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Generation of human organs in pigs via interspecies blastocyst complementation

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Contents

More than eighteen years have passed since the first derivation of human embryonic stem cells (ESCs), but their clinical use is still met with several challenges, such as ethical concerns regarding the need of human embryos, tissue rejection after transplantation and tumour formation. The generation of human induced pluripotent stem cells (iPSCs) enables the access to patient-derived pluripotent stem cells (PSCs) and opens the door for personalized medicine as tissues/organs can potentially be generated from the same genetic background as the patient recipients, thus avoiding immune rejections or complication of immunosuppression strategies. In this regard, successful replacement, or augmentation, of the function of damaged tissue by patient-derived differentiated stem cells provides a promising cell replacement therapy for many devastating human diseases. Although human iPSCs can proliferate unlimitedly in culture and harbour the potential to generate all cell types in the adult body, currently, the functionality of differentiated cells is limited. An alternative strategy to realize the full potential of human iPSC for regenerative medicine is the in vivo tissue generation in large animal species via interspecies blastocyst complementation. As this technology is still in its infancy and there remains more questions than answers, thus in this review, we mainly focus the discussion on the conceptual framework, the emerging technologies and recent advances involved with interspecies blastocyst complementation, and will refer the readers to other more in-depth reviews on dynamic pluripotent stem cell states, genome editing and interspecies chimeras. Likewise, other emerging alternatives to combat the growing shortage of human organs, such as xenotransplantation or tissue engineering, topics that has been extensively reviewed, will not be covered here.

1 | INTRODUCTION

Human pluripotent stem cells (hPSCs) can be derived from preimplantation blastocysts (Thomson et al., 1998) or generated through nuclear reprogramming such as somatic cell nuclear transfer (SCNT) (Tachibana et al., 2013; Wakayama, Perry, Zuccotti, Johnson, & Yanagimachi, 1998) and transcription factor-induced pluripotent stem cells or iPSCs (Aasen et al., 2008; Takahashi et al., 2007; Yu et al., 2007). Two characteristic properties of hPSCs, the unlimited proliferative capability in culture and the ability to differentiate into all cell types in the adult body, hold great potential to provide unlimited source material for cell-based therapies. In addition, patient-specific SCNT PSCs or iPSCs are also important for the advancement of personalized medicine. To realize their potential, more efforts have been

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dedicated to the development of strategies for generating mature and functional cells and tissues for transplantation from hPSCs. Although some therapies are already in advanced clinical trials, to date, no hPSCs-based therapy is available in the clinic.

Current strategies for obtaining cells and tissues from hPSCs are largely based on in vitro differentiation, although effective on many fronts, have several limitations: (i) in vitro differentiation is not synchronized and there still remain undifferentiated hPSCs present in the differentiation cultures, which will raise safety concerns as they are prone to generate teratomas; (ii) different hPSC lines are known to have variable efficiency in differentiating towards a specific lineage (Osafune et al., 2008); (iii) cells differentiated from hPSCs in vitro are mostly immature cell types similar to cells of foetal or neonatal origin (Hrvatin et al., 2014); (iv) large-scale production of hPSCs derivatives is still not in place for many lineages; (v) current differentiation protocols are not amenable for the generation of three-dimensional transplantable tissues and organs. To overcome these problems, better and more efficient protocols, strategies to functionally mature differentiated cells and scaling up differentiation methods are needed.

