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Lessons from Interspecies Mammalian Chimeras

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Abstract

As chimeras transform from beasts of Greek mythology into tools of contemporary bioscience, secrets of developmental biology and evolutionary divergence are being revealed. Recent advances in stem cell biology and interspecies chimerism have generated new models with extensive basic and translational applications, including generation of transplantable, patient-specific organs.

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INTRODUCTION

Definition

The monstrous chimera conjured in Greek mythology was a fire-breathing beast, with two heads and the tail of a serpent. Although sighting a chimera was a bad omen in ancient lore, the contemporary definition of chimerism need not be associated with such egregious abominations. In modern biology, a chimera refers to an organism or tissue composed of at least two genetically distinct populations of cells originating from different zygotes (Tippett 1983). A chimera can occur naturally in utero when nonidentical twins share a common blood supply. Each of these twins will be partially chimeric with the blood of his or her respective sibling. Alternatively, twin zygotes can fuse into one embryo early during development, forming one fetus with chimerism in all tissues. In both cases, these chimeric people will enjoy healthy lives and may never learn of their dual cell composition. This modern definition of a chimera is disparate from the images conjured in Greek mythology.

Terminology

Genetic mosaicism differs from chimerism. In a mosaic organism, all cells are derived from the same zygote, but there are at least two populations of cells with distinct genotypes (Lee & Luo 1999). This situation often occurs due to genetic mutations during early development, after which

all the daughter cells arising from the mutated parent cell inherit the new genotype. Because we are all mosaics of random mutations that occur after cell damage or division, mosaicism is commonly used to refer to a specific gene or chromosome of interest.

Chimeras can differ significantly depending on the type and distribution of donor cells. In human transplants, the donated organs or cells are usually restricted to a specific region or tissue. After transplants or blood transfusions, the recipient becomes partially chimeric. However, if two embryos fuse early during development and produce one fetus, cells from both embryos can become evenly distributed throughout all tissues. The resulting fetus is a systemic chimera. On the opposite spectrum is microchimerism, in which a very small number of donor cells contribute to a host's tissues. This phenomenon is often seen in pregnancy, when a few fetal cells cross the placenta and enter the mother's bloodstream (Nelson et al. 1998). Although low in number, these cells may survive and engraft for decades.

In the context of artificial chimeras, primary chimeras and secondary chimeras are formed, depending on how, when, and what kind of cells are introduced. Traditionally, primary chimeras are formed if chimerism is initiated during early embryogenesis, often resulting in systemic contribution. In contrast, secondary chimeras are partially chimeric and are usually generated well after the onset of gastrulation (Mascetti & Pedersen 2016a).

Another important distinction is between intraspecies and interspecies chimerism, in which a chimera is composed of cells from the same species and different species, respectively. However, a zygote produced by mating or in vitro fertilization of two different species is not an interspecies chimera. After fertilization, the DNA from the egg and sperm joins into one nucleus of a single cell. The resulting zygote is a hybrid of the two species (Rossant et al. 1983). All the cells in a hybrid originate from one zygote and have the same genotype. Therefore, a hybrid is not a chimera.

Finally, the temporal and spatial properties of transplanted cells impact the level of chimerism. Human pluripotent stem cells (PSCs) are often injected into adult mice to assess their teratoma-forming capabilities. Because a teratoma is a tumor composed of multiple tissues not normally present at the site, the resulting chimera is termed heterotopic to reflect the abnormal anatomical location of the differentiating human cells. The chimera can also be classified as heterochronic following cell injection because PSCs represent an earlier, less restricted, developmental lineage that does not exist in somatic tissues. However, transplantation of cells into a tissue matching their own spatial origin and temporal origin would be considered orthotopic and isochronic, respectively (Yntema 1939).

Purpose

Chimera research is growing in popularity for both basic and translational applications. Developmental limitations of interspecies chimeras can be used to study species divergence or to probe signaling pathways in the context of evolutionary and developmental biology. Also, proof of cellular potency and function can be determined by generating chimeric organisms.

One significant translational use of experimental mammalian chimeras is disease modeling. Progression and treatment of human diseases cannot always be safely tested in humans, whereas mice may not perfectly model the disease of interest. However, partially chimeric mice with both mouse and human cells could be an effective model. Another promising use of chimeras is the production of human organs and blood in livestock or other large animals. These organs could potentially be used for drug testing or transplants, eliminating the need for organ donors. Interspecies organogenesis is discussed more extensively below in the section titled Interspecies Organogenesis.

