivatives and immature $CD34^+CD38^-CD45^+CD90^+$ cells derived from the CML-iPS cells were not sensitive to imatinib. Yet mature hematopoietic cells ($CD34^-CD45^+$) derived from the CML-iPS cells restored their sensitivity to imatinib. Given that the

expression of the proto-oncogene C-ABL is high at mouse embryonic day 10 when the first definitive hematopoietic stem cells are generated (Muller *et al*, 1982; Sanchez *et al*, 1996), the CML-iPS cells, corresponding to mouse blastocyst cells at embryonic day 3–5, may express regulatory factors that suppress C-ABL signaling. Thus, understanding the underlying mechanisms that counteract BCR-ABL signaling in CML-iPS cells and their immature, newly differentiated progeny could provide insight into the resistant to imatinib. Furthermore, it may be possible to screen new drugs that can inhibit CML clones at this stage and then treat CML patients with such drugs in combination with classical inhibitors. Taken together, these studies show how reprogramming to pluripotency can modulate oncogene expression and recapitulate the initial chronic phase of CML.

Reprogramming of gastrointestinal cancer cell lines

Miyoshi *et al* (2010) hypothesized that reprogramming of gastrointestinal cancer cell lines into iPS would allow the cells to undergo differentiation and enhanced sensitivity to therapeutics. The iPS cells arising in their experiments were capable of differentiation into cells of the three germ layers *in vitro*. Interestingly, while the parental gastrointestinal cancer cell lines generated tumors within four months of injection into NOD/SCID mice, such tumorigenesis was not seen with the differentiated cells arising from the iPS cells derived from the cell lines. In accord with these observations, the gastrointestinal cancer-derived iPS cells, upon differentiation, expressed higher levels of the tumor suppressor genes $p16^{Ink4\alpha}$ and *p53,* slower proliferation and were sensitive to differentiation-inducing treatment (Miyoshi *et al*, 2010). These findings show that the pluripotency state imposed by the OSKM factors can partially suppress the cancer phenotype in the gastrointestinal cell lines.

Reprogramming of glioblastoma (GBM) neural stem cells and human sarcoma

To determine whether cancer-specific epigenetic changes can be altered or erased by reprogramming and how such might correlate with transcriptional changes and suppression of malignancy, primary GBM-derived neural stem cells (GNS) were reprogrammed using piggyBac transposon vectors expressing OCT4 and KLF4 (Stricker *et al*, 2013). Widespread resetting of epigenetic methylation occurred in the GNS-iPS cells in cancer-specific methylation variable positions (cMVPs) and also at the GBM tumor suppressor genes *CDKN1C* (cyclin-dependent kinase inhibitor 1C) and *TES* and was associated with the genes' derepression. Interestingly, teratomas from the GNS-iPS cells generated compact and non-infiltrative cells of all three germ layers. The majority of cells in the teratomas developed into highly proliferating neural progenitors, showing epigenetic memory of the cell type used to generate iPS cells.

Primary GBM develops rapidly from neural precursors, apparently without clinical or histopathological evidence of less malignant precursor lesions (Ohgaki & Kleihues, 2013). In accordance with the human cancer, neural progenitor cells that were differentiated from iPS-GNS recapitulated aggressive glioblastoma when transplanted into the adult mouse brain (Stricker *et al*, 2013). In contrast, non-neural mesodermal progenitors, differentiated from two independent GNS-iPS clones *in vitro*, sustained the expression of TES and CDKN1C formed benign tumors and failed to infiltrate the surrounding brain (Stricker *et al*, 2013).

