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A critical look: Challenges in differentiating human pluripotent stem cells into desired cell types and organoids

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Abstract

Too many choices can be problematic. This is certainly the case for human pluripotent stem cells (hPSCs): they harbor the potential to differentiate into hundreds of cell types; yet it is highly challenging to exclusively differentiate hPSCs into a single desired cell type. This review focuses on unresolved and fundamental questions regarding hPSC differentiation and critiquing the identity and purity of the resultant cell populations. These are timely issues in view of the fact that hPSC-derived cell populations have or are being transplanted into patients in over 30 ongoing clinical trials. While many in vitro differentiation protocols purport to "mimic development," the exact number and identity of intermediate steps that a pluripotent cell takes to differentiate into a given cell type in vivo remains largely unknown. Consequently, most differentiation efforts inevitably generate a heterogeneous cellular population, as revealed by single-cell RNA-sequencing and other analyses. The presence of unwanted cell types in differentiated hPSC populations does not portend well for transplantation therapies. This provides an impetus to precisely control differentiation to desired ends—for instance, by logically blocking the formation of unwanted cell types or by overexpressing lineage-specifying transcription factors—or by harnessing technologies to selectively purify desired cell types. Conversely, approaches to differentiate three-dimensional "organoids" from hPSCs intentionally generate heterogeneous cell populations. While this is intended to mimic the rich cellular diversity of developing tissues, whether all such organoids are spatially organized in a manner akin to native organs (and thus, whether they fully qualify as organoids) remains to be fully resolved.

This article is categorized under:

Adult Stem Cells > Tissue Renewal > Regeneration: Stem Cell Differentiation and Reversion

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Early Embryonic Development: Gastrulation and Neurulation

KEYWORDS

developmental biology, germ layer, organoid, pluripotent stem cell, stem cell differentiation

1 | INTRODUCTION

The ability of human pluripotent stem cells (hPSCs)—which include embryonic (Thomson et al., 1998) and induced pluripotent stem cells (Park et al., 2008; Takahashi et al., 2007; Yu et al., 2007)—to differentiate into all the hundreds of diverse cell types within the human body is both a blessing for, and the bane of, regenerative medicine. The blessing is evident: the pluripotency of hPSCs, combined with their ability to prodigiously divide in culture (expanding $>10^{100}$-fold within several months; Levenstein et al., 2006), has led to the oft-quoted aspiration that it should be possible to manufacture limitless numbers of a given human cell-type in vitro for transplantation therapies or other applications (Cohen & Melton, 2011; Murry & Keller, 2008; Tabar & Studer, 2014).

However, the very same pluripotency is also a bane: given that hPSCs have a panoply of hundreds of lineage options ahead of them, it has been challenging to exclusively differentiate hPSCs down any one developmental route to yield a pure population of a single lineage. As they differentiate, hPSCs navigate multiple, poorly understood developmental lineage decisions in stepwise fashion as they progressively segue into more differentiated fates. Directing differentiation is reminiscent of the labors of Odysseus—navigating a narrow course for his ship between the aquatic monstrosities Scylla and Charybdis in Homer's *The Odyssey*. Much like Odysseus, hPSCs can easily stray from an intended lineage trajectory, differentiating into unwanted cell types that could in turn cause deleterious effects in therapeutic settings.

Consequently, the field of stem cell differentiation has seen alternating progress over the past few decades. Most differentiation methods yield a range of lineage outcomes in differing proportions, with the desired lineage often comprising a subset of the whole population (Cohen & Melton, 2011; McKnight, Wang, & Kim, 2010). In heterogeneous differentiating cultures, commingled lineages likely reciprocally signal among one another, rendering differentiation difficult to control. This has been further complicated by the use of undefined animal serum or feeder coculture in some embodiments (Murry & Keller, 2008). Moreover, with suboptimal differentiation protocols, differentiation efficiencies have been reported to vary dramatically between individual hPSC lines (Osafune et al., 2008). Here, we critically assess various challenges in hPSC differentiation, with the view that it should be possible to more precisely guide differentiation toward desired ends by understanding the means through which differentiation occurs.

2 | FINDING THE RIGHT PATH: ACCESSING DESIRED LINEAGES THROUGH THE CORRECT INTERMEDIATE PROGENITORS

Many differentiation approaches purport to "mimic development" to some degree and entail treatment with a sequence of various signaling modulators. However, for many mature cell types, we do not know the exact number or identity of steps through which they develop from pluripotent cells in vivo. Identifying the complete sequence of lineage steps needed to differentiate hPSCs into a desired cell type remains a major challenge for stem cell and developmental biology. Consequently, certain differentiation protocols claim to yield a terminal cell type in only a few steps that likely fail to recapitulate the full number and sequence of steps leading to cell-type specification in vivo, with a number of ensuing consequences (see below).

There is thus an urgent need for comprehensive lineage maps of mammalian development to guide hPSC differentiation. These maps may materialize soon, for instance, by randomly labeling single progenitors in mouse embryos using Cas9-inflicted genetic barcodes and then creating detailed lineage maps of their progeny (Chan et al., 2019). In any case, precisely mapping intervening developmental intermediates is crucial to effectively differentiate hPSCs into any lineage, as the below vignettes demonstrate.

2.1 | Primitive streak

Take, for instance, the initial differentiation of hPSCs into the endoderm, mesoderm, and ectoderm germ layers. This was initially conceptualized as a "three way" lineage decision (Figure 1ai). Yet, it is now widely recognized that these three lineages are instead generated through two nested lineage bifurcations: first, hPSCs bifurcate into primitive streak intermediates or ectoderm, and such primitive streak intermediates subsequently bifurcate into endoderm versus mesoderm intermediates (Figure 1ai). Therefore, hPSCs cannot be directly differentiated into endoderm or mesoderm in one step. Many differentiation protocols (of which only several are mentioned here for the sake of brevity) now employ some combination of bone morphogenetic protein (BMP), fibroblast growth factor (FGF), transforming growth

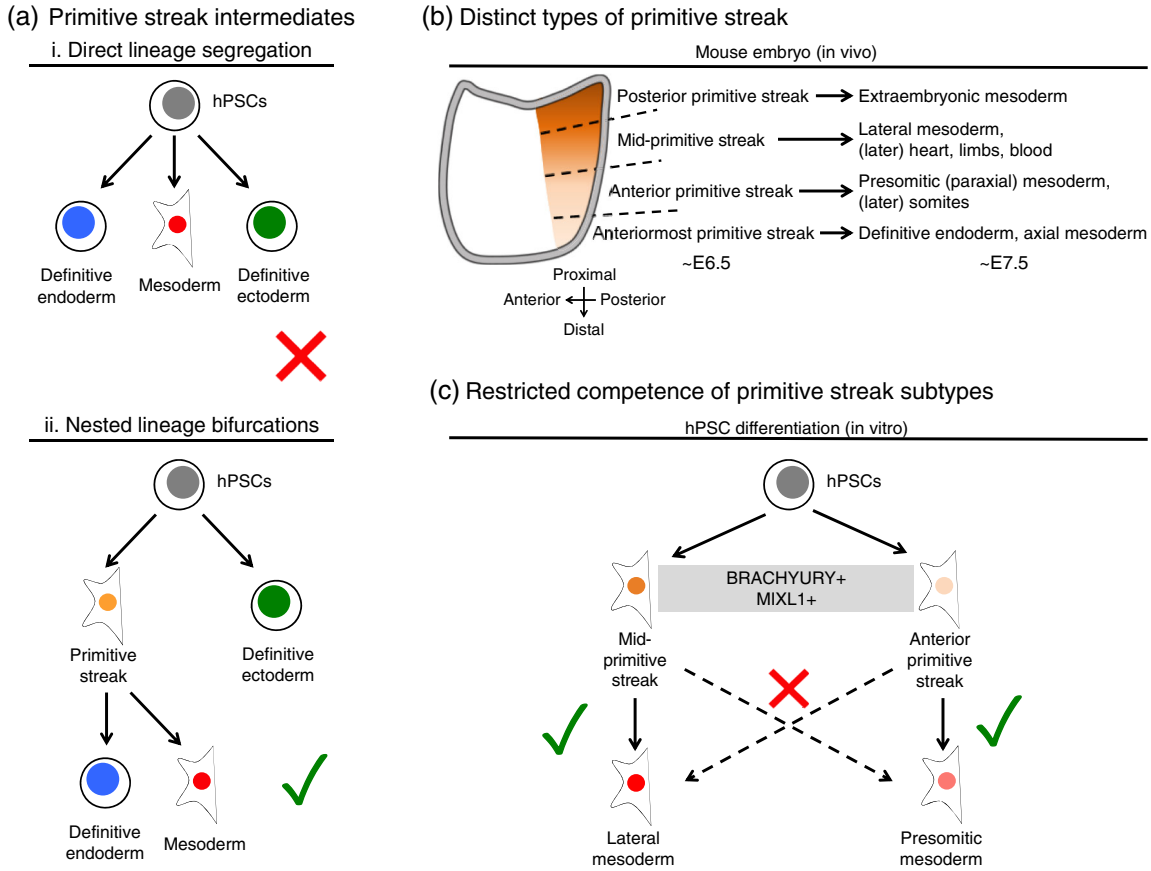

FIGURE 1 Primitive streak differentiation and the importance of the very first steps of hPSC differentiation. (a) Human pluripotent stem cells (hPSCs) do not directly differentiate into definitive endoderm or mesoderm (i), but first must differentiate through a transitory primitive streak intermediate (ii). (b) In the ~6.5-day-old (~E6.5) mouse embryo, there is no "pan-mesoderm" precursor; rather distinct primitive streak lineages give rise to different types of mesoderm (Lawson, Meneses, & Pedersen, 1991; Rosenquist, 1970; Tam & Beddington, 1987). (c) hPSC-derived anterior and mid primitive streak populations are broadly marked by both BRACHYURY and MIXL1; however, each primitive streak subtypes has a distinct lineage potential in terms of its ability to further differentiate into downstream cell types

factor-beta (TGFβ), and/or Wingless-related integration site (WNT) to differentiate hPSCs toward primitive streak-containing populations as a prelude to downstream endoderm or mesoderm formation (e.g., Ang et al., 2018; Bernardo et al., 2011; Chu et al., 2019; D'Amour et al., 2006; Gertow et al., 2013; Li et al., 2019; Loh et al., 2014; Loh et al., 2016; Mendjan et al., 2014; Rao et al., 2016; Yu, Pan, Yu, & Thomson, 2011). Differentiation of hPSCs through the intermediacy of the primitive streak is paramount to efficiently generate endoderm or mesoderm at later stages of differentiation, as omitting primitive streak induction (e.g., by withholding WNT) leads to a total failure to generate endoderm (Li et al., 2019) or mesoderm from hPSCs (Rao et al., 2016).

