Cell Stem Cell
Perspective

Dynamic Pluripotent Stem Cell States and Their Applications

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Embryonic pluripotency can be recapitulated in vitro by a spectrum of pluripotent stem cell states stabilized with different culture conditions. Their distinct spatiotemporal characteristics provide an unprecedented tool for the study of early human development. The newly unveiled ability of some stem cell types for crossing xeno-barriers will facilitate the generation of interspecies chimeric embryos from distant species, including humans. When combined with efficient zygote genome editing technologies, xenogeneic human pluripotent stem cells may also open new frontiers for regenerative medicine applications, including the possibility of generating human organs in animals via interspecies chimeric complementation.

Introduction

Following a precisely choreographed and spatiotemporally controlled developmental program, pluripotent cells, which are initially contained in the embryonic epiblast, can give rise to all cell lineages of the developing and adult organism. Embryonic pluripotency is short lived but can be captured in vitro under artificial culture conditions. Unlike the epiblast, pluripotent cells in culture can self-renew indefinitely while retaining multilineage differentiation abilities. Pluripotency can also be reinstated in cells of later developmental stages through culture adaptation (e.g., embryonic germ cells) (Matsui et al., 1992; Resnick et al., 1992), somatic cell nuclear transfer (SCNT) (Gurdon, 1962; Wilmut et al., 2002), or cellular reprogramming with defined transcription factors (iPSCs) (Takahashi and Yamanaka, 2006). These artificially converted pluripotent cells exhibit molecular and functional properties similar to and characteristic of the embryonic epiblast. Additionally, recent studies have introduced a new twist by unveiling subtle but functionally important, molecular differences among stem cells from distinct temporal and spatial domains within the epiblast (Hackett and Surani, 2014; Kojima et al., 2014; Nichols and Smith, 2009; Wu et al., 2015).

Thanks to these and other observations, our understanding of pluripotency has been greatly broadened in the past decade. Pluripotency, as we see it today, is no longer a singular property. The recent discovery of distinct spatiotemporal pluripotent states has brought us one step closer to grasping the essence of how the intrinsic developmental program is orchestrated among ephemeral epiblast cells in preparation for setting up the whole body plan. Moreover, discrete pluripotent states with unique molecular and functional features have expanded the utility of pluripotent stem cells (PSCs) for both fundamental and clinical research.

In this Perspective, we will provide a brief account of the distinct pluripotent states identified to date in both rodents and primates, their molecular features, functional properties, and potential applications. To build upon these insights, we also propose the concept of xeno-pluripotency, which we define as the capability of PSCs from one species to enter into the early embryonic developmental program of another species and contribute to chimera formation. Finally, we summarize previous work on interspecies chimeras and elaborate on an emerging application, interspecies chimeric complementation, for regenerative medicine applications.

Naive and Primed Pluripotent States

Mouse ESCs (mESCs) were the first pluripotent cell type isolated from early embryos. In 1981, Evans and Kaufman (1981) and Gail R. Martin (Martin, 1981) independently reported the successful establishment of cultured ESC lines from mouse blastocysts. mESCs were first grown on mitotically inactivated feeder cells in the presence of serum. Later studies identified LIF and BMP4 to be sufficient to liberate mESCs from serum and feeders without compromising their chimeric and germline competency (Smith et al., 1988; Ying et al., 2003). Further refinements led to the establishment of the ground state culture: a minimal condition devoid of extrinsic stimuli and only containing two small molecule inhibitors (2i): a GSK3 inhibitor, CHIR99021, which activates the canonical Wnt pathway and promotes self-renewal, and a MEK inhibitor, PD0325901, which blocks differentiation (Ying et al., 2008). 2i culture supports robust derivation, propagation, and pluripotency of mESCs from a variety of genetic backgrounds, including non-permissive strains, which are resistant to ESC derivation using conventional cultures (Kawase et al., 1994). More importantly, 2i culture also supports the derivation of authentic ESCs from rat blastocysts, a feat achieved 27 years after the initial mESC derivation (Buehr et al., 2008; Li et al., 2008). These milestone studies of rodent ESCs paved the way toward the derivation of ESCs from other species, including humans.

Since the initial reports of mESCs, derivation of ESC lines has been attempted in several non-rodent species with limited success (Chen et al., 1999; Evans et al., 1990). These putative ESCs could generate tissues representative of all three germ lineages in culture; however, their developmental potential wasn't evaluated using in vivo assays. In 1995, the derivation of the first stable ESC line from a primate, the rhesus macaque, was reported (Thomson et al., 1995), observations which ultimately led to the successful derivation of ESCs from human blastocysts (hESCs) (Ludwig et al., 2006; Reubinoff et al., 2000; Thomson et al., 1998). Despite similar embryonic origins, there are several

noticeable differences between hESCs and mESCs: (1) the colony morphology of mESCs is "dome" shaped, while hESCs appear flattened; (2) some signature pluripotent markers differ between human and mouse ESCs; e.g., hESCs express SSEA-3 and SSEA-4 instead of SSEA-1, which are expressed by mESCs; and (3) unlike mESCs, hESCs are sensitive to single-cell dissociation and thus need to be passaged as small clumps. Signaling pathways involved in the maintenance of the human and mouse ESC pluripotency programs are also different: instead of LIF/BMP4, hESCs are dependent on FGF/TGFβ signaling pathways for their maintenance in an undifferentiated state (Vallier et al., 2005). These differences were initially attributed to the divergent pre-implantation developmental programs between primate and rodent. This notion, however, was challenged when another pluripotent cell line designated as epiblast stem cells (EpiSCs) was derived from the post-implantation mouse epiblast (Brons et al., 2007; Tesar et al., 2007). EpiSCs exhibit features resembling the salient characteristics of hESCs including colony morphology, low single-cell cloning efficiency, and signaling dependency, among others. Notably, like hESCs, EpiSCs could also be obtained directly from pre-implantation blastocysts (Najm et al., 2011). The similarities shared between hESCs and EpiSCs suggest that, during derivation, isolated human inner cell mass (ICM) likely continued on their developmental trajectory in culture to a developmentally more advanced state and acquired an EpiSC-like identity. In support of this notion, Sutter and colleagues identified a transient post-ICM intermediate (PICMI) during the transition from human ICM to ESCs in culture. PICMI displays features characteristic of the post-implantation epiblast, such as X chromosome inactivation and high expression of genes of the NODAL/ACTIVIN signaling pathway (O'Leary et al., 2012).

These and other differences between pluripotent mESCs and EpiSCs led to the realization of the existence of distinct pluripotent states in vitro (Nichols and Smith, 2009). In a way these in vitro states are reminiscent of the pluripotency continuum, which exists within a short time window during early embryogenesis (Solter et al., 1970; Stevens, 1970). mESCs resemble early epiblasts from pre-/peri- implantation embryos and thus exist in a developmentally earlier or more "naive" state. On the other hand, EpiSCs were captured and stabilized from egg cylinder epiblast cells, and thus were exposed to inductive signals emanating from surrounding tissues, and consequently, are instructively specified or "primed" for differentiation. The concept of naive and primed pluripotent states allows distinguishing in vitro cultured mESCs and EpiSCs through developmental timing and helps us gain novel insights into the molecular intricacies underlying developmental regulation of pluripotency in vivo.

Single-cell analyses demonstrated that mESCs most closely resemble naive epiblasts of mature E4.5 blastocysts (Boroviak et al., 2014; Martello and Smith, 2014). Although EpiSCs could be isolated from pre-gastrulation (E5.5) to late-bud (E8.25) -stage embryos, they display gene expression profiles more similar to epiblasts of late-gastrula-stage embryos (Kojima et al., 2014), suggesting that FGF2/TGFβ signaling corralled post-implantation epiblasts into self-renewal at this stage. These findings helped pinpoint the in vivo counterparts of mESCs and EpiSCs and confirmed their existence in two temporally distinct pluripotent states. Apart from their molecular differences, naive mESCs and primed EpiSCs also differ in their timing ability to reenter early embryo development. After being injected into pre-implantation embryos, mESCs could colonize the blastocyst ICMs and contribute to chimera formation. EpiSCs, however, were inefficient in being integrated into blastocyst ICMs. Interestingly, chimeric competency of EpiSCs could be robustly demonstrated after their grafting into post-implantation E7.5 epiblasts followed by in vitro whole-embryo culture (Huang et al., 2012). In contrast, grafted mESCs did not proliferate properly and failed to differentiate in post-implantation E7.5 epiblasts. Also, when grafted to embryos of a later developmental stage (E8.5), by which time pluripotency has been lost, EpiSCs could not integrate, proliferate, and differentiate. Overall, and in agreement with transcriptomic studies, these observations helped establish a functional equivalency between mESCs and naive epiblasts as well as mEpiSCs and late-gastrula-stage epiblasts. They also highlighted how matching developmental timing is a key factor for PSCs to colonize and integrate into the developing embryo.

Conventional hESCs are also classified as primed PSCs. Although similar, hESCs do exhibit molecular signatures distinct from EpiSCs (Chia et al., 2010) and after being grafted into E7.5 mouse epiblasts, unlike mEpiSCs, hESCs did not survive for an extended period of time and failed to proliferate and differentiate (Wu et al., 2015). This may suggest species differences or they may represent different types of primed pluripotent states. It is worth taking into consideration that differences in genetic constituents, as well as post-implantation epiblast morphogenesis in humans (flattened embryonic disc) and mice (cup-shaped egg cylinder), may have allowed stabilization of distinct populations of epiblast cells from different developmental stages upon exposure to FGF2/TGFβ signaling in vitro. In line with this idea, Bernemann et al. (2011) revealed that EpiSC lines from different genetic backgrounds displayed features of distinct developmental states. Due to ethical considerations, the exact developmental potential of primed hESCs could not be functionally evaluated using blastocyst chimeric formation. A supportive argument for the chimeric-incompetent status of hESCs was raised by Tachibana et al. (2012) after they demonstrated that rhesus macaque ESCs propagated in hESC culture could not colonize rhesus blastocysts and failed in contributing to chimera formation. Of note is that rhesus ICM explants also failed in their chimeric contribution capability, but instead could form separate viable fetuses, presumably due to the formation of independent hypoblast layers that separated donor and host ICMs. It is thus unclear from this experiment the relationship between rhesus ESCs and the in vivo ICM. For conceptual as well as for practical considerations, finding conditions that can stabilize human PSCs (hPSCs) in a naive state of pluripotency similar to that of mESCs is critical (Table 1). It will enrich our understanding of embryonic pluripotency across evolutionarily divergent species, as well as offer an attractive source of PSCs able to overcome several practical barriers of conventional primed hESCs, including low cloning efficiency, limited scalability, and putatively less amenability for multilineage differentiation.

The first successful attempt at achieving a naive state in hPSCs relied on continued transgene expression where cells restored LIF responsiveness and could be propagated in mouse

ground state culture (2i) (Hanna et al., 2010). However, these cells could not be maintained long-term independent of transgene expression, suggesting that the achieved state was likely synthetic in nature. A wave of recent reports have claimed the stabilization of transgene-free naive-like hPSCs (Chan et al., 2013; Duggal et al., 2015; Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014; Valamehr et al., 2014; Ware et al., 2014). Features of mESCs were observed in some of these naive-like hPSCs, including colony morphology, expression of naive-related genes, high cloning efficiency, LIF dependency, and epigenetic and metabolic signatures. It is worth noting that different human naive cultures vary considerably and many of them still retain factors that activate FGF/TGFβ signaling pathways, which are indispensable for maintaining primed pluripotency in mice. This can potentially be attributed to discrepancies in the ICM's responses to FGF and NODAL/ACTIVIN signaling between mouse and human (Blakeley et al., 2015; Kuijk et al., 2012; Roode et al., 2012). Also, in many cases single-cell passaging of the naive-like hPSCs was assisted by ROCK kinase inhibition, a well-adopted strategy to boost the poor cloning efficiency of primed hPSCs. Thus, it remains unclear whether these human naive PSCs are the true counterpart of mESCs. An encouraging study recently claimed a modest chimeric contribution with naive cynomolgus monkey ESCs converted from primed cells using a modified human naive culture (Chen et al., 2015). Although further analyses of live births and germline contribution are needed to confirm their true naive status, the possibility of using ESCs for generating chimeric primates is indeed exciting and may facilitate the generation of non-human primate (NHP) models (Izpisua Belmonte et al., 2015). Interestingly, Huang et al. (2014) recently took a systems biology approach and performed weighted gene co-expression network analysis (WGCNA) of human and mouse PSCs. WGCNA revealed that unlike murine PSCs, hPSCs exhibit a high degree of variation, likely resulting from distinct culture conditions used by different studies. More importantly, it was found that naive gene networks between human and mouse are more divergent than expected and both showed resemblance to blastocysts of their own species origin. This suggests that species-specific pre-implantation development strategies might have imposed different features on naive pluripotency.

With the recognition of naive and primed pluripotent states, our understanding of pluripotency has been temporally enriched. The ability to capture pluripotency in culture from different time points provides us with invaluable tools to model early developmental processes in vitro. State transitions between naive and primed conferred by genetic and epigenetic forces have facilitated our molecular understanding of how embryonic pluripotency is harnessed for ensuing proper lineage specification.

