Minireview

Interspecies chimeras as a platform for exogenic organ production and transplantation

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Impact statement

This work provides a brief review, which addresses the scientific and ethical challenges associated with interspecies chimeric animals and humanized organs.

Abstract

Chronic diseases are associated with considerable morbidity and mortality. Therefore, new therapeutic strategies are warranted. Here, we provide a brief review outlining the rationale and feasibility for the generation of intraspecies and interspecies chimeras, which one day may serve as a platform for organ transplantation. These strategies are further associated with consideration of scientific and ethical issues.

Keywords: Chimera, somatic cell nuclear transfer, ETV2, MYOD, MYF5, MYF6, hiPSCs

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Introduction

Chronic illnesses such as diabetes, cardiovascular, and blood diseases, as well as trauma-induced volumetric muscle loss have considerable morbidity and mortality.[1] The only curative therapy for these endstage, terminal, and debilitating diseases is transplantation. Although curative, transplantation is limited due to the shortage of donor organs or donor site morbidity. Less than 1% of adults with these morbidities are able to receive organ transplantation and therefore alternative therapies have been examined to address this shortage.[2] One of the alternative therapies is the engineering of interspecies chimeric organs (Figure 1). One approach for the generation of interspecies chimeric organs relies on the deletion of an entire organ or lineage within the host and the complementation of the host embryo with donor stem cells. This platform technology provides the donor stem cells (derived from a species distinct from the host) with a competitive advantage as the host is unable to contribute to a specific organ or lineage due to the genetic deletion of lineage-specific master regulators (Figure 1). Therefore, an entire organ or lineage will be host-derived. This strategy has been accelerated by the advent of techniques such as somatic cell nuclear transfer (SCNT), CRISPR gene editing, and with the development of hiPSCs and other stem cell populations. Recent reports provide convincing support for these technologies in small and large animal models. Furthermore, these studies provide information regarding the cues and the regulators that govern cellular proliferation, apoptosis, organ development, and size. Collectively, these strategies hold tremendous promise for the generation of new animal models and one day they may serve as a potential source for transplantable organs.

Humanized mouse models

Previous studies have engineered mouse models, which were transplanted with human cells or engineered to express human proteins.[3] These humanized mouse models have been used to gain mechanistic insights in human-specific physiology and pathologies. Typically, these studies have utilized immunodeficient mice (NOG mice,[4] NSG mice,[5] BRG mice,[6] irradiation, etc.) to allow for the engraftment of the human cell populations. For example, humanized mouse models have been generated that have a human thymus, a human spleen, a human-murine chimeric spleen, human-murine chimeric lymph nodes, human hematopoietic stem cells, human immune system, or a human liver. These humanized mouse

