L LECCIÓN CONMEMORATIVA
JIMÉNEZ DÍAZ

Madrid, 17 mayo 2018

"Medicina regenerativa, enfermedad y envejecimiento"

Juan Carlos Izpisua Belmonte
The Salk Institute for Biological Studies
La Jolla, California, EE.UU.

In Greek mythology, a chimaera is a fearsome fire-breathing beast composed of different parts of more than one animal, vividly depicted in Homer's Iliad as a lion-headed creature with the body of a goat and the tail of a serpent. In modern bioscience, chimaeras are entities made up of cells from different organisms. They are extremely valuable basic biology research tools with the potential for future clinical use. Experimental chimaeras generated from cells of more than one individual of the same species, particularly the mouse, have been widely used for many biomedical studies. Here, however, I will focus on mammalian chimaeras generated from different species, also known as interspecies chimaeras, which have recently garnered attention among researchers and the public, owing to their potential for providing replacement human organs. Interspecies chimaeras in mammals were first developed in the 1970s as tools to aid the study of cell lineage and cell fate during embryonic development.

These early studies paved the way for understanding evolutionarily conserved and divergent developmental processes in an interspecies setting in vivo. The derivation of pluripotent stem (PS) cells from early embryos, as well as from differentiated cells through cellular reprogramming, has renewed the interest in generating interspecies chimaeras.

Improvement in mouse ES cell culture eventually enabled the derivation of germline-competent rat ES cells, rekindling the interest in generating rat–mouse chimaeras. A milestone study by Nakauchi and colleagues reported the generation of viable adult rat–mouse chimaeras by the injection of rat PS cells into mouse blastocysts or by injection of mouse PS cells into rat blastocysts. Chimerism in interspecies fetuses varied among individuals and tissues, but was lower than that in the mouse–mouse or rat–rat intraspecies chimaeras. The production of viable rat PS-cell-derived rat–mouse chimaeras has been confirmed by other studies. We further showed that rat PS-cell-derived rat–mouse chimaera aged normally and lived an entire mouse life span (about 2 years). The higher chimaerism rate of rat PS cells versus rat ICMs suggest that rat PS cells may have growth advantages over rat ICMs, perhaps as a result of their in vitro culture. The phenotype of rat–mouse chimaeras provides several insights. First, the chimaeras born from rat surrogates were generally rat-sized and those born from mouse surrogates were mouse-sized unless there were high contributions by the xenogenic PS cells, a factor which was typically associated with morphological abnormalities and embryonic lethality. Second, the chimeric contribution of xenogenic cells seems to display lineage bias. Although a high percentage of mouse CD45+ cells were detected in rat-blastocyst-derived chimeric livers, rat CD45+ blood cells were rarely detected in mouse-blastocyst-derived chimeric fetal livers. This observation was specific to interspecies chimaeras, as the same rat PS cell line efficiently contributed to CD45+ cells in intraspecies chimeric fetal livers. Third, as mice have a gallbladder whereas rats do not, the presence or absence of the gallbladder in rat–mouse chimaeras appeared to be determined by the host species, suggesting that the donor PS cell derivatives are subject to regulation by the host programs that drive

organogenesis. Intriguingly, we recently found rat PS cells could contribute to mouse gallbladder, suggesting that the mouse embryonic microenvironment was able to unlock a gallbladder developmental program in rat cells that is normally suppressed during rat development. Whether or not high contributions of rat PS cells to mouse-blastocyst-derived chimaeras would interfere with mouse gallbladder development remains an interesting, unresolved issue.

Unlike rodents, germline-competent ES cells have yet to be isolated from other species. Stable ES cell lines could be derived from both nonhuman primate and human blastocysts; however, not one of these lines was able to generate intra- or interspecies blastocyst chimaeras. It was later realized that the rodent and primate ES cells represent different phases of pluripotency, naive and primed, respectively. Mouse ES cells represent the naive epiblast state, whereas epiblast stem cells (EpiS cells), another PS cell type derived from the post-implantation epiblast of rodent embryos, are primed for differentiation. Many defining features of EpiS cells were frequently found in primate ES cells, suggesting that primate ES cells were stabilized at the primed pluripotent state. Consistent with their post-implantation identity, mouse EpiS cells can engraft into egg cylinders in culture and differentiate into all three embryonic germ lineages, including primordial germ cells. Similarly, human PS cells grown in different primed cultures can also integrate into mouse gastrula-stage embryos and form ex vivo interspecies chimaeras. We have since shown that primed human PS cells could chimerize gastrula stage chick embryos as well. Thus, gastrula-stage embryos, rather than pre-implantation blastocysts, permit primed human PS cells to engraft and cross the xenobarrier.

