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Chimeras for the twenty-first century

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Abstract

Recent advances in stem cell biology and molecular engineering have improved and simplified the methodology employed to create experimental chimeras, highlighting their value in basic research and broadening the spectrum of potential applications. Experimental chimeras have been used for decades during the generation of murine genetic models, this being especially relevant in developmental and regeneration studies. Indeed, their value for the research and modeling of human diseases was recognized by the 2007 Nobel Prize to Mario Capecchi, Martin Evans, and Oliver Smithies. More recently, their potential application in regenerative medicine has generated a lot of interest, particularly the enticing possibility to generate human organs for transplantation in livestock animals. In this review, we provide an update on interspecific chimeric organogenesis, its possibilities, current limitations, alternatives, and ethical issues.

Concepts and classifications

A chimera is defined as an organism or tissue that is composed by at least two genetically different popula- tions that originate from different zygotes [1,2]. Although sometimes used interchangeably, *mosaics* are individuals in which genetically different cellular popu- lations derive from the same zygote, as a result of mutations that arise later in development and propa- gate in some lineages [3,4].

Primary or systemic chimeras are generated during the early stages of embryogenesis, as the genetically different cell populations contribute to all tissues and organs, causing systemic chimerism. When they are formed after gastrulation, the contribution is more lim- ited and thus they are called *secondary or partial chime- ras* [5]. Recipients of allogeneic and xenogeneic transplants represent a special case of partial chimer- ism, as it occurs after morphogenesis is completed.

Chimeras are also classified attending to other crite- ria such as location and chronological age, which are two critical aspects that affect the developmental suc- cess of the chimeric organism. According to the loca- tion, partial chimeras are classified as orthotopic or heterotopic; orthotopic chimeras are generated when cells are transplanted into their cognate site and can participate in the natural developmental process of the organ or tissue. On the other hand, heterotopicchimeras develop and integrate in a different (ectopic) location. Regarding chronological states, chimeras are isochronic when formed by donor and host cells which are at the same developmental stage and heterochronic when there is a mismatch.

Finally, chimeras are also classified as intra- or inter- specific depending on whether contributing cells come from individuals from the same species or belonging to different species.

Developmental chimeras

Primary chimeras can be generated by combining blas- tomeres isolated from a minimum of two embryos, aggregating two or more sectioned embryos or inject- ing embryonic stem cells (ESCs) into a blastocyst, under the zona pellucida or into the blastocyst cavity [5]. Tarkowski et al. were the first to demonstrate the for- mation of a chimeric blastocyst through the aggrega- tion of two sectioned mouse embryos that could then be implanted and develop in the uterus of a foster mother [6]. Using this method, the chimerism precedes blastulation, the first event in differentiation, and most cells are totipotent. Aggregation of these cells results in a complete systemic chimerism that includes both the internal cell mass (ICM) that will give rise to the embryo and the trophectoderm that generates the extraembryonic tissues. Systemic chimeras can also be generated after blastulation, by combining an ICM with a second embryo [7]. These chimeras formed by ICM transfer present a lesser donor contribution to the pla- centa and extraembryonic tissues than those formed by aggregation of embryoblasts, morulas, or early blasto- meres [8].

The eruption in basic research of ESCs, isolated and established from mice embryos began in 1981 [9]. It fueled an authentic revolution in the field of chimeras – which, reciprocally, has been instrumental for advances in the stem cell field, by providing a measure of pluri- potency for novel types of stem cells. Indeed, systemic chimeras can be generated by injecting pluripotent stem cells (PSCs) in early embryos, thus confirming their equivalence to the ICM (from which ESC originate) [10]. These cells contribute to all tissues in the embryo but make a limited contribution to extraembryonic tissues (in contrast to totipotent cells). From an experimental point, PSCs have many advantages given that they can be expanded and propagated *in vitro* and can be derived from adult somatic cells by nuclear transfer (NT-PSC) [11] or by cellular reprograming (iPSC) [12–14]. In contrast, ICM cells can only be obtained from the blastocyst [15]. The development of mutated ESC lines by homologous recombination opened the field to gen- etic manipulation in mammals. From Evan's pioneering experiments [16], the production of murine (m)ESC lines with mutations in

target disease genes rapidly expanded the use of chimeric mice to model human disease [17–24]. The most widely used strategy for generating knockout and knockin mice is to target 129- derived ES cells and microinject them into C57BL/6 blastocysts [25]. The resulting mice generally are back- crossed with C57BL/6, in order to obtain a constant genetic background. However, the 129 genetic back- ground will always exist close to the target gene [26]. Thus, animals generated with this strategy are a genetic mixture, so it has been proposed to use C57BL/6 ES cells and blastocysts to avoid this problem [25].

