The shortage of human organs for transplantation is a devastating medical problem. One way to expand organ supply is to derive functional organs from patient-specific stem cells. Due to their capacity to grow indefinitely in the laboratory and differentiate into any cell type of the human body, patient-specific pluripotent stem (PS)† cells harbor the potential to provide an inexhaustible supply of donor cells for transplantation. However, current efforts to generate functional organs from PS cells have so far been unsuccessful. An alternative and promising strategy is to generate human organs inside large animal species through a technique called interspecies blastocyst complementation. In this method, animals comprised of cells from human and animal species are generated by injecting donor human PS cells into animal host embryos. Critical genes for organ development are knocked out by genome editing, allowing donor human PS cells to populate the vacated niche. In principle, this experimental approach will produce a desired organ of human origin inside a host animal. In this mini-review, we focus on recent advances that may bring the promise of blastocyst complementation to clinical practice. While CRISPR/Cas9 has accelerated the creation of transgenic large animals such as pigs and sheep, we propose that further advances in the generation of chimera-competent human PS cells are needed to achieve interspecies blastocyst complementation. It will also be necessary to define the constituents of the species barrier, which inhibits efficient colonization of host animal embryos with human cells. Interspecies blastocyst complementation is a promising approach to help overcome the organ shortage facing the practice of clinical medicine today.

INTRODUCTION

The shortage of viable organs for transplantation impedes the treatment of organ failure. Despite considerable efforts, thousands of patients continue to die while awaiting organ transplant each year [1]. The increase of organ failure in aging societies has worsened the problem of organ shortage. To address this issue, various strategies are being pursued. Transplantation of organs formerly deemed undesirable is being considered. Attempts to increase organ awareness aim to target the potential of willing but unregistered donors [2]. Porcine organs are also considered a potentially favorable source [3,4]. Despite

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†Abbreviations: PS, pluripotent stem cells; iPS, induced pluripotent stem cells; NHPs, non-human primates; LIF, leukemia inhibitory factor; 2i, kinase inhibitors; EpiS cells, epiblast stem cells.

Keywords: pluripotent stem cells, interspecies chimeras, chimeras, interspecies blastocyst complementation, blastocyst complementation, organ shortage, organ generation, organ transplantation, Pdx1, pluripotency, reprogramming, mouse pluripotent stem cells, human pluripotent stem cells, naive pluripotent stem cells, naive pluripotency, primed pluripotent stem cells, primed pluripotency, CRISPR, Cas9

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different approaches to resolve this devastating medical problem, increasing the number of allografts available for transplant remains the central challenge.

One strategy to expand the number of organs available for transplantation is to generate functional organs from patient-specific induced pluripotent stem (iPS) cells [5]. The defining features of human iPS cells – indefinite self-renewal in culture as well as capacity to differentiate into any cell type – in principle, allows for access to an endless supply of therapeutically relevant cells [6,7]. Further, iPSC-derived organs would be genetically identical to their intended patients and recipients. Autologous or allogeneic transplantation of iPSC-derived organs is anticipated to avoid immune rejection or complications of allogeneic transplantation of iPSC-derived organs is anticipated to avoid immune rejection or complications of immunosuppression regimens [8-10].

Classical strategies for obtaining desired cells from human PS cells involve differentiation in a dish. However, in vitro differentiation possesses key disadvantages, including: the danger of remnant undifferentiated human PS cells developing into teratomas post-transplantation [11] and failure to achieve functional maturation of in vitro generated human PS cell derivatives which typically manifest immature (typically fetal-like) features. Current methodologies are not compatible with producing complex three-dimensional tissues, such as transplantable organs. Consequently, new approaches to cell differentiation are needed to overcome these barriers.