Yet, even the primitive streak concept is an oversimplification. Historically, the primitive streak has been morphologically defined as a structure within the gastrulating embryo. Nevertheless, it is now evident that it harbors a spectrum of related yet molecularly and functionally distinct cell types known as various primitive streak subtypes. Such complexities first surfaced when in vivo analyses failed to discover a singular "primitive streak" intermediate that has the full potential to generate all types of endoderm and mesoderm in vivo. Rather, within the early primitive streak, different subdomains of the primitive streak are each fated to give rise to distinct derivatives in vivo (Figure 1b). Specifically, anterior-most (distal-most) primitive streak gives rise to definitive endoderm and axial mesoderm; the anterior primitive streak generates presomitic mesoderm; the mid-primitive streak conceives lateral mesoderm; and finally, posterior (proximal) primitive streak yields extraembryonic mesoderm (Lawson et al., 1991; Rosenquist, 1970; Tam & Beddington, 1987) (Figure 1b).

Thus, in a strict sense, hPSCs are not differentiated into "primitive streak"—there is a molecular and functional diversity in primitive streak subtypes, each the starting point for a different downstream cell type. Within the first 24 hr of differentiation or so, hPSCs initially differentiate into different subtypes of primitive streak (e.g., anterior-most,

anterior, mid, and posterior primitive streak) (Loh et al., 2016; Mendjan et al., 2014). These primitive streak subtypes are molecularly distinct, with *FOXA2*, *GSC*, and *HHEX* enriched in anterior, and *CDX2* and *FOXF1* enriched in posterior primitive streak populations generated from hPSCs in the first 24 hr of differentiation (Bernardo et al., 2011; Loh et al., 2014; Loh et al., 2016; Mendjan et al., 2014; Sumi, Tsuneyoshi, Nakatsuji, & Suemori, 2008). These diverse primitive streak populations are functionally distinct: they have separate lineage potentials. Twenty four hours later, hPSC-derived anterior-most primitive streak differentiates into definitive endoderm and hPSC-derived anterior primitive streak progresses into presomitic mesoderm, whereas hPSC-derived mid-primitive streak forms lateral mesoderm (Loh et al., 2016; Mendjan et al., 2014) (Figure 1c).

Hence, even at the incept of hPSC differentiation, induction of a particular type of primitive streak is imperative for the subsequent generation of endoderm or different types of downstream mesoderm. For instance, if mid-primitive streak is inadvertently generated, it cannot be efficiently differentiated into anterior primitive streak derivatives (e.g., presomitic mesoderm) and vice versa (although some degree of differentiation is still possible, suggesting a limited inherent plasticity; Loh et al., 2016) (Figure 1C).

The diversity of primitive streak subtypes and their restricted lineage potentials is an important point for hPSC differentiation protocols, because pan-primitive streak markers *BRACHYURY* and *MIXL1* are broadly expressed across all primitive streak subtypes in vivo (Rivera-Pérez & Magnuson, 2005; Robb et al., 2000) and in vitro (Loh et al., 2016; Mendjan et al., 2014). Therefore, despite encouraging progress in generating nearly pure cultures of hPSC-derived MIXL1$^+$ "primitive streak" (Chu et al., 2016; Takasato et al., 2014), it is critical to assess whether these protocols actually produce the specific primitive streak subtype (e.g., anterior, mid, or posterior) poised to produce a desired downstream differentiation outcome. Broadly speaking, beyond the examples mentioned here, whether other subtypes of primitive streak exist; their respective developmental potentials; and whether they can be efficiently derived from hPSCs remain outstanding questions.

2.2 | Endoderm

After the endoderm germ layer arises (at embryonic day 7–7.5 [~E7–7.5] of mouse development), it is compartmentalized along the anterior–posterior axis into the anterior endoderm (foregut) and posterior endoderm (midgut/hindgut, ~E8.5), which presages the subsequent emergence of specific endodermal organ progenitors (~E9.5) (Zorn & Wells, 2009) (Figure 2ai). Efforts to optimize hPSC differentiation into these endodermal organ progenitors have been availed by markers that identify distinct progenitor domains along the anterior–posterior length of the endoderm in vivo (Grapin-Botton, 2005; Sherwood, Chen, & Melton, 2009).

A general concept is that endoderm anterior–posterior patterning must precede organ progenitor specification, both in vivo and in vitro. For instance, endoderm must first be posteriorly patterned into CDX2$^+$ mid/hindgut through the influence of BMP, FGF, and/or WNT signals (Loh et al., 2014; Sherwood, Maehr, Mazzoni, & Melton, 2011; Spence et al., 2011) before it can be further differentiated into intestinal cell types (Figure 2aii). Similarly, endoderm must first be anteriorized into anterior foregut endoderm intermediates before one can subsequently access lung or thyroid fates (Green et al., 2011) (Figure 2aii). Hence, while the in vitro chronology broadly reflects the in vivo developmental sequence, a number of issues remain unresolved.

There is considerable ambiguity regarding the precise sequence of nested lineage choices and the exact identity of the intermediate progenitors through which the endoderm germ layer eventually becomes diversified into a dozen different endodermal organs (Zorn & Wells, 2009) (Figure 2ai). This point is illustrated by the directed differentiation of hPSCs into pancreatic cell types, which is of immense importance to develop cell therapies for diabetes. Prevailing strategies typically differentiate hPSC-derived endoderm into "primitive gut tube", "posterior foregut," and then "pancreatic progenitors" (Kroon et al., 2008; Pagliuca et al., 2014; Rezania et al., 2014) (Figure 2bi).

Yet, many questions surround the precise developmental origin and identity of posterior foregut endoderm and pancreatic progenitors. First, how is the posterior foregut specified from hPSCs? Does endoderm have to be initially differentiated into a "pan-foregut progenitor" or "primitive gut tube" that bifurcates to form the anterior foregut (Green et al., 2011) versus the posterior foregut (Figure 2bii)? Alternatively, the anterior foregut and posterior foregut might be totally distinct lineages that arise separately from hPSC-derived endoderm without passing through a common bipotent intermediate (Figure 2bii). This is not an idle intellectual exercise: in fact, understanding how to appropriately specify hPSC-derived posterior foregut is of paramount importance, as manipulating signals at the posterior foregut stage of differentiation has far-reaching effects on later stages of pancreatic differentiation (Russ et al., 2015; Veres et al., 2019).