Alternative Temporally Distinct Pluripotent States

In addition to naive and primed states, a number of studies have suggested the existence of other temporally distinct states (Figure 1 and Table 1).

Post-implantation epiblasts between E5.5 and E6.25 are competent to form primordial germ cells (PGCs) under inductive signals from the surrounding extra-embryonic tissues (Ohinata et al., 2009). PGC competency is largely lost in EpiSCs (Hayashi and Surani, 2009). A transient cellular state highly similar to the pre-gastrulating epiblast (designated as epiblast-like cells or EpiLCs) could be generated from naive ESCs (Hayashi et al., 2011). Unlike EpiSCs, EpiLCs could be efficiently induced to a PGC fate, thus constituting an ideal starting material for gaining molecular insights into PGC specification, the first critical step of germ cell development (Aramaki et al., 2013; Nakaki et al., 2013). Moreover, robust induction of PGC-like cells (PGC-LCs) from EpiLCs enables generation of functional gametes using mESCs and marks the first step in reconstituting complete germ cell development in vitro, the Holy Grail in mammalian germ cell biology (Hayashi et al., 2011, 2012). With the arrival of naive hESCs, a similar strategy has been adopted for the induction of human PGC-LCs (Irie et al., 2015). Interestingly, however, a recent report by Sasaki et al. (2015) demonstrated highly efficient hPGC-LCs induction directly from primed hiPSCs through an incipient mesoderm-like state (iMeLCs). This observation suggests that primed hiPSCs bear a property intermediate between mouse EpiSCs and EpiLCs. In spite of their advantages for germ cell studies, EpiLCs are transient and not a clonogenic entity.

In another study, Han et al. (2010) identified two cell populations within EpiSCs that could be distinguished by GFP signals driven by the entire 18 kb regulatory region of the *Oct4* gene (GOF18). While it is not fully clear why some EpiSCs do not express the reporter construct, this probably relates to differential enhancer usages that can be used empirically to define separate epiblast states. Interestingly, although the *Oct4*-GFP+ population gradually diminished upon extended culture, they could readily integrate and contribute to chimera formation, contrary to *Oct4*-GFP− cells, upon blastocyst injection. Germline transmission, however, was not observed with *Oct4*-GFP+ cells. From this study it was suggested that a transient population resembling the early-stage epiblast, and able to retain chimeric competency, may exist within EpiSC cultures (Gardner et al., 1985). Indeed, a follow-up study from the same group demonstrated stabilization of this transient *Oct4*-GFP+ population with a modified EpiSC culture condition containing FGF4 (Joo et al., 2014).

Several other reports have also claimed the isolation of chimeric-competent EpiSCs, a feature normally associated with naive ESCs. Chang and Li isolated intermediate epiblast stem cells (IESCs) displaying dual responsiveness to LIF-STAT3 and ACTIVIN-SMAD2/3 signaling. IESCs could efficiently incorporate into the ICM, although they altered further normal embryo development (Chang and Li, 2013). By introducing CHIR99021, a GSK3 inhibitor that activates the canonical WNT pathway and one of the components of ground state 2i culture, into FGF2/Activin (F/A) EpiSC culture medium, Tsukiyama and Ohinata (2014) obtained intermediate pluripotent stem cells (INTPSCs) either from naive ESCs or through reprogramming. Remarkably, after blastocyst injection, INTPSCs contributed efficiently to chimeras, including the germline. Gene expression analysis indicated that INTPSCs retain expression of both naive and primed specific genes, suggestive of an intermediate pluripotent state between ESCs and EpiSCs. Whether INTPSCs correspond to the natural pre-gastrulating epiblast remains unexplored. Moreover, it is still unknown whether INTPSCs can be directly stabilized from post-implantation epiblasts. Intriguingly, Kurek et al. (2015) recently showed that WNT inhibition,

Table 1. Summary of the Different Flavors of Mouse and Human PSCs and Their Properties

Species	States	References	Culture Condition	Features
Mouse	totipotent-like	Macfarlan et al., 2012 (2C-like)	LIF/Serum	transient; OCT4−/NANOG−/SOX2−; *MuERV-L*+; embryonic and extra-embryonic lineage contributions
		Morgani et al., 2013 (*Hex*+ ESCs)	2i[a]/LIF	transient; single *Hex*+ ESCs co-expresses epiblast and extra-embryonic genes; embryonic and extra-embryonic lineage contributions
	naive	Evans and Kaufman, 1981; Martin, 1981; Smith et al., 1988; Ying et al., 2003	Serum; LIF/Bmp4	"dome"-shaped colony morphology; high cloning efficiency; XaXa; germline chimera
		Ying et al., 2008 (ground state)	2i	"dome"-shaped colony morphology; high cloning efficiency; XaXa; germline chimera
	intermediate	Ohinata et al., 2009 (EpiLCs)	FGF2/Activin-A/KSR (1%)	transient; high PGC induction efficiency
		Han et al., 2010 (*Oct4*-GFP+ EpiSCs)	FGF2/Actvin-A	transient; high ESC reversion efficiency; gene expression resembles early epiblast; chimera without germline contribution
		Joo et al., 2014 (FGF4-EpiSCs)	FGF4/Serum	XaXi; chimera without germline contribution
		Tsukiyama and Ohinata, 2014 (INTPSCs)	FGF2/Activin-A/CH	germline chimera
		Kurek et al., 2015 (IWP2-EpiSCs)	FGF2/Activin-A/IWP2	high ESC reversion efficiency; gene expression resembles pre-gastrula epiblast; chimera without germline contribution
		Kim et al., 2013 (CX-EpiSCs)	CH/XAV/Serum	high cloning efficiency; self-renewal depends on stabilized cytoplasmic β-catenin activity
		Chang and Li, 2013 (IESCs)	Actvin-A/Serum	dual responsiveness to LIF-Stat3 and Activin-Smad2/3 signaling; global gene expression intermediate between ESCs and EpiSCs; chimera (low contribution and most show developmental retardation)
	primed	Brons et al., 2007; Tesar et al., 2007 (EpiSCs)	FGF2/Actvin-A	"flattened" colony morphology; XaXi; low cloning efficiency; little to no blastocyst chimera; broad engraftment to late epiblast
		Wu et al., 2015 (rsEpiSCs)	FGF2/IWR1/Serum free	high cloning efficiency; short doubling time; XaXi; no blastocyst chimera; posterior biased engraftment to late epiblast
Human	heightened	Yang et al., 2015	transient treatment with BAP (BMP4, A83-01 and PD173074)	insensitive to trypsin passage; express CDX2; sensitive to high FGF2 concentration; trophoblast differentiation in absence of BMP4 treatment
	naive	Hanna et al., 2010	2i/LIF + DOX PD/CH/LIF/FK	transgenes-dependent: Klf4/Oct4 or Klf4/Klf2; "dome"-shaped colony morphology; XaXa; LIF-dependent and TGF β/Actvin independent

(*Continued on next page*)

Table 1. *Continued*

Species	States	References	Culture Condition	Features
		Gafni et al., 2013	NHSM[b]	high cloning efficiency; shortened doubling time; XaXa; DNA hypomethylation; de novo derivation from human blastocyst; blastocyst interspecies embryonic chimera[e]
		Chan et al., 2013	PD/BIO/DOR/LIF	LIF-dependent; coexpression of GATA6 and NANOG
		Ware et al., 2014	PD/CH/FGF2 or PD/CH/SU/LIF	XaXa; de novo derivation from human blastocyst (low efficiency); high cloning efficiency; shortened doubling time; less matured mitochondria
		Theunissen et al., 2014	5iLA[c]	de novo derivation from human blastocyst; XaXi; elevated and homogeneous expression of NANOG, KLF4, and REX1; reduced level of H3K27me3; no blastocyst interspecies embryonic chimera formation
		Wang et al., 2014	PD/CH/LIF/FGF2	*HERVH*+; XaXa; high cloning efficiency
		Takashima et al., 2014 (reset state)	T2ILGO[d]	self-renew independent of ERK signaling; DNA hypomethylation; lower levels of H3K27me3 and H3K9me3; depletion of TFCP2L1 or KLF4 collapses the reset state; mitochondrial activation
		Duggal et al., 2015	FGF2/LIF/PD/CH/FK/AA	high cloning efficiency; reduced doubling time; XaXa; DNA hypomethylation
	primed	Thomson et al., 1998; Reubinoff et al., 2000; Ludwig et al., 2006	FGF2/KSR; Serum; mTeSR	"flattened" colony; XaXi; low cloning efficiency; little to no blastocyst or late epiblast interspecies chimeric contribution
		Wu et al., 2015 (rsESCs)	F2/IWR1/Serum free	high cloning efficiency; XaXi; late-epiblast interspecific embryonic chimera

CH, CHIR99021; KSR, Knockout Serum Replacement; XAV, XAV939; DOX, Doxycyclin; PD; PD0325901; FK, Forskolin; DOR, Dorsomorphin; SU, SU5402; AA, Ascobic Acid; CX, CHIIR99021 and XAV939.
[a]2i: CHIR99021 and PD0325901.
[b]NHSM: LIF, TGFβ1, FGF2, ERK1/2i (PD0325901), GSK3βi (CHIR99021), JNKi (SP600125) and p38i (SB203580).
[c]5iLA: LIF, PD0325901, IM-12, SB590885, WH-4-023, Y-27632, Activin-A.
[d]T2ILGO: LIF, PD0325901, CHIR99021, Gö6983.
[e]Results of interspecies embryonic chimera were not reproduced in Theunissen et al.'s 2014 study using either NHSM or 5iLA cultures.

rather than activation, stabilizes EpiSCs at a pre-gastrula epiblast state. In this study, a porcupine inhibitor IWP2 that blocks WNT secretion was used. IWP2-EpiSCs could revert to naive ESCs with higher efficiency and contribute to chimeric embryo formation. Live chimeras and germline transmission were not analyzed in this study. These seemingly contradictory observations can potentially be explained by the intricate role of WNT signaling in controlling pluripotency. WNT activation promotes self-renewal of naive ESCs and its inhibition leads to rapid transition to the primed state (ten Berge et al., 2011). Modulation in the strength of the Wnt signaling pathway can potentially help "dial" the pluripotency back and forth throughout the early stages of embryogenesis. Indeed, a combination of a Wnt activator (CHIR99021) and a Wnt inhibitor (XAV939 or IWR1, but not IWP2) arrested EpiSCs in a developmental state closer to ESCs than to EpiSCs grown in conventional F/A culture (Kim et al., 2013), putatively via a novel cytoplasmic β-catenin activity. It should also be noted that naive ESCs, particularly under LIF/Serum culture and primed EpiSCs/hESCs, are heterogeneous and display sub-states with distinct transcriptional and developmental potentials (Hough et al., 2014; Kumar et al., 2014; Tsakiridis et al., 2014). Therefore, other yet-to-be-identified intermediate states that sit between mESCs and EpiSCs might be uncovered.

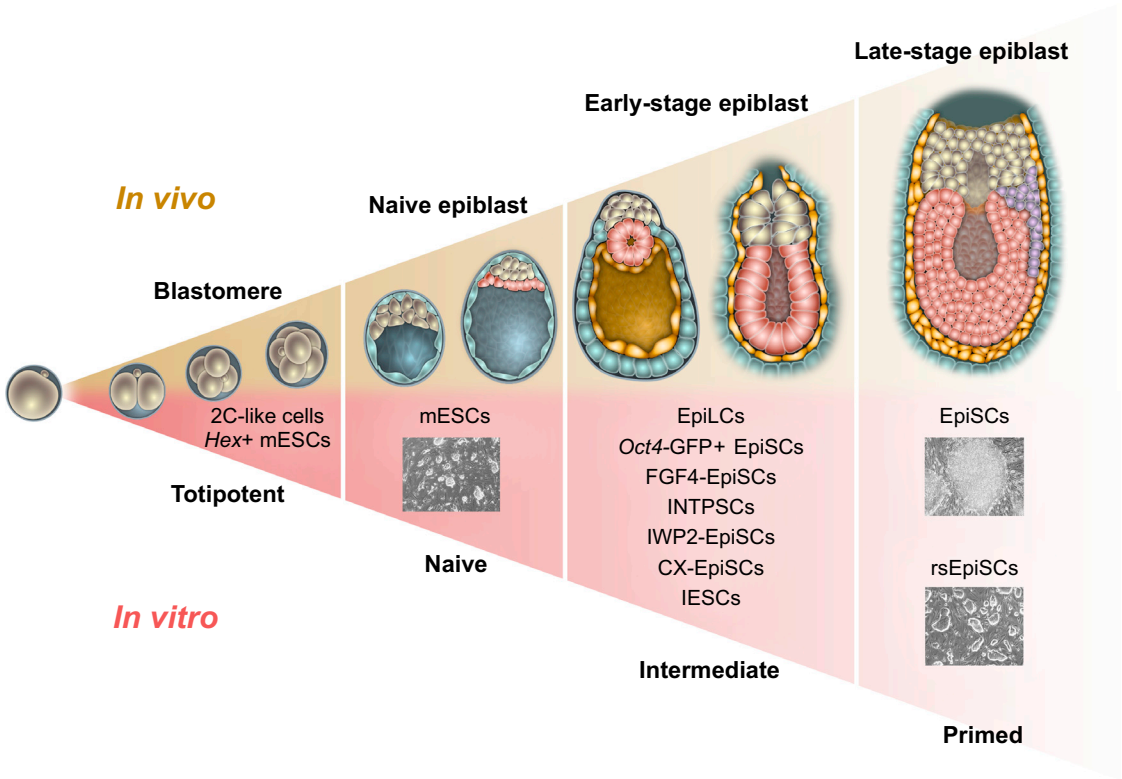

Figure 1. Spatiotemporally Distinct PSC States

PSCs showing different timing and spatial properties have been isolated from the early mouse embryos. Top: illustrations of mouse embryos at different stages of early development. Embryonic cells with totipotent or pluripotent potentials are indicated. Bottom: in vitro cultured cells showing functional features resembling in vivo embryonic cells and categorized into different pluripotent states accordingly. Cell morphologies of mESCs, EpiSCs, and rsEpiSCs are shown.