To determine whether human cancer cells can be reprogrammed to pluripotency and then terminally differentiated with concomitant loss of tumorigenicity, human sarcoma cell lines were reprogrammed by infecting with human OSKM along with NANOG and LIN28 (Zhang *et al*, 2013). In xenograft assays in non-immune mice, the reprogrammed sarcoma cells formed tumors at slower rates than their parental cell lines. The sarcoma-iPS-derived tumors were of lower grade, exhibited more necrosis, reduced staining for a marker of proliferation, and reduced expression of the vimentin mesenchymal marker than tumors from the sarcoma parental cell lines. Thus, reprogramming decreased the aggressiveness of the cancer compared to the cells' parental counterparts. All 32 oncogenes and 82 tumor suppressor genes whose promoter DNA was initially methylated, were demethylated as a result of reprogramming, indicating that the reprogramming process was accompanied by major epigenetic changes in growth- and cancer-related genes. By ANOVA of gene expression profiles and principal component analysis, the reprogrammed sarcoma cells, though only partially reprogrammed, were more like embryonic stem cells compared to mesenchymal stem cells (MSCs) and partially reprogrammed fibroblasts, demonstrating that the sarcoma cell line was de-differentiated into a pre-MSC state (Zhang *et al*, 2013).

The above studies with cancer cell lines showed that the pluripotency factors and the pluripotency state can suppress features of the cancer phenotype, restore differentiation potential, perturb epigenetics via DNA methylation, and alter cancer-related gene expression.

Reprogramming of human primary pancreatic ductal adenocarcinoma

Taking together the principles learned from SCNT and iPS studies of cancer cell lines, it seemed possible that generating iPS cells from primary human cancer cells would allow the cells to be propagated indefinitely in the pluripotent state and that, upon differentiation, a subset of the cells would undergo early developmental stages of the human cancer, thereby providing a live cell human model to study cancer progression (Kim *et al*, 2013). However, generating iPS had not been achieved with cancer epithelial cells from primary human adenocarcinomas. To test this idea, primary pancreatic epithelial cells isolated from pancreatic ductal adenocarcinoma (PDAC) cells and normal cells at the margin of the tumors were reprogrammed by introducing OSKM. Colonies came up frequently from the margin epithelial cultures and very rarely from the cancer epithelial cultures. Once OSKM factors were suppressed, all ES-like colonies differentiated or died; therefore, low level of OSKM expression was retained and the ES-like colonies that arose were called 'iPS-like', as they were apparently unable to completely sustain a pluripotency program.

One cancer iPS-like line, designated 10–22 cells, harbored classical PDAC mutations, including an activated *KRAS* allele, a *CDKN2A* heterozygous deletion, and decreased *SMAD4* gene copy levels, as well as retained the gross chromosomal alterations seen in the parental, primary cancer epithelial cells, demonstrating that the PDAC-iPS line was derived from advanced PDAC cells (Kim *et al*, 2013). The 10-22, PDAC-iPS-like line differentiated into all three germ layer descendants during *in vitro* embryoid body differentiation, though neuronal lineages were under-represented. *In vivo* teratoma assays in non-immune mice showed that the 10–22 cells generated multiple germ layer tissues, but preferred to generate endodermal ductal structures. Notably, the ductal structures at

3 months resembled pancreatic intraepithelial neoplasias (PanIN), regarded as a potential precursor to PDAC, and by 9 months the cells progressed to the invasive stage. By contrast, an isogenic line of iPS-like cells derived from the tumor margin gave rise to ductal teratomas, but not PanINs or invasive ductal features. Thus, reprogramming of the PDAC created an experimental model in which human PDAC progression could be studied in live cells.

A major cause for the lethality of PDAC is that there are no reliable biomarkers or clinical phenotypes of the early stages of the disease. To discover potential biomarkers for early PDAC progression, PanIN lesions at 3 months were isolated from non-immune mouse teratomas and cultured in serum-free medium as organoids (Kim *et al*, 2013). After 6 days of culture, the medium was harvested and subjected to mass spectroscopic analysis, in order to discover protein markers that are secreted or released from early-stage human PDAC. By subtracting the secreted and released proteins of the organoids from such proteins of contralateral control tissue and of the 10–22 cells grown under pluripotency conditions, 107 proteins specific to the PanIN-stage samples were discovered. Of these, 64% of released or secreted proteins were seen in databases of genes, proteins, and networks expressed within human PanIN and PDAC cells, further validating the PanIN status of the organoids derived from the 10–22 cell teratomas.