Experimental chimeras have been instrumental to define and measure different degrees of stem cell potency. Mouse systemic chimeras are easily established by injection into the blastocyst of mESC derived from preimplantation embryos (ICM) but not from the cells taken from the epiblast (EpiSC) which are nevertheless pluripotent and capable of differentiation into the three germ layers and to form teratomas [27]. Thus, mEpiSC are considered "primed" and less potent than "naïve" mESC from the ICM. Human ESC (hESC) are derived from the ICM of preimplantation embryos [28], but are functionally more similar to primed mESC [27,29] and fail to efficiently integrate into the mouse blastocyst [30,31]. Ethical restrictions do not allow examining the capacity of hESC to form chimeras in a human blastocyst and thus it has been difficult to dissipate doubts about their actual potency. However, a recent study has demonstrated that technical modifications are sufficient to enable non-human primate PSC (primed ESC, NT-ESC, and iPSC from Cynomolgus monkeys) to efficiently establish viable intraspecific chime- ras, although the resulting chimerism was very low [32]. This study suggests that current limitations in the formation of human chimeras may not be due to a lack of pluripotency and could be overcome with technical modifications.

Finally, injection of donor cells after gastrulation gives rise to partial or secondary chimeras [33]. Theoretically, in this case, it is mandatory that donor cells are at the same developmental stage

than the embryo (isochronic) to produce viable chimeras, although this requirement needs to be understood in a broad developmental con- text. For example, matching could be more permissive at the phylotypic stage [34]. Another aspect to be considered is that the chronological mismatch causes massive apoptosis of the donor cells and in fact inhibition of apoptosis of donor cells by overexpression of BCL2 [35] or BMI1 [36] has been shown to overcome this barrier. It is interesting that in the case of cell transplantation into adult organs, transplanted cells also suffer massive apoptosis, which could partly be due to the heterochronicity, given that most cells used for cell therapy are at an earlier developmental stage (stem or progenitor cells) than the recipient tissue. Also interestingly, we and others have observed that proper integration and functional connectivity, for example between neurons, is established once the cells reach an isochronic stage, explain- ing the delay in functional restoration when grafting immature cells and the self-innervation phenomena in embryonic grafts transplanted into adult hosts [37].

Partial chimeras present several advantages over systemic chimeras, particularly for the formation of interspecific chimeras. For instance, it is useful when a high degree of chimerism is embryonically lethal due to developmental disparities. It is also more important to study organogenesis and to model diseases in a single tissue or region. Importantly, it reduces donor contributions to organs such as brain and gonads which poses ethical considerations, particularly in human–animal chimeras [38].

Interspecific chimeras

Generation of avian quail-chick interspecies chimeras set a revolution in experimental embryology as differences in the nuclear structure allowed the tracking of cellular lineages and to establish fate maps [39]. That same year, the first mammalian interspecies chimera was generated by the injection of rat ICM in a mouse blastocyst [7], followed by other attempts [8,40]. However, those interspecies chimeras did not progress further than early post-implantation stages.

It was not until the experiments were conducted in closely related species (*Mus caroli* in *Mus Musculus*) that viable chimeric animals were generated [41]. These initial observations pointed to the existence of interspecies barriers, which are briefly discussed below.

Fetal–maternal incompatibility. Given the failure to develop mammalian aggregated chimeras (which have a high degree of extraembryonic tissue chimerism), it was inferred that for a correct implantation and embryo development, the trophoectoderm needed to be compatible with the uterus to avoid an immunological rejection of the embryo. MacLaren et al. went further and demonstrated that there are species-specific differences in the signaling guiding the proper implantation and interaction between mother and fetus, which also needs to be compatible [42].

Evolutionary distance. The impact of the evolutionary distance is highlighted by the fact that viable goat- sheep "geep" chimeras (10 million years apart [43], http://www.timetree.org) were obtained in 1984 using preimplantation embryos [44] while for mouse–rat chi- meras, which are 20 million years apart, we had to wait for PSC that do not contribute to extraembryonic tissue to obtain a chimeric offspring [45].