Natural selection has produced intricate developmental programs within organisms. Rather than attempting to replicate this complexity in vitro, it may be possible to exploit such developmental programs to generate human organs inside animal hosts directly [12-14]. This would entail the production of interspecies chimeras -- animals comprised of cells from two different species (i.e., human patient and a pig or sheep host). Overcoming the limitations posed by in vitro differentiation, development of human PS cells in interspecies chimeras with the animal host would, if successful, enable the generation of functionally mature, complex three-dimensional transplantable organs. A tractable method for establishing the development of human cells inside animal hosts would lay the foundation for producing transplantable organs from patient-specific stem cells.

In this mini-review, we highlight recent findings that advance the goal of generating human organs inside large animal hosts such as pigs and sheep. Interspecies blastocyst complementation requires the generation of genetically edited animals that can be chimerized by human donor PS cells. While much success has been achieved in creating transgenic pig or sheep, we suggest that interspecies chimer generation will require addressing two major challenges: first, resolving the lack of chimera-competent human PS cells; and second, understanding the species barrier that causes poor chimeric contribution of human donor PS cells. Surmounting these challenges will be necessary for chimeric contribution of human PS cells to distantly related large animal hosts, such as pigs and sheep.

**blastocyst complementation: an introduction**

Perturbing the genetic programs underlying organogenesis can produce organisms lacking entire organs [15-17]. When organ generation is disrupted through genetic intervention, the remaining host cells and tissue will still persist, continuing to provide extrinsic factors and inductive interactions necessary for instructing organ formation [15-17]. A vacant developmental niche forms; donor wild-type PS cells are introduced into host blastocysts. The resulting chimeric embryos are transferred into a pseudopregnant foster mother for subsequent development. Meanwhile, the introduced chimera-competent PS cells developmentally compensate and colonize the empty niche, generating a donor cell-derived organ [15-17]. This complementation of organogenesis-disabled host blastocysts with wild-type donor cells is termed blastocyst complementation (Figure 1) [12,15-19].

**Interspecies Blastocyst Complementation in Rodents**

The first report of interspecies blastocyst complementation for creating organs involved a study where Pdx1-deficient mouse blastocysts were complemented with rat PS cells [16]. The resulting rat-mouse interspecies chimeras possessed an entirely rat pancreas [16,17,19]. It is important to note that interspecies chimeras generated between rats and mice possessed vessels, nerves, and some interstitial elements that were blends of mouse and rat cells that may pose a problem for clinical translation [16]. Despite this caveat, this study was the first to report generation of a PS cell-derived functional organ from one species inside the animal of another species.

**Large Animal Hosts**

Adapting blastocyst complementation for human organ production will require the use of sufficiently sized animals. Pigs and sheep may represent particularly suitable large animal hosts because of their similarity to humans with regard to organ size as well as other advantages, such as breeding potential, period to reproductive maturity, and number of offspring unlike other animals such as non-human primates (NHPs) like baboons. Additionally, pigs have a lower cost of maintenance compared with NHPs such as baboon [20]. It is worth noting that the amenability of sheep for interspecies chimera formation has been demonstrated and extensively investigat-
ed through studies of sheep-goat interspecies chimeras [21,22].

The possibility of interspecies blastocyst complementation for organ generation in large animal hosts was illustrated in a study where pancreatogenesis-disabled pigs were complemented with allogeneic blastocysters [23]. The resulting adult pig chimeras possessed entirely donor-derived pancrea. Moreover, gene-edited Pdx1-knockout sheep that can potentially serve as a host for interspecies organ generation have also been generated [24]. As noted above, xeno-generation of human organs inside animal hosts will require production of human organs without animal host-derived nerves and vasculature. In this regard, additional blastocyst complementation experiments are needed to identify the correct strategy for removal of host-derived vasculature and nerves. As interspecies blastocyst complementation approaches continue to improve, it will be desirable to create customized pig or sheep hosts with appropriately complemented nerves and vasculature.