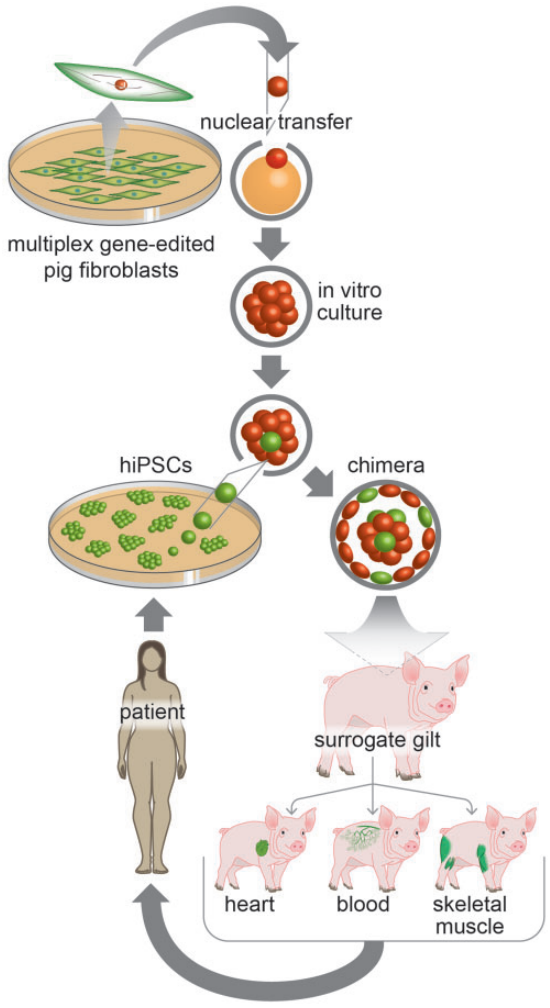

Figure 1. Overall schematic outlining a strategy to engineer humanized organs as a platform for xenotransplantation. As outlined, porcine embryonic fibroblasts are gene edited using CRISPR/Cas9 and used for somatic cell nuclear transfer to produce a porcine embryo. Blastocyst complementation using hiPSCs and the gene-edited porcine embryo (which lacks an entire lineage or organ) is performed and the chimeric embryo is surgically transferred to a surrogate gilt. These humanized (chimeric) gene-edited pigs are intended to serve as donors to patients with advanced or terminal diseases.

models have been utilized as translational models for the studies of infectious diseases (HIV, Herpes simplex virus type 2, Human herpes virus 6, Human cytomegalovirus, Leishmania major virus, Varicella-zoster virus, Chlamydia, Mycobacterium, Malaria and others), liver diseases (Hepatitis C virus, Hepatitis B virus, etc.), tumor growth, and cancer immunology.[7] Furthermore, humanized mice that have a functional human immune system have been used as preclinical models to study the mechanisms that govern human immune responses to human islet allografts and human cardiac allografts.[8] Finally, the characterization of human stem cells has historically used the formation of teratomas in immunodeficient mice as an indicator of their pluripotent capacity.[9] Collectively, these human-murine chimeras have demonstrated the feasibility of producing interspecies chimeras and these models have enhanced our understanding of the fundamental mechanisms of human physiology, diseases, and therapies.

Master regulators and pioneer factors govern lineage development

Precise, context-dependent transcriptional networks orchestrate embryogenesis and fate decisions. These developmental trajectories are governed by a hierarchy of transcriptional networks as outlined in Waddington's epigenetic landscape whereby pluripotent stem cells daughter progenitors as they roll down bifurcating paths toward their ultimate fate (cardiovascular vs. myogenic vs. hematopoietic, etc.) (Figure 2(a)).[11] The transcription factor that occupies the very top of the hierarchical cascade, which governs these fate decisions are referred to as master regulators.[11] Master regulators are both necessary and sufficient for lineage development. Consequently, gene disruption studies of master regulators result in the deletion of the lineage of interest, while misexpression of the master regulator (in a somatic cell population such as fibroblasts) reprograms or respecifies the cell and directs it to a completely different (but specific) lineage.[10] Hundreds of master regulators have been defined in plants, lower organisms, and mammals. Several examples of master regulators include members of the MyoD family that promote myogenesis,[12–14] Etv2 which promotes hematoendothelial development,[15–25] and Pdx1 which promotes pancreatogenesis (Figure 2(b)).[26,27] A very small subset of master regulators, called pioneer factors, reprogram cell fate by binding transcriptionally silent genes—those that have a closed chromatin state (nuclease resistant), which are not accessible to other transcription factors (nonpioneers) (Figure 2(c)).[28,29] By relaxing the chromatin state, these pioneer factors (which are capable of binding nucleosomal DNA) enable other transcription factors to access their binding motifs through histone modifications to collectively activate gene expression resulting in the reprogramming of cell fate (Figure 2(c)). Importantly, pioneer factor binding occurs prior to lineage specification/commitment. Examples of pioneer factors include: OCT3/4, SOX2, and KLF4 (O/S/K) for iPSC reprogramming,[28] FOXA for hepatocyte reprogramming,[30] ASCL1 for neurogenesis[31] and others. Therefore, the criteria for a pioneer factor are the following:[32] (1) they must have the ability to bind nucleosomal DNA; (2) they must have the ability to promote chromatin relaxation and expose transcription factor motifs, and (3) they must have the ability to recruit other transcription factors and promote gene expression resulting in lineage specification.[32] These studies, which have extensively and elegantly defined master regulators and pioneer factors provide a platform to engineer embryos that completely lack an entire lineage and/or organ.