In addition to intermediate states, totipotent-like states with the potential to contribute to both embryonic and extra-embryonic lineages have been described. In one study, Macfarlan et al. (2012) identified a rare transient population of mESCs with embryonic two-cell (2C) -like features that could be identified by MERV-L retrotransponson expression. These 2C-like cells lack some key pluripotency proteins, including OCT4, SOX2, and NANOG, and surprisingly, they acquired the developmental potential reminiscent of totipotency. Remarkably, nearly all ESCs cycle in and out of this totipotent-like state, a process partially controlled by histone-modifying enzymes. Induction of 2C-like cells could be facilitated by chromatin reprogramming through downregulation of the chromatin-assembly activity of CAF-1 (Ishiuchi et al., 2015). Most recently, however, an in-depth single-cell RNA-sequencing analysis showed that 2C-like cells are globally more similar to blastocysts than to two-cell-stage embryonic cells (Kolodziejczyk et al., 2015). Thus, the true identity of 2C-like cells remains unclear. In a separate study, Morgani et al. (2013) found a *Hex*-positive (Hex is an extra-embryonic endoderm marker) fraction within ground state mESC cultures that not only co-expressed epiblast and extra-embryonic marker genes, but also contributed to both lineages in chimeric embryos. In addition to these rodent studies, hPSCs with heightened potency have been also described with transient BMP4 treatment; however, their developmental potential remains elusive (Yang et al., 2015). The temporal identities of these totipotent-like states likely precede that of naive ESCs in developmental terms. It remains unknown whether totipotent stem cells or cells with expanded developmental potentials can be stabilized in culture.

The experimental accessibility of most developmental stages of mouse embryogenesis has made derivation of pluripotent cell types from different time points possible. Also, recent studies have demonstrated that the derived cells are highly plastic and can interconvert in response to extracellular signals (Pera and Tam, 2010). Although discrete states could be stabilized in vitro, it should be noted that animal development is a continuous process and temporal states captured in vitro likely only represent a small group of cells frozen in time in specific cell culture environments. Future investigations of PSCs with different timestamps will help delineate the regulatory programs underlying ontogenesis in vivo.

Spatially Distinct Pluripotent States

Animal development is a dynamic process that not only moves forward in time but also expands in space. Cells at different topological locations are exposed to diverse external stimuli that, together with intrinsic cellular cues, lead to specific fate lineage commitments in the developing embryo. Embryonic cells with distinct spatial attributes first emerge after the compaction of eight-cell embryos where an outer polarized epithelial monolayer encircles a group of inner apolar cells (Stephenson et al., 2012).

This spatial allocation of cells coincides with the first lineage specification: outside cells are committed to form the trophectoderm (TE) and the inside cells become the ICM of the blastocyst. The ICM further segregates into epiblast and primitive endoderm (PE) lineages with PE facing the blastocoel and epiblast apposed to the polar trophectoderm. Following implantation into the uterine tissue, the blastocyst goes through a rapid phase of proliferation and morphogenesis into an elongated cup-like structure, the egg cylinder. Further into post-implantation development, the distally positioned epiblast undergoes cavitation and reorganization into an epithelium surrounding a central pro-amniotic cavity. These morphogenetic events are accompanied by regionalization and embryonic patterning in preparation for the subsequent establishment of the whole body plan. It is conceivable that, influenced by local cues, individual epiblast cells bear distinctive features reflective of their spatial origins.

An in-depth analysis of the grafting outcomes of EpiSCs has led to the realization that they more readily colonize the anterior primitive streak of the late-streak-stage embryo (Kojima et al., 2014). The spatial property of pluripotency became evident after the recent discovery of a novel class of EpiSCs with distinct spatial characteristics (Wu et al., 2015). These newly derived EpiSCs were named region-selective EpiSCs or rsEpiSCs largely based on their unique ability to selectively engraft into the posterior proximal part of post-implantation epiblast, distinct from conventional EpiSCs. Following in vitro whole-embryo culture, grafted rsEpiSCs were able to further proliferate and differentiate into the three primary germ lineages. In line with this, global transcriptomic comparison of cultured rsEpiSCs with four dissected regions (anterior-proximal, anterior-distal, posterior-proximal, and posterior-distal) of the post-implantation epiblasts revealed a higher correlation between rsEpiSCs with posterior-proximal epiblast than other epiblast quadrants. Distinct grafting outcomes and global transcriptome profiles between EpiSCs and rsEpiSCs allude to the existence of spatially distinct pluripotent states. Likely other spatially unique pluripotent states may exist in the post-implantation epiblast, and future studies into this direction will certainly help enrich our understanding of epiblast patterning and early lineages commitment. In addition to mouse, rsPSCs have also been obtained from human and rhesus PSCs. While functional evaluation of human rsPSCs using a post-implantation human epiblast is not possible, grafting human rsPSCs into a mouse embryo surprisingly resulted in the robust integration, proliferation, and differentiation of human cells in the posterior epiblast, a similar outcome to mouse rsEpiSCs. This suggests that epiblast cells across different species could be spatiotemporally synchronized in a way that allow human and mouse cells to intermix during early development.

Xeno-Pluripotency and Interspecies Chimeras

Interspecies approaches, such as mammalian hybrids and heterokaryons (Blau et al., 1983), have provided key knowledge that would have been otherwise impossible to obtain by traditional means. Interspecies chimera formation is probably the only ethically acceptable way to study the developmental potential of hPSCs in an in vivo context. Primed hESCs were first evaluated for their xeno-developmental potential following injection into mouse blastocysts and embryo transfer (James et al., 2006). In their study, Brivanlou and colleagues found that the majority of human-mouse embryonic chimeras showed developmental retardation and human cells were rarely found in morphologically normal embryos. A similar finding was reported from a study using NHP ESCs (Simerly et al., 2011). The presumed EpiSC-like identity led to the test for grafting hESCs to the post-implantation mouse epiblast, a permissive environment for EpiSCs to thrive. Surprisingly, however, hESCs were found incompatible with the host tissue (Wu et al., 2015). These observations indicate that primate PSCs grown in conventional F/A-based media are inefficient in contributing to normal development of early mouse embryos (Table 2).

With the advent of naive-like primate PSCs, enthusiasm was rekindled for examining their interspecies chimeric competency, or what we refer to here as xeno-pluripotency. The outcomes, however, are inconsistent. Gafni et al. (2013) reported robust human-mouse embryonic chimera formation using naive cells cultured in NHEM medium. In contrast, Theunissen et al. (2014) did not observe any chimera formation among 860 injected embryos using naive-like hESCs derived in their own 5iLA medium, or in 436 injected embryos using NHEM cultured naive-like hESCs previously reported by Gafni et al. To add to the puzzle, using yet another set of naive culture condition (4i/L/b), Fang et al. (2014) demonstrated that naive rhesus iPSCs were able to generate rhesus-mouse chimeric embryos (Table 2). Despite these seemingly contradicting results, a common observation that can be drawn from these studies is that naive-like primate PSCs are more efficient than primed cells in integrating into the ICM of mouse blastocysts, observations which are supported by a couple of other studies using reset naive cells (Masaki et al., 2015; Takashima et al., 2014). It remains an unresolved issue whether current naive-like hPSCs are able to cross the xeno-barrier and efficiently contribute to the later developmental stages of another species. While all of the mentioned studies used mouse as the host species, since the evolutionary distance between human and mouse is about 90 million years (http://www.timetree.org), it will be interesting to test the developmental potential of naive-like hPSCs in animal hosts that are evolutionarily closer to humans.

Notwithstanding naivety, the xeno-pluripotent property of primed human rsPSCs to differentiate into all three embryonic germ lineages in an interspecies chimeric embryo opens a window of opportunity to study early human developmental events and understand human versus mouse developmental differences that are otherwise inaccessible (Table 2). With further improvement of embryo culture, some remaining questions could be properly addressed. For example, it remains unknown to what extent human rsPSCs can differentiate in a developing mouse embryo. Additionally, it will be important to test whether such differentiation is efficient enough to enable robust examination of developmental differences. Alternatively, grafting rsPSCs in a more accessible model system, such as the developing chick embryo, and analyzing their fate at desired developmental stages could potentially help further reveal the xeno-developmental potential of human rsPSCs (Izpisúa-Belmonte et al., 1992, 1993; Stern, 2005).

As far as interspecies chimeras are concerned, and due to a lack of authentic ESCs, earlier work has relied on mixing early embryos or isolated embryonic cells from two different species for generating chimeras (Table 2). Initial trials with *Mus musculus*

Table 2. Summary of the Current State of Research on Interspecies Chimeras

Species	Evolutionary Distance[a]	Method	Surrogate	Outcome	Reference
Mus musculus, *Myodes glareolus*	30.4 Mya	morula-morula aggregation	*Mus musculus*	majority of embryos were abnormal; two normal-looking embryos at E9 and E10 with low chimerism; no live embryos beyond E11	Mystkowska, 1975
Mus musculus, *Mus caroli*	6.5 Mya	blastocyst injection of ICMs	*Mus musculus*	live-born chimeras developed to adulthood	Rossant and Frels, 1980
Ovis aries, *Capra hircus*	9.9 Mya	aggregation of four-cell or eight-cell embryos; blastocyst injection of ICMs	*Ovis aries* or *Capra hircus*	live-born chimeras developed to adulthood	Fehilly et al., 1984
Bos Taurus, *Bos indicus*	0.5 Mya	aggregation of morulae, compact morulae or early blastocyst demi-embryos	*Bos Taurus* or *Bos indicus*	live-born chimeras developed to adulthood	Williams et al., 1990
Mus musculus, *Rattus*	17.9 Mya	morula-morula aggregation; morula-ICM aggregation; blastocysts injection of ICMs	*Mus musculus*	chimeric blastocysts and embryonic chimeras; no viable live-born chimeras	Mulnard, 1973; Stern, 1973; Zeilmaker, 1973; Gardner and Johnson, 1973; Rossant, 1976
		blastocyst injection of rat PSCs	*Mus musculus*	live-born chimeras developed to adulthood	Kobayashi et al., 2010; Isotani et al., 2011
		blastocyst injection of mouse PSCs	*Rattus*	live-born chimeras developed to adulthood	Kobayashi et al., 2010
Mus musculus, *Apodemus sylvaticus*	11.4 Mya	blastocyst injection of apodemus ESCs	*Mus musculus*	live-born chimeras developed to adulthood	Xiang et al., 2008
Mus musculus, *Homo sapiens*	90.1 Mya	blastocyst injection of hESCs	*Mus musculus*	developmental retardation; poor and low efficient contribution to normal embryos	James et al., 2006
		morula injection of naive hPSCs	*Mus musculus*	interspecies chimeric embryos at E8.5–E10.5 (Gafni et al.); no chimeric contribution in recovered E10.5 embryos (Theunissen et al.)	Gafni et al., 2013; Theunissen et al., 2014
		epiblast grafting of human rsESCs	*Mus musculus*	chimeric contribution after grafting to posterior part of E7.5 mouse epiblast followed by 36 hr in vitro embryo culture	Wu et al., 2015
Mus musculus, *Macaca mulatta*	90.1 Mya	eight-cell or blastocyst injection of rhesus naive iPSCs	*Mus musculus*	interspecies chimeric embryos at E10.5 and E16	Fang et al., 2014

[a]http://www.timetree.org.