The above experiments used pre-existing cell lines to create animal hosts that can be complemented with wild-type cells. It will be ideal to employ optimized genome editing strategies to create customized animal hosts with target lineages disrupted, which will permit colonization of the devoid niche with donor cells. Applying CRISPR/Cas9 genome editing in zygotes may achieve this end [25,26]. The use of CRISPR/Cas9 in zygotes has enabled production of knockout animals in various species, including large animals such as pigs and sheep [24]. Furthermore, targeting of multiple lineages will be needed to achieve the production of human organs without animal host-derived nerves and vasculature. In these regards, highly multiplexed genetic engineering by CRISPR/Cas9 may prove useful [3,4].

**IDENTIFYING HUMAN CHIMERA-COMPETENT PLURIPOTENT STEM CELLS**

The studies above suggest that developing an interspecies blastocyst complementation platform will require creating genetically modified animal hosts and human chimera-competent PS cells. Hence, an essential technology needed for successful complementation of pig or
cells grow as two-dimensional flat colonies. Perhaps the most significant difference between mouse and human PS cells relates to whether each cell type corresponds to a cellular state competent to form chimeras. While mouse ES cells reliably form chimeras, it is unlikely conventional human PS cells correspond to a chimera-competent cellular state. Mouse and rat ES cells propagated in 2i exhibit high-grade chimeric contribution and germline transmission [33,34]. Although evaluating the chimera competency of human ES cells is ethically constrained, primate ES cells grown in FGF-containing human ES cell culture conditions also have a flat morphology and notably fail to generate chimeras after blastocyst injection [35]. Given the chimera-competency of rodent PS cells, it has been speculated that the application of 2i to PS cells from other species may lead to the generation of chimera-competent ES cells from other species, including primates and humans [32-36].

Conventional Mouse and Human PS cells

While both manifest potential to form all three germ layers – ectoderm, mesoderm, and endoderm – conventional human PS cells show strikingly distinct characteristics when compared to mouse PS cells. Although both mouse and human PS cells can be derived from the inner cell mass and by direct reprogramming of somatic cells, human ES and iPS cells require radically distinct conditions from mouse ES and iPS cells for their continuous propagation in vitro [5-7,27]. Human PS cells are typically derived and cultured in FGF-containing medium [28]. In contrast, the standard culture conditions for derivation and maintenance of mouse iPS cells involve an optimal combination of leukemia inhibitory factor (LIF) and two kinase inhibitors (2i) – PD0325901 and CHIR99021, small molecule inhibitors of the MEK and GSK3 kinases, respectively – that sustain mouse PS cells in a pre-implantation inner cell mass-like state with high-grade chimera-competency [29,30]. In striking contrast, LIF and inhibitors of MEK and GSK3 kinases induce differentiation of human PS cells [31,32]. Another obvious difference between conventional human PS cells and mouse PS cells is their appearance – mouse ES cells grow as three-dimensional domed colonies whereas human PS cells grow as two-dimensional flat colonies.

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Naive and Primed Pluripotent States

Current evidence suggests these differences between mouse and human stem cells may be ascribed to discrete developmental identities. The generation of a novel type of PS cell from post-implantation rodent embryos, termed epiblast stem cells (EpiS cells), using FGF-containing human ES cell culture conditions suggested that human ES cell pluripotency likely reflects a later stage of development [37,38]. Like human ES cells, EpiS cells also grow as flat colonies and depend on FGF signaling. No-

Figure 2. Mouse and human naive and primed pluripotent stem cells. (Top left) Mouse naive embryonic stem (ES) cells; (top right) Mouse primed epiblast stem (EpiS) cells; (bottom left) putative human naive induced pluripotent stem (iPS) cells; (bottom right) human primed iPS cells. Mouse ES cells were grown in N2B27-2i/LIF conditions. Mouse EpiS cells and human iPS cells were grown in FGF-containing medium. Human naive iPS cells were grown in a modified 2i/LIF medium (ADLA, data unpublished).
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features of chimera-competent mouse ES cells or chimaera-incompetent EpiS cells. Today, it is commonly accepted that PS cells exhibit features associated with different stages of embryonic development. The terms “naive” and “primed” were introduced by Nichols and Smith to designate PS cells with “pre-implantation” or “post-implantation” character (Figure 2) [36,43].