The proteins released or secreted from the PanIN organoids fell into three major networks, including interconnected networks for TGF-β and integrin signaling, and for the transcription factor HNF4α. Notably, HNF4a is not or barely expressed in normal pancreatic ductal cells, poorly expressed in the PanIN1 stage, but is activated in PanIN2 and PanIN3 stages, invasive stages, and in early, well-differentiated human pancreatic cancer (Kim *et al*, 2013). HNF4a then decreases markedly and becomes undetectable in advanced or undifferentiated PDAC. Thus, being able to reconstitute PDAC progression with 10–22 cells revealed the activation of an HNF4a network distinctive for the late PanIN stages and well-differentiated PDAC; this network activated during PDAC progression would have been missed in studies of late-stage tumor cells.

In conclusion, although handful of reports have demonstrated the reprogramming of cancer by iPS technology and only a subset of cancer cells are amenable to the process, common lessons can be drawn. (1) The differentiation potential of the cancer cells can be restored, at least in part. (2) Epigenetic states are altered markedly during reprogramming to pluripotency or near-pluripotency. (3) The reprogrammed cells exhibit a reduced aggressive cancer phenotype in teratoma assays, which may be concomitant with suppression of oncogenes from the original cancer and activation of tumor suppressor genes. (4) The reprogrammed cells can re-acquire the cancer phenotype when they differentiate into the lineage from which the iPS line was derived. Indeed, the iPS or iPS-like reprogrammed cancer cells retain a propensity to do so, compared to other lineages. (5) The iPS-like reprogrammed cancer cells can be used to study the progression of the cancer phenotype (Fig 2).

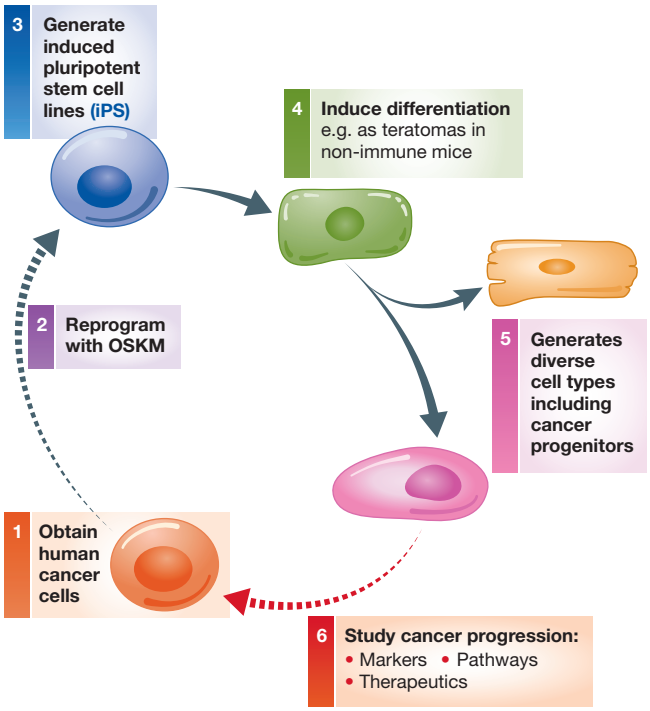

Figure 2. Cancer cell reprogramming by iPS and recapitulation of cancer progression.
Scenario for developing iPS lines from human cancer cells (1–3), inducing their differentiation to generate cancer progenitors (4–5), and using the cancer progenitors to study features of cancer progression (6). By this means, late-stage human cancers can be reprogrammed to recapitulate early-stage disease and progression for discovering markers, pathways, and therapeutics.

Questions for the future

Many questions remain. How do the pluripotency factors and reprogramming process partially suppress the cancer phenotype? How might such suppression be restricted to the lineage from which the cancer iPS cells were derived? During the differentiation of cancer iPS cells, how do the cells harboring the late-stage cancer genome undergo disease progression, as opposed to immediately developing the late-stage, parental cancer phenotype? What is the basis by which cancer iPS lines are so difficult to generate? How can the process be improved, to create more models for human cancer progression? While definitive answers to these questions are not in hand, hints about underlying mechanisms are arising in different areas, as discussed next.

