Review Article

Human-animal chimeras for autologous organ transplantation: technological advances and future perspectives

Yingfei Lu[1], Yu Zhou[1,2], Rong Ju[2], Jianquan Chen[1,2]

[1]Central Laboratory, Translational Medicine Research Center, [2]Department of Obstetrics and Gynecology, The Affiliated Jiangning Hospital with Nanjing Medical University, Nanjing 211100, China

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Correspondence to: Jianquan Chen. Central Laboratory, Translational Medicine Research Center, The Affiliated Jiangning Hospital with Nanjing Medical University, Nanjing 211100, China. Email: jqchen68@hotmail.com.

Abstract: Organ transplantation is the most promising curation for end-stage organ disease. However, the donor organ shortage has become a global problem that has limited the development of organ transplantation. Human-animal chimeras provide the ability to produce human organs in other species using autologous stem cells [e.g., induced pluripotent stem cells (iPSCs) or adult stem cells], which would be patient-specific and immune-matched for transplantation. Due to the potential application prospect of interspecies chimeras in basic and translational research, this technology has attracted much interest. This review focuses primarily on technological advances, including options of donor stem cell types and gene editing in donor cells and host animals, in addition to perspectives on human-animal chimeras in clinical and basic research.

Keywords: Autologous stem cells; embryo complementation; gene editing; human-animal chimeras; organ transplantation

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Introduction

For patients suffering from end-stage organ failure, organ transplantation is their only hope for gaining a new life and has saved millions of lives worldwide. However, organ shortage and substantial risk of rejection have limited the development of organ transplantation. Even in the US, where over 50% of adults are registered as organ donors, more than 20 patients on the waiting list die every day. According to the speech delivered by Huang Jiefu, the Director of China National Organ Donation and Transplantation Committee, although China has become the world's second largest country for organ transplantation, the 20,201 transplant surgeries are mostly inadequate to meet the demand of over 300,000 organs per year. In 2015, voluntary donations became the only legitimate source for organ transplants in China. Meanwhile, the traditional culture makes recruitment of organ donors more challenging in China than other countries. Therefore, finding more resources for organ transplantation is urgently needed.

Emerging stem cell technology is promising for the development of tissue engineering and regenerative medicine as it can provide abundant biological materials. Using organs derived from the patients' cells [fibroblasts, adult stem cells, or induced pluripotent stem cells (iPSCs), etc.] as personalized medicine, is one of the trends in healthcare development. Organs generated with autologous cells minimize immune rejection, accordingly obviating the costs of the long-term administration of immunosuppression agents and reducing the risks of infection and tumorigenesis.

Scientists are trying to produce artificial organs by *in vitro* differentiation or 3-dimensional printing (1-3). Although stem cells can differentiate into many cell types

in vitro, it is next to impossible to induce differentiation into multiple cell types simultaneously in the same dish. Moreover, the organ structures that consist of various cells and organ-specific molecules is too complex to simulate *in vitro*. Thus, the existing technology for artificial organs cannot meet the demand of transplantation, and is only suitable for disease modeling (4,5) and drug screening (2). Before we can completely unveil the mysteries of reproduction and development, it is almost impossible to generate human organs *in vitro* for transplantation.

Fortunately, animal fetuses can provide a suitable environment for human organogenesis. Producing human cells, tissues, and whole organs in animals has been proven to be feasible. The animals bearing human organs or cells are known as human-animal chimeras. Using animals as biological incubators for human organs has become a new research trend because there are several benefits: (I) animals are plentiful and ready-to-use, and can allow both the patient and doctor to be well-prepared before transplantation; (II) the animal body provides a suitable niche for organogenesis, so the generated organs would be more natural and biological than the artificial organs produced *in vitro*; (III) the personally tailored organ derived from patient's stem cells may theoretically minimize the risk of immune rejection. Chimera research, was therefore, thought to be one of the hottest research areas according to *Science Magazine* in 2016. In this review, we summarize the experimental advances of interspecies chimeras, especially human-animal chimeras, including generation technique, options of donor stem cell types and gene editing in donor cells and host animals, in addition to perspectives on the potential roles in overcoming the limitations in organ transplantation by from organ shortage.