Naive-like Human PS Cells

The existence of distinct murine PS cell states stimulated interest in generating human naive PS cells (Figure 2) [36]. Various groups have claimed generation of hu-
man naive PS cells by combining 2i with different experimental methods (Table 1) [32,44-52]. Human naive-like cells in 2i-containing culture conditions exhibit some mouse naive features, such as domed colony morphology and self-renewal in 2i [32,44-53]. However, such cells remained FGF-dependent, reflecting species differences or possibly indicating failure to reach a fully naive state [45,48,49,51,53-56]. While still unclear, it is possible that the difficulties in achieving a rodent-like naive pluripotent state may be linked to differences in the signaling mechanisms governing rodent and primate embryogenesis [57], as well as the absence of diapause in humans, a phenomenon thought to provide the basis for rodent naive pluripotency propagation in vitro [58,59]. At the same time, while signaling mechanisms may diverge across species, it appears that core aspects of the transcription factor network governing rodent naive pluripotency are manifest in primate embryos and some reported human naive cells [46,47,51,57,60-63]. Whether capture of human PS cells with such transcription factor governance actually results in chimera-competent human PS cells is a very interesting question that has not been resolved [17].

Efforts to Generate Human-animal Interspecies Chimeras

Human chimera-competent PS cells, if they exist, may be able to contribute to the embryos of other species. Indeed, experiments involving human primed PS cells and mouse post-implantation embryos suggest feasibility. Consistent with the generation of chimeras by grafting murine primed PS cells onto egg-cylinder stage embryos, transplantation of conventional human PS cells into mouse egg-cylinder embryos results in differentiation into multiple fates, although it is unclear whether cooperative morphogenesis between donor and host cells occurred [41,64]. For generation of blastocyst stage chimeras, various groups have introduced alternative naive-like PS cells into mouse pre-implantation embryos [17,45,46,52,65]. However, in all cases the degree of chimeric contribution was essentially non-existent, especially when compared with higher-grade interspecies chimerism observed in mouse-rat interspecies chimeras [46,66]. Limited but detectable contribution of human cells to pig embryos has also been observed [17]. It is worth noting that the Belmonte study, unlike other studies evaluating interspecies chimerism, detected expression of lineage-affiliated markers in chimeric embryos, suggesting that differentiation of human PS cells may have occurred in vivo [17].

Synchronization of developmental stage between transplanted PS cells with host embryos seems to be needed for efficient chimera formation [41,42,64]. However, the introduction of human naive-like PS cells into murine and porcine pre-implantation embryos still fails to generate interspecies chimeras [17,46,66]. It is possible that “truly naive” human PS cells have not yet been generated, and this may account for the failure to generate blastocyst-stage chimeras [60,67]. Another possibility is that human naive pluripotent stem cells, classified as naive based on rodent molecular criteria, may not be developmentally equivalent to or synchronized with pig host embryos at the time of blastocyst injection [68]. It has been proposed that alternative human PS cells into late pig blastocysts may favor their development into chimeras [68]. Indeed, while introduction of certain naive-like human PS cells such as 4i and NHSM cells generated higher percentages of pig blastocysts containing human cells, introduction of an alternative human PS cell type cultured in FAC medium rather than other reported naive-like cells resulted in the formation of human-pig interspecies chimeras in the few instances it was observed [17,69].