(mouse) -*Rattus* (rat) (evolutionary distance: 17.9 Mya) (Gardner and Johnson, 1973; Rossant, 1976; Stern, 1973; Zeilmaker, 1973) and mouse-*Myodes glareolus* (bank vole) (evolutionary distance: 30.4 Mya) (Mystkowska, 1975) mostly yielded non-viable embryos. In 1980 Rossant and Frels reported the first interspecies chimeras undergoing normal development in mammals (Rossant and Frels, 1980). In this study they used two rodent species: *Mus musculus* and *Mus caroli* (ryukyu mouse), species that are closely related with an evolutionary distance of only about 6.5 Mya. Since then, live chimeras have been generated between *Ovis aries* (sheep) and *Capra hircus* (goat), 9.9 Mya apart (Fehilly et al., 1984); and *Bos Taurus* (cow) and *Bos indicus* (zebu), 0.5 Mya apart (Williams et al., 1990). Of note is that the pairs of species used in these studies are evolutionarily close in distance, sharing more than 97% of their genomic sequences. These early studies led to the conclusion that irreconcilable differences in the course of embryogenesis precluded formation of viable interspecies chimeras from

evolutionarily divergent species. While this assumption is likely true, the question of how much evolutionary divergence could be tolerated by pluripotent cells from two species was recently challenged by the successful generation of live chimeras between *Mus musculus* and *Apodemus sylvaticus* (wood mouse), which diverged about 11.4 million years ago, a feat achieved with the use of naive ESCs (Xiang et al., 2008). In addition, live mouse-rat chimeras were also obtained via injection of naive mouse or rat PSCs into host rat or mouse blastocysts, respectively, following embryo transfer to surrogates of the host species (Kobayashi et al., 2010). These studies suggest that in vitro cultured rodent PSCs may acquire new features that allow them to cross xeno-boundaries that are normally not possible by mixing in vivo embryonic cells. It will be interesting to see whether this property of cultured PSCs can be further harnessed for creating interspecies chimeras of more divergent evolutionary origins, such as the mouse and bank vole. Also, de novo derivation of other xeno-pluripotent stem cells (xPSCs) or artificial pluripotent states created through cellular reprogramming (Tonge et al., 2014; Wu and Izpisua Belmonte, 2014) will offer further important evolutionary insights.

Engineering-minded approaches may help increase the efficiency and extend the degree of PSCs for crossing xeno-boundaries during embryonic development. These approaches will require a deeper understanding of the molecular and cellular events unleashed by interspecies cell mixing in early development. Two key processes are potentially involved, heterochrony and cell competition. Heterochrony, a change in the relative timing or rate of a developmental process, may account for many of the evolutionary divergences observed. Examining heterochrony at genetic, molecular, and cellular levels will help us understand how development is modified to produce evolutionary changes and explain the inefficiency observed with the formation of interspecies chimeras (Smith, 2003). Cell-cell competition, the process of eliminating unfit or unwanted cells, is gaining increasing recognition as an evolutionarily conserved mechanism for development, tissue homeostasis, organ size control, and stem cell maintenance (Clavería et al., 2013; Johnston, 2009). Cell competition was first studied in *Drosophila* where cells carrying a *Minute* mutation were outcompeted by wild-type cells with metabolic advantages (Morata and Ripoll, 1975). Later studies in mammalian systems revealed that this process is universal and highly conserved (Amoyel and Bach, 2014). In addition to the classical model, myc-induced super-competition constitutes another mode of cell competition whereby cells with higher Myc expression outcompete neighboring wild-type cells (Amoyel and Bach, 2014). Both types of cell competition have thus far only been examined in the intraspecific setting and their roles in interspecies chimera formation await to be explored. Interestingly, by using a genome-wide cheater screening, Zwaka and colleagues identified a network of genes whose downregulation confers embryonic cells with the ability to out-compete wild-type cells in development, a feature reminiscent of myc-driven super-competition (Dejosez et al., 2013). Another form of cell competition that is relevant in an interspecific context is cell cycle differences. Faster dividing cells from one species will likely dominate and out-compete slower dividing cells from the other species during development, affecting the degree of chimerism. Armed with this mechanistic information, synthetic biology approaches to program mammalian cell behavior (Lienert et al., 2014) or modulation of cell-cell competition during early development (Clavería et al., 2013) may expand the repertoire of viable interspecies chimeras and offer invaluable insights into animal development in an evolutionary context.

The generation of human-animal chimeras, if achieved, will offer tremendous advantages for regenerative medicine. One possible application is in vivo drug screening. The current approaches for drug development include in vitro screening, in vivo animal models, and eventually multiphase clinical trials in humans. For in vitro screening patient samples and immortalized cell lines are conventionally used. Compound screening using patient samples is limited by their availability and expansion in culture. Alternatively, immortalized cell lines provide an unlimited number of cells but their use is often complicated by genetic and metabolic abnormalities introduced by immortalization. In vivo transgenic mouse models have been widely adopted for modeling human diseases and consequently serve as "in vivo" drug screening platforms. However, there are considerable anatomical, physiological, and behavioral differences between mice and humans that limit the degree to which insights derived from the mouse models can be applied to understanding human biology. The drawback of using animal models has been underscored by the failure of translating several successful preclinical animal tests into human clinical trials. Interspecies chimeras draw strength from both in vitro human-cell-based screening and in vivo animal models and hold the potential to be a superior preclinical testing platform for more accurate prediction of clinical outcomes.

Another future therapeutic application is the potential to obtain more mature and functional cells, tissues, and even organs from hPSCs in an in vivo environment. Despite the enormous potential that has been unleashed by pluripotent hESCs and hiPSCs, current in vitro strategies for differentiation to obtain functional and mature cell types for transplantation have been met with several major limitations: (1) only limited immature cell types of fetal or neonatal origin can be produced (Hrvatin et al., 2014) and in most cases are unsuitable for transplantation (Wu and Hochedlinger, 2011); (2) differentiation efficiencies vary across cell lines and often necessitate laborious optimization (Osafune et al., 2008); (3) differentiating cultures often contain undifferentiated pluripotent fractions that pose tumor risks; (4) large-scale production to meet the clinical demand remains challenging; and (5) we are still far from generating highly complex tissues and organs in vitro (Lancaster and Knoblich, 2014). Differentiating hPSCs through the normal course of embryo development in an in vivo environment of an animal host offers potential solutions to some of these challenges. Stochastic contribution of donor PSCs in chimera generation, however, is not ideal for organ generation, where minimal host cell contamination is imperative.

Interspecies Chimeric Complementation

Throughout evolution, nature has evolved a sophisticated and robust system to generate functional tissues and organs during the normal course of embryo development. The intrinsic genetic program works seamlessly with extrinsic developmental niches in a highly regulated spatiotemporal manner to enable embryonic cells to commit to specific cell lineages and be organized

into higher-order tissue architectures. A better understanding of these developmental principles has been possible thanks to the powerful combination of gene-targeting technologies with germline competent ESCs for the generation of genome-edited rodent models. These and other technologies have provided unprecedented insights into how specific genetic and epigenetic factors orchestrate organismal embryonic development. Alterations in the expression of these factors during embryonic development, and despite the existence of an intact extrinsic embryonic niche, leads to tissue and organ impairment, generating in some cases embryos lacking entire cell lineages and/or organs. To name a few: homozygous deletion of the *Pdx1* gene in mice disables the pancreatic developmental program and results in the generation of apancreatic mice that will die soon after birth (Offield et al., 1996), mouse embryos homozygous for the *Lhx1*-null allele lack kidney development, and targeted disruption of the *Nkx2.5* gene in mice leads to embryonic lethality around E10.5 with retarded cardiac development (Lyons et al., 1995).

By genetically disabling organogenesis, the extrinsic developmental niches become ''empty'' due to the inability of gene-altered progenitors to populate the embryonic niches. Donor wild-type PSCs can then be used for the generation of chimeric animals and to ''fill'' these empty developmental niches. A pioneer technique, blastocyst complementation (named so because donor cells were introduced to the host at the blastocyst stage), was introduced by Alt and colleagues in 1993 (Chen et al., 1993) when they demonstrated that wild-type mouse ESCs could colonize *Rag2*$^{-/-}$ mouse blastocysts and generated normal T and B lymphocytes exclusively of donor origin. For cells other than lymphocytes, Wu et al. (2002) employed *Hprt*-deficient blastocyst complementation to derive embryonic fibroblasts from donor mutant ESCs without a selection marker. This approach is particularly useful for mutations in donor ESCs that lead to early embryonic lethality. In addition to cells, organ complementation was first attempted by Douglas Melton and colleagues in 2007 (Stanger et al., 2007). In this study they used wild-type mouse ESCs to complement *Pdx1*-deficient mouse blastocysts. As a result, the entire pancreatic epithelium was derived from the donor ESCs. Interestingly, in the same study, an alternative complementation approach in which conditional progenitor cell ablation based on diphtheria toxin A (DTA) was used to eliminate PDX1+ pancreatic or LAP+ (liver-enriched transcriptional activator) hepatic progenitors during development and donor ESCs were able to successfully complement these progenitor deficiencies. For liver, another study by, Espejel et al. (2010) complemented *Fah*-deficient blastocysts with wild-type iPSCs to demonstrate that iPSCs could differentiate into hepatocytes independent of cell fusion. DTA-based cell ablation has been used most recently to eliminate NKX2.5+ cardiac progenitors followed by introduction of wild-type ESCs. These wild-type ESCs were able to successfully compensate for the loss of progenitors in the developing heart (Sturzu et al., 2015).

Interspecies blastocyst complementation was first suggested in a study reporting the generation of live mouse-wood mouse chimeras (Xiang et al., 2008). It was not until 2010 that a milestone paper from Nakauchi's group (Kobayashi et al., 2010) demonstrated this potential between mouse and rat. In their study rat PSCs were used to complement mouse *Pdx1* null blastocysts and as a result, an entire rat pancreatic epithelium could be generated in the mouse host. Afterward, Isotani et al. (2011) successfully complemented blastocysts from nude mice lacking a thymus with rat ESCs and generated a functional xenogenic rat thymus. Usui et al. (2012) tried complementing *Sal1* null blastocysts to generate kidneys. When mouse PSCs were used, the kidney was successfully generated via blastocyst complementation; however, rat iPSCs failed in this context, suggesting that key molecules involved in the interaction between mesenchyme and the ureteric buds during kidney development might not be conserved between mice and rats.

Despite only two successful reports to date, the interspecies blastocyst complementation platform has raised an intriguing possibility for the generation of functional human cells/tissues/organs in animal hosts. Due to its resemblance to humans in anatomy, physiology, organ size, and cell cycle characteristics, the pig could be a possible candidate.

With the recent completion of swine genome sequencing (Groenen et al., 2012), together with the successful development of SCNT technologies (Lai et al., 2002; Park et al., 2001), the pig has emerged as one of the most popular large animal models in biomedical research (Prather et al., 2013). This has been further enhanced by the advancement of genetic engineering technologies such as homologous recombination (Lai et al., 2002), zinc finger nucleases (ZFNs) (Hauschild et al., 2011; Whyte and Prather, 2012), transcription-activator-like effector nuclease (TALEN) (Carlson et al., 2012), and the clustered regularly interspaced short palindromic repeats (CRISPR) with RNA-guided nucleases, such as Cas9 (CRISPR-Cas9) (Hai et al., 2014; Wang et al., 2015; Whitworth et al., 2014). The combination of SCNT with genetically modified pig somatic cells has produced a number of valuable porcine models of human diseases, including diabetes (Renner et al., 2010; Umeyama et al., 2009), cystic fibrosis (Rogers et al., 2008), retinitis pigmentosa (Petters et al., 1997; Ross et al., 2012), spinal muscular atrophy (Lorson et al., 2011), and Alzheimer's disease (Kragh et al., 2009). Intraspecific blastocyst complementation in the pig has also been achieved by Nakauchi and colleagues (Matsunari et al., 2013). In their study, the authors cloned fibroblasts overexpressing HES1 under the *Pdx1* promoter. *Pdx1-Hes1* transgene expression suppressed the pancreatic program, thus leading to the creation of a pancreatogenesis-disabled pig blastocyst. Since chimeric-competent pig PSCs were not available, the authors cloned fibroblasts expressing the huKO fluorescent protein and used blastomeres isolated from huKO embryos to complement the *Pdx1-Hes1* blastocysts. As a result, huKO blastomeres were able to contribute to chimera formation and generated an entire huKO+ pancreatic epithelium. Moreover, the chimeric pigs generated by complementation were able to grow into adulthood with a functional pancreas.

Despite the success, it is difficult to implement SCNT in a standard laboratory. With the advent of programmable nucleases including ZFNs, TALENs, and CAS, genome editing has become more precise and efficient (Doudna and Charpentier, 2014; Gaj et al., 2013). These nucleases can recognize specific DNA sequences and generate double strand breaks (DSBs) at predetermined genomic loci. Once DSBs are created two major cellular DSB repair mechanisms (non-homologous end joining [NHEJ] and homology-directed repair [HDR]) are activated through which targeted genome modification can be achieved. These

programmable nucleases not only facilitate genome editing using cultured cell lines, but also, more importantly, allow direct genome editing in early embryos for the fast generation of transgenic animals. Error-prone NHEJ produces indels in the genome that will lead to loss-of-function of genes of interest. A NHEJ-based knockout strategy is highly efficient and thus has been successfully achieved in a wide variety of species including mouse, rat, pig, sheep, cow, and NHP (Geurts et al., 2009; Hai et al., 2014; Hauschild et al., 2011; Niu et al., 2014; Sung et al., 2013; Wang et al., 2013). Moreover, the CRISPR-Cas9 system allows multiplex gene editing, which is advantageous for the generation of multiple-gene knockouts simultaneously. Therefore, nuclease-mediated one-cell gene editing is potentially a more accessible approach for editing host embryos for blastocyst complementation.