METHODS TO GENERATE CHIMERAS

Primary Embryonic Cells

Some of the first systemic mammalian chimeras were created by mixing primary cells from embryos. Morula aggregation is a technique in which two or more morulae lacking their zona pellucidae are forced to contact in a dish. The resulting morulae fuse and form a chimeric blastocyst (Tarkowski 1961). A similar method involves aggregating isolated blastomeres from preblastulation embryos. Alternatively, the blastomeres can be injected into a morula or the subzonal space between the morula and zona pellucida of a second embryo. In all these methods, chimerism starts before the onset of blastulation, the first differentiation event.

Systemic chimeras can also be created after blastulation by mixing the inner cell mass (ICM) of a blastocyst with a second embryo (Gardner & Johnson 1973). This technique requires first removing the zona pellucida and trophectoderm to isolate the ICM. The ICM can then be microinjected into a different blastocyst or aggregated with another morula. Chimeras formed through ICM transfer differ from the chimeras produced with morula or early blastomere aggregation. Prior to blastulation, most of the cells in the embryo are considered totipotent. Aggregation of totipotent cells can result in chimeric trophectoderm and ICM, leading to chimerism in all extraembryonic tissues and the embryo proper, respectively. Chimeras formed by ICM transfer are restricted from trophectoderm engraftment and will therefore contribute less to the placenta and extraembryonic tissues than chimeras produced with morula or early blastomere aggregation (Rossant 1976). Thus, if an interspecies chimeric trophectoderm is found to inhibit implantation or development, totipotent aggregation methods may not be preferred (MacLaren et al. 1992). However, aggregation methods are technically easier; do not require micromanipulation equipment; and, depending on the species, may work better than injection methods (Tachibana et al. 2012).

Pluripotent Stem Cell Injection: Naive and Primed

Systemic chimeras can also be created by injecting PSCs into early embryos. In these experiments, PSCs are termed the donor cells, whereas the embryo is the host. Because PSCs functionally resemble the ICM and epiblast (Tang et al. 2010), PSCs have a limited contribution to extraembryonic tissues but can contribute to all tissues of the embryo proper. Relative to ICMs, genetic modification of PSCs is more routinely accomplished. Additionally, PSCs can be derived from specific individuals, whereas ICMs are usually derived from genetically unique blastocysts.

Functional quality of PSCs is often assessed by their ability to contribute to all tissues, including the germline, in an intraspecies chimera. Some mouse PSCs have been validated by using tetraploid complementation assays, in which the entire embryo proper is derived from donor cells (Nagy et al. 1993). These experiments also revealed that late-passage PSCs have less developmental potential than do early-passage PSCs, likely due to changes in the epigenetic landscape (Li et al. 2007). A few differentially methylated regions in the genome are especially sensitive to multiple passages (Zhong et al. 2015). This sensitivity can result in abnormal development or weak chimeric contribution in certain tissues. Because PSC quality and culture conditions may vary between species, each new cell line should be functionally evaluated until validation of robust derivation and culture condition.

Not all PSCs resemble the same developmental time point. Traditionally, cultured mouse embryonic stem cells (ESCs), a type of PSC, are derived from the preimplantation mouse epiblast. They require leukemia inhibitory factor (LIF) in the culture medium, grow in dome-shaped colonies, survive single-cell passaging, and readily produce systemic chimeras following blastocyst

injection (Nichols & Smith 2009). Mouse postimplantation epiblast stem cells (EpiSCs) are derived from the postimplantation epiblast. Although these cells are only a couple days later in development than are mouse ESCs, they do not respond to LIF, grow in a monolayer, often die during single-cell passaging unless supplemented with additional inhibitors, and do not form chimeras following blastocyst injection (Tesar et al. 2007). Mouse EpiSCs are said to be primed, to distinguish them from the more developmentally naive mouse ESCs. Primed and naive PSCs can differentiate into all three germ layers and form diverse teratomas, and thus they are classified as pluripotent.

Human ESCs are derived from the preimplantation human epiblast in a manner similar to that of mouse ESCs (Heins 2004) but are functionally more analogous to the primed state. Human ESCs are cultured with the same growth factors as are the primed mouse EpiSCs, die during single-cell passaging, and form colonies with flat morphology. Obvious ethical restrictions prevent scientists from evaluating human ESCs in vivo by creating a human chimera. However, human ESCs will likely fail to survive and integrate into a human blastocyst because both human ESCs and mouse EpiSCs fail to efficiently integrate into a mouse blastocyst. Traditionally cultured human PSCs are therefore considered to be primed. Multiple groups have published methods for resetting human cells into a naive-like state (see Ware 2016), although conclusive, functional validation based on primate blastocyst injection is difficult due to ethical restrictions.