Through millions of years of evolution nature has established robust developmental programmes for each living organism, and some of these developmental processes are well conserved among species. By taking advantages of these conserved developmental programmes, we may consider differentiating hPSCs in an in vivo environment of an animal host. Complex tissues and organs are routinely formed in vivo. In vivo differentiation is highly synchronized and guided by spatiotemporal dynamic developmental signals; embryonic cells know exactly where to go and what to become. Uncommitted cells are normally eliminated during the fast-paced and efficient developmental processes. In addition, in vivo differentiation likely will yield functional and mature cell types suitable for transplantation. Although this is an exciting possibility, two major challenges have to be overcome for targeted in vivo tissue and organ generation using hPSCs: first, we need to have a robust protocol for the generation and propagation of chimeric-competent hPSCs; second, we need to solve the issue of stochastic chimeric contribution of PSCs. The goal of this review is to examine the recent advances in the field of interspecies chimeric complementation, highlighting the two main approaches: zygote genome editing and pluripotent stem cells. Other strategies for the generation of transplantable organs such as xenotransplantation, tissue engineering and 3D printing, which have been extensively reviewed (Badylak, Weiss, Caplan, & Macchiarini, 2012; Murphy & Atala, 2014; Yang & Sykes, 2007), will not be discussed here.

2 | BLASTOCYST COMPLEMENTATION

Mammalian development is a highly regulated process for the precise generation of tissues and organs that provide vital life support for an adult organism. Organ and tissue generation is the result of seamless coordination of intrinsic genetic programme and extrinsic niche factors during different stages of development. Embryonic cells, starting from a single zygote, follow a comprehensive blueprint to turn on specific genetic programmes for lineage specification and tissue formation. The derivation of germline-competent embryonic stem cells (ESCs) and development of gene-targeting technologies have facilitated the generation of thousands of mouse genetic models and consequently help us gain a deeper understanding of genetic principles underlying embryogenesis. Now we know genetic programmes are governing many aspects of tissue and organ formation, from specification to maturation. Some of these genetic programmes, if disrupted and despite the existence of intact developmental niche factors, can lead to the generation of embryos, foetuses or neonates lacking entire tissues or organs. For example, *Pdx1* knockout mice are deficient in pancreatic development. *Pdx1* knockout mouse neonates lack entire pancreases and die soon after birth (Offield et al., 1996); mice lacking *Sal1* gene cannot survive long after birth due to kidney agenesis (Nishinakamura et al., 2001); *Runx1* knockouts are embryonic lethal at approximately E12.5 that is characterized by a complete absence of definitive haematopoiesis (Van Deursen, Hiebert, Grosveld, & Downing, 1996; Wang et al., 1996); *Nkx2.5* deficiency in mice leads to embryonic lethality around E10.5 with retarded cardiac development (Lyons et al., 1995).

Via gene knockouts or other genetic strategies differentiation capabilities can be disabled in lineage progenitors, thereby preventing them from contributing to tissue and organ generation. Meanwhile, the extrinsic niche factors necessary for tissue and organ formation remain intact. In this regard, the developmental niche is considered "empty" due to lack of commitment from progenitor cells. The chimeric capability of donor wild-type PSCs can thus be harnessed to "fill" these empty niches, and as a result, the generated organ will mostly consist of donor cells. This approach is often referred to as blastocyst complementation, named so largely because donor cells are typically delivered into the host at the blastocyst stage, and was first introduced in 1993 in a study by Chen et al. (Chen, Lansford, Stewart, Young, & Alt, 1993) where the authors used wild-type mouse ESCs (mESCs) to complement Rag2-deficient recipient mouse blastocysts. Rag2 knockout mice lack the development of T and B lymphocytes and thus successfully complemented animal by wild-type mESCs will have T and B lymphocytes of exclusive donor origin. Later, blastocyst complementation has also been applied for the study of lens development. When wild-type mESCs were used for the complementation of homozygous aphakia mutant mouse strain, normal lens were generated; in contrast, Rb-deficient mESCs generated an aberrant lens phenotype (Liégeois, Horner, & DePinho, 1996). Blastocyst complementation was first used for organ generation in 2007 by Douglas Melton's group (Stanger, Tanaka, & Melton, 2007). In this study, the authors used wild-type mESCs to complement mouse blastocysts deficient in *Pdx1*, a key gene in the pancreas development. As a result, the donor mESCs populated the entire pancreatic epithelium in the *Pdx1*-deficient host (Stanger et al., 2007). In another study, Espejel et al. complemented *Fah*-deficient blastocyst with wild-type mouse iPSCs (miPSCs) to demonstrate that donor miPSCs could contribute to normal hepatocytes differentiation in *Fah*-deficient host independent of cell fusion (Espejel et al., 2010).