Approaches to generation techniques

Commonly used experimental approaches to acquire interspecies chimeras include aggregation of cleavage stage embryos, injection of pluripotent stem cells into blastocysts or morulae, and transplantation of adult stem cells into in utero fetus (*Figure 1*).

One way to introduce xenogeneic stem cells into an embryo is by blastocyst injection, which refers to injecting stem cells into *in vivo/vitro*-cultured blastocyst cavities. Rat-mouse (6), human-mouse (7), and sheep-goat (8) chimeras with systemic chimerism are reported to be produced via blastocyst injection. However, blastocyst injection has failed to introduce stem cells into primate (such as rhesus monkey) embryos (9).

Chimeras may also result from the aggregation of two or more embryos either at equal or unequal stages. The quick and straightforward operation and low equipment cost make embryo aggregation an entry-level technique for chimera generation. Aggregation of rat-mouse (10), sheep-goat (11), and cattle-buffalo (12) embryos all form interspecies chimeras. Despite the lower survival rate of chimeric embryos produced by aggregation than by blastocyst injection, the chimerism rates have been observed to be higher (13,14). Considering that chimerism rates decide whether the human-animal chimeras can be an organ resource, aggregation is also a desirable choice when the embryo and stem cells are in a good growth condition.

The in utero transplanting of stem cells was an in utero treatment for congenital diseases at first (15). Now, it has become the most widely used experimental technique in human-animal chimera study. Human adult stem cells, including mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs), can form chimeras in mouse, rat, pig, sheep or goat fetuses and contribute to multiple organs of the chimeras (16-21). In utero transplantation is reported to be the most efficient way to limit human cells in the target organ of human-animal chimera (22).

However, the interspecies barrier between human and other animals is much thicker than that between rat and mouse. Even a minimal number of human cells may disrupt the development and growth of host animals. Consequently, the chimerism rates using the mentioned techniques are still extremely low. Contribution from human stem cells in human-mouse chimera generated by blastocyst injection was as low as 0.01% (23). Pigs, sheep, and goats are universally acknowledged as the preferred species for xenotransplantation into human body. However, due to the interspecies barriers, human cells only contributed 0.001% of the total cells in human-pig chimeric embryo (24) and up to 0.01% in human-sheep embryo according to the report in the 2018 AAAS annual meeting. This is far away from producing organs for transplantation into human bodies. Consequently, researchers have made much efforts to increase the chimerism rates by gene editing in host embryos and stem cells.

Host embryo gene editing: embryo complementation

One of the main ethical concerns raised by human-animal chimeras is how to avoid stem cell contribution

