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Hurdles to Generating Human Islets in Animals via Blastocyst Complementation

Tomoyuki Yamaguchi [1]

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Abstract
Purpose of Review To clarify the hurdles to generation of human islets via blastocyst complementation and to identify techniques to overcome them.
Recent Findings Blastocyst complementation is a promising method for generating functional islets from pluripotent stem cells which are identical to in vivo islets. Studies have reported successful generation of mouse pancreas in rats and rat pancreas in mice via interspecies blastocyst complementation and have shown the possibility for generation of human organs in xenogeneic animals. However, there remain hurdles to generating human islets in animals.
Summary The major hurdles to generating human islets include difficulty in engineering human–animal chimeras due to the cellular status of human pluripotent stem cells, immunological rejection of donor tissue in xenogeneic animals, and ethical concerns.

Keywords Blastocyst complementation · Interspecies chimera · Naïve pluripotent stem cell

Introduction

Pluripotent stem cells can differentiate into any body tissue, except for extraembryonic tissues such as the placenta, and are expected to be the resources for generating organs in regenerative medicine. Among them, induced pluripotent stem cells (iPSCs) can be generated by introducing certain genes into somatic cells; thus, their use has enabled generation of substitute cells or tissues from an individual's own cells indefinitely for his or her own treatment [1]. The development of an in vitro differentiation method for cells derived from pluripotent stem cells and used for these treatments has made remarkable progress; furthermore, it has become possible to generate cells with a similar functionality to cells in vivo. However, it is not yet possible to generate three-dimensional structured organs in vitro with functionality identical to their in vivo counterparts. This may be due to the fact that very complicated intercellular interactions accompanying individual development as well as signal transduction by secretory factors cannot be faithfully reproduced in vitro. On the other hand, pluripotent stem cells possess a unique function called chimera formation ability, and when these cells are injected into preimplantation embryos, this ability contributes to host animal development and organ formation becomes possible. The "blastocyst complementation method" involves generating organs and tissues in vivo by utilizing the chimera-forming ability of these pluripotent stem cells.

Human organ production is expected to be accomplished using this blastocyst complementation method, but there are several hurdles to achieving this goal. In this review, we will outline one such hurdle to the preparation of human organs using the blastocyst complementation method and discuss how to overcome it.

Blastocyst Complementation

Blastocyst complementation is an in vivo organ or tissue generation method making use of the chimera-formation ability of pluripotent stem cells. A chimera (i.e., chimeric animal) is an

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✉ Tomoyuki Yamaguchi
tomoyama@ims.u-tokyo.ac.jp

[1] Division of Stem Cell Therapy, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo, Japan

animal comprising two or more types of cells with different genetic backgrounds. The term "chimera" has been derived from a monster of the same name in Greek mythology that had the body of a goat and the head of a lion with a poisoned tail on it. A chimera can be experimentally generated by aggregating two embryos (agglutination method) or injecting inner cell mass (ICM) or pluripotent stem cells into an embryo (injection method) [2, 3]. In 1993, Chen et al. reported a blastocyst complementation technology to generate fully pluripotent stem cell-derived lymphocytes in vivo: they generated chimeric mice by injecting wild-type mouse embryonic stem cells (mESCs) into blastocysts of *Rag2*-knockout (KO) mice. Since mice lack the Rag2 enzyme, which is necessary for the rearrangement of immunoglobulins, and are deficient in mature lymphocytes, injected ESCs complemented the deficient lymphocytes in the peripheral blood of $Rag2^{-/-}$ chimeric mice [4]. Such injected ESCs can complement genetically deficient tissues through a normal developmental process in the host animal. Thus, "blastocyst complementation" is used for generating fully pluripotent stem cell-derived tissues or organs in a chimeric animal by injecting wild-type pluripotent stem cells into blastocysts derived from a genetically deficient animal (Fig. 1). Several studies have reported the use of this allogeneic blastocyst complementation method for the generation of the kidney in *Sall1* knockout mice [5], the thymus in nude mice (i.e., *Foxn1* mutant mice) [6], vascular endothelial and hematopoietic cells in *Flk1* mutant mice [7], the pancreas in *Pdx1* knockout mice [8], and the forebrain in *Emx1–cre;R26-DTA* mice [9]. In addition to rodents, successful generation of pancreas has been accomplished in *pdx1-Hes1* transgenic pigs [10], which are apancreatic pigs produced using the ICM of the *Kusabira-Orange* transgenic pigs and retinal pigmented epithelial cells of *MITF* mutant pigs [11]. Furthermore, using interspecies blastocyst complementation method, the generation of rat thymus in mice [6], rat pancreas in mice [8], and mouse pancreas in rats have been reported [12••].