Whether or not a human PS cell type satisfies molecular criteria for naive pluripotency, it still remains imperative to explore all culture parameters to maximize the interspecies chimera-competency of donor human PS cells [17,70]. Alternative strategies such as disabling apoptosis may help human stem cells overcome stage-related compatibility barriers to interspecies chimera formation. Indeed, overexpression of BCL2 endows chimera-incompetent rat EpiS cells with capacity to contribute to mouse blastocysts [71]. More generally, it is clear that the generation of naive PS cells may not be sufficient for achieving efficient interspecies chimera formation. Yet-to-be defined impediments may play a significant role.

THE SPECIES BARRIER

An alternative explanation for the inefficient chimeric contribution of existing human naive PS cells to the embryos of other species is that additional unidentified parameters impede interspecies chimera formation [42] (Figure 3).

Developmental Speed

One possible component of the species barrier is differences in the developmental speed between species [72]. Our understanding of how species-specific developmental timing is regulated remains primitive and poor. It has been observed that when mouse EpiS cells and human PS cells are subjected to the same neural differentiation protocol, human PS cell differentiation is comparatively prolonged with differences in the rates of differentiation mirroring differences in development timing in vivo. As a further corroboration, generation of teratomas from human PS cells in immunodeficient mice showed that human cells maintained a human rather than the mouse host developmental timing. These in vitro and in vivo data
Additional evolutionary differences may also play a role. Primates and large animals such as pigs and sheep have undergone evolutionary divergence with regard to peri-implantation development. Unlike primates that possess a short pre-attachment period, both pigs and sheep exhibit a long pre-attachment period. During this pre-attachment period, both pigs and sheep embryos undergo a process in which the blastocyst evolves into a filamentous structure, extending up to 1 meter long in pigs [73]. Primates, in contrast, do not undergo such a process [67]. Such differences may prove pivotal for achieving efficient interspecies chimerism in post-implantation conceptuses.

A hypothetical strategy to bypass divergent embryology between humans and large animals is to identify more permissive stages for engraftment of human cells.
into animal hosts. One such candidate embryonic stage would be a mid-embryonic stage called “the phylotypic period,” a timepoint in development (i.e., gastrulation) of an animal that resembles other species [74]. This stage may also exhibit increased interspecies conservation of gene expression and signaling milieu. Future comparative embryology studies between human and pigs and sheep may prove fruitful for identifying more permissive stages for cross-species chimera generation.

The existence of viable adult rat-mouse chimeras suggests that the results obtained when introducing human PS cells into mouse and pig host embryos may differ if host embryos from more closely related species such as primates are used. To develop effective strategies to lower species barriers, it may prove informative to study chimerism in early-stage human-monkey embryos cultured to post-implantation stages to identify impediments to human-non-primate interspecies chimera formation (Figure 3B). Such experiments could inform strategies to improve human chimerism in a distantly related animal host.

FUTURE DIRECTIONS

Advances in the generation of interspecies chimeras and blastocyst complementation methodology will lay a foundation for generating transplantable patient-specific organs inside large animal hosts such as pigs or sheep. While CRISPR/Cas9 will enable the generation of appropriately customized animal hosts, there remain many barriers to regular adoption of this practice. Whether extensive chimerism can be obtained between humans and more closely related species such as primates is likely but still remains undemonstrated. It is not known whether it will be possible to engineer human organs without contaminating animal host-derived nerves and vasculature, which is clinically problematic. Fundamental problems remain, such as how best to resolve differences in developmental speed and to overcome distinct developmental processes that have arisen from evolution. Moreover, as the xenogenic immune response is one of the most robust, it will be instructive to understand the mechanism of xenogenic immune tolerance in interspecies chimeras such as those between mouse and rat or sheep and goats [16,75]. Strategies such as “humanization” of host animals may be needed for translation into the clinic. Humanization of host animals raises several contentious ethical questions and guidelines from the International Society for Stem Cell Research (ISSCR) and other regulatory authorities indicate there are ethical issues involved in interspecies chimera research [76]. We will refer readers to various commentary articles that address these issues [77,78]. The community must carefully consider future challenges and proceed forward within ethical, legal, and social guidelines [79].

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