Currently, production of chimeras via PSC microinjection requires a host embryo. However, advancements in stem cell biology may eventually enable the construction of artificial embryos by aggregating trophoblast stem cells or totipotent cells that self-assemble into a blastocyst (Harrison et al. 2017). Therefore, the present distinction between donor and host may become ambiguous if we stop employing primary embryonic cells and start synthesizing embryos.

Partial Chimeras

Partial chimeras can be created by injecting donor cells after the onset of gastrulation in the host (Wood et al. 2012). When donor cells are injected in utero, their type should match the corresponding developmental state of the embryo. This method of chimera formation offers several advantages over the formation of systemic chimeras. A high contribution of donor cells to all tissues may require additional ethical considerations, particularly when human cells contribute to the brain or germline of other mammals (Hyun et al. 2007). Partial chimerism may also be useful if a high degree of systemic chimerism between two species is embryonic lethal due to developmental disparities. In other cases, a researcher may want to model diseases in only one tissue or region.

Not all chimeras are created during embryonic development. In hospitals, allogeneic transplants and blood transfusions are creating chimeric people daily. However, human transplants are carefully screened to ensure minimal immune rejection by the new host. Interspecies partial chimerism therefore works best if initiated prior to the development of the host's immune system, decreasing the chance of donor cell rejection. Accomplishing this procedure is technically challenging because the cells must be delivered in utero to a specific location of the developing embryo or fetus without causing damage. In contrast, blastocyst injection using a micromanipulator is fast and relatively less invasive.

PROGRESS, PROBLEMS, AND LESSONS

Early Attempts

The first interspecies mammalian chimera was reported in the early 1970s, when Gardner & Johnson (1973) injected rat ICM cells into mouse blastocysts. Following embryo transfer into

a mouse uterus, they found significant chimerism in all three germ layers 3 to 5 days later. When Gardner & Johnson later tried aggregating mouse and rat morulae, implantation efficiency dropped.

Rossant & Frels (1980) produced the first full-term, interspecies mammalian chimeras with a high degree of systemic cell contribution. This was performed between two species of rodents by injecting *Mus caroli* ICMs into *Mus musculus* blastocysts and transferring the resulting chimeric embryos into an *M. musculus* uterus. Rossant later described the necessity of species matching the trophectoderm with the uterus. This point was highlighted when an *M. caroli* pup developed normally in an *M. musculus* mother—which was possible only after reconstituting an *M. caroli* ICM with *M. musculus* trophoblasts (Rossant et al. 1983).

The first interspecies goat-sheep chimeras were made in the 1980s (Fehilly et al. 1984). The ruminant results echoed those of rodents in that the trophectoderm and uterus must be from the same species for normal implantation and development. Although the overall phenotype of viable offspring was a blend of both goat and sheep characteristics, the dominant phenotype matched that of the maternal species in which the embryo developed.

Many believed that species matching of the trophectoderm and uterus was necessary to prevent maternal immune rejection of the invading interspecies trophoblasts. Although this belief was partially true, MacLaren et al. (1992) analyzed differences in placentation of sheep and goat embryos in a sheep-goat chimeric uterus. They concluded that there are differences in fetomaternal interactions between the two species and that such differences result in incompatible signaling near the time of implantation (MacLaren et al. 1992). This was the first clear sign of a xenogeneic barrier in a specific tissue that hinders the formation of interspecies mammalian chimeras.

Stage Matching

The most significant functional difference between naive mouse PSCs and primed mouse PSCs is that only the naive PSCs efficiently contribute to chimeras following blastocyst injection. However, it was subsequently found that primed mouse EpiSCs can form efficient chimeras if injected into postimplantation mouse embryos at E7.5 (Huang et al. 2012). At this later time point, mouse EpiSCs could engraft, proliferate, and differentiate into all three germ layers, whereas naive cells could not. If EpiSCs were injected a day later, at E8.5, the donor cells failed to engraft, likely because the host embryo started losing pluripotency. This observation highlighted the importance of isochronic cell injection and advanced the idea of stage matching.

Systemic primate-mouse chimerism was attempted by injecting or aggregating primate PSCs with mouse embryos. In most cases, few donor cells engrafted into the mouse ICM, and fewer cells persisted after implantation (James et al. 2006, Masaki et al. 2015, Simerly et al. 2011). However, like mouse EpiSCs, primed human PSCs were shown to efficiently engraft into the mouse postimplantation epiblast (Mascetti & Pedersen 2016b). This finding validated, for the first time, the pluripotent potential of primed human PSCs in vivo. Unfortunately, injecting directly into an epiblast in utero is technically challenging. The postimplantation PSC injections were all performed ex vivo and were analyzed after 1–2 days of whole-embryo culture. Thus, the utility of these chimeras is limited because we currently do not have methods of efficiently producing live pups after removing the implanted embryos from the uterus.