The pancreas-complemented intra- and interspecies chimeric animals have exhibited normal blood glucose levels and have responded well to the glucose tolerance test. Histological evidence has shown that the endocrine, exocrine, and ductal cells in the generated pancreas were completely derived from the injected pluripotent stem cells and were histologically normal.

Furthermore, it has been confirmed that when pancreatic islets derived from a mouse pancreas generated in a rat were transplanted into a streptozotocin-induced diabetic mouse model, normal blood glucose levels were maintained for > 1 year without any immunosuppressant treatment, except for the initial 5 days following transplantation. Therefore, it has been proven that organs generated using blastocyst complementation are functionally comparable to in vivo organs and are indeed transplantable [12••]. Although the concept of blastocyst complementation-mediated organ replacement therapy among several animal species has been proven, some hurdles still remain to human organ regeneration via blastocyst complementation. In fact, although the generation of human–animal chimeras has been reported, the human cell chimerism in these interspecies chimeras was very low; therefore, it may be impossible to generate human organs using blastocyst complementation. The most critical issue concerning this low

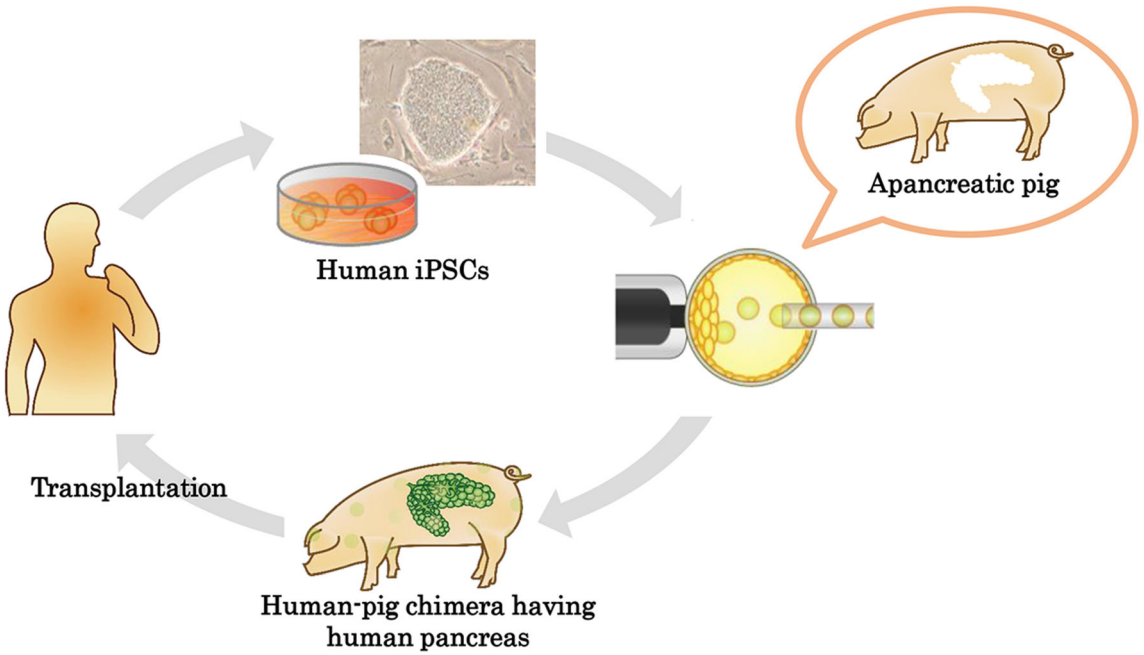

Fig. 1 Generation of the human pancreas in a pig via blastocyst complementation. The human–pig chimera having a human pancreas is generated by injection of human iPSCs established from a patient into blastocysts isolated from apancreatic pigs

chimerism is considered to be the difference between the cellular status of human and rodent pluripotent stem cells.