Control of cancer versus pluripotency

As for SCNT, iPS technology was able to reprogram a subset of cancer cells to the pluripotent or near-pluripotent state and restore differentiation potential to the cells. Pluripotent ES cells and iPS cells have epigenetic features that may be reciprocal to those in cancer cells (Suva *et al*, 2013). For instance, comprehensive high-throughput relative methylation analysis demonstrated the existence of two independent epigenetic mechanisms for cell reprogramming and tumorigenesis. Hypomethylated, 'differentially methylated regions' (DMRs) that can occur in iPS reprogramming were hypermethylated DMRs in colon cancer (Doi *et al*, 2009). Seventy-seven percent of cancer-specific methylation variable positions (cMVPs) were hypermethylated and 23% were hypomethylated in GBM

neural stem cell (GNS) (Stricker *et al*, 2013). More than 44% of these cMVPs were reset during reprogramming of GNS to GNS-iPS along with a majority of targets of the Polycomb complex (Stricker *et al*, 2013). Furthermore, oncogenes and tumor suppressor genes can become demethylated as a result of iPS reprogramming, which is the opposite of the methylation of tumor suppressor genes seen during cancer progression (Zhang *et al*, 2013). ATP-dependent SWI/SNF complexes are considered to be tumor suppressors, since recurrent mutations of Brg1 subunits of the complex have been observed in various human cancers (Wilson & Roberts, 2011), including pancreatic cancer (Jones *et al*, 2008; Shain *et al*, 2012). The loss of the Brg1 BAF complex promoted the formation of intraductal papillary mucinous neoplasms and PDAC (von Figura *et al*, 2014). By contrast, overexpression of Brg1 and Baf155 components of the BAF complex, along with OSKM, enhances reprogramming to iPS (Singhal *et al*, 2010). Together, these findings show that common factors and epigenetic features can differentially elicit tumor formation or reprogramming to pluripotency.

While the coordinate, ectopic expression of OSKM induces reprogramming of somatic cells pluripotency (Takahashi & Yamanaka, 2006), activation of individual pluripotency factors can contribute to tumorigenesis. For instance, the inducible expression of OCT4 in mice initiated dysplasia by preventing the differentiation of multipotent lineages (Hochedlinger *et al*, 2005). The ectopic expression of OCT4 in human melanoma cells produced a more aggressive, cancer stem cell-like melanoma (Kumar *et al*, 2012). Furthermore, whereas reprogramming to pluripotency in the mouse can produce well-differentiated teratomas (Abad *et al*, 2013), partial reprogramming in mice yields tumors (Ohnishi *et al*, 2014). These findings indicate that the pluripotency transcription factors are integrated into networks that govern cancer phenotypes.

An unexplained feature of the use of cancer-derived iPS cells is that the cells harbor the mutations of the late-stage cancer from which the iPS cells were derived, but, as described above, the cancer iPS cells can exhibit disease progression; that is, not simply return to the late-stage phenotype from which they were derived. It follows that the disease progression exhibited by the models may not reflect that seen during the sequential accumulation of cancer driver mutations as might occur in natural tumor development. Yet in human PDAC progression, PanIN2 cells can harbor somatic mutations required for PDAC development, but it can take years for the cells to acquire metastatic activity (Yachida *et al*, 2010; Murphy *et al*, 2013). Thus, even during natural PDAC tumor development, epigenetic or extrinsic factors may be crucial for progression. The attenuation of such factors may occur transiently during reprogramming to pluripotency, and the release from pluripotency can thus mirror the progression to the cancer phenotype seen *in vivo*.

Low efficiency of reprogramming cancer cells to pluripotency

Cancer cells are reprogrammed very inefficiently and only subset of cancers are amenable to reprogramming. Certain mutations might make cancer cells refractory to reprogramming; that is, 'non-suppressible' by pluripotency. One iPS line out of 8 derived from PDAC contained the primary PDAC driver allele $KRAS^{G12D}$, even though 78% of starting primary cancer epithelial cells contained $KRAS^{G12D}$ (Kim *et al*, 2013). Conceivably, secondary mutations arising during iPS formation allowed the $KRAS^{G12D}$ cells to be reprogrammed. Furthermore, aneuploidy in cancer could cause the cellular stress responses such as activation of *p53* through the stress kinase *p38* (Thompson & Compton, 2010), thus possibly impeding reprogramming. PDAC epithelial cells from patients pre-treated with radiation did not produce any iPS colonies, perhaps due to senescence induced by irradiation and DNA damage (J.K. and K.S.Z, unpublished observations). Given that many cancer patients are treated with chemotherapy and irradiation prior to surgical resection, such treatments may prevent the creation of iPS lines.