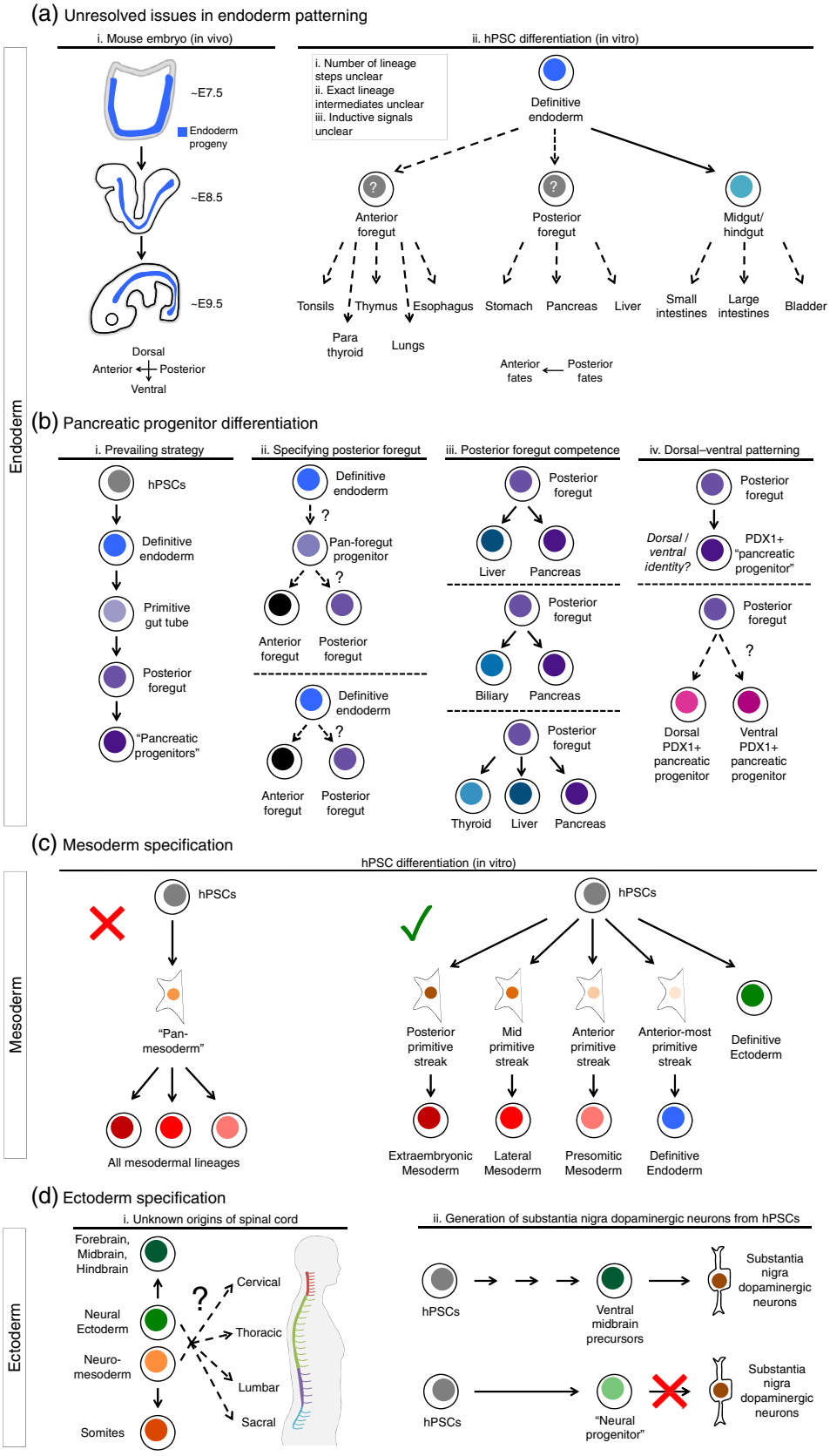

FIGURE 2 Unexpected complexities in hPSC differentiation towards the endoderm, mesoderm and ectoderm germ layers. (a) The process by which the definitive endoderm germ layer develops into >12 different organs in vivo is poorly understood (*left*); areas yet to be fully understood stymie the in vitro differentiation of hPSCs into endodermal derivatives (*right*). (b) Prevailing strategies to differentiate hPSCs toward pancreatic progenitors are ostensibly divided into several intermediate steps (i), yet outstanding questions remain, including: how the posterior foregut is specified (ii); what the exact identity of the posterior foregut is (i.e., what lineages can it differentiate into) (iii); and whether hPSC-derived pancreatic progenitors have a dorsal and/or ventral identity (iv). (c) There is no ubiquitous "pan-mesoderm" progenitor that gives rise to all mesoderm lineages (*left*), but rather hPSCs differentiate into distinct primitive streak subtypes, each of which gives rise to a distinct mesoderm subtype or alternatively the definitive endoderm (*right*). (d) The developmental origins of the spinal cord remain unknown, with neural ectoderm and neuromesoderm both serving as potential intermediates for generating spinal cord in vitro (i); recent success in generating substantia nigra dopaminergic neurons was made possible by differentiating hPSCs through a ventral midbrain precursor intermediate instead of a "pan-neural progenitor" (ii). hPSC, human pluripotent stem cell

Second, while consensus holds that hPSC-derived endoderm has to be differentiated into posterior foregut before generating pancreas, "posterior foregut" progenitors remain to clearly defined in vivo (Figure 2biii). Posterior foregut has been variously described as comprising a bipotent liver-pancreas progenitor (Chung, Shin, & Stainier, 2008), a

bipotent pancreas-biliary progenitor (Spence et al., 2009), and/or a multipotent thyroid-liver-pancreatic progenitor (Angelo, Guerrero-Zayas, & Tremblay, 2012) in vivo within zebrafish and mouse embryos (Figure 2biii). Is there only one "posterior foregut" route to generate pancreas, or are there multiple such routes, and if so, does the choice of intermediate route impact downstream pancreatic differentiation? Indeed, hPSC differentiation studies have suggested the existence of at least two types of posterior foregut, one poised for pancreatic, and the other primed for liver, differentiation in vitro (Ang et al., 2018).

Third, while hPSC differentiation protocols endeavor to generate early $Pdx1^+$ pancreatic progenitors, there is no singular "pancreatic progenitor" at this stage in vivo. Instead, at E9.5–E10 of mouse development, two anatomically distinct dorsal and ventral buds of $Pdx1^+$ pancreatic progenitors emerge (reviewed by Pan & Wright, 2011) (Figure 2biv). Do current hPSC-derived pancreatic progenitors in vitro correspond to the dorsal pancreatic progenitor program, ventral pancreatic progenitor program, both, or neither (Figure 2biv)? Do dorsal versus ventral pancreatic progenitors differ in their potential to generate downstream pancreatic cell types (Pan & Wright, 2011), and if so, should hPSC differentiation efforts attempt to specifically generate one or the other? While many efforts have optimized initial anterior–posterior endoderm patterning to enhance downstream production of hPSC-derived organ progenitors, the process of dorsal–ventral endodermal patterning (Sherwood et al., 2009) remains relatively enigmatic—specifically, how is it temporally coordinated with anterior–posterior endodermal patterning and what signals drive it? Incorporating a step emulating dorsal–ventral patterning in future endodermal differentiation strategies will be of interest.

While this vignette has focused on unknowns surrounding pancreatic progenitor specification, the same issues are germane to the generation of most endodermal organ progenitors. By analogy to pancreas, other endodermal organs may also have unexpected developmental progenitors, which may in turn necessitate alterations to hPSC differentiation protocols. For instance, the hindgut (prospective large intestine) emanates from at least two independent progenitors: definitive endoderm as well as extraembryonic endoderm, although the extent of contribution from both types of progenitor and whether their respective progeny last into adulthood remains to be fully resolved (Chan et al., 2019; Kwon, Viotti, & Hadjantonakis, 2008; Nowotschin et al., 2019). Taken together, the hierarchy of "pre-organ" lineage intermediates that arise during anterior–posterior and dorsal–ventral patterning of endoderm (or for that matter, all germ layers) remains to be resolved. This knowledge is of paramount importance if we are to efficiently manufacture particular endodermal derivatives from PSCs (Figure 2aii).

2.3 | Mesoderm

Even the notion of "germ layers" has needed revisions and embellishments in recent years. The mesoderm germ layer comprises a range of subtypes—including axial mesoderm, presomitic (paraxial) mesoderm, lateral mesoderm, intermediate mesoderm, and extraembryonic mesoderm—and each gives rise to various mature cell types.

One longstanding supposition of many mesodermal differentiation protocols has been that hPSCs must first be differentiated into a common "pan-mesoderm" progenitor (that can form any type of mesodermal lineage) which can be subsequently directed into a desired mesodermal subtype such as lateral/cardiac mesoderm (Burridge, Keller, Gold, & Wu, 2012) (Figure 2c). In contrast, lineage tracing and grafting experiments in mouse and chick embryos (Lawson et al., 1991; Rosenquist, 1970; Tam & Beddington, 1987) have argued against the existence of such a "pan-mesoderm" progenitor in vivo. As aforementioned, the primitive streak does not seem to constitute a "pan-mesoderm" precursor, and rather, distinct anterior, mid, and posterior primitive streak subsets are established early, and each primitive streak subset has the restricted competence to only produce a specific mesodermal subtype (Figures 1c and 2c).

There is no evidence for a ubiquitous "pan-mesoderm" progenitor that can be diversified into all downstream mesodermal derivatives (Mendjan et al., 2014), and this insight has significant ramifications for hPSC differentiation. Instead of differentiating hPSCs into a common "pan-mesoderm" intermediate before generating cardiac or presomitic mesoderm, hPSCs must be differentiated into the appropriate primitive streak intermediate (e.g., mid-primitive streak) before being further differentiated into cardiac mesoderm (Loh et al., 2016; Mendjan et al., 2014) (Figure 1c). Passage through the wrong primitive streak intermediate precludes the ability to efficiently further differentiate into a desired mesoderm subtype (Loh et al., 2016; Mendjan et al., 2014) (Figure 1c).

Hence describing mesoderm as a "germ layer" may be a historical anachronism (Baxter, 1977). Axial, presomitic, intermediate, lateral, and extraembryonic mesoderm subtypes are anatomically contiguous in the form of a "germ

layer," yet they do not seem to directly emerge from a common "pan-mesoderm" precursor—each mesoderm subtype has its own distinct developmental origin in the primitive streak (Figure 2c). This raises an interesting concept: mesoderm may constitute a collection of lineages that do not share a common immediate precursor (i.e., they are lineally distinct) yet they are anatomically juxtaposed with one another, which gave rise to the initial assumption that they were lineally related (Figure 2c). Mesoderm specification therefore differs from endoderm; in the latter, hPSCs must first be differentiated into a common intermediate precursor (definitive endoderm) that subsequently can be diversified into seemingly all endodermal organ derivatives (see above) (Figure 2aii).

That notwithstanding, our understanding of mesoderm patterning remains incomplete. While a number of protocols have been reported to generate presomitic, intermediate, and lateral mesoderm derivatives from hPSCs, production of axial or extraembryonic mesoderm in vitro is generally underexplored.

2.4 | Ectoderm

Unexpected complexities have also surfaced for ectoderm germ layer development. Here we focus on the spinal cord and substantia nigra dopaminergic neurons as two examples to illustrate how identifying intervening developmental intermediates is of paramount importance to guide hPSC differentiation.

The neural ectoderm was previously construed to constitute a uniform precursor population that underwent anterior–posterior patterning to form the forebrain, midbrain, hindbrain and spinal cord (reviewed by Lumsden & Krumlauf, 1996). However, a new emerging hypothesis is that the brain and spinal cord have distinct developmental origins and that they do not originate from a single, monolithic "neural ectoderm" precursor (Henrique, Abranches, Verrier, & Storey, 2015; Metzis et al., 2018). It has been hypothesized that neural ectoderm forms the brain, whereas a distinct "neuromesoderm" progenitor (variously referred to as "caudal lateral epiblast" or "axial progenitors") conceives the spinal cord in addition to the presomitic mesoderm (Takemoto et al., 2011; Tzouanacou, Wegener, Wymeersch, Wilson, & Nicolas, 2009) (Figure 2di). The existence of a joint spinal cord and presomitic mesoderm progenitor argues against a decisive division in mesoderm and ectoderm "germ layers"; consequently, the germ layer model is potentially oversimplified.

In light of this, understanding the potentially unique developmental origins of spinal cord is critical to efficiently generate sought-after spinal cord motor neurons from hPSCs. Prevailing methods differentiate hPSCs into spinal cord motor neurons proceed through neural ectoderm intermediates (reviewed by Sances et al., 2016), or more recently, presumptive neuromesoderm intermediates (Denham et al., 2015; Gouti et al., 2014; Lippmann et al., 2015; Verrier, Davidson, Gierliński, Dady, & Storey, 2018). Can spinal cord progenitors truly be derived via two independent routes (Tao & Zhang, 2016)? in vivo analyses are warranted to test whether both neural ectoderm and/or neuromesoderm are legitimate progenitors to the spinal cord; to quantify how extensively each might contribute to the spinal cord and to assess whether each progenitor pool might preferentially contribute only to specific spinal cord regions (e.g., cervical, thoracic, lumbar, and sacral) (Figure 2di). Indeed, although spinal cord differentiation is rightly regarded as the exemplar for directed differentiation efforts (Wichterle, Lieberam, Porter, & Jessell, 2002), the current controversies over spinal cord origins underscore the dire need for comprehensive lineage maps as the field revisits the cellular origins of seemingly "well-understood" cell types.