Degree of chimerism. The study of mouse–rat chimeras revealed that the percentage of systemic contribution of rat cells was correlated with fetal anomalies. Viable offspring never had above 25% of rat cells suggesting embryonic lethality of higher degrees of chi- merism [46]. Similar to implantation, signaling differences and incompatibilities in surface receptor expression and response to cytokines may exist, alter- ing migration, proliferation, and differentiation in specific tissues and organs [46].

Developmental age and pace. Many studies have highlighted the importance of matching the develop- mental stages of donor cells and blastocyst (isochronic chimeras) to obtain successful chimerism. For example, EpiSC which did not form chimeras in the blastocyst, could colonize day 7.5 post-implantation embryos [47]. Likewise *primed* human PSC can form chimeras when

injected at the gastrula stage [48]. However, naive human PSC injected into mice blastocyst only rarely contribute to the mouse embryo, casting some doubts on the actual naiveté of the cells. Indeed, in one study, human cells could be detected at mid-gestational stage only in seven out of 3000 embryos, and in a very low proportion (one in 10,000) [30]. In another study, the injection of human naive PSC into pig blastocyst resulted in an even lower contribution, one in 100,000 [49]. These studies underlie the importance of the chronological barrier beyond the injection time, extending to the different developmental pace in different species, with critical signaling events occurring at different times or in a different temporal sequence [34]. Gene editing has been proposed as a useful tool to synchronize the development of donor and receptor cells, overcoming this barrier [50].

Chimeric organogenesis

Research on interspecies chimeras is gaining traction due to the broad range of basic and translational applications of chimeric models. Recently, it has been particularly helpful for studies with human tumor progression and response to immunotherapy in immunocompetent mice [51]. Another promising application that we discuss below in more detail is the pro- duction of human chimeric organs in livestock animals for transplantation, using human PSC (Figure 1).

In the last few years, several reports have shown that, following genetic manipulation of the host, it is possible to generate whole tissues and organs almost entirely derived from the donor cells. The first step is to create an *empty niche* – analogous to myeloablation to facilitate engraftment before bone marrow hematopoietic stem cell transplantation – so that donor cells lack endogenous competition and a complete contribution to the organ or tissue can be achieved. Knocking down "master" genes that regulate specific developmental pathways such as *Pdx1* for pancreas [52], *Sall1* for kid- ney [53], *Nkx2.5* for heart [54], *Pax6* for the eye [55], etc. can efficiently

disrupt organogenesis in the host, creat- ing the corresponding empty niche. The next step is the *complementation of the blastocyst* by injecting PSC from the species of interest to generate a chimera of the missing organ. Blastocyst complementation can produce systemic chimerism, which can be reduced by target complementation (i.e. creating an empty niche), local injection at a later developmental stage and by delayed complementation with lineage-restricted cells.

Interspecies organogenesis by blastocyst complementation has been achieved for both mice and rats using rat and mouse PSC, respectively. Nakauchi's group generated a rat pancreas in *Pdx1* knockout apancreatic mice [45] and later reported the reciprocal experiment [56]. More recently, generation of mouse kidneys in a $Sall1^{mut/mut}$ rat model has also been reported [57]. Importantly, these experiments have shown that the host determines the size and morph- ology of the chimeric organ, thus corroborating that these features are regulated by non-cell autonomous signaling. In classic "heteroplastic" (between different species within the same genus) transplantation experiments in amphibians in the early twentieth century, the difference in transplanted limb size was restored to that corresponding to the donor species when feeding was maximized, suggesting an impact of nutrient availability in the host effect on organ size [58]. Regarding function, critical for future applications, transplantation of (mouse) pancreatic islets from rat chimeric pancreas into diabetic mice could maintain normal blood glucose levels for over a year [56].

Combination of interspecies complementation with CRISPR-Cas9 genomic editing of the host has simplified the generation of these chimeras. Importantly, these proof of concept experiments could be translated to larger animals in order to generate human organs, a necessary transition as the chimeric organ size is dictated by the organ environment in the host species [46]. However, an efficient interspecies blastocyst complementation has only been achieved in rodents. Human

PSC injection in pig embryos achieved very low levels of chimerism [49]. Notably, the evolutionary distance between pigs and humans is >90 million years (www.timetree.org). Presumably, selection of a closer

host species, such as primates, would increase the likelihood of successful chimera formation, but these kinds of experiments are forbidden in many countries [59].