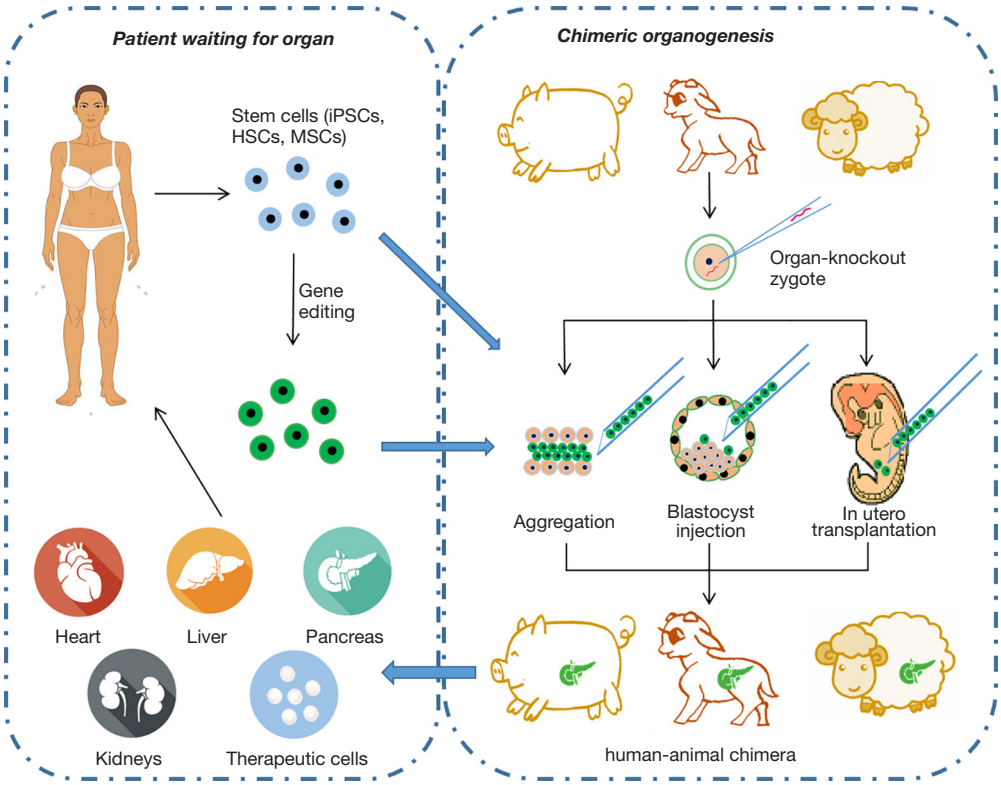

Figure 1 Interspecies chimeras may provide patient-specific organs for transplantation. The patient's stem cells (e.g., iPSCs, HSCs, MSCs) are injected into farm animal embryos to produce a human-animal chimera bearing needed organ for transplantation back into the patient's body. The chimeric animals can be made by inducing stem cells into the embryo through aggregation, blastocyst injection or in utero transplantation. Knocking out key regulators for organogenesis in zygote results in organ deficiency and provides vacancy for stem cells to produce a xenogenic organ. Meanwhile, gene editing in stem cells can trigger pathogenic gene switch-off, as well as the directed differentiation, contribution, and increased post-transplantation survival of the cells. iPSCs, induced pluripotent stem cells; HSCs, mesenchymal stem cells; MSCs, hematopoietic stem cells.

to the nervous or reproductive system. Modifying host embryos by knocking out the essential regulatory genes for target organs (e.g., Pdx1 for pancreas, Sal1 for kidneys, Rag2 for lymphocytes, Dcx for forebrain) in the zygotes leads to organ deficiency. This vacancy could be filled during development by introducing exogenous stem cells, producing chimeras in which the target organs mostly consist of injected stem cells (25-27). This technique, known as embryo complementation, was first used to search for the critical regulators of organ development (27). Through embryo complementation, researchers can limit the contribution of stem cells in a specific organ. This not only increases chimerism rates in target organs, but also minimizes the detrimental influence on host embryos from xenogenic cells and subsequently reduces the abortion and malformation rates.

If xenogenic stem cells are used for embryo complementation, a xenogenic organ would be developed and could form an interspecies chimera. In 2010, Nakauchi's group injected rat ESCs into Pdx1 knockout mice blastocysts. The rat ESCs were limited mostly to the pancreas and contributed to 81.9%±3.4% of all cells in the pancreas (6). Chimeric mice remained alive and reached adulthood with no pancreas dysfunction. In 2017, this group successfully generated mouse pancreas in rats, the islets of which were transplanted into diabetic mice. No immune rejection was observed, and the blood glucose maintained at normal level (28).

However, it seems that blastocyst injection is not a suitable way for large human-animal chimeras. Although preliminary data confirmed that intraspecies porcine chimeras could be produced through blastocyst

injection (29), injecting human PSCs resulted in systemic chimerism (unpublished data by Nakauchi's group). The cells at unexpected positions might cause malformation and provoke ethical concerns. On the contrary, in utero injection of PSCs to the site of organogenesis resulted in mainly local chimerism and less systemic chimerism (22).

To date, stem cell isolation and expansion *in vitro* is growing into maturity. Meanwhile, gene editing in sheep and porcine embryos are available using CRISPR-Cas9 (30,31). Moreover, eGenesis Bio has claimed that up to 62 genes could be batch edited, which would provide the feasibility of constructing host animals with deficiencies of all cell types in specific organs. These have all laid the roots for producing human organs in human-animal chimeras.