A recent success in the field has been the successful generation of substantia nigra dopaminergic neurons from hPSCs. There are multiple dopaminergic neuron subtypes, including those in the substantia nigra that developmentally originate from the ventral midbrain and principally control motor functions; these neurons are lost in Parkinson's disease and are priority targets for cell replacement therapies (reviewed by Arenas, Denham, & Villaescusa, 2015). While many efforts endeavored to differentiate hPSCs into "pan-neural progenitors" and then into dopaminergic neurons, the resultant neurons often lacked archetypic substantia nigra transcription factors and failed to properly engraft in vivo (reviewed by Arenas et al., 2015) (Figure 2dii). Access to hPSC-derived substantia nigra dopaminergic neurons was only made possible by identifying crucial intervening lineage intermediates—namely recognizing their specific origin in the ventral midbrain. Recent protocols have now differentiated hPSCs into ventral midbrain precursors through the manipulation of HEDGEHOG and WNT signals, and such precursors were subsequently competent to differentiate into substantia nigra dopaminergic neurons (Fasano, Chambers, Lee, Tomishima, & Studer, 2010; Kirkeby et al., 2012; Kriks et al., 2011) (Figure 2dii). In addition, the production of substantia nigra dopaminergic neurons proved intractable until these cells were accessed through the correct developmental intermediate.

3 | MAKING A FIRM DECISION: ACCURATELY DIRECTING CELL FATE AT LINEAGE SEGREGATION POINTS

How can hPSCs be coerced down a desired lineage route in preference to other possible developmental endpoints? The realization that development is organized as a cascade of "branching track" lineage choices (Waddington, 1940) has important corollaries. One recurrent principle is that at each binary lineage choice (also known as a lineage bifurcation), it is possible to exclusively differentiate progenitors into a given cell type through a two-pronged approach to promote the formation of a desired lineage while actively inhibiting formation of the alternative fate. This can be achieved by providing the relevant inductive signal(s) to specify the desired outcome, while *of equal importance* inhibiting signal(s) that would otherwise promote the alternate fate.

Indeed, this strategy has enabled efficient negotiation of the lineage choices leading from pluripotency to early germ layer fates. The first lineage bifurcation encountered by hPSCs leads them to either differentiate into primitive streak (which is the developmental precursor of endoderm and mesoderm) or ectoderm. BMP, FGF, TGFβ, and WNT promote primitive streak while inhibiting ectoderm formation (Bernardo et al., 2011; Blauwkamp, Nigam, Ardehali, Weissman, & Nusse, 2012; Chambers et al., 2009; Gadue, Huber, Paddison, & Keller, 2006; Loh et al., 2014; Loh et al., 2016; Yu et al., 2011). Hence, simultaneous application of these four signals (or combinations thereof) can generate a > 98% pure MIXL1$^+$ primitive streak population within 24 hr of hESC differentiation by blocking ectoderm formation (Loh et al., 2014; Loh et al., 2016). (Different levels of these four signals generates the graded primitive subtypes described above; Loh et al., 2016; Mendjan et al., 2014.) In contrast, complete blockade of primitive streak-inducing BMP and TGFβ signals suppresses primitive streak formation and instead diverts cells into ectoderm (Chambers et al., 2009).

Subsequently, primitive streak cells face another fork in the road to become either definitive endoderm or different subtypes of mesoderm. High levels of TGFβ specify endoderm (D'Amour et al., 2005; Loh et al., 2014), whereas BMP and WNT, respectively, promote lateral and presomitic mesoderm formation (Cheung, Bernardo, Trotter, Pedersen, & Sinha, 2012; Loh et al., 2016; Umeda et al., 2012) (Figure 3ai). Hence treating primitive streak intermediates with high TGFβ while concurrently inhibiting pro-mesodermal BMP signaling can "force" primitive streak cells to exclusively differentiate into SOX17$^+$ endoderm and not mesoderm (Loh et al., 2014; Sumi et al., 2008) with up to 99% purity by Day 2 of hESC differentiation (Loh et al., 2014; Loh et al., 2016) (Figure 3ai). Importantly, since differentiating hPSCs seemingly produce BMP, active inhibition of *endogenous* BMP signaling is crucial to fully suppress mesoderm formation and

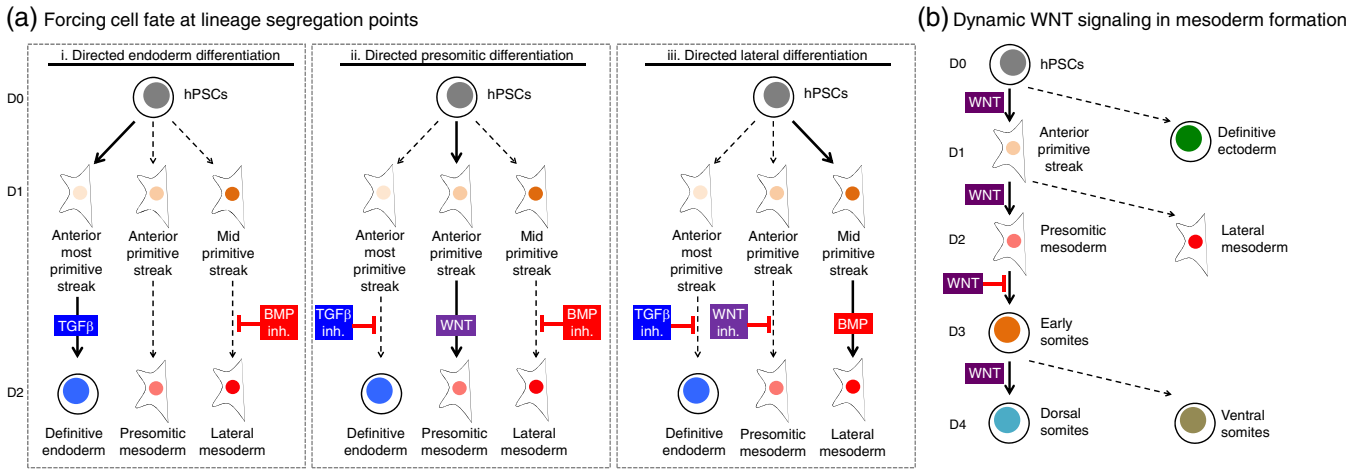

FIGURE 3 Reconstituting cell differentiation and assaying the products thereof. (a) In the first day of hPSC differentiation to endoderm and mesoderm lineages, hPSCs differentiate into anteriormost primitive streak, anterior primitive streak, and mid-primitive streak, which, respectively, have the competence to further differentiate into definitive endoderm, presomitic mesoderm, or lateral mesoderm, respectively. Subsequent to the primitive streak, manipulation of BMP, TGFβ, and WNT allows guided differentiation into one of these three lineages while suppressing differentiation into unwanted fates (i–iii) (Loh et al., 2016). (b) Over the course of 4 days of hPSC differentiation into dorsal somites (dermomyotome/future skeletal muscle precursors), WNT specifies four distinct cell types (Chal et al., 2015; Loh et al., 2016) and hence dynamic control of WNT signals every 24 hr of differentiation is crucial to progressively differentiate cells along this developmental trajectory

consolidate endodermal fate (Loh et al., 2014) (Figure 3ai). Hence while the factors exogenously added to coax differentiation are important, differentiating cells also endogenously signal to one another and manipulating such endogenous signals is critical. Differentiation protocols that principally specify endoderm through TGFβ addition (but do not include a BMP inhibitor to repress mesoderm) tend to yield endoderm with lower efficiency or consistency (Rostovskaya, Bredenkamp, & Smith, 2015).

Conversely, blockade of endoderm-inducing signals can instead steer differentiating primitive streak cells into mesoderm. Inhibiting TGFβ suppresses endoderm differentiation from primitive streak and instead broadly promotes mesodermal fate. WNT activation together with simultaneous blockade of TGFβ and BMP pathways efficiently differentiates primitive streak into presomitic mesoderm while respectively inhibiting differentiation towards endoderm and lateral mesoderm (Chu et al., 2019; Loh et al., 2016) (Figure 3aii). Along the alternate developmental route, BMP activation together with dual inhibition of TGFβ and WNT pathways specifies lateral mesoderm from primitive streak (Loh et al., 2016) (Figure 3aiii). The need to concomitantly manipulate these three pathways (BMP, TGFβ, and WNT) at this developmental stage emphasizes that, at any given point of development, there is no "single" dominant signaling pathway but rather the combinatorial integration of multiple cues is needed for the specification of most lineages.

In addition, by reducing the complex process of development into a sequence of simple lineage choices, it is possible at each stage to "force" progenitors at each juncture to exclusively differentiate toward a single desired outcome while suppressing extraneous differentiation to mutually exclusive, unwanted fates. By understanding the signals that specify one fate or the other at each lineage choice, it is possible to apply "paired inhibitor/agonist combinations" (Kyba, 2016) to more precisely guide differentiation toward a given lineage while deferring the competing dangers of other potential lineage outcomes—analogous to Odysseus precisely charting a path between Scylla and Charybdis. However, in vertebrate development, it is evident that developmental lineage choices are usually, but not always, binary (Davidson, 2010; Graf & Enver, 2009). Single-cell transcriptional analyses have suggested that certain lineage decisions may not be sharp segregations but rather may be construed as gradually diverging continua (Laurenti & Göttgens, 2018; Wagner et al., 2018). Validation of whether complex "multiway" lineage decision points truly exist—and whether cellular fate can be precisely directed at such junctures—warrants further attention.

4 | TEMPORALLY DYNAMIC SIGNALING AND RAPID CELL-FATE TRANSITIONS

Another prevailing principle is how single developmental signals are dynamically re-interpreted to specify distinct lineages within a short span of time during in vitro differentiation (Rao & Greber, 2016). By way of example, while in the first 24 hr of differentiation, WNT initially promotes hPSC differentiation into primitive streak, 24 hr later, it promotes the progression of primitive streak into presomitic mesoderm (Chal et al., 2015; Loh et al., 2016). Then 24 hr later, it inhibits presomitic mesoderm differentiation into early somites, and finally 24 hr later, it blocks somite differentiation into dorsal somites while promoting a ventral somite fate (Chal et al., 2015; Loh et al., 2016). Hence, during the short span of 4 days of in vitro differentiation, WNT specifies four distinct lineages (Figure 3b). This closely parallels the respective emergence of these lineages in the mouse embryo, wherein ~E5.5 post-implantation epiblast differentiates into ~E6.5 primitive streak, forming presomitic mesoderm by ~E7.0–E7.5 which then segments into early somites (~E8.0) and then forms dorsal somites (~E8.0–E8.5), with lineages segueing into one another every ~12–24 hr.