Since 2010, many groups have published methods for resetting human PSCs into a naive-like state (see Ware 2016). After injecting nearly 3,000 mouse embryos with various naive human PSCs, Theunissen et al. (2016) found that only 7 of the implanted embryos had human cells remaining at mid-gestation. Additionally, the ratio of human cells to mouse cells was often less than 1 out of 10,000. A similar experiment attempted to form a human-pig chimera (Wu et al.

Figure 1

Rat-mouse chimeras generated by pluripotent stem cell (PSC) microinjection. Rat-mouse chimeras generated by injecting PSCs into blastocysts survive into adulthood (*second from left* and *third from left*). Black and white contributions to coat color are from mouse and rat cells, respectively. The blastocyst will develop into viable offspring only when implanted into the same species' uterus. The size of the chimera conforms to that of the blastocyst and uterus. Therefore, the chimera's overall phenotype resembles either a mouse (*left*) if derived from a mouse blastocyst or a rat (*right*) if derived from a rat blastocyst.

2017). Again, although human cells were detected, their contribution was close to 1 in 100,000. This result reflects the 90-million-year evolutionary distance between humans and both mice and pigs (**http://timetree.org**) and suggests that a barrier to efficient systemic chimerism other than stage matching exists between species.

Evolutionary Distance and the Xenogeneic Barrier

Live rat-mouse systemic chimeras were first produced in 2010 by injecting rat PSCs into mouse embryos (Kobayashi et al. 2010). This development was remarkable due to the dissimilarities between the two species. Rats and mice evolutionarily diverged approximately 20 million years ago (MYA), more than double the divergence of goats and sheep (**http://timetree.org**). Additionally, rats are nearly ten times larger than mice, but the overall body size of rat-mouse chimeras tends to match that of the host blastocyst and the corresponding species in which they develop (**Figure 1**). Further analysis of rat-mouse chimeras revealed that the mouse fetuses with a high systemic contribution of rat cells had more developmental abnormalities, and the overall chimerism of live pups was usually less than 25%. Chimerism much greater than 25% in rat-mouse chimeras is likely embryonic lethal, suggesting the presence of a xenogeneic barrier that impedes the development of interspecies chimeras.

The effect of a xenogeneic barrier may be proportional to the evolutionary distance between two species. To test this theory, Sato and colleagues derived PSCs from a prairie vole and injected them into mouse embryos (T. Yamaguchi, H. Sato, T. Kobayashi, M. Kato-Ito & T. Goto, et al., manuscript in progress). Prairie voles diverged from mice more than 30 MYA (**http://timetree.org**). As expected, prairie vole–mouse chimeras had fewer donor cells and were more difficult to produce than rat-mouse chimeras.

In the context of cell signaling, a xenogeneic barrier could be due to mismatches between orthologous surface receptors, cytokine incompatibilities, or misaligned dose responses. These differences may be present only in certain tissues at key time points, leading to improper

migration, proliferation, and differentiation. In basic coculture experiments, we have found that PSCs from different species vary in their ability to form homogeneously mixed colonies, suggesting early discrepancies in cell contact and migratory cues. Although this simple assay may reflect manifestation of an in vitro xenogeneic barrier only at the pluripotent stage, other cell-intrinsic factors could present at later stages. For example, differentiation speed, doubling time, and cell division limits may contribute to an interspecies xenogeneic barrier if the two species vary significantly in developmental rate or size.

INTERSPECIES ORGANOGENESIS

Background

Takahashi et al. (2007) described the production of human induced PSCs (iPSCs) from adult fibroblasts. Because iPSCs have the potential to differentiate into any cell in the body, many researchers hope that iPSC technology could be used to manufacture patient-specific organs. These organs could have profound impacts in the field of regenerative medicine if they are used for autologous transplantation or disease modeling. Although some progress has been made to generate tissues and organoids, assembly of cells into functional organs in vitro has had limited success (Grapin-Botton 2016).