Other limitations, as mentioned above, are the degree of chimerism and disparities in developmental regulatory gene expression patterns. Regarding the first one, it appears that too many donor cells can interfere with the developmental program, so only those embryos with a low degree of chimerism would survive. In such a case, donor cells may be insufficient (too few or not close enough) to successfully complement the target organ [49].

A final interspecies compatibility issue arises at the time of transplantation back into the donor species because the chimeric organs are not completely made of donor cells. In particular, with current approaches, vasculature, nerves and stroma are derived from the host and are highly immunogenic. Therefore, additional genetic modification of the host would be necessary, either to extend chimerism at least to blood vessels, creating another empty (vascular) niche [60], or to sup- press antigen expression in the host, or to modify antigen expression introducing genes from the donor species (humanized models) that can act as immuno- modulators [61]. In this regard, progress in genetic editing and engineering provides almost unlimited opportunities to modify the host genome.

Despite all these limitations, livestock and in particular pigs, are currently considered an optimal species for hosting chimeric human organ formation: they have a relatively short gestational period (4 months) and rapid postnatal growth, compatible organ size and physiology, and are extensively used in the food industry [62]. Moreover, production of genetically modified pigs

using NT and CRISPR/Cas9 has facilitated the generation of empty niche hosts. For example, Matsunari et al. have generated apancreatic pigs which could be successfully complemented – although so far only with pig cells – to generate a functional chimeric pancreas [63].

Alternative synthetic organs

PSC 3D cultures overcome some of the classical limitations of 2D cell cultures and exploit the ability of the cells to self-organize and form structures that resemble mini organs and show some functionality [64]. *Organoids* represent an outstanding tool in basic research, developmental studies, and disease modeling, but they also have many limitations. Not the least is their small size, which is partly due to the lack of vascularization. Different approaches have been explored to tackle this issue. For example, co-culturing hepatic endoderm, endothelium, and septum mesenchymal progenitors led to vascularized and functional liver tis- sue [65]. Vascularization of brain organoids can be achieved transplanting them directly into a mouse brain where host blood vessels will invade the graft [66,67], or coating brain organoids with human endothelial cells (ECs) before transplantation [68]. Both approaches led to extensive vascularization of the organoid in a couple of weeks, although generated vessels are from different origin and the growth is limited. Another limitation is that organoids require an extracellular matrix (ECM), most often matrigel – although recent approaches tend to replace it with more sophis- ticated synthetic ECM analogs [69], and tissue-specific decellularized matrices [70]. Finally, organoids rarely contain all the characteristic cell types of the tissue and their function is quite rudimentary. Concerning the cell types, studies looking to obtain more complex models have explored the addition of cells derived from different germ layers, such as, for instance, microglial cells into brain organoids to study neuro-immune disorders [71]. Nevertheless, detailed analyses of cell types have reported the presence of mesodermal cells in (neuroectodermal) brain organoids [72,73]. Regarding function,

the development of iPSC-derived human 3D brain microphysiological system (BMPS) that comprises differentiated mature neurons and glial cells (astrocytes and oligodendrocytes) recreates more accurately the brain microenvironment and provides a reliable model to investigate neuron-neuroglia function [71,74]. Using this same strategy, this group developed a spheroid tis- sue microarray (micro TMA) in which they incorporated glioblastoma cells. This method provides a suitable plat- form for personalized anti-cancer drugs testing, reduc- ing the costs and animal use [75].

Aforementioned examples represent useful *in vitro* or, after transplantation, *in vivo* models for testing therapeutic approaches. However, the possibility to use organoids to generate whole viable organs for trans- plantation appears remote.