Stem/progenitor cells for chimera production

Theoretically, all types of cells with differentiation potency can contribute to the chimera. The chimerism rates depend on the potency of the cells. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are the preferred cell types in chimera studies (6,24,28,32). Due to their pluripotency, the incorporated stem cells contribute to multiple organs of the host animals. However, a recent study demonstrated that rhesus macaque ESCs were incapable of generating intraspecies chimeras by blastocyst injection (9). To explain this difference between rodent and primate stem cells, some researchers proposed that only naive ESCs are capable of producing chimeras with pre-implantation embryos (9), while primed ESCs (EpiSCs) are only chimera-competent in the epiblasts of post-transplantation embryos (24,33). That is to say, the stem cells at a certain developmental stage are only competent in those embryos at an equal stage. Similarly, progenitors should be in utero transplanted into embryos at an equal stage (34), while adult stem cells should be injected to postnatal bodies to generate chimeras.

Nevertheless, several studies have demonstrated that human stem cells at asynchronous stages can also generate chimeras in animal embryos. Transplanting human adult stem cells (such as MSCs, neural stem cells and HSCs) into post-transplantation embryos of rat, sheep or goats (17,19,35), and into murine blastocysts can all generate chimeras (36). Moreover, compared to iPSCs, autologous adult stem cells have advantages such as easy accessibility, simple culture conditions, and less tumorigenicity (37).

Several recent studies also demonstrated that despite the equality of the developmental stage, the survival of injected stem cells is also crucial for chimerism. Both rat and human EpiSCs overexpressing anti-apoptotic genes (e.g., BCL2, BCL-XL, CRMA, BMI1, etc.) can form interspecies chimeras in mouse embryos (38-40). Meanwhile, adding anti-apoptotic reagent Y27632 (ROCK1 inhibitor) into culture medium also gives rise to chimerism of primate EpiSCs into morulae (41). Therefore, other proliferation-inducing and anti-apoptotic treatment, such as forced expression of SIRT1 (42), neurotrophin 3/4 (43) or Rho signaling pathway (44), might also impel chimera formation. Worryingly, however, apoptosis blockage in iPSCs might be more prone to tumorigenesis. In that case, more research is needed to assess the safety of increasing the chimerism rate through improving stem cell survival.

Gene editing in injected stem cells may trigger directed differentiation and guide contribution to specific tissues or organs. For example, Mixl1-overexpressed ESCs or iPSCs tended to contribute to endodermal organs after blastocyst injection (25). We speculate that overexpression of the critical regulators of organogenesis, such as Pdx1 and Sall1 mentioned above, may also give rise to directed differentiation of stem cells. Moreover, gene editing has the capacity for pathogenic mutation correction in autologous stem cells. This would be beneficial for patients suffering from malignancy hematologic disease (45-47), and for those with end-stage organ failure caused by genetic disorders, such as polycystic renal disease and hepatolenticular degeneration, to receive autologous organs for transplantation.

Facing challenges

Low rates of chimera and chimerism

It is never easy to cross the xenobarrier. The more xenogenic cells contribute to the chimera, the higher risk of abortion and malformation (28). Only 20% of rat blastocysts injected with mouse ESCs became chimeric pups (6), while only 35% of all embryos transplanted in utero with human HSCs were eventually born alive (18,35). By improving stem/progenitor cell survival, as we mentioned above, the rate of chimeric embryo occurrence increased.

Nonetheless, the impairment of host animals by xenogenic cells still exists and triggers adverse pregnancy outcomes. This might be due to undesired immunologic defense activation. In *Ldlr*, *Rag2* and *Il2rg* KO mice, immunodeficiency allows injected human iPSCs and glial progenitor cells to contribute to mouse liver and brain, respectively (48,49). However, the above techniques have

only been proven to be effective in rodents. Methods to generate suitable human organs in livestock still lack sufficient evidence. Accordingly, further studies in large animals are urgently needed.

Immune rejection

The contamination of animal cells (e.g., endothelial cells) in the human organs produced by a chimera might lead to immune rejection. Although these cells could be eliminated by multiple gene knockouts in host embryos, the embryo development may also be severely affected. Knockout of α-1,3-galactosyltransferase has been reported to be effective in minimizing immunogenicity of xenograft. The kidneys from α-1,3-galactosyltransferase knockout pig worked properly with slight immunosuppressive therapy in a baboon body for up to 136 days (50). Overexpressing human CD46 exhibited a similar effect on avoiding immunogenicity for xenotransplantation (50-52). Excitingly, the latest research showed that xenotransplantation of a pig heart with α-1,3-galactosyltransferase-knockout combined with human CD46 and thrombomodulin overexpression functioned normally in baboons for up to 195 days (53). Can these genetically engineered pigs produce humanized organs more suitable for transplantation and prolong overall survival? This is a desirable orientation for chimera research.