Yet in certain differentiation schema, the same developmental signals are continuously applied for several consecutive days, weeks, or even months (especially in the case of organoid differentiation [see below]). This might explain why such protocols yield a heterogeneous assembly of lineages: the same signal is dynamically re-interpreted to yield a multitude of outcomes as time progresses.

How is a single signal re-interpreted over such a short span of time by a developing cell to signify different outcomes (Rankin et al., 2017; Wandzioch & Zaret, 2009)? The answer may lie in cell-intrinsic changes in competence to respond to the same signal. Additionally, interpretation of any given signal also likely depends on the context of combinatorial signals delivered in parallel. Whatever the underlying mechanisms, the rapid "re-utilization" of a single signal across consecutive lineage decisions to specify extremely different fates is likely how evolution succeeded in efficiently utilizing a common developmental toolkit of roughly a dozen major pathways (De Robertis, 2008) to encode hundreds of distinct possible fates. In any case, the temporal dynamism with which a single signal (e.g., WNT) is re-interpreted in turn demands equally dynamic modulation of developmental signals to specify desired outcomes as differentiating cells dynamically pass through successive fate transitions (Figure 3b).

5 | FAST FORWARDING DIFFERENTIATION: TRANSCRIPTION FACTOR OVEREXPRESSION

Given that it has been so vexing to efficiently differentiate hPSCs using extracellular signals, others have eschewed the use of such signals and have instead directly overexpressed transcription factors (TFs) of the desired cell type within hPSCs. The goal is to forcibly instantiate the transcriptional program of the desired cell type, thus "short cutting" normal development. This is reminiscent of how in Greek mythology, Athena was reputed to be born "fully fledged" from the head of Zeus, dispensing with the usual protracted process of postnatal development.

While it typically takes weeks or months for extracellular signals to differentiate hPSCs into electrophysiologically active neurons, remarkably the overexpression of the neuronal TF *NGN2* in hPSCs generated neurons within ~1 week and with nearly 100% efficiency (Zhang et al., 2013) (Figure 4a). The unprecedented speed and efficiency of neuronal specification in this system speaks to the power of TF overexpression as a potential strategy to generate desired cell types; other early successes included the rapid derivation of megakaryocytes (Elcheva et al., 2014; Moreau et al., 2016). The surprising ability to "fast forward" differentiation from hPSCs into desired cell types—while skipping intervening steps once thought to be crucial (see above)—raises a host of questions.

First, does developmental history matter: are the TF-induced lineages "normal", though they have forsaken their normal developmental progenitors? That is, do the ends justify the (unusual) means? For instance, while *NGN2*-induced neurons were electrophysiologically functional, it remains to be determined whether they transcriptionally correspond to any neuronal subtype found in the native brain (Zhang et al., 2013) (Figure 4a). Perhaps *NGN2* overexpression may directly shortcut to the end of neuronal commitment by directly transactivating core "pan-neuronal" genes without affecting developmental genes linked with regional identity, which would be normally induced during the natural course of brain anterior–posterior and dorsal–ventral patterning (Figure 4a). This concept that TF-induced neurons are a *tabula rasa* partially lacking regional identity was supported by a recent analysis of motor neurons derived by overexpressing the TFs *Ngn2*, *Isl1*, and *Lhx3* in mouse PSCs (Briggs et al., 2017). These TF-induced motor neurons seemingly lacked an anterior–posterior identity (as indicated by *Hox* genes) that was typically found in motor neurons that were derived from embryos or that were differentiated from PSCs by extracellular signals (Briggs et al., 2017).

Second, what happens when a TF that is typically expressed in a mature cell type is mis-expressed in a hPSC—will it appropriately engage its appropriate target genes within the foreign chromatin landscape of a pluripotent cell? While some TFs constitute versatile "pioneer factors" that can activate their target genes irrespective of their local chromatin state (Iwafuchi-Doi & Zaret, 2016), the binding of most TFs is thought to be constrained by the cell's chromatin landscape (Lambert et al., 2018), which is in turn determined by its lineage. That is, forced expression of a lineage-specifying

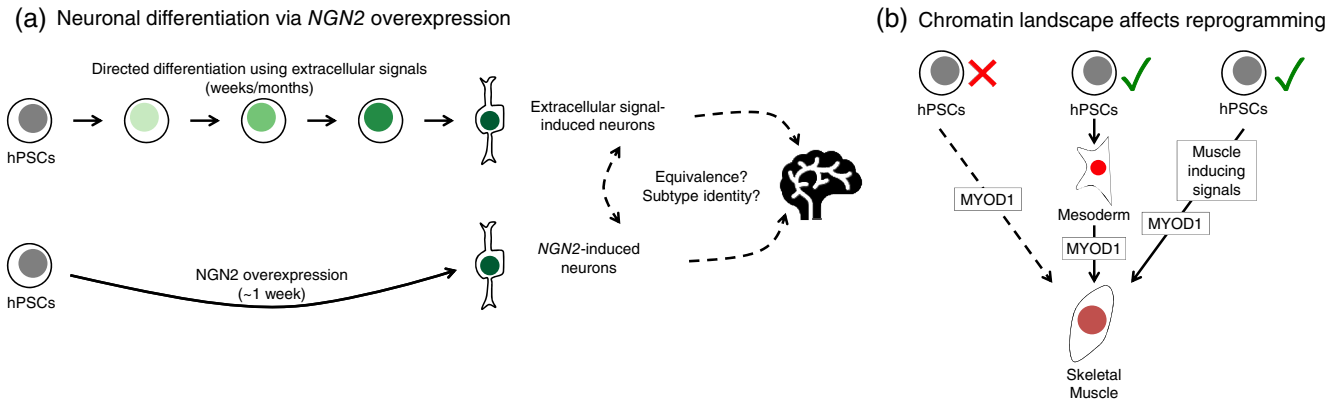

FIGURE 4 Expedited differentiation via the overexpression of lineage-specifying transcription factors. (a) Electrophysiologically active neurons can be generated from hPSCs via directed differentiation using extracellular signals or via overexpression of a neuron-specifying TF (*NGN2*) (Zhang et al., 2013). Do these two differentiation strategies generate equivalent neurons, do the resultant neurons have a clear subtype identity, and how comparable are they to their in vivo counterparts? (b) Overexpression of *MYOD1* in hPSCs fails to induce skeletal muscle in the absence of either first differentiating the cells through a mesoderm intermediate (Albini et al., 2013) or concurrently adding muscle-inducing extracellular signals (Pawlowski et al., 2017), suggesting the chromatin landscape decisively dictates the success of TF-based hPSC differentiation

master TF in hPSCs may fail to elicit the desired lineage, if same TF exerts different effects in different cell states. Indeed, ectopic overexpression of the muscle-specifying TF *MYOD1* in undifferentiated hPSCs fails to drive skeletal muscle differentiation, unless hPSCs are first differentiated into mesoderm prior to *MYOD1* induction (Albini et al., 2013) or are concurrently treated with skeletal muscle-inducing extracellular signals (Pawlowski et al., 2017) (Figure 4b). Nonetheless, the majority of cell types have not yet been successfully generated from hPSCs via TF overexpression, and it remains an open question as to why this is the case.

Perhaps one could obtain the best of both worlds through a collaboration of the two approaches, using TF overexpression to efficiently and rapidly engender the core transcriptional program of a desired cell type while using extracellular signals to concomitantly entrain or refine their regional (anterior–posterior or dorsal–ventral) identity. For instance, neurons have recently been induced from hPSCs by combining *NGN2* overexpression with neural ectoderm-specifying signals (dual BMP and TGFβ blockade) (Nehme et al., 2018). While this broadly increased the expression of pan-forebrain markers (Nehme et al., 2018), it remains unclear whether this strategy could be targeted to precisely induce a specific neuronal subtype in preference to others. One general limitation of TF-based hPSC differentiation is that TF overexpression is typically accomplished using genomically integrated transgenes in hPSCs. This might be less suitable for clinical applications unless mRNA transfection (Warren et al., 2010) or similarly transient transgene delivery techniques were used.

6 | THE VIRTUES OF PURITY

We have thus far emphasized the production of a single cell type in isolation from hPSCs, but is this even worthwhile or practicable to pursue? We opine that generating a pure population of a given lineage from hPSCs is a meaningful pursuit, at least for certain applications. Pioneering early studies differentiated hPSCs into an impure population containing a subpopulation of pancreatic cells. Transplantation of these impure cell populations into mouse models yielded not only human pancreatic tissue, but occasionally also mesodermal derivatives such as bone and cartilage in vivo (Kroon et al., 2008; Rezania et al., 2012; Rostovskaya et al., 2015) (Figure 5ai). Tumors were also observed after transplantation of PSC-derived, heterogeneous cell populations containing either a subset of liver cells (Haridass et al., 2010) or neural cells (Ganat et al., 2012). While subsequent advances have increased the uniformity of these cell populations, it is evident that the presence of unwanted cell-type(s) in heterogeneous cell populations can lead to untoward consequences after transplantation.

The identity and purity of hPSC-derived cell populations are often assessed by quantifying the percentage of cells expressing a "cell-type-specific" marker gene (or several such markers), but classifying cells in such a way inevitably piques questions about the choice and specificity of the marker gene(s). By way of example, while efforts to generate hPSC-derived β-cells routinely relied on INSULIN as a diagnostic marker, an incisive realization was that INSULIN$^+$ cells could spuriously coexpress the α-cell marker GLUCAGON and that these "polyhormonal" cells did not constitute functional β-cells (reviewed by Shahjalal, Abdal Dayem, Lim, Jeon, & Cho, 2018) (Figure 5aii). It is thus imperative to examine expression of *unwanted* lineage markers to ensure that they are not coexpressed with markers of the desired lineage. Taken together, the full battery of markers necessary to confidently assign cell-type identity is debatable.