The apparent preference for pluripotent cells to regenerate the cancer type from which they were derived reflects the tendency of iPS cell lines to preferentially differentiate into their lineages of origin (Bar-Nur *et al*, 2011; Kim *et al*, 2011). This 'deficiency' in exhibiting equal pluripotency for all cell lineages can be an advantage in developing human cell models of cancer progression, whereby the cancer iPS lines preferentially recapitulate stages of the cancer type of interest. Thus, despite the difficulties and caveats in generating human cancer iPS models, the examples covered in this review provide new insights into disease progression. It is hoped that a better understanding of how to create iPS cells from human cancers, and epithelial cancers in particular, will provide more opportunities to model and understand other types of solid tumors.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Programming and reprogramming a human heart cell

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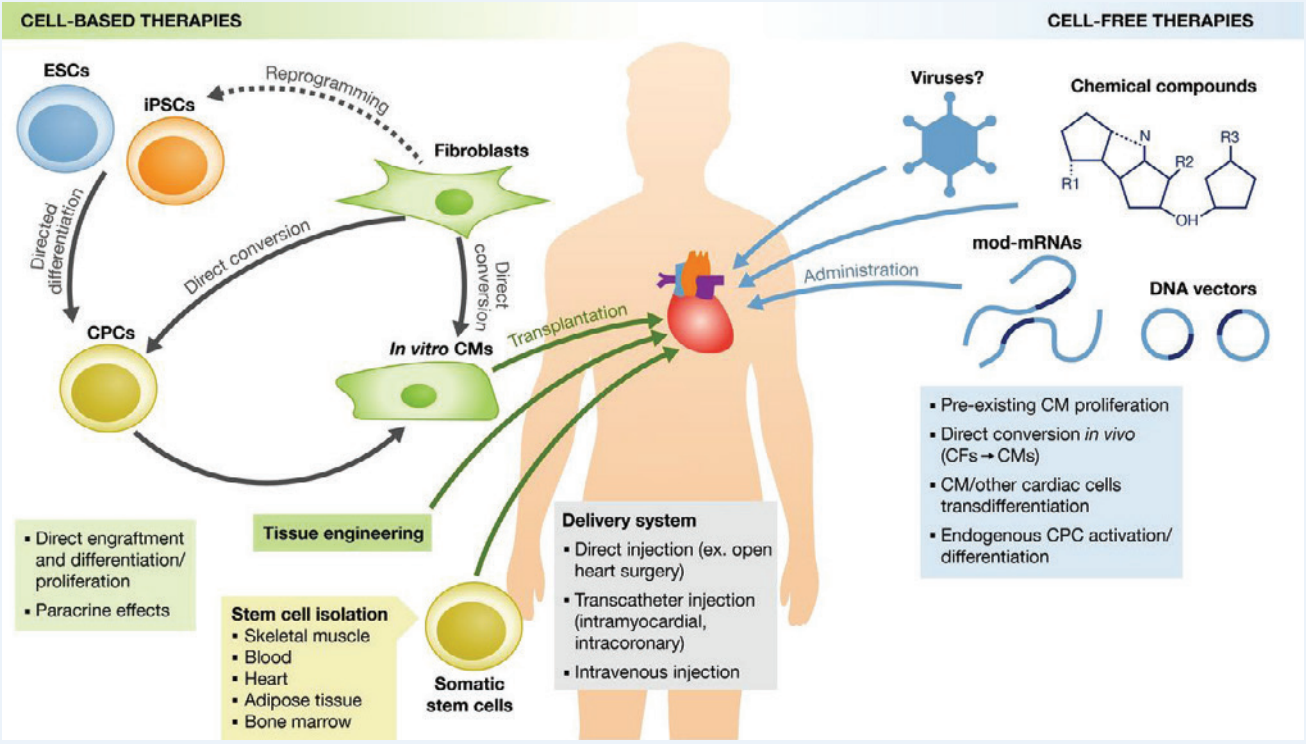
The latest discoveries and advanced knowledge in the fields of stem cell biology and developmental cardiology hold great promise for cardiac regenerative medicine, enabling researchers to design novel therapeutic tools and approaches to regenerate cardiac muscle for diseased hearts. However, progress in this arena has been hampered by a lack of reproducible and convincing evidence, which at best has yielded modest outcomes and is still far from clinical practice. To address current controversies and move cardiac regenerative therapeutics forward, it is crucial to gain a deeper understanding of the key cellular and molecular programs involved in human cardiogenesis and cardiac regeneration. In this review, we consider the fundamental principles that govern the "programming" and "reprogramming" of a human heart cell and discuss updated therapeutic strategies to regenerate a damaged heart.