Differences in the Cellular Status of Pluripotent Stem Cells

The difference between the developmental stages of the host animal's embryo and the human pluripotent stem cells is considered to be one of the hurdles to the generation of a human organ in an animal via interspecies blastocyst complementation. Pluripotent stem cells are classified into two types, naïve and primed, depending on their developmental stage.

mESCs were developed in 1981 by Evans et al. by culturing the ICM of mouse preimplantation embryos [13]. These mESCs were shown to be pluripotent, like embryonic carcinoma cells, because they formed a teratoma comprising cells from the three germ layers when transplanted into immunodeficient mice.

Moreover, mESCs possess chimera-forming ability. In practice, the chimeric mice can be generated by injecting mESCs into early mouse embryos or by aggregating mESCs and early embryos. These mESCs can contribute not only to the development of the three germ layers but also to the generation of germ cells in the chimera [14].

This establishment of mESCs and the technology for generation of chimeric mice has facilitated the generation of genetically modified mice and has led to dramatic advancements in biomedical research. Similar to mESCs, rat ESCs (rESCs) with chimera-forming ability have been established from rat blastocysts by Smith et al. in 2008 [15].

Human ESCs (hESCs) were established in 1998 by Thomson et al. Similar to mESCs, these were established from human preimplantation embryos and were shown to be pluripotent because they formed teratomas when implanted into immunodeficient mice; however, there were some key differences [16]. Morphologically, hESC colonies are characterized by a flat shape, whereas mESCs form domed colonies. In addition, mESCs are dependent on leukemia inhibitory factor (LIF) for maintenance of their undifferentiated state, whereas hESCs are dependent on fibroblast growth factor (FGF) and activin. Mouse epiblast stem cells (mEpiSCs) established from mouse post-implantation embryos are pluripotent stem cells capable of forming teratomas; however, they cannot differentiate into germ cells [17, 18]. Thus, they are thought to correspond to E6.5 epiblasts.

Because these mEpiSCs are cultured under the same conditions as hESCs (i.e., critical dependence on basic FGF and activin instead of LIF) and their colony morphology is flat, the developmental stages of hESCs are considered to correspond to those of post-implantation embryos. Moreover, because mEpiSCs do not possess a chimera-forming ability, hESCs are considered to be non-chimera–forming stem cells.

Recently, pluripotent stem cells have been categorized into two groups based on their phenotype: naïve and primed [19]. Cells such as mESCs, mouse iPSCs (miPSCs), rESCs, and rat iPSCs (riPSCs) in the ground state are phenotypically close to the epiblasts of preimplantation embryos (i.e., pluripotent stem cells capable of gametization as well as three germ layer contribution) and are categorized as "naïve pluripotent stem cells." On the other hand, the cells derived from post-implantation embryos or those phenotypically close to post-implantation epiblasts such as hESCs and human iPSCs (hiPSCs) are classified as "primed pluripotent stem cells."

Although there are differences between "naïve" and "primed" pluripotent stem cells in terms of colony morphology, signals for maintenance of an undifferentiated state, DNA methylation, X chromosome activation, etc., the most critical issue involved in performing blastocyst complementation is that the primed pluripotent stem cells do not possess chimera-forming ability.