Single-cell RNA-seq (scRNAseq)—which allows one to determine the coexpression of many genes in individual cells and to estimate the proportions of marker-positive cells—is becoming an objective measure of the composition and heterogeneity of differentiated populations (reviewed by Camp, Wollny, & Treutlein, 2018). For instance, scRNAseq analysis of hPSC-derived endothelial cell populations revealed that less than 7% of cells were endothelial cells, with the bulk of the culture resembling cardiomyocytes, liver cells, or smooth muscle cells (Paik et al., 2018) (Figure 5bi). scRNAseq analysis of hPSC-derived pancreatic populations revealed that less than 20% of cells were β-cells, with other pancreatic cell types predominating in the cultures (Veres et al., 2019) (Figure 5biiBii). scRNAseq analysis of hPSC-derived neural populations revealed that both forebrain and midbrain/hindbrain cell types were simultaneously generated, and intriguingly, that differentiation was asynchronous, with both neural progenitors and neurons co-existing even at later stages of differentiation (Yao et al., 2017). In another instance, scRNAseq was used to quantify the purity of hPSC-derived primitive streak, presomitic mesoderm and lateral mesoderm, revealing them to be 96–100% pure with regard to expression of selected lineage markers (Loh et al., 2016) (Figure 5biii).

However, an oft-overlooked limitation of scRNAseq is that it is an imperfect measurement of a single cell's transcriptome due to dropout: a technical limitation whereby lowly expressed marker genes in a single cell spuriously evade

detection (Kharchenko, Silberstein, & Scadden, 2014). Despite dropout, transcriptome-wide measurements of highly expressed genes can be used to assign putative cell types from scRNAseq data (Pollen et al., 2014), although the details of the clustering algorithm and other computational parameters can affect the number of "cell types" identified in the population. Another drawback is that scRNAseq measures mRNA, not protein levels. Consequently, scRNAseq is an estimation, but not a precise measurement, of cellular identity or purity.

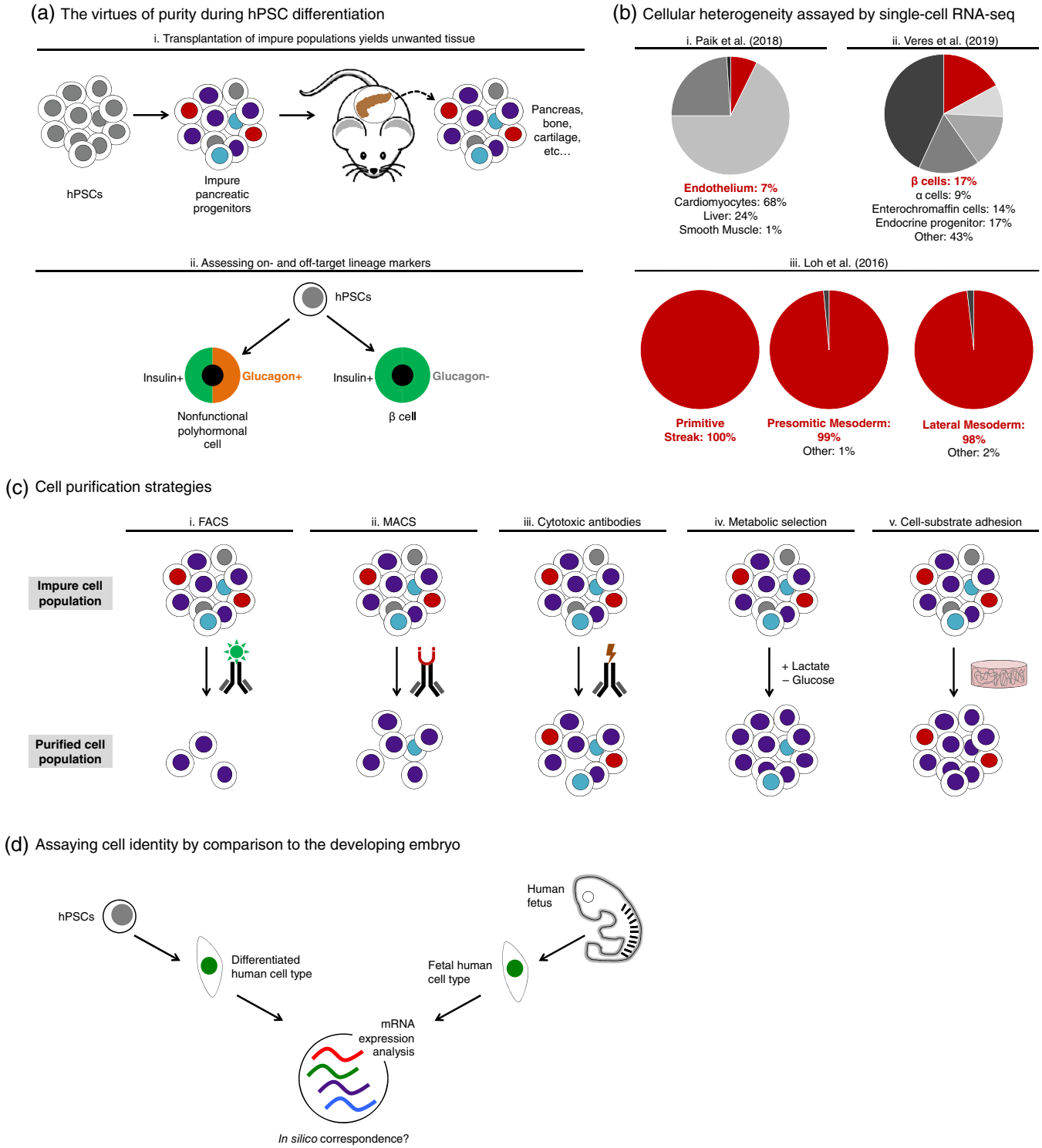

FIGURE 5 Legend on next page.

7 | SEPARATING WHEAT FROM THE CHAFF: CELL PURIFICATION STRATEGIES

A sobering realization is that even if directed differentiation protocols produce increasingly homogeneous cell populations from hPSCs, even a "99% pure" cell population may not be apropos for therapeutic transplantation. Even a frighteningly small number of undifferentiated hPSCs (10,000 cells) can form a tumor (a teratoma) upon transplantation (Lee et al., 2009). Certain hPSC-derived cell therapies may entail the transplantation of billions of cells into a given patient. To attain a desirable safety profile, by inference differentiation may have to exceed 99.99999% efficiency, which is difficult to fathom with extant directed differentiation schema (despite the developmental biology-based improvements detailed above).

Strategies to selectively purify a desired cell type and to eliminate all traces of unwanted cell types—especially undifferentiated hPSCs—from a heterogeneous population urgently warrant further exploration. Fluorescence-activated cell sorting (FACS) can yield highly pure populations but can be lengthy and strenuous on "sensitive" cell types (e.g., hPSC-derived pancreatic progenitors) (Kelly et al., 2011) (Figure 5ci). In contrast, magnetic enrichment is faster and gentler but can yield lower cell purities (Kelly et al., 2011) (5). Both of these strategies sort cells by virtue of their expression of surface markers and their efficacy is thus limited by the cell-type-specificity of the chosen markers; moreover, they entail cell dissociation and fundamentally rely on technologies that sort cells.

Recently, three alternate purification schema have emerged that do not entail the physical sorting of cells. First, treatment of heterogeneous differentiated populations with a cytotoxic anti-PODXL antibody efficiently lyses residual $PODXL^{+}$ hPSCs, thus depleting hPSCs without recourse to cell sorting (Choo et al., 2008) (5). Second, specialized media can be used to selectively ablate certain cell types in culture by exploiting cell type-specific metabolic vulnerabilities. For instance, differentiation in lactate-supplemented media in the absence of glucose significantly enriches for hPSC-derived cardiomyocytes, as cardiomyocytes can efficiently utilize lactate, whereas undifferentiated hPSCs and some other cell types cannot (Tohyama et al., 2013; Tohyama et al., 2016) (Figure 5civ). Third, the culture substrate itself can be used to select for certain lineages—for instance, while neural cells can adhere to laminin-111, while hPSCs cannot (Kirkeby et al., 2017) (Figure 5cv). Cytotoxic antibodies, metabolic selection, and cell-substrate adhesion represent means to enrich for desired cell types without physical cell sorting but have yet to be broadly applied to the enrichment of diverse cell types.

8 | IDENTIFYING THE TARGET: BENCHMARKING HPSC-DERIVED CELL TYPES

Even if the field can generate a "pure" population of a given cell type, how closely will hPSC-derived cell types approximate their in vivo counterparts, as pertains to a combination of molecular and/or functional criteria?

FIGURE 5 The virtues of purity and identity during hPSC differentiation. (a) Transplantation of hPSC-derived heterogeneous populations containing a subset of pancreatic progenitors into rodent models yielded a variety of unwanted cell types including bone and cartilage (Kroon et al., 2008; Rezania et al., 2012; Rostovskaya et al., 2015) (i); at later differentiation stages, assessing the expression of both insulin (an "on-target" marker) and glucagon (an "off-target marker) allows for the distinction between nonfunctional polyhormonal cells and β-cells (ii) (Pagliuca et al., 2014; Rezania et al., 2014) (Russ et al., 2015). (b) Single-cell RNA-sequencing of hPSC-derived endothelial (Paik et al., 2018) (i), pancreatic (Veres et al., 2019) (ii), primitive streak (Loh et al., 2016) (iii), presomitic mesoderm (Loh et al., 2016) (iii) or lateral mesoderm (Loh et al., 2016) (iii) populations estimates the purity and the composition of the respective cultures; percentages and cell-type identities are reported here as indicated in each of the published papers. (c) Enrichment of a particular cell type from a heterogeneous cell population can be accomplished using: (i) FACS, which strictly purifies cell types while adversely affecting cell yield and survival; (ii) magnetic activated cell sorting (MACS), which enriches for cell types with lower purity, but maintains higher cell yield and survival; (iii) cytotoxic antibodies, which generally can deplete one unwanted cell type (e.g., hPSCs), but spare other contaminating cell types; (iv) metabolic selection, which facilitates the selective growth of a desired cell type while also potentially maintaining other contaminating cell types that share the same metabolic growth advantage; (v) and cell-substrate adhesion which provides favorable conditions for target cells to survive while some, but not all, contaminating cells die. (d) The developmental potential of early hPSC-derived cell types might be tested by gene expression comparisons with the analogous cell type derived from the human fetus. FACS, fluorescence-activated cell sorting; hPSC, human pluripotent stem cell

To this end, we must benchmark hPSC-derived cell types against freshly derived primary human tissue. It is possible to obtain cells from mid- to late-gestation human embryos or from adult humans as comparators for terminally differentiated cell types from hPSCs. For instance, scRNAseq and other transcriptional assays have been used to directly compare neural cells isolated from a human fetus against their hPSC-derived counterparts (Kirkeby et al., 2012; La Manno et al., 2016; Pollen et al., 2019) (Figure 5d).