Small animal models for interspecies chimerism

Recent studies have successfully undertaken interspecies blastocyst complementation in rodent models. For example, GFP-labeled rat pluripotent stem cells were injected into the *Pdx1* deficient mouse blastocyst resulting in viable chimeric offspring.[33] Similarly, the injection of GFP-labeled mouse pluripotent stem cells into the *Pdx1*

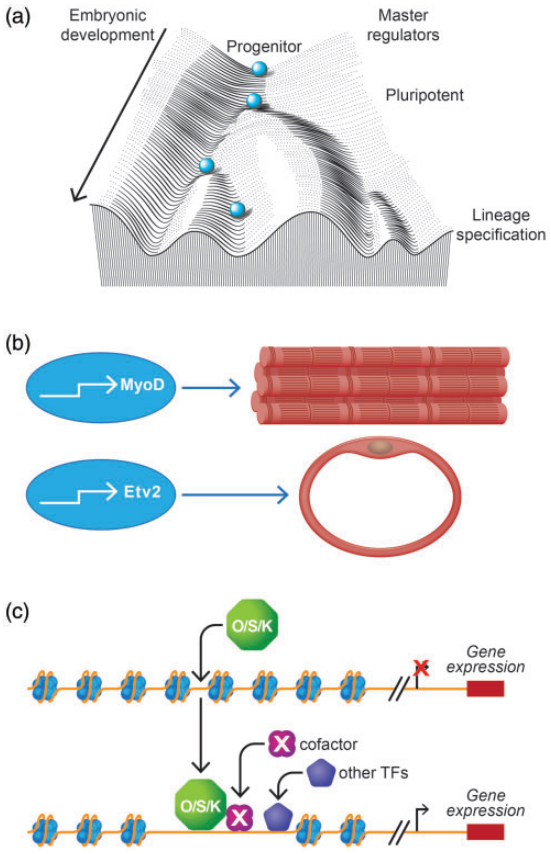

Figure 2. Master regulators and pioneer factors are important reprogramming factors and specify lineage development. (a) Adaptation of Waddington's developmental depiction of master regulators which govern pluripotent cells to specific lineages during embryogenesis.[10] (b) Schematic representation of conversion assays demonstrates the capacity of master regulators which, by themselves, are able to convert (or reprogram) differentiated cells (fibroblasts) to a specific lineage (MyoD converts fibroblasts to skeletal myocytes and Etv2 converts fibroblasts to the hematoendothelial lineages). (c) Adaptation of a schematic demonstrating the mechanisms whereby pioneer factors function. Oct4/Sox2/Klf4 (O/S/K) are pioneer factors which bind nucleosomal DNA and relax the chromatin which exposes the binding motifs allowing lineage specific transcription factors to bind and activate lineage specific gene expression.

deficient rat blastocyst resulted in viable chimeric offspring.[33] Although the *Pdx1* deficient mouse or rat lacked a pancreas (the islets and exocrine acini), the interspecies chimeras had a fully functional pancreas which responded appropriately with an oral glucose tolerance test and reversed streptozotocin-induced diabetes after the transplantation of the chimeric islets beneath the kidney capsule of the diabetic mouse.[33] These studies demonstrated that while there was a considerable size discrepancy between the two species (i.e. mouse vs. rat) the size of the interspecies chimeric organ was determined by the host (and not the donor stem cells). These results support the notion that external cues govern the size of organs during embryogenesis and the postnatal period. Furthermore, while the interspecies chimeric organ contained residual host cells (i.e. vasculature, fibroblasts, etc.), these cells had little immunogenicity and were replaced over time with donor derivatives following transplantation. For these reasons, the transplant recipient only received immunosuppression for less than a one-week period following transplantation.[34]

In addition to these studies, ongoing efforts focused on the engineering of chimeric vasculature, hearts, kidneys, lungs, and others have been pursued using interspecies blastocyst complementation and small rodents.[35–37] The advantages of using rodent models are the relatively short gestational period, the extensive familiarity of the mouse as a genetic model, and the extensive phenotyping of gene disruption models in mice. Importantly, the characterization and definition of which master regulators are required for the deletion of entire lineages and organs to provide a supportive niche for interspecies donor stem cells is an advantage.