Virus infection risk

Another fear is that human organs produced in animals might be transporters of new zoonoses, transferring endogenous retrovirus (ERV) to humans. Therefore, non-human primates, harboring pathogens that easily infect humans, are no longer thought to be promising organ donors for humans. In contrast, large farming animals, including pigs, sheep and goats are acknowledged as suitable xenogeneic donors for humans. Although there are still several kinds of ERVs in pigs which are infective for humans, scientists have been able to generate ERV-free pigs by using somatic cell nuclear transfer and CRISPR-Cas9 techniques (54). The ERV-free animals address this safety concern and offer novel options for animals bearing human organs.

Economic benefits and future prospective

Organ transplantation has proven to be the most economical treatment for organ failure. In Shanghai, kidney transplantation can save up to ¥150,000 for each patient suffering from end-stage renal disease in the first 8 years (55). However, organ shortage and transplant rejection keep post-transplantation medical costs inflated. Interspecies chimeras provide the capability to produce adequate donor organ; thus, more patients on the waiting list can choose organ transplantation, and, more importantly, at earlier stage of disease. This would reduce the risk of severe complications.

Meanwhile, the autologous stem cell-derived organs may minimize the risk of rejection and consequently save the vast expense of immunosuppression and anti-infectives, which may cost up to ¥5,000–10,000 per month. Moreover, the convenience of operating on animals would not only ensure full preoperative preparation of removing organs for chimeric animals and transplantation into the patients' bodies but also avoid organ necrosis during transportation. Also, producing patient-specific organs from autologous stem cell-animal chimera do not need *in vitro* organ culture, and thus may save significant expense compared to *in vitro* culture and differentiation of autologous iPSCs.

Apart from providing autologous organs, human-animal chimeras have additional benefits in clinical and experimental research. We might be able to produce therapeutic cells and functional proteins for clinical use. For instance, autologous HSCs in which pathogenic genes are edited might cure malignant hematological diseases with less immune rejection than allogeneic transplantation. Human cells overexpressing coagulation factor IX have been enriched in human-mouse chimeras, with the proteins being properly modified, secreted, and cleaved *in vivo*, thus being suitable for hemophilia B treatment (56). Although human-animal chimeras are still far away from clinical therapy, their application value in basic research has been undeniably proven in studies for human stem cell potency (20,21), cell fate during embryo early development and organogenesis (21), as well as *in vivo* models of human diseases (35) for drug metabolism study (57,58) and drug screening (48).

Crossing the species boundary between human and animals is always connected with many ethical issues. The possibility of inducing human cells into animal neural or reproductive systems has met particularly strong public resistance. However, human-animal chimera is not only a promising strategy to alleviate organ shortage, but also a reliable model for studies on organ development, pathogenesis, immunologic defense, and drug screening. Respecting the significance of human-animal chimera in basic and translational research, the National Institutes

of Health (NIH) restarted funding for studies on adding human stem cells to animal embryos. Certainly, this research still needs to be cautiously carried out only after adequate studies in animal models and after undergoing more ethical review. US researchers are attempting to incubate human neurons for Parkinson's disease treatment in human-pig embryo, while a human-glial-progenitor-cell-mouse chimera was used as a research model for neurological and mental disorders several years ago (49,59). Furthermore, experiments have proven that human gametes crossing the interspecies reproductive barrier and hybridizing with gametes of animals, including anthropoid apes, is virtually impossible. In other words, even if human sperm or oocytes were to be produced in chimeras, no embryo with human-like features would be capable of development.

Moreover, oocyte-like cells produced through *in vitro* differentiation have an impaired ability to achieve meiotic maturation, while the production of human gametes in animals would fill this gap. This would not only provide new tools for research on germ cell development and maturation but help patients with azoospermia or premature ovarian failure as well. In conclusion, rather than complete prohibition, which would obstruct the developmental possibilities of human-animal chimeras, continuing research under strict ethical review would be more beneficial to human society.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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