Chen et al. (1993) were the first to describe blastocyst complementation. When wild-type mouse ESCs were injected into blastocysts derived from *Rag2*$^{-/-}$ immunodeficient mice, chimeric pups had both B and T cells that were derived only from the injected ESCs. When immunoglobulin heavy chain joining (*JH*) gene segment–deficient ESCs were injected into *Rag2*$^{-/-}$ blastocysts, the chimeras had T cells but no B cells, indicating that the *JH* gene segment is essential for the development of B cells but not of T cells. Chen et al. developed this system to analyze the function of a gene for lymphoid development. From a stem cell biology viewpoint, one could conclude that the ESCs developed into T and B cells by using the vacant niche in the RAG2$^{-/-}$ environment. We extended this interpretation from lymphoid cell niche to organ niche and attempted an in vivo approach to organogenesis by using organogenesis-disabled hosts. By creating the organ niche, donor cells can receive developmental cues for organogenesis while limiting competition from host cells. This possibility was first tested in an allogeneic setting by using anephritic *Sall1*$^{-/-}$ mice complemented with ESCs. The resulting chimeric pups had normal kidneys, although they did not grow into adulthood due to a suckling defect (Usui et al. 2012). Interspecies organogenesis was accomplished later by injecting rat PSCs into a mouse blastocyst that was genetically modified to prevent pancreatic development (Kobayashi et al. 2010). At the onset of pancreatogenesis, the rat cells filled the empty niche, resulting in a mouse-sized pancreas entirely derived from xenogeneic cells. Ultimately, interspecies blastocyst complementation could be performed in large animals to make human-sized organs for transplantation (**Figure 2**).

Creating the Niche

Disabling the organ niche in a host is necessary to ensure full contribution of donor cells to the targeted organ. This approach is especially important with interspecies chimeras, in which the donor cells may have a selective disadvantage and are often low in number. Creating a niche allows the donor cells to proliferate without competition while placing selective pressure on embryos to allow for the development of xenogeneic organs.

Creating the niche can be done through genetic knockouts (KOs). PDX1 is a conserved mammalian transcription factor that is responsible for pancreatic development (Offield et al. 1996). Multiple *Pdx1*$^{-/-}$ mammalian species have been shown to develop normally in utero

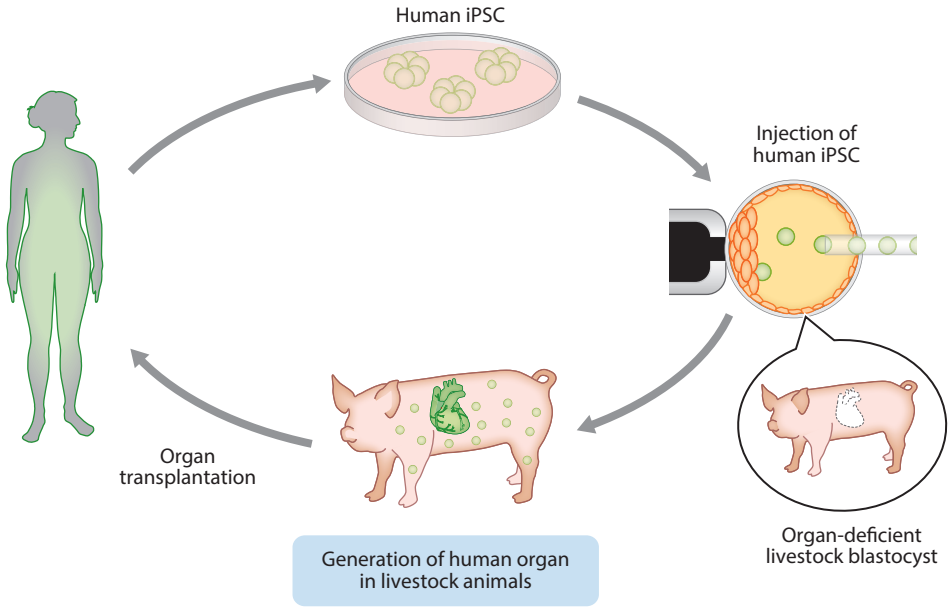

Figure 2

In vivo human organ generation cycle using blastocyst complementation. Human induced pluripotent stem cells (iPSCs) are derived from a patient in need of an organ. The iPSCs are injected into a livestock blastocyst that has been genetically modified to prevent development of the organ needed by the patient. The iPSCs create a chimera and complement the empty organ niche, generating an organ with the patient's cells. The organ is then transplanted into the patient.

without a pancreas. A KO phenotype will often manifest only after homozygous disruption. Thus, heterozygous animals can be maintained and crossed to produce many KO embryos with no additional gene manipulation. The disadvantage of this strategy is that only one in four of the embryos will have a homozygous KO.

Homozygous gene KO can also be efficiently achieved by using RNA-guided endonucleases or TALENs. Cytoplasmic injection of *Cas9* mRNA and the corresponding guide RNA into mouse zygotes has produced KOs with up to 80% efficiency (Yang et al. 2014). Such injection can be done with a single cut to produce a nonhomologous end joining–induced frameshift or with multiple cuts to remove a large exonic region. However, the efficiency may depend on the accessibility of the target locus and can vary with chromatin structure.