As mentioned above, it remains unknown which types of hPSCs can efficiently cross xeno-barriers. Also it should be pointed out that there is a larger evolutionary distance between humans and pigs (95 Mya) than between humans and mice (90.1 Mya). The choice of pigs as hosts for production of human organs with hPSCs is rather based on organ size, physiology, ample supply, and their amenability to be raised in a clean environment. From a developmental point of view, however, pigs have features distinct from both humans and mice which may be advantageous or disadvantageous; for example: (1) the presence of porcine ICM lasts for a longer time period (about 6–7 days) compared to that of mice (1 day) and humans (3 days) (Oestrup et al., 2009). (2) Both human and pig epiblasts assume a disk-shaped epiblast layer whereas mice develop a cup-shaped epiblast. (3) Pigs have epitheliochorial placentae, which are less invasive than haemochorial placentae typical of humans and mice. To address the key question of whether hPSCs can cross species barriers and contribute to early pig development, it is imperative that hPSCs are empirically tested following injection into early pig embryos and embryo transfer to pseudopregnant sows. Practically speaking this is not trivial and calls for collaborative efforts with researchers across many disciplines including embryologists, veterinarians, stem cell biologists, genome editing experts, clinicians, and bioethicists. Moreover, pigs may not be the right host, as currently there is lack of information regarding how divergent the developmental programs, cell-cell communications, signaling for lineage specifications, and allocations are shared between humans and pigs. In addition to pigs, we also need to consider other animal species such as sheep (95 Mya), goat (95 Mya), cow (95 Mya), and rabbit (90.1 Mya), among others. Evolutionarily closer NHPs (e.g., common marmoset, 41.8 Mya; rhesus macaque, 27.3 Mya; Chimp, 6.2 Mya), however, are unlikely to be considered due to practical and ethical reasons.

In addition to blastocyst complementation, there are other forms of chimeric complementation (Figure 2): (1) tetraploid complementation, the most stringent in vivo pluripotency test, is probably the ultimate chimeric complementation with donor ESCs contributing to all structures in the fetus (Nagy et al., 1993). It will be intriguing to know whether this can work in an interspecies scenario where PSCs from one species can generate an entire living organism inside the tetraploid embryo of another species. (2) Since human rsPSCs can be incorporated and differentiated in the epiblast of gastrula mouse embryos, interspecies epiblast complementation may help enrich human cells in early peri-gastrula developmental niches and generate early human progenitor cells. (3) In utero conceptus complementation with human lineage progenitors offers an alternative way to generate human organs in organogenesis-disabled livestock (Rashid et al., 2014). Previous studies on grafting human primary cells, or cell derivatives generated from hPSCs, to a wide variety of experimental animals have paved the way for gaining important insights into key parameters—among them, the cellular and molecular host niche environment, cross-species signaling interplays, and developmentally permissive spatiotemporal attributes—that are important for successful human cell engrafting following in utero injection (Fisher et al., 2013; Nicholas et al., 2013; Si-Tayeb et al., 2010; Zhang et al., 2001).

Ethical Considerations

The isolation of different types of hPSCs and their potential to contribute to interspecies chimera formation have, on one hand, opened new avenues to study human biology and unveil novel regenerative medicine applications; on the other hand, however, they also unleash new ethical challenges. Human-animal chimera research involves the transfer of totipotent, pluripotent, or multi-potent stem cells or their derivatives, into animals in embryonic, fetal, or postnatal stages of development (Hyun et al., 2007). According to this definition, teratoma assays, the grafting of pluripotent hPSCs into immunodeficient animals (mouse prevalently) to evaluate their in vivo differentiation potential, can be considered as one type of human-animal chimera (Lensch et al., 2007). Teratomas are generated heterotopically and thus pose different ethical issues compared to chimeras created via mixing cells at the pre-implantation blastocyst stage of development. Orthotopic human-animal chimeric embryos generated by integrating human cells into different developmental stages of animal embryos also have different ethical implications and should be evaluated case by case.

From an ethical perspective, three main categories of orthotopic human-animal chimera research need to be considered (Hermerén, 2015; Hyun, 2015). One involves in vitro studies using early embryos. A case in point is the implantation of human rsPSCs into isolated early post-implantation mouse embryos followed by short-term in vitro culture (Wu et al., 2015). Since with current technologies the chimeric embryos generated are non-viable and cannot be carried to term, there are minimal concerns of animal health and welfare as well as ethical issues. The second involves the generation of in vivo embryonic chimeras. Chimeras generated with naive hPSCs by Hanna's group fall into this category (Gafni et al., 2013). In their experimental set up, pregnancy was stopped 10 days into mouse gestation, a period within the limit allowed for research on human embryos (Hermerén, 2015). Although it is possible that the fetal mouse brain might have had some degree of human contribution, ethical concerns are in this case limited. The third category includes in vivo studies with sentient animals, which raises additional ethical challenges (Hermerén, 2015). Although no hPSCs have been reported, multi-potent stem cells and their derivatives are commonly being injected into live animals including NHPs for evaluation of their differentiation potential or function (Kriks et al., 2011; Pagliuca et al., 2014; Zhu et al., 2014). In a study by Goldman and collaborators in 2013, mouse forebrain glial cells were replaced

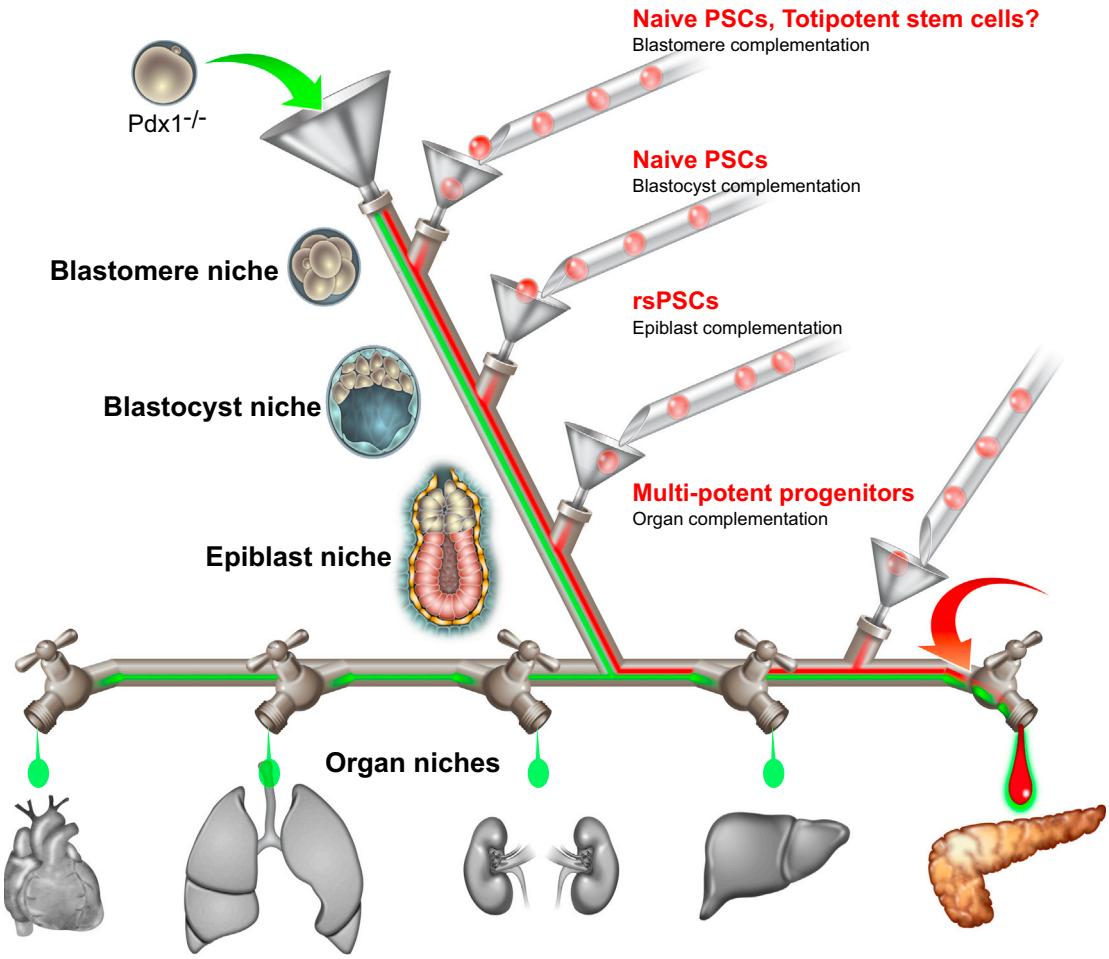

Figure 2. Interspecies Chimeric Complementation
By genetically altering the host embryos (e.g., *Pdx1*$^{-/-}$), developmental programs specific for certain lineages and organs can be disabled. Totipotent, pluripotent, or multi-potent stem cells from one species can potentially be used to rescue the organ defects of the host species at different time points during development. And as a result, organs enriched with cells derived from donor stem cells are generated in a xeno environment.

by human glia (Han et al., 2013). The "humanized" mice showed elevated cognitive capability with enhanced plasticity and learning, and thus raised the question of whether moral humanization accompanys biological humanization in these chimeras, a concept not readily accommodated by existing ethical guidelines (Hyun, 2015).

Human tissue and organ generation using animal hosts needs to be approached with the appropriate precautions. Guidelines on human-animal chimeras put forward by the National Academy of Sciences (NAS) (National Research Council, 2005; National Research Council, 2007) and the International Society for Stem Cell Research (ISSCR) (http://www.isscr.org/docs/default-source/hesc-guidelines/isscrhescguidelines2006.pdf) should be strictly followed. Both guidelines made the following recommendation: "All research involving the introduction of hES cells into nonhuman animals at any stage of embryonic, fetal, or postnatal development should be reviewed by the ESCRO committee. Particular attention should be paid to the probable pattern and effects of differentiation and integration of the human cells into the nonhuman animal tissues." The NAS and ISSCR also recommended limits on interspecies chimera research involving human cells. Currently, it is commonly agreed that no hPSCs should be allowed to be implanted in NHP embryos and that human-animal chimeras should not be allowed to breed. Current NIH funding guidelines follow these recommendations and prohibit experiments on breeding human-animal chimeras and mixing hPSCs with NHP embryos. These recommendations, however, do not preclude injecting hPSCs into early embryos of other species, such as the pig. Most recently, on September 23, 2015, the NIH issued a notice stating that research in which hPSCs are introduced into non-human vertebrate animal pre-gastrulation stage embryos will not be funded while the agency considers a possible policy revision in this area (https://grants.nih.gov/grants/guide/notice-files/NOT-OD-15-158.html). The NIH has invited scientists and bioethicists to a workshop on November 6, 2015 to evaluate the state of scientific and ethical issues in animal-human chimera research and a revised guideline is expected to be in place afterward.

Strategies to ease some of the ethical concerns, especially those related to brain contributions, can be envisioned: due to

marked differences in differentiation bias among hPSC lines (Osafune et al., 2008), selecting lines that are inefficient for neural differentiation could be considered. It has been proposed that modulation of certain lineage transcription factors can influence PSCs' in vivo differentiation propensity (Kobayashi et al., 2015), thus offering a path for avoiding neural contribution. Other methods that could be worth considering include genetic inactivation of key genes for human neural development (Zhang et al., 2010) and/or implementation of safety switches similar to what has been used for adoptive T cell therapy, which can trigger apoptosis in hPSC-derived neurons (Di Stasi et al., 2011; Straathof et al., 2005).

Conclusion

We have come a long way since the first capture of embryonic pluripotency in culture. The derivation of mESCs has transformed modern biology. Their abilities to indefinitely expand in vitro and generate all adult lineages in vivo, combined with gene editing technologies, have provided us with a vast treasure of human disease models. The quest for understanding extrinsic and intrinsic cues underlying pluripotency has contributed to the recent isolation of various spatiotemporally divergent pluripotent states. Chimeric competency is no longer a privilege of mESCs. Naive ESCs capable of contributing to germline chimeras have been derived from other rodents and most recently from the cynomolgus monkey. Chimeric competency has also been expanded into the realm of interspecies with naive ESCs efficiently crossing xeno-boundaries among rodents for the generation of live interspecies chimeras. The discovery of a spectrum of pluripotent states across intra- and inter-species domains will open new avenues for uncovering novel and thought-provoking areas of investigation in embryonic development, pathogenesis, aging, and evolution.

These advances in animal models go hand in hand with the rapidly evolving field of hPSCs and regenerative medicine. hPSCs hold great potential to revolutionize the practice of medicine since they constitute the source from where unlimited cells, tissues, or even organs could be derived to treat numerous debilitating disorders. Despite substantial progress, to date, no hPSC-based therapies have transitioned from experimental to clinical practice. Infused with novel concepts in pluripotency and equipped with the unique properties of naive and region-specific hPSCs, regenerative medicine applications unlocking the full potential of hPSCs can be envisioned. Empowered by interspecies chimeric-competent rsPSCs, naive hPSCs, or other novel PSC types, complex tissue and organ generation may, in a not too distant future, become feasible.

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REFERENCES

Amoyel, M., and Bach, E.A. (2014). Cell competition: how to eliminate your neighbours. Development *141*, 988–1000.

Aramaki, S., Hayashi, K., Kurimoto, K., Ohta, H., Yabuta, Y., Iwanari, H., Mochizuki, Y., Hamakubo, T., Kato, Y., Shirahige, K., and Saitou, M. (2013). A mesodermal factor, T, specifies mouse germ cell fate by directly activating germline determinants. Dev. Cell *27*, 516–529.