A study has reported that because mEpiSCs injected into mouse preimplantation embryos, such as morula, died due to apoptosis, a chimera was not formed. This suggests that hiPSCs also die in embryos and cannot form chimeras; in fact, hiPSCs have been confirmed to die in mouse embryos around the implantation stage when injected into a mouse preimplantation embryo. Thus, differences in developmental stages between the host embryo and injected pluripotent stem cells may be the first hurdle to blastocyst complementation using hiPSCs. Attempts to convert from a "primed" to "naïve" pluripotency for hiPSCs have been reported from many groups; in particular, Takashima et al. [20] and Theunissen et al. [19] have established cells exhibiting a gene expression pattern similar to that of preimplantation epiblasts, and these cells were anticipated to form chimeras. Naïve human pluripotent stem cells established by Theunissen et al. [19], Gafni et al. [21], or Yang et al. [22] contributed to mouse embryonic development (Table 1). Meanwhile, Wu et al. reported that human naïve or intermediate (the cells exhibited features both primed and naïve phenotypes) pluripotent stem cells could not contribute to mouse embryonic development, but could contribute to pig embryonic development [23] (Table 1). Although human–animal chimeras were generated in those reports, the contribution of human cells was quite low and may not be enough to generate a human organ by blastocyst complementation. Because there remains no experimental system in humans that can clearly demonstrate the ability to form chimeras and only the formation of an interspecies chimera can be used as an indicator, it is difficult to confirm the chimera-forming ability of human pluripotent stem cells.

Attempts have also been made into forming chimeras with primed pluripotent stem cells. A study has reported that the forced expression of Bcl2 in mEpiSCs could overcome apoptosis and contribute to mouse embryonic development, forming adult mouse chimeras [26•]. Moreover, Bcl2

Table 1 Trials for the generation of human–animal chimeras

Pluripotent state of injected human PSCs (Culture medium)	Host animal species (The embryonic stage of PSCs injected zygotes)	Reference
Naïve (NHSM)	Mouse (morula)	Gafni et al., 2013 [21]
Naïve (5i/L/A, 4i/L/A)	Mouse (morula, blastocyst)	Theunissen et al., 2016 [19]
Naïve by inducible expression of NANOG and KLF2 (2i)	Pig (blastocyst)	Wu et al., 2017 [23]
Naïve (NHSM)	Pig (blastocyst)	
Naïve (4i)	Pig (blastocyst)	
Naïve (FAC)	Pig (blastocyst)	
Naïve (LCDM)	Mouse (8-cell)	Yang et al., 2018 [22]
Primed with inducible expression of BCL2 (E8)	Mouse (4-cell)	Wang et al., 2018 [24]
Primed with inducible expression of BMI1 (mTeSR1)	Mouse (8-cell, morula or Blastocyst)	Huang et al., 2018 [25]

5i/L/A: MEK inhibitor, GSK3beta inhibitor, BRAF inhibitor, ROCK inhibitor and SRC inhibitor, hLIF and activin A in N2B27 medium

4i/L/A: exclude GSK3beta inhibitor from 5i/L/A

NHSM: MEK inhibitor, GSK3beta inhibitor, JNK inhibitor, p38 inhibitor, PKC inhibitor, ROCK inhibitor, FGF2, TGFbeta, hIGF1 and hLIF in KnockOut DMEM with N2 supplement

4i: exclude hIGF1 and LDN193189 from NHSM

FAC: GSK3beta inhibitor, FGF2 and activin in N2B27 medium

2i: MEK inhibitor, GSK3beta inhibitor, hLIF and doxycycline in N2B27 medium

E8: Essential 8 with doxycycline

mTeSR1: mTeSR1 with doxycycline

overexpressed in mEpiSCs contributed to embryonic development in both rats and mice and formed a rat–mouse interspecies chimera [26•]. Furthermore, Wang et al. have reported that hiPSCs with Bcl2 overexpression contributed to mouse embryonic development [24] (Table 1). Huang et al. reported that BMI1-forced expression in hiPSCs also overcomes apoptosis and contributes to mouse embryonic development [25] (Table 1). To generate the human pancreas using interspecies blastocyst complementation, it is critical to achieve higher chimerism of human pluripotent stem cells in an interspecies chimera. To accomplish this, technological advancements for preventing cell death during the development of primed pluripotent stem cells would be effective in addition to the conversion of primed to naïve human pluripotent stem cells.

Ethical Concerns

hPSCs show a great potential in regenerative medicine and developmental biology research because of their pluripotency and infinite proliferation capacity, but ethical issues need to be considered around their use.