A transgenic knockin can be used to create an organ niche. Because PDX1 is expressed primarily during pancreatic development, a *Pdx1* promoter upstream of a suicide cassette will cause apoptotic elimination of the hosts developing pancreatic cells. The transgene can be inserted anywhere in the accessible genome because it operates in *trans* with *Pdx1*. An inducible suicide cassette can be used if the experimenter requires temporal control over apoptosis (Straathof 2005). However, suicide cassettes have the potential of inducing a bystander effect, in which the dying cells induce apoptosis or differentiation in nearby cells (Belyakov et al. 2002). Another potential drawback is that low levels of the target gene may be expressed in other tissues, during which the suicide cassette could damage unintended regions of the developing embryo.

Instead of using suicide cassettes, genes responsible for lineage bifurcation can be placed downstream of the targeted promoter. This was accomplished in pigs by inserting a *Pdx1* promoter sequence followed by the *Hes1* gene. Low and high *Hes1* expression acts as a determinant

switch for pancreatic and biliary differentiation, respectively (Sumazaki et al. 2003). The targeted expression of *Hes1* in early PDX1-positive cells prevented development of the pig pancreas (Matsunari et al. 2013).

Homozygous KOs permanently disrupt an endogenous gene, and such disruption cannot be undone by the organism. However, knockins, especially those that are integrated randomly in the genome, may suffer from silencing (Redberry 2006). Such silencing could lead to an incomplete emptying of the host organ niche, resulting in the unintended contribution of host cells to the targeted organ. Regardless of the method used, additional stress is placed on the zygote if physical manipulation is used to deliver gene editing components, which may result in a low development rate for some species. All these factors must be considered when one is creating the organ niche.

Complementing the Niche

Delivery of donor cells must be completed prior to the development of the targeted organ by creating either a systemic or a partial chimera. Because initiation of organogenesis happens early during development, manual cell delivery directly to the organ niche requires precise surgery on a miniscule embryo to achieve isochronic and orthotopic engraftment. This technique, termed conceptus complementation (Rashid et al. 2014), has not yet been used to successfully complement solid organs. Blastocyst complementation is an alternative approach in which a primary chimera is created at the preimplantation stage, leading to systemic donor cell engraftment. PSC injection may be preferred to ICM injection because PSCs are simple to maintain and genetically modify in culture, and iPSCs can be derived from specific patients. However, large-animal organogenesis has been achieved only using morula aggregation (Matsunari et al. 2013) and has not yet been accomplished in an interspecies manner.

Interspecies organogenesis via blastocyst complementation has been accomplished in mouse and rat hosts with reciprocal rat and mouse donor PSCs, respectively (Isotani et al. 2011, Kobayashi et al. 2010, Wu et al. 2017, Yamaguchi et al. 2017). The pancreas has been a frequent target due to its proven KO history with no other developmental consequences, as well as its translational appeal. Even though rats are nearly an order of magnitude larger than mice, the resulting pancreas is always the size of the host and is almost entirely derived from donor cells. Yamaguchi et al. (2017) went a step further and transplanted mouse pancreatic islets that were produced in a rat host into a diabetic mouse. Because 10–20% of the transplanted cells were found to be of rat origin, likely from supporting tissues that were chimeric, immunosuppression drugs were administered over 5 days to prevent acute graft rejection. Remarkably, the mouse transplanted with only 100 islets maintained normal blood glucose levels for more than a year without any further immunosuppression. When the graft was removed 260 days posttransplantation, there were no remaining rat cells. The results confirm the functionality and safety of organs generated in a xenogeneic environment.

For successful complementation, a minimum number of donor cells near the developmental niche are likely required (Stanger et al. 2007). These cells will receive the cues necessary for organogenesis. However, primary chimeras formed from highly divergent species suffer from low or no donor cell engraftment. Methods to enhance donor cell contribution while maintaining embryo viability are needed.