A landmark paper from Hiromitsu Nakauchi's group in 2010 demonstrated the feasibility of using blastocyst complementation for the xeno-generation of organs (Kobayashi et al., 2010). In their study, unlike Stanger et al., the author used rat PSCs to complement *Pdx1*-deficient mouse blastocysts. Interestingly, rat PSCs could successfully chimerize mouse development and generated viable rat-mouse interspecies chimeras. Importantly, rat PSCs could also generate an entire rat pancreatic epithelium inside the *Pdx1*-null mouse hosts. The generated rat pancreases expressed proper molecular markers and were functional as evidenced by their ability to maintain normal serum glucose levels in adult chimeras. Later, as a second successful attempt for using rat-mouse complementation system for organ generation, Isotani et al. complemented blastocysts from nude mouse that lack thymus with rat ESCs and obtained a functional rat thymus in nude mouse host (Isotani, Hatayama, Kaseda, Ikawa, & Okabe, 2011). In another attempt, Usui et al. tested blastocyst complementation for the generation of kidneys. In this study, however, the authors found that unlike mouse PSCs rat PSCs could not complement kidney agenesis defect of mouse *Sal1* knockout embryos, suggesting key molecules involved in the interaction between mesenchyme and the ureteric buds during kidney development are not conserved between the two species (Usui et al., 2012).

The success of rat-mouse organ complementation also raises the intriguing possibility to generate functional human organs in host species other than humans. Considering its resemblance to humans in anatomy, physiology, organ size, genomic similarity and cell cycle characteristics, pig constitutes a good candidate for such purpose. Pig genome has been recently sequenced (Groenen et al., 2012), and SCNT is also available in the pig (Park et al., 2001; Lai 2002), which have made the pig one of the most popular large animal models in biomedical research (Prather, Lorson, Ross, Whyte, & Walters, 2013). Recently, rapid evolving field of genome editing has also embraced pig (Carlson et al., 2012; Hai, Teng, Guo, Li, & Zhou, 2014; Hauschild et al., 2011; Wang et al., 2015; Whitworth et al., 2014; Whyte & Prather, 2012). By combining SCNT and genome editing, a number of useful pig models for human diseases have been created, such as cystic fibrosis (Rogers et al., 2008), diabetes (Renner et al., 2010; Umeyama et al., 2009), Alzheimer's disease (Kragh et al., 2009), retinitis pigmentosa (Petters et al., 1997; Ross et al., 2012) and spinal muscular atrophy (Lorson et al., 2011). Most recently, Hiromitsu Nakauchi's group has also demonstrated the feasibility of organ generation using blastocyst complementation in pig (Matsunari et al., 2013). To obtain organ-disabled hosts, the authors cloned fibroblasts expressing a transgene *Hes1* under the *Pdx1* promoter (*Pdx1-Hes1*). *Pdx1-Hes1* transgene expression suppresses pancreatic programme thus cloned embryos' pancreatic development was disabled, similar to *Pdx1* knockout, which resulted in the creation of apancreatic pig. *Pdx1-Hes1* embryos were thus used as recipients for the complementation with wild-type donor cells. In contrast to rodents, there is lack of chimeric-competent pig ESCs/iPSCs. Instead, the authors cloned fibroblasts expressing huKO fluorescent protein and used their blastomeres as donor cells to complement the *Pdx1-Hes1* embryos via aggregation chimera formation. As expected, huKO blastomeres could successfully contribute to chimera formation in *Pdx1-Hes1* host. Importantly, huKO blastomeres could generate entire pancreatic epithelium in *Pdx1-Hes1* foetuses. Moreover, chimeric pigs generated by complementation could grow into adulthood with functional pancreases.