Bernemann, C., Greber, B., Ko, K., Sterneckert, J., Han, D.W., Araúzo-Bravo, M.J., and Schöler, H.R. (2011). Distinct developmental ground states of epiblast stem cell lines determine different pluripotency features. Stem Cells *29*, 1496–1503.

Blakeley, P., Fogarty, N.M.E., Del Valle, I., Wamaitha, S.E., Hu, T.X., Elder, K., Snell, P., Christie, L., Robson, P., and Niakan, K.K. (2015). Defining the three cell lineages of the human blastocyst by single-cell RNA-seq. Development *142*, 3151–3165.

Blau, H.M., Chiu, C.-P., and Webster, C. (1983). Cytoplasmic activation of human nuclear genes in stable heterocaryons. Cell *32*, 1171–1180.

Boroviak, T., Loos, R., Bertone, P., Smith, A., and Nichols, J. (2014). The ability of inner-cell-mass cells to self-renew as embryonic stem cells is acquired following epiblast specification. Nat. Cell Biol. *16*, 516–528.

Brons, I.G.M., Smithers, L.E., Trotter, M.W.B., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S.M., Howlett, S.K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R.A., and Vallier, L. (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature *448*, 191–195.

Buehr, M., Meek, S., Blair, K., Yang, J., Ure, J., Silva, J., McLay, R., Hall, J., Ying, Q.-L., and Smith, A. (2008). Capture of authentic embryonic stem cells from rat blastocysts. Cell *135*, 1287–1298.

Carlson, D.F., Tan, W., Lillico, S.G., Stverakova, D., Proudfoot, C., Christian, M., Voytas, D.F., Long, C.R., Whitelaw, C.B.A., and Fahrenkrug, S.C. (2012). Efficient TALEN-mediated gene knockout in livestock. Proc. Natl. Acad. Sci. USA *109*, 17382–17387.

Chan, Y.-S., Göke, J., Ng, J.-H., Lu, X., Gonzales, K.A.U., Tan, C.-P., Tng, W.-Q., Hong, Z.-Z., Lim, Y.-S., and Ng, H.-H. (2013). Induction of a human pluripotent state with distinct regulatory circuitry that resembles preimplantation epiblast. Cell Stem Cell *13*, 663–675.

Chang, K.-H., and Li, M. (2013). Clonal isolation of an intermediate pluripotent stem cell state. Stem Cells *31*, 918–927.

Chen, J., Lansford, R., Stewart, V., Young, F., and Alt, F.W. (1993). RAG-2-deficient blastocyst complementation: an assay of gene function in lymphocyte development. Proc. Natl. Acad. Sci. USA *90*, 4528–4532.

Chen, L.-R., Shiue, Y.-L., Bertolini, L., Medrano, J.F., BonDurant, R.H., and Anderson, G.B. (1999). Establishment of pluripotent cell lines from porcine preimplantation embryos. Theriogenology *52*, 195–212.

Chen, Y., Niu, Y., Li, Y., Ai, Z., Kang, Y., Shi, H., Xiang, Z., Yang, Z., Tan, T., Si, W., et al. (2015). Generation of Cynomolgus Monkey Chimeric Fetuses using Embryonic Stem Cells. Cell Stem Cell *17*, 116–124.

Chia, N.-Y., Chan, Y.-S., Feng, B., Lu, X., Orlov, Y.L., Moreau, D., Kumar, P., Yang, L., Jiang, J., Lau, M.-S., et al. (2010). A genome-wide RNAi screen reveals determinants of human embryonic stem cell identity. Nature *468*, 316–320.

Clavería, C., Giovinazzo, G., Sierra, R., and Torres, M. (2013). Myc-driven endogenous cell competition in the early mammalian embryo. Nature *500*, 39–44.

Dejosez, M., Ura, H., Brandt, V.L., and Zwaka, T.P. (2013). Safeguards for cell cooperation in mouse embryogenesis shown by genome-wide cheater screen. Science *341*, 1511–1514.

Di Stasi, A., Tey, S.-K., Dotti, G., Fujita, Y., Kennedy-Nasser, A., Martinez, C., Straathof, K., Liu, E., Durett, A.G., Grilley, B., et al. (2011). Inducible apoptosis as a safety switch for adoptive cell therapy. N. Engl. J. Med. *365*, 1673–1683.

Doudna, J.A., and Charpentier, E. (2014). Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science *346*, 1258096.

Duggal, G., Warrier, S., Ghimire, S., Broekaert, D., Van der Jeught, M., Lierman, S., Deroo, T., Peelman, L., Van Soom, A., Cornelissen, R., et al. (2015). Alternative Routes to Induce Naïve Pluripotency in Human Embryonic Stem Cells. Stem Cells *33*, 2686–2698.

Espejel, S., Roll, G.R., McLaughlin, K.J., Lee, A.Y., Zhang, J.Y., Laird, D.J., Okita, K., Yamanaka, S., and Willenbring, H. (2010). Induced pluripotent stem cell-derived hepatocytes have the functional and proliferative capabilities needed for liver regeneration in mice. J. Clin. Invest. *120*, 3120–3126.

Evans, M.J., and Kaufman, M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature *292*, 154–156.

Evans, M.J., Notarianni, E., Laurie, S., and Moor, R.M. (1990). Derivation and preliminary characterization of pluripotent cell lines from porcine and bovine blastocysts. Theriogenology *33*, 125–128.

Fang, R., Liu, K., Zhao, Y., Li, H., Zhu, D., Du, Y., Xiang, C., Li, X., Liu, H., Miao, Z., et al. (2014). Generation of naive induced pluripotent stem cells from rhesus monkey fibroblasts. Cell Stem Cell *15*, 488–496.

Fehilly, C.B., Willadsen, S.M., and Tucker, E.M. (1984). Interspecific chimaerism between sheep and goat. Nature *307*, 634–636.

Fisher, J.E., Lillegard, J.B., Mckenzie, T.J., Rodysill, B.R., Wettstein, P.J., and Nyberg, S.L. (2013). In utero Transplanted Human Hepatocytes Allow Postnatal Engraftment of Human Hepatocytes in Pigs. Liver Transpl. *19*, 328–335.

Gafni, O., Weinberger, L., Mansour, A.A., Manor, Y.S., Chomsky, E., Ben-Yosef, D., Kalma, Y., Viukov, S., Maza, I., Zviran, A., et al. (2013). Derivation of novel human ground state naive pluripotent stem cells. Nature *504*, 282–286.

Gaj, T., Gersbach, C.A., and Barbas, C.F., 3rd (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol. *31*, 397–405.

Gardner, R.L., and Johnson, M.H. (1973). Investigation of early mammalian development using interspecific chimaeras between rat and mouse. Nat. New Biol. *246*, 86–89.

Gardner, R.L.R., Lyon, M.F.M., Evans, E.P.E., and Burtenshaw, M.D.M. (1985). Clonal analysis of X-chromosome inactivation and the origin of the germ line in the mouse embryo. J. Embryol. Exp. Morphol. *88*, 349–363.

Geurts, A.M., Cost, G.J., Freyvert, Y., Zeitler, B., Miller, J.C., Choi, V.M., Jenkins, S.S., Wood, A., Cui, X., Meng, X., et al. (2009). Knockout rats via embryo microinjection of zinc-finger nucleases. Science *325*, 433.

Groenen, M.A.M., Archibald, A.L., Uenishi, H., Tuggle, C.K., Takeuchi, Y., Rothschild, M.F., Rogel-Gaillard, C., Park, C., Milan, D., Megens, H.-J., et al. (2012). Analyses of pig genomes provide insight into porcine demography and evolution. Nature *491*, 393–398.

Gurdon, J.B. (1962). The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. J. Embryol. Exp. Morphol. *10*, 622–640.

Hackett, J.A., and Surani, M.A. (2014). Regulatory principles of pluripotency: from the ground state up. Cell Stem Cell *15*, 416–430.

Hai, T., Teng, F., Guo, R., Li, W., and Zhou, Q. (2014). One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. Cell Res. *24*, 372–375.

Han, D.W., Tapia, N., Joo, J.Y., Greber, B., Araúzo-Bravo, M.J., Bernemann, C., Ko, K., Wu, G., Stehling, M., Do, J.T., and Schöler, H.R. (2010). Epiblast stem cell subpopulations represent mouse embryos of distinct pregastrulation stages. Cell *143*, 617–627.

Han, X., Chen, M., Wang, F., Windrem, M., Wang, S., Shanz, S., Xu, Q., Oberheim, N.A., Bekar, L., Betstadt, S., et al. (2013). Forebrain engraftment by human glial progenitor cells enhances synaptic plasticity and learning in adult mice. Cell Stem Cell *12*, 342–353.

Hanna, J., Cheng, A.W., Saha, K., Kim, J., Lengner, C.J., Soldner, F., Cassady, J.P., Muffat, J., Carey, B.W., and Jaenisch, R. (2010). Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. Proc. Natl. Acad. Sci. USA *107*, 9222–9227.

Hauschild, J., Petersen, B., Santiago, Y., Queisser, A.-L., Carnwath, J.W., Lucas-Hahn, A., Zhang, L., Meng, X., Gregory, P.D., Schwinzer, R., et al. (2011). Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases. Proc. Natl. Acad. Sci. USA *108*, 12013–12017.

Hayashi, K., and Surani, M.A. (2009). Self-renewing epiblast stem cells exhibit continual delineation of germ cells with epigenetic reprogramming in vitro. Development *136*, 3549–3556.

Hayashi, K., Ohta, H., Kurimoto, K., Aramaki, S., and Saitou, M. (2011). Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. Cell *146*, 519–532.

Hayashi, K., Ogushi, S., Kurimoto, K., Shimamoto, S., Ohta, H., and Saitou, M. (2012). Offspring from oocytes derived from in vitro primordial germ cell-like cells in mice. Science *338*, 971–975.

Hermerén, G. (2015). Ethical considerations in chimera research. Development *142*, 3–5.

Hough, S.R., Thornton, M., Mason, E., Mar, J.C., Wells, C.A., and Pera, M.F. (2014). Single-cell gene expression profiles define self-renewing, pluripotent, and lineage primed states of human pluripotent stem cells. Stem Cell Reports *2*, 881–895.

Hrvatin, S., O'Donnell, C.W., Deng, F., Millman, J.R., Pagliuca, F.W., Dilorio, P., Rezania, A., Gifford, D.K., and Melton, D.A. (2014). Differentiated human stem cells resemble fetal, not adult, β cells. Proc. Natl. Acad. Sci. USA *111*, 3038–3043.

Huang, Y., Osorno, R., Tsakiridis, A., and Wilson, V. (2012). In Vivo Differentiation Potential of Epiblast Stem Cells Revealed by Chimeric Embryo Formation. Cell Reports *2*, 1571–1578.

Huang, K., Maruyama, T., and Fan, G. (2014). The naive state of human pluripotent stem cells: a synthesis of stem cell and preimplantation embryo transcriptome analyses. Cell Stem Cell *15*, 410–415.

Hyun, I. (2015). From naïve pluripotency to chimeras: a new ethical challenge? Development *142*, 6–8.

Hyun, I., Taylor, P., Testa, G., Dickens, B., Jung, K.W., McNab, A., Robertson, J., Skene, L., and Zoloth, L. (2007). Ethical standards for human-to-animal chimera experiments in stem cell research. Cell Stem Cell *1*, 159–163.

Irie, N., Weinberger, L., Tang, W.W.C., Kobayashi, T., Viukov, S., Manor, Y.S., Dietmann, S., Hanna, J.H., and Surani, M.A. (2015). SOX17 is a critical specifier of human primordial germ cell fate. Cell *160*, 253–268.

Ishiuchi, T., Enriquez-Gasca, R., Mizutani, E., Bošković, A., Ziegler-Birling, C., Rodriguez-Terrones, D., Wakayama, T., Vaquerizas, J.M., and Torres-Padilla, M.-E. (2015). Early embryonic-like cells are induced by downregulating replication-dependent chromatin assembly. Nat. Struct. Mol. Biol. *22*, 662–671.

Isotani, A., Hatayama, H., Kaseda, K., Ikawa, M., and Okabe, M. (2011). Formation of a thymus from rat ES cells in xenogeneic nude mouse↔rat ES chimeras. Genes Cells *16*, 397–405.

Izpisúa-Belmonte, J.C., Brown, J.M., Crawley, A., Duboule, D., and Tickle, C. (1992). Hox-4 gene expression in mouse/chicken heterospecific grafts of signalling regions to limb buds reveals similarities in patterning mechanisms. Development *115*, 553–560.

Izpisúa-Belmonte, J.C., De Robertis, E.M., Storey, K.G., and Stern, C.D. (1993). The homeobox gene goosecoid and the origin of organizer cells in the early chick blastoderm. Cell *74*, 645–659.

Izpisua Belmonte, J.C., Callaway, E.M., Caddick, S.J., Churchland, P., Feng, G., Homanics, G.E., Lee, K.-F., Leopold, D.A., Miller, C.T., Mitchell, J.F., et al. (2015). Brains, genes, and primates. Neuron *86*, 617–631.