TARGETED ORGANOGENESIS

Problems with Systemic Chimeras

Creating systemic chimeras for interspecies organogenesis has both ethical and practical concerns. The contribution of human cells to an interspecies chimeric brain must be considered in the

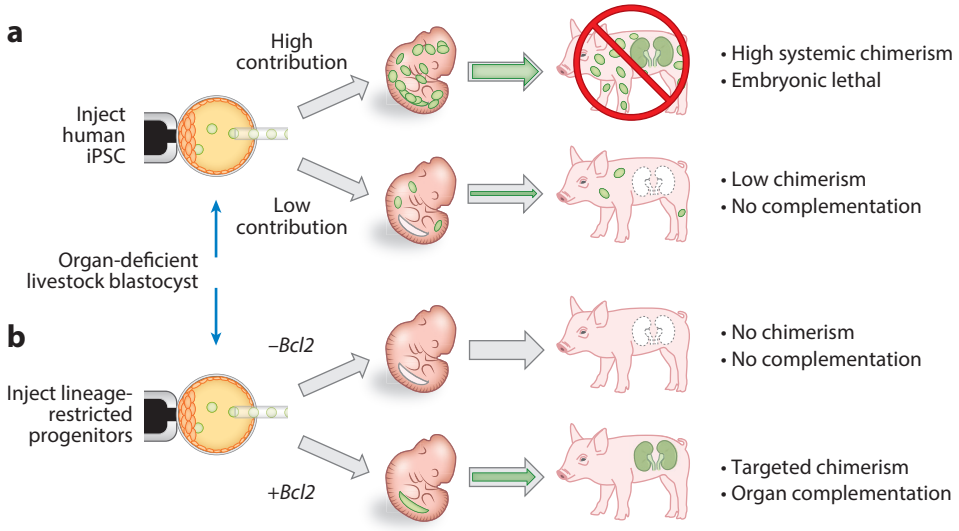

Figure 3

Targeted organogenesis. Human cells are injected into livestock blastocysts that have been genetically modified to prevent development of a specific organ (e.g., a homozygous disruption of *Sall1* to prevent kidney development). (*a*) Human pluripotent stem cells (PSCs) may create systemic chimeras. High chimerism could lead to embryonic lethality. If chimerism is low, there may not be enough cells in close proximity to the disabled organ niche to complement the organ. (*b*) Transient inhibition of apoptosis through *Bcl2* expression is necessary to enable lineage-restricted progenitor cells to survive after injection into the heterochronic environment of the blastocyst. The cells will later engraft in a region-specific manner. A high local concentration of cells may enable organ complementation without causing embryonic death.

context of human sentience. Although minimal gain in sentience is expected in a mouse brain with some human cells due to size and morphological restrictions, human cells composing a nonhuman primate brain warrant extra attention. Germline contribution is another concern if nonhuman mammals are complemented with human cells (Hyun et al. 2007, Rashid et al. 2014).

Practical problems arise when attempting blastocyst complementation in highly divergent species. Xenogeneic incompatibilities could exist between specific tissues during embryogenesis. Too many engrafted donor cells in a given tissue at a particular time point could therefore inhibit development (**Figure 3*a***). This scenario would create selective pressure in which only embryos with minimal chimerism would survive. Later in development during formation of the organ niche, the few remaining donor cells in surviving embryos might not be enough or in reasonable proximity for complementation (Stanger et al. 2007, Wu et al. 2017). These mutually exclusive requirements could prove uncompromising between certain species. Conceptus complementation should enhance complementation by delivering a high number of cells only to the niche, but it is inundated by the technical difficulties associated with precise in utero injection (Rashid et al. 2014). An alternative approach is to inject lineage-restricted cells into preimplantation embryos, thus targeting specific tissues for partial chimerism. Blastocyst complementation with this approach is termed targeted organogenesis.

Targeted Chimerism

Forced expression of differentiation factors can persuade donor PSCs to follow a targeted lineage. Transient expression of MIXL1 in blastocyst-injected mouse PSCs results in donor cell

contribution primarily in the endoderm (Kobayashi et al. 2015). Additionally, chimeric contribution in the endoderm is sometimes higher than in chimeras formed without forced MIXL1 expression. However, using this method, there is still potential for mild chimerism in other tissues. Another approach is to use inducible suicide cassettes that are activated in all donor cells except those in the targeted region. Although decreasing chimerism in nontargeted regions, this approach is not expected to increase chimerism in the region of interest. Additionally, massive cell death may inhibit further embryogenesis if chimerism is high prior to the activation of apoptosis. Because transgenes have the potential to be silenced, one cannot guarantee full lineage restriction or ablation by using these methods. A final approach could utilize multiple genetic KOs in the PSCs to restrict their ability to differentiate into the unintended tissues. Although this method may prove to be safe and efficient, it could be difficult to disable all the necessary genes while minimizing off-target effects.