Synopsis:



Kenneth Chien and colleagues review molecular principles of cardiogenesis, which hold great promise for regenerative "programming" or "reprogramming" of human heart conditions.

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Reprogramming of cell fate: epigenetic memory and the erasure of memories past

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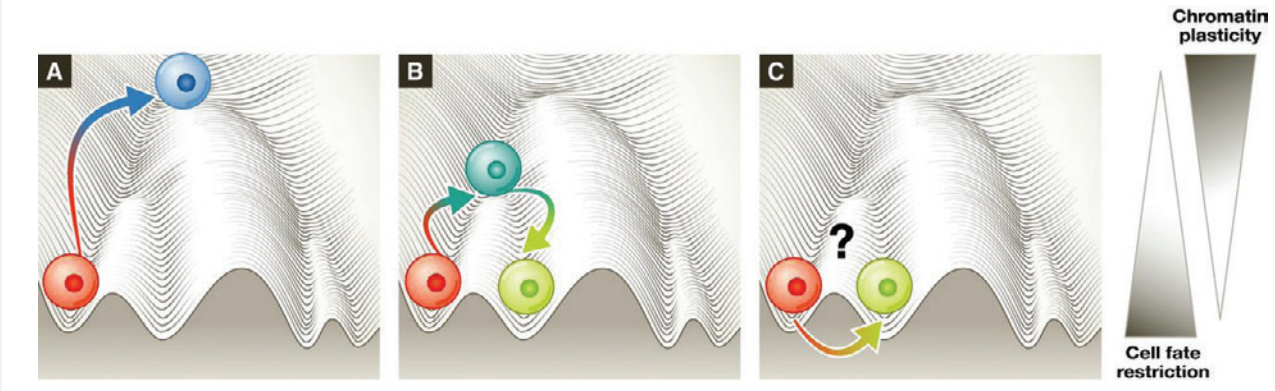
Cell identity is a reflection of a cell type-specific gene expression profile, and consequently, cell type-specific transcription factor networks are considered to be at the heart of a given cellular phenotype. Although generally stable, cell identity can be reprogrammed *in vitro* by forced changes to the transcriptional network, the most dramatic example of which was shown by the induction of pluripotency in somatic cells by the ectopic expression of defined transcription factors alone. Although changes to cell fate can be achieved in this way, the efficiency of such conversion remains very low, in large part due to specific chromatin signatures constituting an epigenetic barrier to the transcription factor-mediated reprogramming processes. Here we discuss the two-way relationship between transcription factor binding and chromatin structure during cell fate reprogramming. We additionally explore the potential roles and mechanisms by which histone variants, chromatin remodelling enzymes, and histone and DNA modifications contribute to the stability of cell identity and/or provide a permissive environment for cell fate change during cellular reprogramming.

Synopsis:



Petra Hajkova & colleagues feature the interplay of chromatin structure with cell fate determining transcription factors as therapeutic opportunity in the context of cellular reprogramming.