The first ethical issue to be discussed on the use of human pluripotent stem cells regards the fact that the human embryo is destroyed during hESC establishment, which was the reason for placing limits around hESC research from the viewpoint of human dignity in the USA. iPSCs, which can be generated from somatic cells, greatly advanced stem cell research because there are no such ethical issues. However, even in studies using iPSCs, there exist other ethical issues in producing human–animal chimeras.

The first issue regarding the production of chimeras is the concern that non-human animals having human cells might acquire personhood. In 2013, Han et al. reported that the transplantation of human glial progenitor cells into the mouse brain results in the replacement of most mouse glial cells with human astrocytes and enhancement of long-term potentiation, indicating that the learning ability of mice with human astrocytes was enhanced [27]. Although this result is not directly equivalent to the acquisition of human personhood, it does indicate that human–non-human animal chimeras in which human cells contribute to the formation of the brain might have more human-like characteristics than do wild-type mice. Thus, producing an animal with high cognitive function similar to humans but with an unclear borderline with humans should be avoided from the viewpoint of human dignity.

The second problem is that human–non-human animal chimeras have the potential to generate human gametes. Isotani et al. reported that rESCs-derived spermatozoa were formed in a rat–mouse xenogeneic chimera generated by injecting rESCs into mouse embryos [6]. This result suggests that human germ cells can be formed in human–non-human animal chimeras, and breeding these animals may lead to the generation of human embryos. Thus, the International Society for Stem Cell Research (ISSCR) impose a ban on the breeding of chimeric animals having human cells.

Recently, in Japan, the guidelines for human–non-human chimeric animal research have been relaxed, and the ban on

the transplantation of embryos with hiPSCs into the uterus of a non-human animal was lifted [28]. Although the new guidelines in Japan have lifted the ban on the generation of interspecies chimeras with hiPSC-derived tissues via blastocyst complementation, researchers must perform the experiments in compliance with the guidelines and with maximum concern for ethical issues. Therefore, genetically modifying hiPSCs so as not to contribute to the generation of brain or germ cells could be an effective safeguard.

Immunological Rejection

Immunological rejection in interspecies chimeras also regarding the generation of human–animal chimeras is an additional hurdle that must be overcome in this field of research.

Interspecies blastocyst complementation has the ability to generate a functionally and structurally normal mouse pancreas in a rat. However, lymphoid infiltration in the generated pancreas and hyperglycemia symptoms similar to type I diabetes mellitus have been observed in several interspecies chimeras [12••]. In addition, lymphoid infiltration in the synovium, epidermis, and other tissues, which are symptoms similar to those of various autoimmune diseases such as synovitis and dermatitis, has been reported in mouse–rat interspecies chimeras [29]. Because the innate immune system recognizes antigens based on the information encoded in the genome, it is possible that the xenogeneic tissue in interspecies chimeras is recognized as an antigen for rejection. Moreover, it is considered that the acquired immune system is also activated depending on the chimerism in the thymus, and xenogeneic tissues are recognized as antigens for rejection. Therefore, utilizing an immunodeficient animal as a host or using an immunosuppressant may be more effective for developing an interspecies chimera with normal xenogeneic organs.

Furthermore, in terms of ethics, researchers must strive not to compromise human dignity in the generation of human–animal chimeras; for example, a high contribution of human cells to the brain or to germ cells must be avoided during the development of these chimeras. To overcome these hurdles, it may be effective to use genetically modified human pluripotent stem cells, which cannot contribute to brain or germ cells.

Conclusion

Currently, blastocyst complementation is the only available method for generating functional organs with three-dimensional structures from pluripotent stem cells, but its success has been limited to rodent species.

As described in this review, there are several problems associated with overcoming the differences in the developmental stages and processes between humans and host animals in order to achieve the development of interspecies chimeras with human organs. Nonetheless, the development of human–animal chimeras has been previously reported.

Overcoming these hurdles and generating human organs could resolve the shortage of organs in transplant cases and save many lives. Moreover, there is no doubt that chimeric animals containing human organs would be very useful in many fields, including drug screening and research on human development. The blastocyst complementation is a study that should be greatly enhanced as well as in vitro differentiation of pluripotent stem cells.

Compliance with Ethical Standards

Conflict of Interest The author declares that he has no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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