As discussed above (in the section titled Progress, Problems, and Lessons), matching donor cells to their developing tissues in an isochronic manner seems necessary for robust chimerism (Mascetti & Pedersen 2016a,b). Masaki et al. (2015) discovered that the heterochronic injection of mouse EpiSCs into mouse blastocysts fails to make chimeras because of donor cell apoptosis. This problem can be overcome by transient expression of antiapoptotic genes, enabling EpiSCs to form efficient systemic chimeras following blastocyst injection. Masaki et al. then injected apoptosis-disabled, lineage-restricted progenitor cells into mouse blastocysts. Remarkably, these cells survived and contributed only to their corresponding lineages, although not always efficiently (Masaki et al. 2016). Because disabling apoptosis is transient, it has not yet led to tumor development.

Although the migration, survival, and proliferation of the heterochronic progenitors over multiple days in vivo are yet to be clarified, the evidence suggests the existence of a system by which stage-unmatched, outlier cells are eliminated by apoptosis. Although disabling apoptosis may increase progenitor cell survival, heterotopically located cells may fail to engraft when host embryogenesis catches up to the donor cell's developmental state, perhaps leading to low chimeric efficiency. Alternatively, the donor cells may lie dormant for a few days, after which they correctly migrate to their corresponding tissues during temporal synchronization. Understanding the underlying mechanism of engraftment is an extremely intriguing biological question.

CONCLUDING REMARKS

Summary

For many years, interspecies mammalian chimeras were considered difficult to generate. Early experiments revealed complications with interspecies placentation. However, use of PSCs with restricted trophectoderm development instead of morula aggregation has greatly enhanced chimera research. Generation of interspecies chimeras between evolutionarily close species, particularly rodents, is relatively easy, yet provides a useful tool to study mechanisms of organogenesis such as organ size, body size, and xenogeneic immunity.

Incompatibilities arising from a xenogeneic barrier could inhibit interspecies chimerism in some or all tissues at various developmental stages. Evolutionary distance may play a role in the xenogeneic barrier, resulting in chimeric inefficiency. This obstacle becomes clear during the formation of interspecies chimeras between the mouse and its closely related cousin, *M. caroli*, or between the mouse and a more distant relative, the rat. Following blastocyst injection, a healthy pup nearly 100% derived from *M. caroli* cells can be born from a mouse blastocyst. In contrast, a mouse embryo with much greater than 25% rat cells is generally not viable. A high host cell contribution may be essential to viability by ensuring that the embryo correctly follows developmental cues from

the placenta and uterus. Failure to properly interact with these developmental signals could result in resorption. Remarkably, complemented organ size also matches that of the host, suggesting that developmental morphology in organs is a cell-extrinsic property.

Targeted chimerism not only diminishes ethical concerns regarding human-animal chimeras, but also may enable chimerism between distant species by eliminating the detrimental effects of pervasive xenogeneic cell engraftment (**Figure 3*b***). However, chimeric inefficiency may limit the use of lineage-restricted, apoptosis-disabled progenitors until methods are firmly established.

Future Milestones

More than 100,000 people in the United States alone are awaiting solid organ transplant—1 in 5 will die within 5 years due to organ rejection or failure (NIDDK 2012, Organdonor.gov 2017). Those that survive require lifelong immune suppression, often leading to chronic infections and illnesses. Advances in stem cell and developmental biology may finally enable the production of patient-specific human organs in large animals via blastocyst complementation. These autologous transplants would require minimal immunosuppression and would not elicit graft-versus-host disease, a deadly problem during which residual T cells in an allogeneic transplant attack the recipient. One out of every 1,000 large animals currently destined for the food industry could eliminate the organ shortage (see **https://www.nass.usda.gov/Statistics_by_Subject/index.php?sector=ANIMALS & PRODUCTS**). However, a few milestones in interspecies organogenesis must first be reached.

Large-animal interspecies chimeras have not yet been created with PSCs. If successful, organ complementation should be attempted. These experiments may highlight the advantages and disadvantages of various large-animal hosts. Additionally, human PSCs have not efficiently created chimeras in rodent or large-animal hosts. Less divergent hosts could be explored to validate human PSC developmental potency. At minimum, chimerism and blastocyst complementation could be tried among nonhuman primates. Exploring the limitations of the xenogeneic barrier, as well as mitigating its effect by humanizing host embryos, also warrants consideration. Finally, complementation using lineage-restricted cells should be achieved to limit ethical concern and to possibly enhance viability.

Producing an organ in vitro, creating an artificial uterus, and attenuating the effects of aging have been distant dreams of many stem cell and developmental biologists. These paradigm-shifting accomplishments will require mastery of many developmental cues that may be hard to discover in a dish. By highlighting conserved signaling pathways and elucidating critical differences, these fantasies may approach reality with the lessons learned from interspecies mammalian chimeras.

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