While it is possible to directly compare more developmentally advanced cell types in this way, molecularly benchmarking hPSC-derived germ layer or early tissue progenitors is currently impossible. It is technically and ethically infeasible to obtain their in vivo counterparts—which arise in Weeks 2–4 of human embryonic development (O'Rahilly & Müller, 1987)—for molecular comparisons. Even if a given cell type can be isolated from the human fetus or adult, any comparison against in vitro-derived cell types will likely be imperfect, as the genetic background of the hPSC line under investigation will almost certainly differ from that of the tissue donor unless an isogenic hiPSC line was derived and used for differentiation. Comparisons matched in both genotype and developmental stage are logistically easier for mouse PSC-derived cell types, as recently performed for in vivo- versus in vitro-derived mouse motor neurons (Ichida et al., 2018).

But what degree of concordance would one expect to see between in vivo cell types that have experienced a physiological environment by comparison to in vitro lineages mostly grown on plastic (Figure 5d)? Even naïve mouse PSCs (an extremely well-characterized in vitro cell type) have transcriptional differences by comparison to their in vivo counterparts within the pre-implantation blastocyst, mostly related to metabolism (Boroviak, Loos, Bertone, Smith, & Nichols, 2014). This might reflect metabolic adaptation to cell culture and as such may be less significant. Nonetheless, it raises an interesting precedent as to how precisely in vitro-differentiated counterparts (or, for that matter, any cultured cell) will resemble their presumed in vivo counterparts. Indeed, scRNAseq recently revealed that while hPSC-derived substantia nigra dopaminergic neurons approximated their in vivo fetal counterparts, they also shared certain attributes with other mutually exclusive lineages (La Manno et al., 2016). Expanding this scRNAseq analysis to additional hPSC-derived neural lineages revealed that in vitro-derived cell types consistently displayed a perturbed metabolic signature entailing elevated glycolysis and an unfolded protein response by comparison to primary human fetal cell types (Pollen et al., 2019).

Of course, it is likely that hPSC-derived lineages will never be a perfect replica of authentic cell-types in vivo; at best, they will constitute a facsimile. From a pragmatic point of view, minor transcriptional differences between in vivo- and in vitro-derived lineages may be inconsequential. The unresolved question is whether any discrepancies from the in vivo transcriptional program might compromise the functionality of differentiated cells. By way of example, current-generation hPSC-derived β-cells express cornerstone markers of β-cell identity but minimally express transcription factors that delineate mature, adult β-cells (e.g., *MAFA* and *SIX3*). (Veres et al., 2019). It remains to be determined whether the lack of these "mature" β-cell transcription factors might underscore functional defects in hPSC-derived β-cells.

9 | DIVIDENDS FROM DIVERSITY: DELIBERATELY GENERATING A HETEROGENOUS CELL POPULATION AND WHAT IT MEANS TO BE AN "ORGANOID"

While many in vitro differentiation strategies endeavor to create a homogeneous cell population, actual tissues are not homogeneous: rather, they comprise a rich diversity of cell types. To imitate this diversity, some hPSC differentiation efforts intentionally generate a *heterogeneous* cell population, often in the form of three-dimensional "organoids" (defined below) (Figure 6a).

Cellular heterogeneity has its advantages: development entails reciprocal signaling between multiple cell types that decide cellular fate in addition to mechanical interactions between multiple cell types that fashion a tissue's final shape (Figure 6a). Hence, a single cell type in isolation might be unlikely to fully develop in monoculture. This speaks to the importance of cellular heterogeneity in some contexts, as embodied by hPSC-derived intestinal and kidney organoids.

The intestine contains commingled epithelial and mesenchymal cell types of endodermal and mesodermal origin, respectively. In vivo, intestinal mesenchyme is strictly required to instruct proper development of the adjacent intestinal epithelium, both by sculpting its morphogenetic shape and by serving as a source of critical extracellular signals (Roberts, Smith, Goff, & Tabin, 1998; Shyer, Huycke, Lee, Mahadevan, & Tabin, 2015). Thus efforts to differentiate hPSC-derived, three-dimensional intestinal organoids deliberately produce a heterogeneous population of endoderm together with a subset of mesoderm at an early stage of differentiation, with the objective of generating intestinal

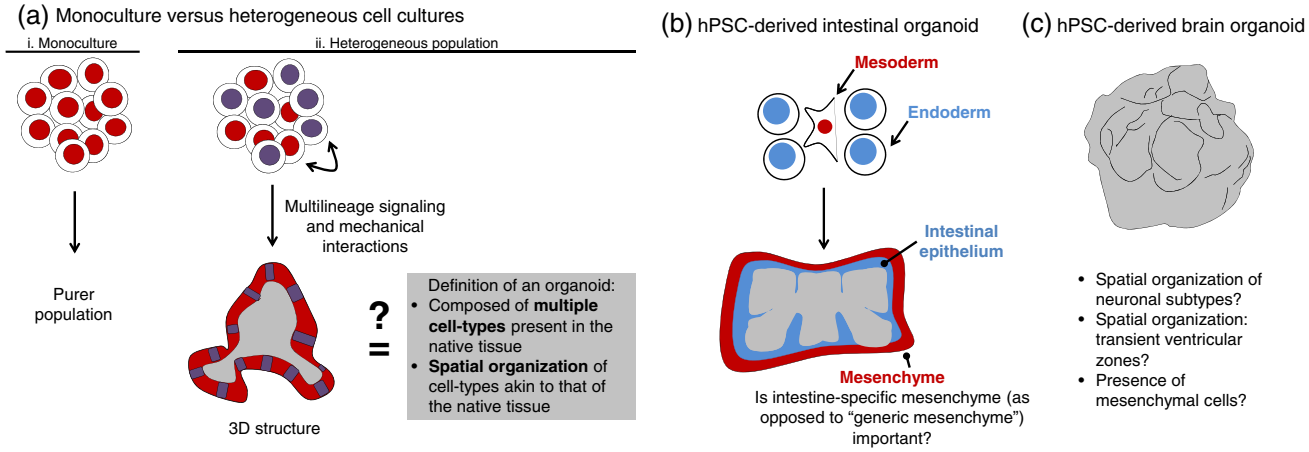

FIGURE 6 The merits of heterogeneous cell populations and their relationship to organoids. (a) Differentiating hPSCs into a homogenous monoculture creates purer populations of a given cell type (i), while differentiating cells in a heterogeneous 3D culture provides different cell types the opportunity to reciprocally signal, and mechanically interact, with one another (ii); however whether all 3D cultures meet the strict definition of an "organoid" (Lancaster & Knoblich, 2014) remains to be determined. (b) Current-generation hPSC-derived intestinal organoids rely on the codifferentiation of endoderm and mesoderm derivatives to generate appropriate cellular diversity and spatial organization akin to the native intestine. (c) Current-generation hPSC-derived brain organoids possess some key features of early brain development, but various questions remain. hPSC, human pluripotent stem cell

epithelial cells as well as their ensconcing mesenchyme (Spence et al., 2011) (Figure 6b). While it remains to be formally proven, it seems likely that the coexistence of endodermal and mesodermal components is indispensable for the formation of spatially complex intestinal organoids.

The kidney is also constructed from the spatial juxtaposition of two lineages—the uretic epithelium and metanephric mesenchyme—whose coalescence forms a ramified tree of collecting ducts decorated with nephrons. hPSC differentiation into a heterogeneous population comprising both uretic epithelium and metanephric mesenchyme eventually generates three-dimensional kidney organoids with convoluted tubules resembling part of the kidney (Takasato et al., 2015). Again, the simultaneous generation of both uretic epithelium and metanephric mesenchyme in vitro harkens to the developmental coexistence of these cell types and how reciprocal signaling between them is required for proper kidney formation in vivo.

With such remarkable progress in generating organoids, it is worth querying how closely they approximate actual tissues. This speaks to the deeper question of what is the precise definition of the term "*organoid*"? If, as their namesake implies, "organoids" are to be reminiscent of organs, we suggest that "organoids" meet two critical criteria. First, they should comprise multiple cell types that are found in the native organ (Lancaster & Knoblich, 2014). Second, these cell types should be spatially organized in a manner analogous to that of the native organ (Lancaster & Knoblich, 2014) (Figure 6a).

We therefore urge caution that not all three-dimensional cell cultures should be referred to as organoids: while many three-dimensional cultures contain multiple cell types, if they are not spatially organized analogous to the native tissue, they should not be given the moniker "organoid." For instance, certain three-dimensional differentiation strategies generate heterogeneous cell populations that do not show appropriate spatial organization and therefore are better classified as "spheroids" or "aggregates." Such deviations from normal tissue architecture are fascinating and informative, as they suggest gaps in our understanding of tissue assembly and potential strategies for improvement. For instance, hPSC-derived brain organoids often harbor neurons radially organized around ventricular zone-like progenitor regions (Kadoshima et al., 2013; Lancaster et al., 2013; Pasca et al., 2015). However, these ventricular zone regions have been reported to disperse after several weeks (Velasco et al., 2019) (Figure 6c). Moreover, the positions of $TBR1^+$/$SATB2^+$ deep and $BRN2^+$ middle cortical layer neurons appears to be reversed in hPSC-derived brain organoids (Qian et al., 2016) (Figure 6c). Therefore, an improved understanding of cortical neuron subtype migration and/or cell sorting could be harnessed to rationally engineer better-organized organoids.

As a key step toward generating organoids, how can one simultaneously generate multiple cell types in the same culture and control the relative frequencies of these cell types? To meet this challenge, a recent method generated

hPSC-derived liver precursors and then, at an intermediate step of differentiation, introduced defined numbers of generic endothelial cells (e.g., human umbilical vein endothelial cells) and generic mesenchymal cells to produce three-dimensional cultures (Takebe et al., 2013; Takebe et al., 2017). Incorporation of endothelial and mesenchymal cells is an important step to engineer increasingly sophisticated organ simulacra.