Likewise, and despite recent, and quite remarkable, advances in tissue engineering [76], generation of whole *engineered organs* with complex 3D structure is not an easy task. The use of scaffolds is required to sup- port a correct tridimensional distribution and enable the functional integration of different cellular types. Broadly, biomaterials used as scaffolds are classified into natural and synthetic materials. In either case, it is crucial that they mimic the ECM so that they provide not only mechanical but also biochemical and signaling tissue-specific patterns. To this end, sophisticated manufacturing technologies are now available such as *micropatterning* and self-assembly of ECM components, *electrospinning* or 3D bioprinting [77]. These techniques are expanding the clinical applicability of biomaterials including synthetic hydrogels [78], ECM derivatives [79] and also tissue [80] and organ components [81]. Indeed, one of the most promising alternatives is to use decellularized organs as the scaffold [82]. Currently, it is based on the use of either human organs that are not suitable for transplantation or animal organs that have a (human) compatible size and morphology. The decellularization process is achieved through intravenous perfusion of different reagents such as detergents and enzymatic solutions that preserve the

vasculature and tridimensional structure of the organ. These decellularized matrices need to be maintained in bioreactors that simulate physiological conditions (physical and mechanical forces and gradients, pH, electric conductance, etc.) [83]. Following the successful decellularization and functional recellularization of a rat heart in 2008 [84], a great number of studies have used this approach for different organs including human organs [70,82,85]. However, the recellularization process is still rather challenging as it needs to combine all the different cell types at the right proportion and differentiation stages to reconstruct the native distribution in the organ [82]. Therefore, although the potential of tissue engineering is definitely huge, whole organ engineering is still far from a therapeutic application and most probably it will require the use of animal organs as scaffolds.

Ethical issues

According to Nakauchi and coworker, eradicating organ scarcity for transplantation would require using just one out of 1000 large animals currently employed in the food industry [46]. Thus, explained in these terms, the use of livestock animals as hosts for human organs should hardly pose any ethical concern in today's society. Notwithstanding, crossing interspecies barriers between animals and humans has always created public discomfort (commonly referred as the "*yuck factor*") [86]. Besides this emotional factor, the bioethical debate about human–animal chimeras can be summarized in three major factors: (1) the development of "human consciousness", (2) human-like appearance, and (3) human gamete production [87].

Development of human consciousness. Simply put, our brain is what makes us human when compared with the rest of animals. Due to that, most authors con- sider that, if human cells were to have any chance of triggering any kind of consciousness in an animal host, these experiments would become ethically unacceptable [88]. In fact, paradoxically, those hypothetical chimeric

animals could no longer be used for hosting human organs and should be treated as one of our kind [89].

Nowadays, interspecies barriers are too strong for this to happen. Nevertheless, potential risks should be taken into account and minimized. For example, a maximum threshold of human chimerism in an animal brain must be defined, especially in large animals, which could be ideal to understand neuropsychiatric disorders, but are also more susceptible to cause concern [59,88]. Furthermore, targeted blastocyst complementation should be used as it greatly reduces the possibility of injected human cells to colonize other organs like the brain, as a consequence of their competitive advantage to colonize the empty niche [88]. However, it should not be dismissed that some experiments have shown intrinsic competitive advantage of human cells over their animal counterparts, clearly exemplified by Goldman's experiments of human glial mice chimeras [90,91].

Human-like appearance. Another ethical concern regarding human–animal chimeras is the potential impact that human stem cells could have on the animal's physical appearance. The creation of a living being that explicitly shows its chimeric condition could blur the limits between humans and animals and challenge the concept of human identity. As Robert and Baylis stated in their dissertation on ethical aspects of chimera generation: "*the most plausible objection to the creation of novel interspecies creatures rests on the notion of moral confusion*" [86]. Therefore, and despite the chances of this happening being extremely unlikely, preemptive actions should be implemented, for instance, by defining a maximum threshold of systemic chimerism for human cells, or through the implementation of prenatal systematic diagnosis in order to identify any indication of human features in chimeric fetuses [88].

Human gamete production. Humanization of chimeric animals could lead, in theory, to the production of human gametes. In fact, the US National Academy of Sciences (NAS) prohibits the

cross-breeding of animals having received human cells or tissues [92]. However, once again, the odds for this to occur are extremely low, given that the interspecies reproductive barrier is too high. Moreover, sterilization of animals carrying human material would be enough to prevent their reproduction [88]. Other alternatives to minimize this risk have been proposed, such as genetically manipulating human cells to incorporate a suicide gene which could be activated upon germinal differentiation, or to directly inhibit their potential to differentiate toward reproductive cells [93].

In addition to, proper ethical issues discussed above, an epidemiological perspective should also be considered. Organs developed in animal hosts could constitute a source of zoonosis, and there is concern about endogenous viruses integrated into the genome of breeding animals, such as porcine endogenous retroviruses. Undoubtedly, this also raises ethical concerns regarding the obligation to ensure patient safety in potential clinical trials in the future [88].