THE EMBO JOURNAL

Application of biomaterials to advance induced pluripotent stem cell research and therapy

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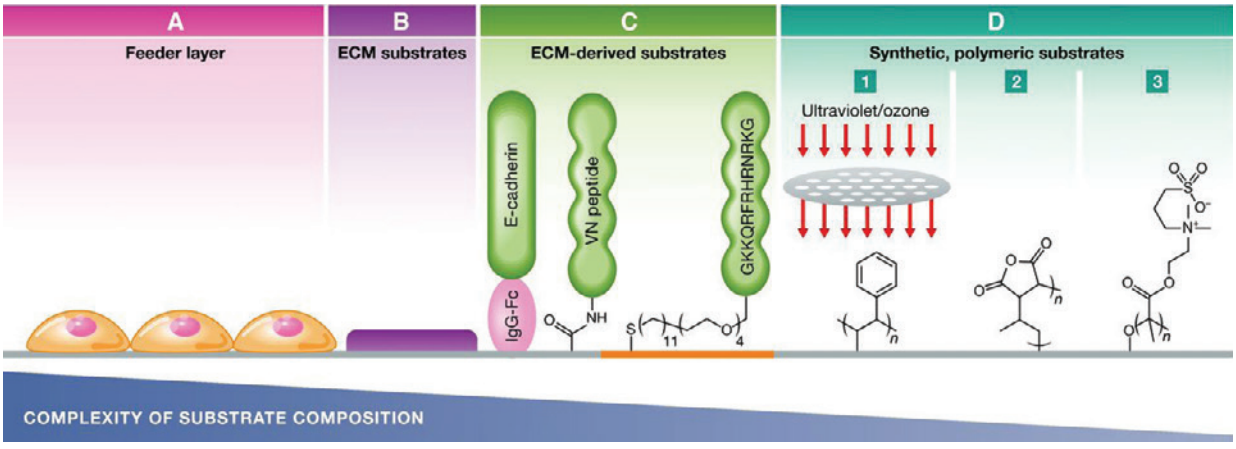
Derived from any somatic cell type and possessing unlimited self-renewal and differentiation potential, induced pluripotent stem cells (iPSCs) are poised to revolutionize stem cell biology and regenerative medicine research, bringing unprecedented opportunities for treating debilitating human diseases. To overcome the limitations associated with safety, efficiency, and scalability of traditional iPSC derivation, expansion, and differentiation protocols, biomaterials have recently been considered. Beyond addressing these limitations, the integration of biomaterials with existing iPSC culture platforms could offer additional opportunities to better probe the biology and control the behavior of iPSCs or their progeny *in vitro* and *in vivo*. Herein, we discuss the impact of biomaterials on the iPSC field, from derivation to tissue regeneration and modeling. Although still exploratory, we envision the emerging combination of biomaterials and iPSCs will be critical in the successful application of iPSCs and their progeny for research and clinical translation.

Synopsis:



Karp and colleagues critically review state-of-the-art biomaterials essential to fully exploit the translational potential of the iPS technologies.

THE EMBO JOURNAL

Forward engineering neuronal diversity using direct reprogramming

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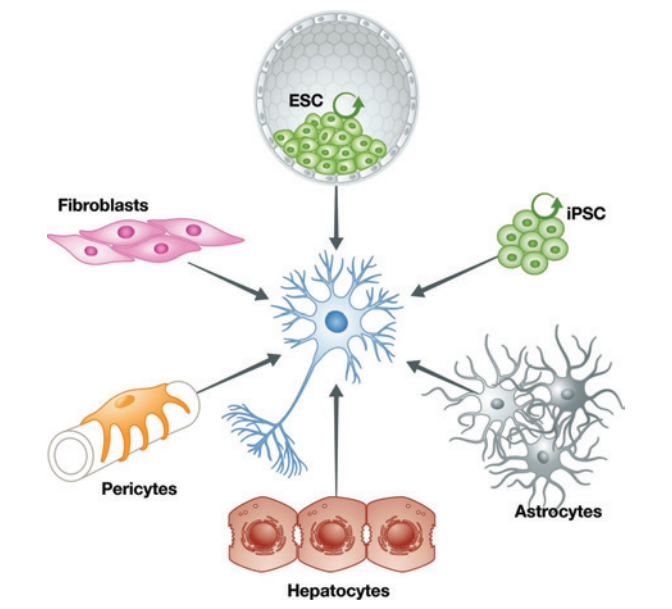
The nervous system is comprised of a vast diversity of distinct neural cell types. Differences between neuronal subtypes drive the assembly of neuronal circuits and underlie the subtype specificity of many neurological diseases. Yet, because neurons are irreversibly post-mitotic and not readily available from patients, it has not been feasible to study specific subtypes of human neurons in larger numbers. A powerful means to study neuronal diversity and neurological disease is to establish methods to produce desired neuronal subtypes *in vitro*. Traditionally this has been accomplished by treating pluripotent or neural stem cells with growth factors and morphogens that recapitulate exogenous developmental signals. These approaches often require extended periods of culture, which can limit their utility. However, more recently, it has become possible to produce neurons directly from fibroblasts using transcription factors and/or microRNAs. This technique referred to as direct reprogramming or transdifferentiation has proven to be a rapid, robust, and reproducible method to generate mature neurons of many different subtypes from multiple cell sources. Here, we highlight recent advances in generating neurons of specific subtypes using direct reprogramming and outline various scenarios in which induced neurons may be applied to studies of neuronal function and neurological disease.