James, D., Noggle, S.A., Swigut, T., and Brivanlou, A.H. (2006). Contribution of human embryonic stem cells to mouse blastocysts. Dev. Biol. *295*, 90–102.

Johnston, L.A. (2009). Competitive interactions between cells: death, growth, and geography. Science *324*, 1679–1682.

Joo, J.Y., Choi, H.W., Kim, M.J., Zaehres, H., Tapia, N., Stehling, M., Jung, K.S., Do, J.T., and Schöler, H.R. (2014). Establishment of a primed pluripotent epiblast stem cell in FGF4-based conditions. Sci. Rep. *4*, 7477.

Kawase, E., Suemori, H., Takahashi, N., Okazaki, K., Hashimoto, K., and Nakatsuji, N. (1994). Strain difference in establishment of mouse embryonic stem (ES) cell lines. Int. J. Dev. Biol. *38*, 385–390.

Kim, H., Wu, J., Ye, S., Tai, C.-I., Zhou, X., Yan, H., Li, P., Pera, M., and Ying, Q.-L. (2013). Modulation of β-catenin function maintains mouse epiblast stem cell and human embryonic stem cell self-renewal. Nat. Commun. *4*, 2403.

Kobayashi, T., Yamaguchi, T., Hamanaka, S., Kato-Itoh, M., Yamazaki, Y., Ibata, M., Sato, H., Lee, Y.-S., Usui, J., Knisely, A.S., et al. (2010). Generation of rat pancreas in mouse by interspecific blastocyst injection of pluripotent stem cells. Cell *142*, 787–799.

Kobayashi, T., Kato-Itoh, M., and Nakauchi, H. (2015). Targeted organ generation using Mixl1-inducible mouse pluripotent stem cells in blastocyst complementation. Stem Cells Dev. *24*, 182–189.

Kojima, Y., Kaufman-Francis, K., Studdert, J.B., Steiner, K.A., Power, M.D., Loebel, D.A.F., Jones, V., Hor, A., de Alencastro, G., Logan, G.J., et al. (2014). The transcriptional and functional properties of mouse epiblast stem cells resemble the anterior primitive streak. Cell Stem Cell *14*, 107–120.

Kolodziejczyk, A.A., Kim, J.K., Tsang, J.C.H., Ilicic, T., Henriksson, J., Natarajan, K.N., Tuck, A.C., Gao, X., Bühler, M., Liu, P., et al. (2015). Single Cell RNA-Sequencing of Pluripotent States Unlocks Modular Transcriptional Variation. Cell Stem Cell *17*, 471–485.

Kragh, P.M., Nielsen, A.L., Li, J., Du, Y., Lin, L., Schmidt, M., Bøgh, I.B., Holm, I.E., Jakobsen, J.E., Johansen, M.G., et al. (2009). Hemizygous minipigs produced by random gene insertion and handmade cloning express the Alzheimer's disease-causing dominant mutation APPsw. Transgenic Res. *18*, 545–558.

Kriks, S., Shim, J.-W., Piao, J., Ganat, Y.M., Wakeman, D.R., Xie, Z., Carrillo-Reid, L., Auyeung, G., Antonacci, C., Buch, A., et al. (2011). Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. Nature *480*, 547–551.

Kuijk, E.W., van Tol, L.T.A., Van de Velde, H., Wubbolts, R., Welling, M., Geijsen, N., and Roelen, B.A.J. (2012). The roles of FGF and MAP kinase signaling in the segregation of the epiblast and hypoblast cell lineages in bovine and human embryos. Development *139*, 871–882.

Kumar, R.M., Cahan, P., Shalek, A.K., Satija, R., DaleyKeyser, A.J., Li, H., Zhang, J., Pardee, K., Gennert, D., Trombetta, J.J., et al. (2014). Deconstructing transcriptional heterogeneity in pluripotent stem cells. Nature *516*, 56–61.

Kurek, D., Neagu, A., Tastemel, M., Tüysüz, N., Lehmann, J., van de Werken, H.J.G., Philipsen, S., van der Linden, R., Maas, A., van IJcken, W.F.J., et al. (2015). Endogenous WNT signals mediate BMP-induced and spontaneous differentiation of epiblast stem cells and human embryonic stem cells. Stem Cell Reports *4*, 114–128.

Lai, L., Kolber-Simonds, D., Park, K.W., Cheong, H.T., Greenstein, J.L., Im, G.S., Samuel, M., Bonk, A., Rieke, A., Day, B.N., et al. (2002). Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science *295*, 1089–1092.

Lancaster, M.A., and Knoblich, J.A. (2014). Organogenesis in a dish: modeling development and disease using organoid technologies. Science *345*, 1247125.

Lensch, M.W., Schlaeger, T.M., Zon, L.I., and Daley, G.Q. (2007). Teratoma formation assays with human embryonic stem cells: a rationale for one type of human-animal chimera. Cell Stem Cell *1*, 253–258.

Li, P., Tong, C., Mehrian-Shai, R., Jia, L., Wu, N., Yan, Y., Maxson, R.E., Schulze, E.N., Song, H., Hsieh, C.-L., et al. (2008). Germline competent embryonic stem cells derived from rat blastocysts. Cell *135*, 1299–1310.

Lienert, F., Lohmueller, J.J., Garg, A., and Silver, P.A. (2014). Synthetic biology in mammalian cells: next generation research tools and therapeutics. Nat. Rev. Mol. Cell Biol. *15*, 95–107.

Lorson, M.A.M., Spate, L.D.L., Samuel, M.S.M., Murphy, C.N.C., Lorson, C.L.C., Prather, R.S.R., and Wells, K.D.K. (2011). Disruption of the Survival Motor Neuron (SMN) gene in pigs using ssDNA. Transgenic Res. *20*, 1293–1304.

Ludwig, T.E., Levenstein, M.E., Jones, J.M., Berggren, W.T., Mitchen, E.R., Frane, J.L., Crandall, L.J., Daigh, C.A., Conard, K.R., Piekarczyk, M.S., et al. (2006). Derivation of human embryonic stem cells in defined conditions. Nat. Biotechnol. *24*, 185–187.

Lyons, I., Parsons, L.M., Hartley, L., Li, R., Andrews, J.E., Robb, L., and Harvey, R.P. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. Genes Dev. *9*, 1654–1666.

Macfarlan, T.S., Gifford, W.D., Driscoll, S., Lettieri, K., Rowe, H.M., Bonanomi, D., Firth, A., Singer, O., Trono, D., and Pfaff, S.L. (2012). Embryonic stem cell potency fluctuates with endogenous retrovirus activity. Nature *487*, 57–63.

Martello, G., and Smith, A. (2014). The nature of embryonic stem cells. Annu. Rev. Cell Dev. Biol. *30*, 647–675.

Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. USA *78*, 7634–7638.

Masaki, H., Kato-Itoh, M., Umino, A., Sato, H., Hamanaka, S., Kobayashi, T., Yamaguchi, T., Nishimura, K., Ohtaka, M., Nakanishi, M., and Nakauchi, H. (2015). Interspecific in vitro assay for the chimera-forming ability of human pluripotent stem cells. Development *142*, 3222–3230.

Matsui, Y., Zsebo, K., and Hogan, B.L.M. (1992). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell *70*, 841–847.

Matsunari, H., Nagashima, H., Watanabe, M., Umeyama, K., Nakano, K., Nagaya, M., Kobayashi, T., Yamaguchi, T., Sumazaki, R., Herzenberg, L.A.L., and Nakauchi, H. (2013). Blastocyst complementation generates exogenic pancreas in vivo in apancreatic cloned pigs. Proc. Natl. Acad. Sci. USA *110*, 4557–4562.

Morata, G., and Ripoll, P. (1975). Minutes: mutants of drosophila autonomously affecting cell division rate. Dev. Biol. *42*, 211–221.

Morgani, S.M., Canham, M.A., Nichols, J., Sharov, A.A., Migueles, R.P., Ko, M.S.H., and Brickman, J.M. (2013). Totipotent embryonic stem cells arise in ground-state culture conditions. Cell Rep. *3*, 1945–1957.

Mulnard, J. (1973). Formation of chimeric blastocysts by fusion of rat and mice embryos at stage 8. C. R. Acad. Sci. Hebd. Seances Acad. Sci. D. *276*, 379–381.

Mystkowska, E.T. (1975). Development of mouse-bank vole interspecific chimaeric embryos. J. Embryol. Exp. Morphol. *33*, 731–744.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J.C. (1993). Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc. Natl. Acad. Sci. USA *90*, 8424–8428.

Najm, F.J., Chenoweth, J.G., Anderson, P.D., Nadeau, J.H., Redline, R.W., McKay, R.D.G., and Tesar, P.J. (2011). Isolation of epiblast stem cells from preimplantation mouse embryos. Cell Stem Cell *8*, 318–325.

Nakaki, F., Hayashi, K., Ohta, H., Kurimoto, K., Yabuta, Y., and Saitou, M. (2013). Induction of mouse germ-cell fate by transcription factors in vitro. Nature *501*, 222–226.

National Research Council (2005). Guidelines for human embryonic stem cell research, First Edition (Washington, D.C.: National Academies Press).

National Research Council (2007). Amendments to the National Academies' Guidelines for Human Embryonic Stem Cell Research, First Edition (Washington, D.C.: National Academies Press).

Nicholas, C.R., Chen, J., Tang, Y., Southwell, D.G., Chalmers, N., Vogt, D., Arnold, C.M., Chen, Y.-J.J., Stanley, E.G., Elefanty, A.G., et al. (2013). Functional maturation of hPSC-derived forebrain interneurons requires an extended timeline and mimics human neural development. Cell Stem Cell *12*, 573–586.

Nichols, J., and Smith, A. (2009). Naive and primed pluripotent states. Cell Stem Cell *4*, 487–492.

Niu, Y., Shen, B., Cui, Y., Chen, Y., Wang, J., Wang, L., Kang, Y., Zhao, X., Si, W., Li, W., et al. (2014). Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell *156*, 836–843.

O'Leary, T., Heindryckx, B., Lierman, S., van Bruggen, D., Goeman, J.J., Vandewoestyne, M., Deforce, D., de Sousa Lopes, S.M.C., and De Sutter, P. (2012). Tracking the progression of the human inner cell mass during embryonic stem cell derivation. Nat. Biotechnol. *30*, 278–282.

Oestrup, O., Hall, V., Petkov, S.G., Wolf, X.A., Hyldig, S., and Hyttel, P. (2009). From zygote to implantation: morphological and molecular dynamics during embryo development in the pig. Reprod. Domest. Anim. *44* (*Suppl 3*), 39–49.

Offield, M.F., Jetton, T.L., Labosky, P.A., Ray, M., Stein, R.W., Magnuson, M.A., Hogan, B.L., and Wright, C.V. (1996). PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. Development *122*, 983–995.

Ohinata, Y., Ohta, H., Shigeta, M., Yamanaka, K., Wakayama, T., and Saitou, M. (2009). A signaling principle for the specification of the germ cell lineage in mice. Cell *137*, 571–584.

Osafune, K., Caron, L., Borowiak, M., Martinez, R.J., Fitz-Gerald, C.S., Sato, Y., Cowan, C.A., Chien, K.R., and Melton, D.A. (2008). Marked differences in differentiation propensity among human embryonic stem cell lines. Nat. Biotechnol. *26*, 313–315.

Pagliuca, F.W., Millman, J.R., Gürtler, M., Segel, M., Van Dervort, A., Ryu, J.H., Peterson, Q.P., Greiner, D., and Melton, D.A. (2014). Generation of functional human pancreatic β cells in vitro. Cell *159*, 428–439.

Park, K.W., Cheong, H.T., Lai, L., Im, G.S., Kühholzer, B., Bonk, A., Samuel, M., Rieke, A., Day, B.N., Murphy, C.N., et al. (2001). Production of nuclear transfer-derived swine that express the enhanced green fluorescent protein. Anim. Biotechnol. *12*, 173–181.

Pera, M.F., and Tam, P.P.L. (2010). Extrinsic regulation of pluripotent stem cells. Nature *465*, 713–720.

Petters, R.M., Alexander, C.A., Wells, K.D., Collins, E.B., Sommer, J.R., Blanton, M.R., Rojas, G., Hao, Y., Flowers, W.L., Banin, E., et al. (1997). Genetically engineered large animal model for studying cone photoreceptor survival and degeneration in retinitis pigmentosa. Nat. Biotechnol. *15*, 965–970.

Prather, R.S., Lorson, M., Ross, J.W., Whyte, J.J., and Walters, E. (2013). Genetically engineered pig models for human diseases. Annu. Rev. Anim. Biosci. *1*, 203–219.

Rashid, T., Kobayashi, T., and Nakauchi, H. (2014). Revisiting the flight of Icarus: making human organs from PSCs with large animal chimeras. Cell Stem Cell *15*, 406–409.

Renner, S., Fehlings, C., Herbach, N., Hofmann, A., von Waldthausen, D.C., Kessler, B., Ulrichs, K., Chodnevskaja, I., Moskalenko, V., Amselgruber, W., et al. (2010). Glucose intolerance and reduced proliferation of pancreatic beta-cells in transgenic pigs with impaired glucose-dependent insulinotropic polypeptide function. Diabetes *59*, 1228–1238.