Large animal models for interspecies chimerism

The overall goal for interspecies chimeric organ production is to engineer an unlimited source of organs for clinical transplantation as curative therapy for chronic debilitating diseases such as cardiovascular, diabetes, vascular, kidney and muscular injury and diseases (Figure 1).[38] In allogeneic (or xenogeneic) transplantation, typically, the donor organ needs to be approximately the same size as the diseased recipient organ. Therefore, large animal models such as sheep, baboon, chimpanzee, pig, and others have emerged as possible candidates.[39] While a number of large animal models have been examined, the pig is an attractive host candidate for a number of reasons. First, the clinical use of porcine heart valves, porcine insulin, and porcine skin grafts have long been used clinically for therapeutic purposes.[40] Second, the pig organs (or the minipig) are relatively the same size as human organs.[41] Third, the pig has relatively large litter sizes (10–15 offspring) and a gestational period marked in months (average gestational period for the pig is 114–118 days). The pig can be genetically engineered using gene editing technologies and has a comparable physiology to humans.[42] Fourth, porcine herds are plentiful, the species-specific pathogens are well known and have been monitored extensively, and barrier facilities have been established for porcine herds. Fifth, porcine organs have been used for preclinical xenotransplantation studies.[39,43]

While there are a number of advantages of using the pig as a host, there are also some challenges. First, the pig has a distinct genome with 38 chromosomes. Second, the average temperature for the pig is 101.5°F–104°F. Third, humans and pigs are relatively evolutionarily divergent (they last shared a common ancestor approximately 80 million years ago).[44,45] Fourth, pigs (like any animal models) can transmit infectious pathogens to humans (zoonoses).[46] Overall, despite these challenges, the pig is a good model (host) to pursue interspecies organ production.

Engineering humanized vasculature in a gene-edited pig

Having established that Ets variant transcription factor 2 (ETV2) was a master regulator for the hematoendothelial lineages in the mouse,[16–20,22–24,47,48] the Garry laboratories used CRISPR/Cas9 gene editing to delete *ETV2* in porcine