3 | PROGRAMMABLE NUCLEASE-BASED GENOME EDITING

Genome editing with programmable nucleases including ZFNs, TALENs and CAS has spawned a new revolution in biomedical research. These nucleases can recognize and target-specific DNA sequences. Once bind to DNA, they can generate double-strand breaks (DSBs) and the repair of which depends on two main cellular DSBs repair pathways: the error-prone non-homologous end joining (NHEJ) and error-free homology-directed repair (HDR). Unlike HDR, NHEJ is high efficient and is active in all cell cycles. NHEJ mostly produces indels in the genome that will lead to loss-of-function of gene(s) and is useful for the generation of gene knockout(s). Importantly, NHEJ-based gene knockout strategy proves to be highly efficient when combined with programmable nucleases in the zygote and has been successfully used for the creation of a variety of knockout animals in many species including mouse, rat, pig, sheep, cow and non-human primates (Geurts et al., 2009; Hauschild et al., 2011; Sung et al., 2013; Wang et al., 2013; Hai et al., 2014; Niu et al., 2014; Liu et al., 2014). Thus, zygote genome editing potentially can obviate the need for pre-existing gene knockout strains and serve as a robust platform for interspecies blastocyst complementation. For detailed discussion on programmable nuclease-based genome editing technologies, the readers are referred to a couple of excellent recent reviews (Doudna & Charpentier, 2014; Gaj, Gersbach, & Barbas, 2013).

4 | PLURIPOTENT STEM CELLS

To generate human organs in pigs using blastocyst complementation, another key to success is the right human PSCs that can contribute to the early pig development for the generation of chimeric human–pig embryos. Of note is that the successful capturing and culturing authentic rat PSCs (Buehr et al., 2008; Li et al., 2008) was the key to success for interspecific blastocyst complementation in rodents. Although we have gained considerable mechanistic understanding of pluripotency, the majority of information was derived from rodent studies and we have yet to achieve the derivation of chimeric- and germline-competent PSCs from species other than rodents.

Mouse ESCs were the first pluripotent cell type captured from a developing embryo (Evans & Kaufman, 1981; Martin, 1981). Initially, mESCs were derived and cultured on mitotically inactivated mouse embryonic fibroblasts (MEFs) in the presence of serum. Better understanding of the self-renewal of mESCs led to the refinement of culture parameters and identified LIF and BMP4 as key factors to maintain mESCs identity. Further studies identified a ground state culture composed of two chemical inhibitors: CHIR99021, a GSK3 inhibitor

that activates the canonical Wnt signalling pathway and PD0325901; FGF/Erk inhibitor that enabled efficient derivation of mESCs from a variety of non-permissive strains and the capturing of authentic ESCs from rat (Buehr et al., 2008; Li et al., 2008; Ying et al., 2008). ESCs are well known for two defining properties: self-renewal and pluripotency. Self-renewal confers ESCs the ability to proliferate indefinitely in culture, thereby providing unlimited amount of cells for a variety of downstream applications. Pluripotency refers to the ability of ESCs to differentiate into all adult cell types in vivo after them being injected back to early developing embryos. ESCs can also contribute to the germline in a chimeric animal and thus have enabled the generation of thousands of transgenic animal models that have enhanced our molecular and genetic understanding of various biological processes during development and provide us with novel insights into human diseases.