Regarding regulatory and legal aspects many countries have issued specific legislation and created over- view research committees that supervise all experiments involving the introduction of human stem cells or tissues in animals [2]. Useful guidelines are avail- able from the NAS [92], the ISSCR [94,95], and The Academy of Medical Sciences, in the UK [96]. In Spain, the Biomedical Research Law prohibits the creation of human embryos and pre-embryos exclusively for research purposes (Artículo 33 de la Ley de Investigación Biomédica) [97]. In Japan, the Ministry of Education, Culture, Sports, Science and Technology has recently lifted the ban to terminate human–animal chimeras after 14 days thus enabling the potential produc- tion of human organs for transplantation, in animals [98].

Disclosure statement

The authors report no declarations of interest.

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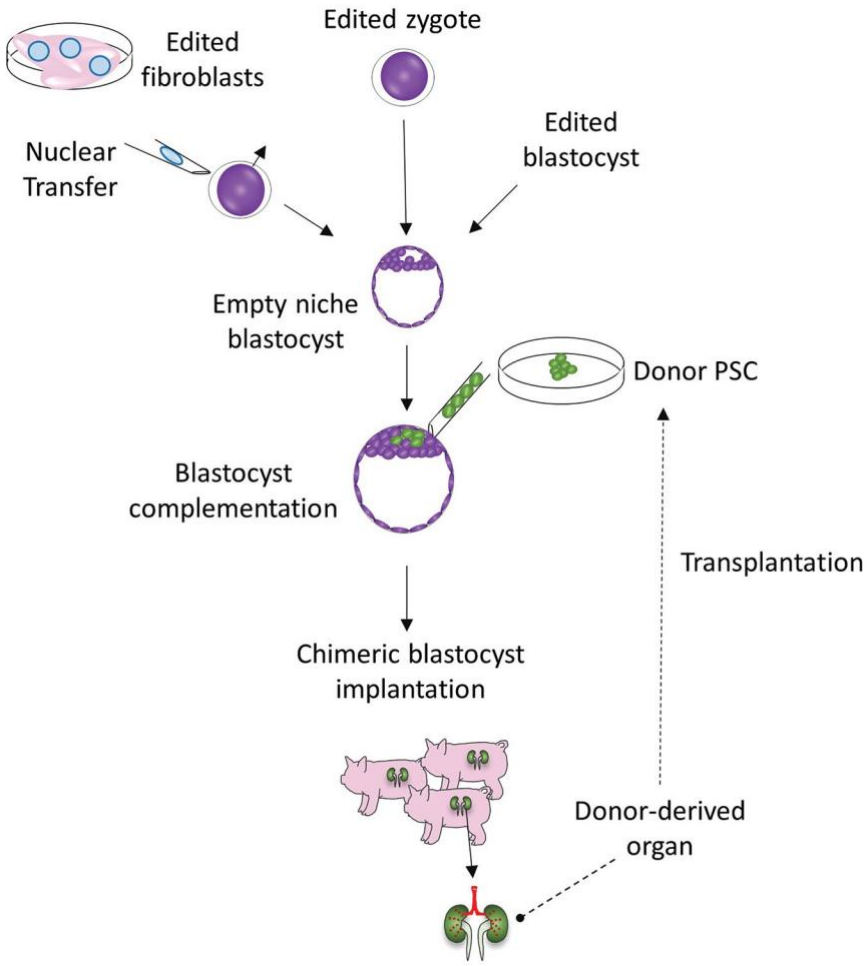

Figure 1. Generation of chimeric organs. The organogenesis ablation in the host by genetic manipulation of key genes leads to the formation of an empty niche. This can be accomplished by editing host somatic cells – followed by nuclear transfer –, zygotes or blastocysts. Subsequent complementation of the modified blastocyst with donor-derived PSC and implantation in a foster mother generates adult individuals with chimeric organs that could then be transplanted back into the donor. These chimeric organs are not completely formed by cells from the donor, since the vasculature, nerves and stroma are host-derived and will be recognized by the immune system as xenogeneic, which mounts a pronounced rejection response. Future refinements are needed to produce functional organs completely derived or compatible with the donor. PSC: pluripotent stem cell.