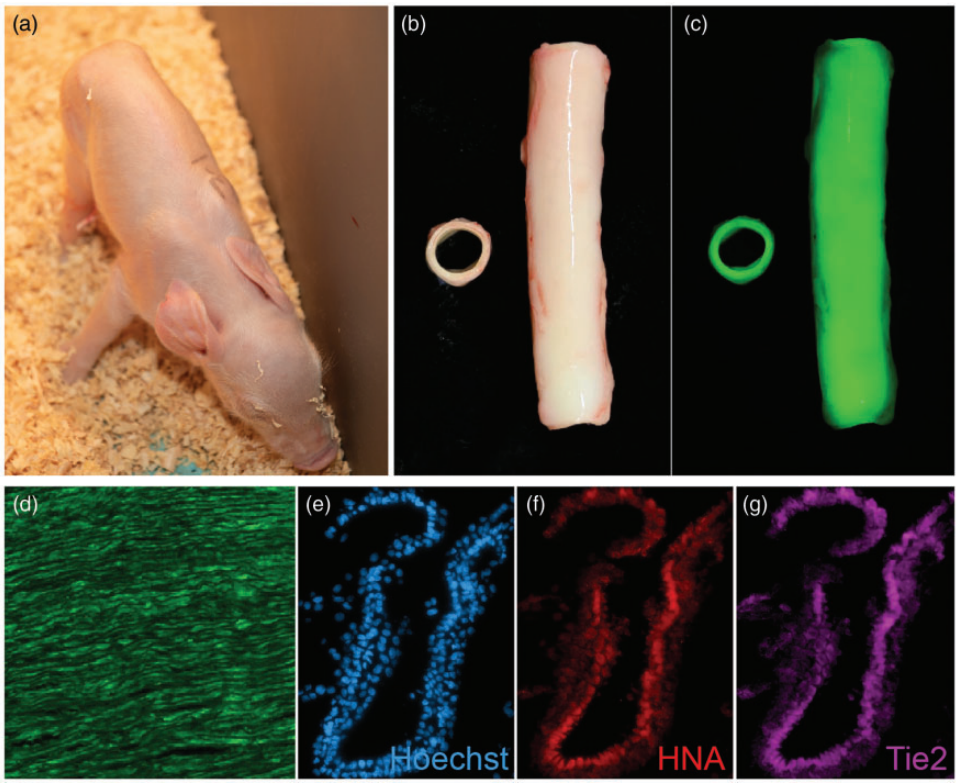

Figure 3. Intra- and interspecies chimeras for endothelial development. (a) Porcine embryonic fibroblasts were gene edited to delete *ETV2*. Following SCNT (cloning) and blastocyst complementation (using GFP-labeled porcine blastomeres) a viable, ambulatory chimeric pig was engineered. (b and c) Analysis of the intraspecies chimeric pig revealed normal dorsal aorta (cross section and longitudinal section; (b), which uniformly expressed GFP using darkfield wholemount immunofluorescence techniques (c). (d) Histologically, the transverse section of the intraspecies chimeric aorta revealed GFP-labeled endothelium and smooth muscle. (e–g) Interspecies (human-porcine) chimeric embryos were generated using the *ETV2* null porcine embryo. E18 human-porcine chimeric embryos were analyzed and sections were stained for Hoechst (e), human nuclear antigen (HNA) (f), and Tie2 (g) demonstrating that all endothelium coexpressed HNA and Tie2.

embryonic fibroblasts.[25] These *ETV2* null porcine embryonic fibroblasts (PEFs) were used in combination with somatic cell nuclear transfer (SCNT) technology to produce *ETV2* null porcine embryos, which were transferred to surrogate gilts and harvested at E18. The *ETV2* null porcine embryos lacked vasculature, blood and were lethal and phenocopied the mouse.[25] To further examine the ability to rescue the *ETV2* null phenotype in the pig, intraspecies complementation was performed to produce pig–pig chimeras (Figure 3). In these studies, porcine GFP-labeled blastomeres were delivered into the *ETV2* null porcine morula and the chimeric embryos were transferred to surrogate gilts. The chimeric piglets delivered via C-section were viable, ambulatory, and completely normal (Figure 3).[25] Histological examination revealed that every endothelial cell and every blood cell was GFP positive (Figure 3).[25] These studies established that the *ETV2* null porcine embryo could be rescued and that each and every blood cell and endothelial cell was GFP positive (i.e. donor derived). Efforts then focused on the engineering of human-porcine chimeras. In these studies, GFP-labeled hiPSCs were delivered into *ETV2* null porcine morulas and surgically transferred to surrogate gilts. At E19, we verified that hiPSCs contributed to the endothelial lineage but the efficiency was relatively low. Therefore, we undertook scRNA-seq of the early porcine morula and blastocyst and compared the molecular signatures with hiPSC single cell RNA-seq datasets. We observed a number of pathways that were dysregulated including BCL2 and TP53.[25] Therefore, we engineered hiPSCs to overexpress BCL2 in order to promote survival and cellular proliferation. The GFP-labeled *BCL2-OE* hiPSCs were complemented with the *ETV2* null porcine embryo and transferred to the surrogate gilt and harvested at E19. These results supported the conclusion that the engineered hiPSCs rescued the *ETV2* null embryo and every endothelial cell was GFP positive, HNA (human nuclear antigen) positive, and TIE2 positive (Figure 3).[25] Collectively, these results supported the conclusion that the efficiency of interspecies chimerism can be increased by targeting specific factors and signaling pathways within the donor cell line. They also support the notion that the pig can be a good host for these interspecies studies.