Synopsis



Kristin Baldwin & colleagues outline most recent routes to generate a huge spectrum of neuronal subtypes *in vitro*. Their functionality and resemblance of endogenous tissue raises hopes for cell replacement therapies.

THE EMBO JOURNAL

Toward beta cell replacement for diabetes

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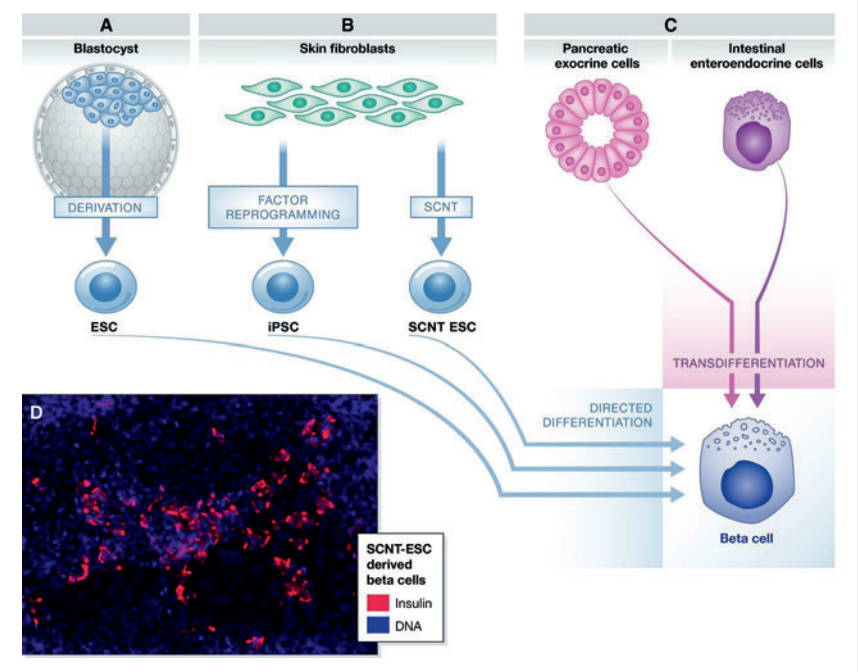
The discovery of insulin more than 90 years ago introduced a life-saving treatment for patients with type 1 diabetes, and since then, significant progress has been made in clinical care for all forms of diabetes. However, no method of insulin delivery matches the ability of the human pancreas to reliably and automatically maintain glucose levels within a tight range. Transplantation of human islets or of an intact pancreas can in principle cure diabetes, but this approach is generally reserved for cases with simultaneous transplantation of a kidney, where immunosuppression is already a requirement. Recent advances in cell reprogramming and beta cell differentiation now allow the generation of personalized stem cells, providing an unlimited source of beta cells for research and for developing autologous cell therapies. In this review, we will discuss the utility of stem cell-derived beta cells to investigate the mechanisms of beta cell failure in diabetes, and the challenges to develop beta cell replacement therapies. These challenges include appropriate quality controls of the cells being used, the ability to generate beta cell grafts of stable cellular composition, and in the case of type 1 diabetes, protecting implanted cells from autoimmune destruction without compromising other aspects of the immune system or the functionality of the graft. Such novel treatments will need to match or exceed the relative safety and efficacy of available care for diabetes.

Synopsis



Dieter Egli & colleagues provide a stem cell perspective on pancreatic beta-cells for diabetes therapies and disease modeling.