The first human ESC line was derived in 1998 by Thomson and colleagues (Thomson et al., 1998). Like mESCs, human ESCs (hESCs) were derived from ICMs of pre-implantation blastocysts. Surprisingly, however, culture conditions for mESCs and hESCs are quite different. hESCs were typically cultured in bFGF/Activin-A-containing medium (FA), and FA medium leads to differentiation of mESCs. Likewise, hESCs could not be stably maintained in mESC culture conditions. Beside cell culture parameters, there are other notable differences between hESCs and mESCs: (i) hESC colonies appear more flatter than mESCs; (ii) self-renewal of hESCs is dependent on bFGF/TGF-β signalling pathways, while LIF/BMP signalling pathways are important for mESCs; (iii) in sharp contrast to mESCs, hESCs survive poorly after trypsinization, indicating a low single-cell cloning efficiency; (iv) female hESCs retain one active and one inactivated copy of X chromosome (XaXi), while both X chromosomes are active in mESCs (XaXa). mESCs can generate germline chimeras, passing a stringent pluripotency test. In this context, however, hESCs cannot be tested for their chimeric competency due to ethical considerations, thus leaving the door open whether hESCs truly represent a genuine ESC line from the human blastocysts. A recent study demonstrated that rhesus macaque ESCs grown in hESC culture were incapable of generating chimeras after being injected into rhesus blastocysts followed by embryo transfer to surrogate females, suggesting hESCs in conventional culture condition are not chimeric-competent (Tachibana et al., 2012).

These differences between hESCs and mESCs were first attributed to species-specific divergence of pluripotency programme. The derivation of another PSC type, the epiblast stem cells (EpiSCs), from post-implantation rodent embryos using hESC-like cultures in 2007 suggests it is not as simple as species differences and hESCs likely resemble post-implantation epiblasts (Brons et al., 2007; Tesar et al., 2007). EpiSCs could be derived from diverse stages of post-implantation epiblasts (Kojima et al., 2014; Wu et al., 2015). Like hESCs, EpiSCs also grow as "flattened" colonies, have low single-cell cloning efficiency and require FGF/TGF-β signalling activation for self-renewal. With modification of derivation method, EpiSCs could also be directly derived from ICMs of blastocysts, further solidify the idea that hESCs are likely in vitro counterpart of post-implantation epiblast cells (Najm et al., 2011). Isolation of EpiSCs has also contributed to the realization that there are different phases of pluripotency during development, which can be captured in culture in at least two distinct pluripotent states: "naive" and "primed," respectively (Nichols & Smith, 2009). Among other differences, the chimeric competency of naive vs. primed PSCs is particularly intriguing. Naïve cells, such as mESCs, can efficiently contribute to germline-competent chimeras following their injection into host blastocysts, while primed EpiSCs rarely do (Kim et al., 2013). On the contrary, when grafted back to stage-matched tissue, the post-implantation epiblasts, EpiSCs could efficiently generate ex vivo chimeric embryos with contribution to all three primary germ layers: mesoderm, endoderm and ectoderm, as well as the primordial germ cells (Huang, Osorno, Tsakiridis, & Wilson, 2012; Kojima et al., 2014; Wu et al., 2015). In this regard, mESCs failed integrating to post-implantation embryos upon grafting (Huang et al., 2012).

Several practical advantages, such as high single-cell cloning efficiency, higher developmental potency and ease with gene targeting associated with naïve state PSCs, have fuelled the search for culture conditions that can stabilize naïve human PSCs. First naive hESCs were stabilized with transgenes including Oct4, Klf4 and Klf2 (Hanna et al., 2010). Following this initial work, a series of recent studies, using different strategies and culture conditions, have claimed the generation of naïve hPSCs from different sources: through de novo derivation from human blastocysts, via nuclear reprogramming or conversion from existing primed hPSCs (Chan et al., 2013; Gafni et al., 2013; Guo, von Meyenn, Santos, Chen, & Reik, 2016; Takashima et al., 2014; Theunissen et al., 2014; Wang et al., 2014; Ware et al., 2014). hESCs grown in these culture showed some features reminiscent of mESCs, such as elevated single-cell cloning efficiency, higher HDR efficiency, hypomethylated genome, bivalent metabolic pathways, expression of naïve signature genes and faster growth kinetics (Wu & Belmonte, 2015a, 2015b). Moreover, cynomolgus monkey ESCs cultured using a modified naïve hESC culture could successfully generate chimeric foetuses, albeit at low efficiency, providing support for the chimeric potential of primate PSCs in naïve culture (Chen et al., 2015). While these studies are informative, the issue of whether a naïve state analogous to mouse truly exists in humans remains unsettled. Besides, there is lack of functional test for human naivety and most of the characterization was carried out at molecular levels. For now, it seems that global comparisons of cultured naïve hPSCs with the ICMs of the human blastocyst will provide the strongest support. It should be noted that there might exist multiple naïve-like states, which explains the diverse culture conditions used for stabilizing naïve hPSCs.