Engineering humanized skeletal muscle in a gene-edited pig

Limited therapies are available for muscle loss due to traumatic injuries, surgical resection of tumors associated with muscle or muscle diseases. Recent studies by Maeng *et al.*[49] in the Garry laboratories further examined intraspecies and interspecies complementation in order to engineer humanized muscle in gene-edited pigs. In these studies, the Garry laboratories engineered pig embryos that lacked native

skeletal muscle by deleting *Myogenic Factor 5/Myogenic Differentiation 1/Myogenic Factor 6* (*MYF5/MYOD/MYF6*) genes using a multiplex gene editing strategy.[49] These mutant porcine embryos were rescued by complementing them with GFP-labeled porcine blastomeres. These intraspecies chimeras were viable. The analysis of the intraspecies chimeric skeletal muscle demonstrated that that the one-month old piglets had normal growth, normal ambulation, and normal skeletal muscle physiological function (Figure 4).[49] Histological assessment verified that every muscle fiber was GFP positive, whereas the neuronal and the vascular elements did not express GFP (Figure 4). Having established these chimeric results, complementation and SCNT strategies were used to generate interspecies chimeras. In these studies, donor *GFP-TP53* null hiPSCs cells were used to complement the *MYF5/MYOD/MYF6* null porcine morula and the chimeric embryos were surgically delivered to surrogate gilts.[49] These interspecies chimeras were harvested at approximately E30 and demonstrated that all the skeletal muscle was of human origin (Figure 4).[49] Extensive analysis verified that no human cells or their derivatives were associated with brain or the germ cell lineages in the chimeric embryos.[49] These studies used multiple techniques to validate the results and importantly the results supported the notion that increased efficiency of chimeric organs could be achieved through targeted expression or deletion of selected genes.[49]

Scientific challenges for the generation of interspecies chimeras

While a number of recent studies support the notion that these technologies will successfully produce a viable and functional humanized organ, several challenges will need to be addressed for these technologies to be broadly available to the scientific and healthcare communities. First, while rat-mouse chimeras have been shown to be successful and efficient, more evolutionary divergent interspecies chimeras have been shown to be less efficient. These barriers will require epigenetic and molecular analyses that will define developmental signals and cues that can be modified to increase the overall efficiency. Second, immunological barriers will need to be defined in the interspecies chimeras during embryogenesis and in the postnatal chimeric animal. Additionally, some organs may have a variable inflammatory response in the chimeric host animal. Third, while studies suggest that host-derived interstitial cells (within a chimeric organ) are largely replaced by donor-derived cells, this process may be organ specific and will require examination.[34] Fourth, while studies suggest that the host animal governs the donor-derived organ (such as the pancreas), this observation will need to be examined for other chimeric organs.[33] Fifth, the maturation and natural aging of the chimeric organs will need to explored and monitored over time. Sixth, for each donor stem cell population used, studies will need to examine their contribution to unintended organs such as the brain or the germ cell lineage. While our recent studies suggested the complete absence of hiPSCs in unintended and nonengineered tissues and organs,[49] these studies will need to be examined for every targeted organ, every host species, and every stem cell population. Collectively, these and other scientific initiatives are receiving intense interest and examination and the sharing of the results from laboratories around the world will further refine the scientific challenges associated with the generation of interspecies chimeric organs.