However, such multilineage coculture strategies also bring their own attendant difficulties, because it has been recently appreciated that there are no "generic" endothelial cells and no "generic" mesenchymal cells in vivo. Instead, endothelium and mesenchyme adopt organ-specific subspecializations and considerably vary in their transcriptomes and functions across different organs (Han et al., 2018; Potente & Mäkinen, 2017). It is unclear whether "generic" endothelial and mesenchymal cells can productively interact with liver progenitors, or whether liver-specific endothelial cells and liver-specific mesenchymal cells might fare better for this application. The same applies for the aforementioned hPSC-derived intestinal organoids (Spence et al., 2011): while they clearly harbor mesenchyme, do these constitute intestinal-specific mesenchyme that might be uniquely suited to promote intestinal epithelium development (Figure 6b)?

Finally, while cellular heterogeneity is integral to the very definition of organoids, excessive or unwanted heterogeneity may be deleterious. By way of example, scRNAseq has revealed the coexistence of dorsal forebrain, ventral forebrain, mesenchymal and other cell types in hPSC-derived brain organoids (Camp et al., 2015; Pollen et al., 2019; Quadrato et al., 2017). Such scRNAseq analyses have revealed substantial variation between individual experiments and among organoids from the same experiment (Quadrato et al., 2017) or amongst organoids derived from different hPSC lines (Pollen et al., 2019), although other analyses suggested less variability (Velasco et al., 2019; Yoon et al., 2019). In any case, the presence of mesenchymal-like cells that express *MYOSIN*, *COLLAGEN*, and *DECORIN* genes (Camp et al., 2015; Pollen et al., 2019; Quadrato et al., 2017) in current-generation brain organoids is unexpected (Figure 6c). It remains to be determined whether these mesenchymal cells foster, or might occlude, proper brain development and the spatial organization of such organoids.

10 | A HUMAN IS NOT A MOUSE (OR A FROG, FISH, OR CHICKEN)

A common critique of current "developmentally guided" hPSC differentiation efforts is that such strategies are not actually guided by *human* development; rather, they are informed by our knowledge of how model organisms such as mouse, frog, fish, or chicken develop (as emphasized by this very review). Yet it is evident that a human is not a mouse. Human and mouse evolutionarily diverged tens of millions of years ago (Perlman, 2016), since which their exact mode of development—including the molecular identity of various embryonic progenitors and the signals that specify them—has diverged.

This issue of evolutionary differences is not simply theoretical; take, for instance, the very practical issue of how long differentiation should take. hPSC differentiation protocols often generate target cell types in weeks or months, and this protracted time course is assumed to arise from, and parallel, the prolonged duration of human embryogenesis, which takes months in vivo (Tao & Zhang, 2016). Speaking to this point, hPSCs differentiating into a specific cell type sometimes demonstrate exquisitely species-specific timings (Chu et al., 2019), which implies that relative slowness might be deeply and genetically ingrained into the process of hPSC differentiation. Yet in other cases, differentiation can be externally accelerated. As aforementioned, alternating activation and inhibition of WNT (and other signals) sequentially differentiates hPSCs into primitive streak, presomitic mesoderm, somites and finally dorsal somites within 4 days in vitro (Loh et al., 2016) (Figure 3b). While this respects the developmental timing of the mouse embryo—wherein the same cell types are produced over the course of 4 days in vivo—it is unclear why human cells have the capacity to differentiate along the "mouse" schedule when human development is ostensibly many times longer (O'Rahilly & Müller, 1987). Perhaps the changing extrinsic signals that induce these respective cell types are provided by the human embryo over a protracted duration, but if they are delivered rapidly in quick succession, the pace of differentiation can be artificially accelerated. This point notwithstanding, timing is only one out of many possible divergences between human vs. model organism development: hence, analyses of human embryogenesis are direly needed to guide hPSC differentiation.

Frankly, the fundamental issue is that there is a paucity of knowledge surrounding early human embryonic development. As aforementioned, human germ layer specification, germ layer patterning, and organ progenitor specification unfold in Weeks 2–4 of human embryogenesis, when it is technically and ethically impossible to obtain human embryos for analysis (O'Rahilly & Müller, 1987). Heroic efforts have analyzed the gastrulating embryos of pig

(Kobayashi et al., 2017) or monkeys (Sasaki et al., 2016), and in rare cases even somite-stage human embryos were obtained (Xi et al., 2017; Zhang et al., 2010). However, we still lack a precise understanding of how germ layer specification and patterning occurs in human embryos, although the core principles may be reminiscent of model organisms.

Recent work has revealed partial differences in the expression and function of genes between the human and mouse blastocyst (reviewed by Wamaitha & Niakan, 2018), raising the question of whether later developmental events might also diverge in significant ways. Indeed, while primordial germ cells in mouse express *SOX2*, their human counterparts do not express *SOX2* but rather express the related family member *SOX17* (Irie et al., 2015; Sasaki et al., 2015). This evolutionary divergence significantly impacts in vitro differentiation, as efforts to generate primordial germ cells from hPSCs leverage *SOX17*, but not *SOX2*, as a diagnostic marker for such cells (Irie et al., 2015; Sasaki et al., 2015). PAX6, a marker of early neural commitment, has been reported to have a different onset of expression in human versus mouse (Zhang et al., 2010). However whether most marker genes (including the ones listed above) show different expression patterns or functions in human versus mouse still awaits unambiguous confirmation owing to the dearth of appropriately stage-matched human embryos for analysis.

11 | FUTURE PERSPECTIVES

On the face of it, the challenge of exclusively differentiating hPSCs into any one out of hundreds of potential fates seems bewildering. This task is evocative of how Odysseus too had to chart a convoluted path back home with incomplete knowledge of the landscape before him, sometimes accessing the correct waypoints and at other times narrowly avoiding side trips leading to unsavory destinations. Despite alternating progress over the past few decades, it is now possible to more effectively generate certain lineages from hPSCs, in part by drawing on the principles outlined above.

One emergent principle to enhance the efficiency of hPSC differentiation is to suppress the production of unwanted cell types. This can be accomplished by logically blocking the extracellular signals that specify unwanted cell types at each developmental lineage decision, thus coercing hPSCs toward a desired developmental end (Figure 3a). A second issue is that as differentiating cells navigate successive lineage choices, the extracellular signals that induce a given cell type change rapidly, even within 24 hr. The same signal can initially promote, and then repress, the formation of a given lineage. These signals must thus be manipulated with equal temporal dynamism to enable cells to segue between successive differentiation states (Figure 3b). However, the coming years will continue to bring ample challenges, four of which we detail below and others that we cannot foresee.

First, there is an ever-increasing proliferation of differentiation protocols to generate various cell types. There will be a need for systematic side-by-side comparisons of these protocols across diverse hPSC lines to assess reproducibility and achieve standardization, as recently done for definitive endoderm specification (Rostovskaya et al., 2015).

Second, developmental roadmaps must be expanded to encompass new *terra incognita*. As this review has emphasized, we are still ignorant of the complete number and identity of steps through which pluripotent cells differentiate into most cell types in vivo. Extant differentiation protocols may entail transition through some, but perhaps not all, of the requisite steps. While it may seem obvious that we must map the steps leading to desired lineages, to logically block the formation of unwanted cell types from hPSCs (Figure 3a), ironically we also have to map the routes and signals leading to these *unwanted* lineages as well. We also must understand, at the last steps of development, how fetal-like differentiated cell types mature into fully fledged, functional cell types (Cohen & Melton, 2011). New high-throughput strategies to determine progenitor-progeny differentiation paths in the mouse embryo (Chan et al., 2019) could revolutionize this field.

Third, beyond measurement of several molecular markers, it will be important to develop new ways to benchmark hPSC-derived cell types against their in vivo counterparts, especially entailing assays of physiological functions. This review has emphasized transcriptional comparisons of hPSC-derived cell types to their in vivo counterparts in the human fetus (Figure 5d). However, orthotopic transplantation of hPSC-derived cell types into non-human embryos (within technical and ethical limits) could constitute a new *functional* assay (Mascetti & Pedersen, 2016). Indeed, in utero transplantation of hPSC-derived neural crest cells into E8.5 mouse embryos led to engraftment in roughly one-quarter of mice (Cohen et al., 2016). However, it will not be trivial to precisely transplant hPSC-derived tissue progenitors into their counterpart tissues within the mouse embryo at most developmental times and places. Interspecific incompatibility could also obscure the ability of hPSC-derived tissue progenitors to develop in vivo: transplanted human cells may intrinsically differentiate at a different pace than the recipient non-human embryo or may not be able to respond to certain developmental signals emanating from it as well (Suchy, Yamaguchi, & Nakauchi, 2018).

Fourth, is it possible to generate hPSC-derived cultures that contain multiple cell types and are also spatially organized in ways that approximate native organs? There is tremendous enthusiasm in creating hPSC-derived 3D multilineage cultures, but in certain cases, the spatial organization of their constituent cells and the reproducibility of such spatial organization warrants further attention. This is paramount to assess whether specific 3D cultures satisfy the complete definition of being "organoids" (Lancaster & Knoblich, 2014) (Figure 6b). Or, if not, how can we reproducibly program their spatial organization? This is especially important because some definitions of "organoids" suggest that they must imitate some functions of their native organ (Lancaster & Knoblich, 2014). Adhering to the general biological principle that form prescribes function, it is likely that the correct spatial organization of multiple cell types relative to one another is necessary for organs (or organoids) to execute many physiological functions. We therefore urgently require high-throughput methods to systematically map the spatial architecture of 3D cultures. This is currently accomplished using immunostaining; yet, analyses that entail cell dissociation (e.g., scRNAseq; Camp et al., 2018) do not capture spatial information. However, for a given tissue section, it is now possible to simultaneously image the expression of hundreds of different mRNAs with great breadth (across large fields of cells) and precision (at single-cell resolution) (Wang et al., 2018). Such technologies may enable the high-throughput interrogation of organoid architecture, thus enabling the optimization of future organoid differentiation protocols that lead to improved spatial organization.

Answers to these questions are of practical import. There are over 30 completed or ongoing clinical trials that have, or are, transplanting patients with hPSC-derived cell populations (Guhr et al., 2018). It stands to reason that the efficacy and safety of future cell replacement therapies will be considerably advanced by understanding, and further improving, the process of hPSC differentiation.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Jonas L. Fowler: Visualization; writing-review and editing. **Lay Teng Ang**: Writing-review and editing. **Kyle M. Loh**: Conceptualization; visualization; writing-original draft, review, and editing.

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