One of the most desirable features of naïve hPSCs is their potential to contribute to interspecies chimeras, which is required for successful blastocyst complementation to generate human organs in animal hosts. Also, interspecies chimeras with naïve hPSCs may provide a system for in vivo disease modelling and drug screening (Wu & Belmonte, 2015a, 2015b, 2016). A previous study showed that primed hESCs could not efficiently contribute to human–mouse chimeric embryos following their injection into the mouse blastocysts (James, Noggle, Swigut, & Brivanlou, 2006). To test whether naïve hPSCs are more efficient in the generation of human–mouse

chimeric embryos, Gafni et al. injected NHSM-cultured naïve hPSCs to mouse blastocysts followed by embryo transfer (Gafni et al., 2013). Interestingly, the authors observed robust chimeric contribution of naïve hESCs in mouse embryos from E8.5–E10.5 developmental stages. Yet, chimeric contribution of human naïve PSCs was not observed by Jaenisch's group. Theunissen et al. used NHSM- and 5iLA-cultured cells and did not detect any human cells in post-implantation E10.5 embryos (Theunissen et al., 2014). A follow-up study by the same group with a more sensitive assay based on the detection of human mitochondrial DNA, however, did detect chimeric contribution, although with limited efficiency, of human naïve PSCs in E10.5 mouse embryos (Theunissen et al., 2016). Several possibilities likely account for this discrepancy: (i) cell injection timing, number and embryo culture may vary among labs; (ii) embryo handling and injection techniques are likely different among researchers; (iii) imaging methods used for fluorescent signals detection in low-grade chimeras. Regardless, human cells detected in the cross-species chimeric embryos by both Gafni et al. and Theunissen et al. were not analysed with lineage-specific markers thus are unclear whether they are properly differentiated. These results suggest that generation of interspecific human–mouse chimeras with naïve hPSCs is inefficient. This inefficiency of intermixing human and mouse cells in early development is likely a result of divergent early developmental processes between primate and rodent; for example, epiblast forms an egg-cylinder shape in rodents but assumes a bilaminar embryonic disc in primates.

In addition to naïve and primed states, there may exist other pluripotent states that can be stabilized with either transgenes or different culture conditions (Wu & Belmonte, 2014; Wu et al., 2015). We have recently uncovered a novel primed pluripotent state that confers interspecific chimeric competency between human and mouse after grafting human cells to gastrulating mouse epiblast (Wu et al., 2015). Human cells were found efficiently incorporated into the posterior part of the developing mouse embryo and contributed to three primary germ lineages. This raises an interesting possibility of "epiblast complementation" for the generation of early human progenitors ex vivo.

5 | CONCLUSIONS

The combination of zygote genome editing and chimeric-competent hPSCs offers an attractive platform for realizing hPSCs' full potential towards generating transplantable organs. If successful, this approach will lead to a paradigm shift in regenerative medicine and will help to overcome the shortage of organ donors. However, the inefficiency of existing naïve hPSCs to contribute to chimeric formation in mouse is casting doubts whether this will be a viable approach. It remains to be seen whether the inefficiency of naïve hPSCs in chimeric contribution observed in mouse is also true with a large animal species such as the pig. Future studies on improving chimeric efficiency of hPSCs in an animal host are warranted to turn the dream of xeno-human organ generation into reality.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

J.W. drafted the review and all the other authors helped editing and writing the review.

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