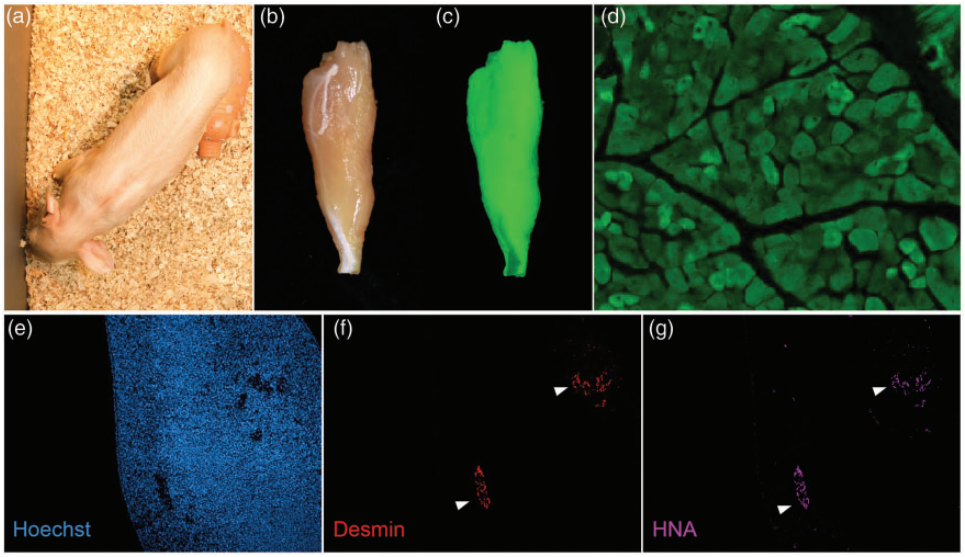

Figure 4. Intra- and interspecies chimeras for skeletal muscle development. (a) Porcine embryonic fibroblasts were gene edited to delete *MYOD/MYF5/MYF6*. Following SCNT (cloning) and blastocyst complementation (using GFP-labeled porcine blastomeres), a viable ambulatory chimeric pig was engineered. (b and c) Analysis of the intraspecies chimeric pig revealed normal skeletal muscle (whole mount; b), which uniformly expressed GFP using darkfield wholemount immunofluorescence techniques (c). (d) Histologically, the intraspecies skeletal muscle (cross section) revealed that every myofiber expressed GFP. (e–g) Interspecies (human-porcine) chimeric embryos were generated using the *MYOD/MYF5/MYF6* null porcine embryo. E28 human-porcine chimeric embryos (limbs) were analyzed and sections were stained for Hoechst (e), desmin-expressing skeletal muscle (f), and human nuclear antigen (HNA) (g) demonstrating that all skeletal muscle coexpressed desmin and HNA (arrowheads).

Ethical issues

As with any paradigm shifting scientific revolution, there are ethical issues that warrant discussion, dialogue, and guidelines. Since 2015, the NIH has instituted a moratorium on the funding of human-animal chimeras using blastocyst complementation.[50] Over this time period, a number of publications have highlighted and addressed ethical issues pertaining to these technologies.[38,51,52] Perhaps the major issue that has prompted debate is the potential, unintended contributions of human stem cells to specific organs such as the brain and the germ cell lineage. Various notions have been raised regarding the possibilities of chimeric animal hosts having unintended humanized brains or cortical human neurons capable of signal transmission or human cells located in the frontal cortex involved in behavior signals. Importantly, scientists need to communicate and follow the guidelines of their institutional Stem Cell Research Oversight (SCRO) committees and proceed in a phased approach during embryogenesis and the postnatal period. Recent studies established that in the absence of a gene disruption strategy that deletes an entire lineage or organ, hiPSCs and their derivatives do not contribute to the brain or germ cell lineage and not significantly to other organs.[49] Additional studies using other human stem cell populations and other host models will need to examine off target human cellular contributions to the brain and germ lineages.

Conclusions

The definition of master regulators and pioneer factors in combination with emerging technologies has demonstrated the feasibility of generating interspecies chimeric organ production. While recent studies have demonstrated strategies to increase the efficiency of interspecies chimeras, further studies and funding are warranted to enhance the production of humanized organs. Ethical issues and guidelines are important to promote ongoing dialogue and guide this field, which holds tremendous promise for novel therapies for patients with chronic and terminal diseases.

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All authors participated in the preparation and the writing of the manuscript.

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