Resnick, J.L., Bixler, L.S., Cheng, L., and Donovan, P.J. (1992). Long-term proliferation of mouse primordial germ cells in culture. Nature *359*, 550–551.

Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A., and Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat. Biotechnol. *18*, 399–404.

Rogers, C.S.C., Stoltz, D.A.D., Meyerholz, D.K.D., Ostedgaard, L.S.L., Rokhlina, T., Taft, P.J.P., Rogan, M.P.M., Pezzulo, A.A.A., Karp, P.H.P., Itani, O.A.O., et al. (2008). Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. Science *321*, 1837–1841.

Roode, M., Blair, K., Snell, P., Elder, K., Marchant, S., Smith, A., and Nichols, J. (2012). Human hypoblast formation is not dependent on FGF signalling. Dev. Biol. *361*, 358–363.

Ross, J.W.J., Fernandez de Castro, J.P., Zhao, J., Samuel, M., Walters, E., Rios, C., Bray-Ward, P., Jones, B.W.B., Marc, R.E.R., Wang, W., et al. (2012). Generation of an inbred miniature pig model of retinitis pigmentosa. Invest. Ophthalmol. Vis. Sci. *53*, 501–507.

Rossant, J. (1976). Investigation of inner cell mass determination by aggregation of isolated rat inner cell masses with mouse morulae. J. Embryol. Exp. Morphol. *36*, 163–174.

Rossant, J., and Frels, W.I. (1980). Interspecific chimeras in mammals: successful production of live chimeras between Mus musculus and Mus caroli. Science *208*, 419–421.

Sasaki, K., Yokobayashi, S., Nakamura, T., Okamoto, I., Yabuta, Y., Kurimoto, K., Ohta, H., Moritoki, Y., Iwatani, C., Tsuchiya, H., et al. (2015). Robust In Vitro Induction of Human Germ Cell Fate from Pluripotent Stem Cells. Cell Stem Cell *17*, 178–194.

Si-Tayeb, K., Noto, F.K., Nagaoka, M., Li, J., Battle, M.A., Duris, C., North, P.E., Dalton, S., and Duncan, S.A. (2010). Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology *51*, 297–305.

Simerly, C., McFarland, D., Castro, C., Lin, C.-C., Redinger, C., Jacoby, E., Mich-Basso, J., Orwig, K., Mills, P., Ahrens, E., et al. (2011). Interspecies chimera between primate embryonic stem cells and mouse embryos: monkey ESCs engraft into mouse embryos, but not post-implantation fetuses. Stem Cell Res. (Amst.) *7*, 28–40.

Smith, K.K. (2003). Time's arrow: heterochrony and the evolution of development. Int. J. Dev. Biol. *47*, 613–621.

Smith, A.G., Heath, J.K., Donaldson, D.D., Wong, G.G., Moreau, J., Stahl, M., and Rogers, D. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature *336*, 688–690.

Solter, D., Škreb, N., and Damjanov, I. (1970). Extrauterine growth of mouse egg-cylinders results in malignant teratoma. Nature *227*, 503–504.

Stanger, B.Z., Tanaka, A.J., and Melton, D.A. (2007). Organ size is limited by the number of embryonic progenitor cells in the pancreas but not the liver. Nature *445*, 886–891.

Stephenson, R.O., Rossant, J., and Tam, P.P.L. (2012). Intercellular interactions, position, and polarity in establishing blastocyst cell lineages and embryonic axes. Cold Spring Harb. Perspect. Biol. *4*, a008235–a008235.

Stern, M.S. (1973). Chimaeras obtained by aggregation of mouse eggs with rat eggs. Nature *243*, 472–473.

Stern, C.D. (2005). The chick; a great model system becomes even greater. Dev. Cell *8*, 9–17.

Stevens, L.C. (1970). The development of transplantable teratocarcinomas from intratesticular grafts of pre- and postimplantation mouse embryos. Dev. Biol. *21*, 364–382.

Straathof, K.C., Pulè, M.A., Yotnda, P., Dotti, G., Vanin, E.F., Brenner, M.K., Heslop, H.E., Spencer, D.M., and Rooney, C.M. (2005). An inducible caspase 9 safety switch for T-cell therapy. Blood *105*, 4247–4254.

Sturzu, A.C., Rajarajan, K., Passer, D., Plonowska, K., Riley, A., Tan, T.C., Sharma, A., Xu, A.F., Engels, M.C., Feistritzer, R., et al. (2015). Fetal Mammalian Heart Generates a Robust Compensatory Response to Cell Loss. Circulation *132*, 109–121.

Sung, Y.H., Baek, I.-J., Kim, D.H., Jeon, J., Lee, J., Lee, K., Jeong, D., Kim, J.-S., and Lee, H.-W. (2013). Knockout mice created by TALEN-mediated gene targeting. Nat. Biotechnol. *31*, 23–24.

Tachibana, M., Sparman, M., Ramsey, C., Ma, H., Lee, H.-S., Penedo, M.C.T., and Mitalipov, S. (2012). Generation of chimeric rhesus monkeys. Cell *148*, 285–295.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663–676.

Takashima, Y., Guo, G., Loos, R., Nichols, J., Ficz, G., Krueger, F., Oxley, D., Santos, F., Clarke, J., Mansfield, W., et al. (2014). Resetting transcription factor control circuitry toward ground-state pluripotency in human. Cell *158*, 1254–1269.

ten Berge, D., Kurek, D., Blauwkamp, T., Koole, W., Maas, A., Eroglu, E., Siu, R.K., and Nusse, R. (2011). Embryonic stem cells require Wnt proteins to prevent differentiation to epiblast stem cells. Nat. Cell Biol. *13*, 1–8.

Tesar, P.J., Chenoweth, J.G., Brook, F.A., Davies, T.J., Evans, E.P., Mack, D.L., Gardner, R.L., and McKay, R.D.G. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature *448*, 196–199.

Theunissen, T.W., Powell, B.E., Wang, H., Mitalipova, M., Faddah, D.A., Reddy, J., Fan, Z.P., Maetzel, D., Ganz, K., Shi, L., et al. (2014). Systematic identification of culture conditions for induction and maintenance of naive human pluripotency. Cell Stem Cell *15*, 471–487.

Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., Becker, R.A., and Hearn, J.P. (1995). Isolation of a primate embryonic stem cell line. Proc. Natl. Acad. Sci. USA *92*, 7844–7848.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. Science *282*, 1145–1147.

Tonge, P.D., Corso, A.J., Monetti, C., Hussein, S.M.I., Puri, M.C., Michael, I.P., Li, M., Lee, D.-S., Mar, J.C., Cloonan, N., et al. (2014). Divergent reprogramming routes lead to alternative stem-cell states. Nature *516*, 192–197.

Tsakiridis, A., Huang, Y., Blin, G., Skylaki, S., Wymeersch, F., Osorno, R., Economou, C., Karagianni, E., Zhao, S., Lowell, S., and Wilson, V. (2014). Distinct Wnt-driven primitive streak-like populations reflect in vivo lineage precursors. Development *141*, 1209–1221.

Tsukiyama, T., and Ohinata, Y. (2014). A modified EpiSC culture condition containing a GSK3 inhibitor can support germline-competent pluripotency in mice. PLoS ONE *9*, e95329.

Umeyama, K., Watanabe, M., Saito, H., Kurome, M., Tohi, S., Matsunari, H., Miki, K., and Nagashima, H. (2009). Dominant-negative mutant hepatocyte nuclear factor 1alpha induces diabetes in transgenic-cloned pigs. Transgenic Res. *18*, 697–706.

Usui, J., Kobayashi, T., Yamaguchi, T., Knisely, A.S., Nishinakamura, R., and Nakauchi, H. (2012). Generation of kidney from pluripotent stem cells via blastocyst complementation. Am. J. Pathol. *180*, 2417–2426.

Valamehr, B., Robinson, M., Abujarour, R., Rezner, B., Vranceanu, F., Le, T., Medcalf, A., Lee, T.T., Fitch, M., Robbins, D., and Flynn, P. (2014). Platform for induction and maintenance of transgene-free hiPSCs resembling ground state pluripotent stem cells. Stem Cell Reports *2*, 366–381.

Vallier, L., Alexander, M., and Pedersen, R.A. (2005). Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. J. Cell Sci. *118*, 4495–4509.

Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F., and Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell *153*, 910–918.

Wang, J., Xie, G., Singh, M., Ghanbarian, A.T., Raskó, T., Szvetnik, A., Cai, H., Besser, D., Prigione, A., Fuchs, N.V., et al. (2014). Primate-specific endogenous retrovirus-driven transcription defines naive-like stem cells. Nature *516*, 405–409.

Wang, Y., Du, Y., Shen, B., Zhou, X., Li, J., Liu, Y., Wang, J., Zhou, J., Hu, B., Kang, N., et al. (2015). Efficient generation of gene-modified pigs via injection of zygote with Cas9/sgRNA. Sci. Rep. *5*, 8256.

Ware, C.B., Nelson, A.M., Mecham, B., Hesson, J., Zhou, W., Jonlin, E.C., Jimenez-Caliani, A.J., Deng, X., Cavanaugh, C., Cook, S., et al. (2014). Derivation of naive human embryonic stem cells. Proc. Natl. Acad. Sci. USA *111*, 4484–4489.

Whitworth, K.M., Lee, K., Benne, J.A., Beaton, B.P., Spate, L.D., Murphy, S.L., Samuel, M.S., Mao, J., O'Gorman, C., Walters, E.M., et al. (2014). Use of the CRISPR/Cas9 System to Produce Genetically Engineered Pigs from In Vitro-Derived Oocytes and Embryos. Biology of Reproduction *91*, biolreprod.114.121723–biolreprod.114.121778.

Whyte, J.J., and Prather, R.S. (2012). Cell Biology Symposium: Zinc finger nucleases to create custom-designed modifications in the swine (Sus scrofa) genome. J. Anim. Sci. *90*, 1111–1117.

Williams, T.J., Munro, R.K., and Shelton, J.N. (1990). Production of interspecies chimeric calves by aggregation of Bos indicus and Bos taurus demi-embryos. Reprod. Fertil. Dev. *2*, 385–394.

Wilmut, I., Beaujean, N., de Sousa, P.A., Dinnyes, A., King, T.J., Paterson, L.A., Wells, D.N., and Young, L.E. (2002). Somatic cell nuclear transfer. Nature *419*, 583–586.

Wu, S.M., and Hochedlinger, K. (2011). Harnessing the potential of induced pluripotent stem cells for regenerative medicine. Nat. Cell Biol. *13*, 497–505.

Wu, J., and Izpisua Belmonte, J.C. (2014). Stem cells: A designer's guide to pluripotency. Nature *516*, 172–173.

Wu, Z., Earle, J., Saito, S., Anderson, C.W., Appella, E., and Xu, Y. (2002). Mutation of mouse p53 Ser23 and the response to DNA damage. Mol. Cell. Biol. *22*, 2441–2449.

Wu, J., Okamura, D., Li, M., Suzuki, K., Luo, C., Ma, L., He, Y., Li, Z., Benner, C., Tamura, I., et al. (2015). An alternative pluripotent state confers interspecies chimaeric competency. Nature *521*, 316–321.

Xiang, A.P., Mao, F.F., Li, W.-Q., Park, D., Ma, B.-F., Wang, T., Vallender, T.W., Vallender, E.J., Zhang, L., Lee, J., et al. (2008). Extensive contribution of embryonic stem cells to the development of an evolutionarily divergent host. Hum. Mol. Genet. *17*, 27–37.

Yang, Y., Adachi, K., Sheridan, M.A., Alexenko, A.P., Schust, D.J., Schulz, L.C., Ezashi, T., and Roberts, R.M. (2015). Heightened potency of human pluripotent stem cell lines created by transient BMP4 exposure. Proc. Natl. Acad. Sci. USA *112*, E2337–E2346.

Ying, Q.-L., Nichols, J., Chambers, I., and Smith, A. (2003). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell *115*, 281–292.

Ying, Q.-L., Wray, J., Nichols, J., Batlle-Morera, L., Doble, B., Woodgett, J., Cohen, P., and Smith, A. (2008). The ground state of embryonic stem cell self-renewal. Nature *453*, 519–523.

Zeilmaker, G.H. (1973). Fusion of rat and mouse morulae and formation of chimaeric blastocysts. Nature *242*, 115–116.

Zhang, S.-C., Wernig, M., Duncan, I.D., Brüstle, O., and Thomson, J.A. (2001). In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat. Biotechnol. *19*, 1129–1133.

Zhang, X., Huang, C.T., Chen, J., Pankratz, M.T., Xi, J., Li, J., Yang, Y., Lavaute, T.M., Li, X.-J., Ayala, M., et al. (2010). Pax6 is a human neuroectoderm cell fate determinant. Cell Stem Cell *7*, 90–100.

Zhu, S., Rezvani, M., Harbell, J., Mattis, A.N., Wolfe, A.R., Benet, L.Z., Willenbring, H., and Ding, S. (2014). Mouse liver repopulation with hepatocytes generated from human fibroblasts. Nature *508*, 93–97.