With the recognition that primate ES cells correspond to the primed state, an unresolved question was whether human PS cells that were more similar to mouse ES cells could be obtained. Derivation of genuine naïve human ES cells, if achieved, would provide several practical advantages over primed cells, including improved single-cell cloning efficiency, ease of genome editing and the potential to generate interspecies chimaeras. The first successful attempt at generating mouse-ES-cell-like human ES cells came from a study by Jaenisch and colleagues in which the forced expression of Oct4 (also known as Pou5f1), Klf4 and Klf2 transcription factors was used to revert primed human ES cells to a more immature state. Although converted cells share many salient features of mouse ES cells, their long-term maintenance was still dependent on ectopic transgene expression. Following this pioneering work, a flurry of recent studies have reported conditions that produce stable, transgene-free immature human PS cells with molecular features resembling mouse ES cells. Some of these cultures have also been used for the de novo derivation of human ES cells from human blastocysts.

It has proven difficult to define the gold standard criteria for assigning the true naive human pluripotent state. Fan and colleagues used a systems biology approach to assess the gene networks of different PS cells and found that the

gene networks of mouse ES cells and EpiS cells were reproducible across all datasets examined; however, naive human PS cells grown in different cultures exhibited high degrees of variation. Naïve gene networks appeared to be poorly conserved between human and mouse PS cells, and both better resembled their respective blastocysts. Thus, a direct comparison of naive human PS cells to pre-implantation human embryos can potentially serve as a more reliable molecular criterion for defining the human naive pluripotent state. In this regard, a recent study showed that the gene expression profiles of naive 5i- and T2iLGöconditioned human PS cells better correspond to that of the human cleavage-stage embryo than cells produced by other protocols.

Validation of the naive state pluripotency in the mouse is achieved by demonstrating the functional contributions made by the mouse PS cells to blastocyst chimaeras. Owing to ethical considerations, the testing of human putative naive cells in such assays is limited to the use of animal host embryos, usually the mouse. Initial attempts at generating human–mouse chimeric embryos using naive human PS cells highlight the potential difficulties that lie ahead in establishing interspecies chimeric models. Hanna and colleagues reported the robust generation of embryonic day (E)10.5 human–mouse chimeric embryos using human PS cells grown in NHSM medium. By contrast, there were several other studies that contradicted this conclusion, outlining the inefficient, limited chimeric contribution of cells cultured in NHSM medium or other naïve conditions. Notably, though, non-human primate PS cells cultured in modified NHSM cultures were reported to contribute to the formation of pre-natal monkey–monkey and monkey–mouse chimaeras, albeit to a modest degree.

Humans and mice differ considerably in various aspects, including post-implantation epiblast development, embryo size, speed of development and gestational period. These and other differences may affect the integration, proliferation and differentiation of human PS cells in the chimeric epiblast. Interspecies chimaera research with large animal hosts that are more similar to humans in anatomy, physiology and organ size could result in an improved research model. Experiments evaluating the chimeric contribution of various human PS cells in large animal hosts are currently lacking. We have injected several existing human PS cell lines into pig blastocysts followed by embryo transfer to surrogate sows and results seem to agree with those from human–mouse chimeric studies. Interestingly, both rat and mouse PS cells, which can robustly generate interspecies chimaeras among rodents, failed in chimeric contribution to day E21–E28 pig embryos. Parallel attempts by Nakauchi and colleagues to inject human PS cells into sheep morulae/blastocysts have found a similarly limited human chimeric contribution 28 days after transfer into surrogate ewes.

To improve the degree of chimerism achieved from the transplantation of xenogeneic stem cells, especially PS cells, several important factors need to be considered.

Transplantation of donor cells into developmental-stage-matched (isochronic) host tissue may be critical for the efficient engraftment of cells into chimaeras. Mouse ES cells were able to gain entry into host development following blastocyst injection but failed to thrive in the post-implantation epiblast. Conversely, although mouse EpiS cells are inefficient at generating blastocyst chimaeras, they are capable of colonizing the E7.5, but not the E8.5, epiblast, a stage at which pluripotency has been lost. Similar observations have been made for human PS cells. Naive human PS cells were readily incorporated into mouse, pig and cow ICMs but later contributions to the developing fetuses were rare and inefficient. Conversely, primed human PS cells efficiently engrafted into E6.5 or E7.5 mouse epiblasts in vitro, but not pre-implantation mouse, pig and cow blastocysts. Several studies reported heterochronic chimaeras, in which cells of early developmental ages were able to respond to a later tissue environment. Gage and colleagues injected human ES cells into the lateral ventricle of E14 mice. The E14 mouse brain allowed some injected human ES cells to differentiate and generate mature, active human neurons in the adult mouse forebrain, unlike the post-natal brain, in which human ES cells generate teratomas. Shinohara and colleagues generated functional spermatozoa after transplantation of E6.5 epiblast or E8.5–E16.5 mouse fetal germ cells into the seminiferous tubules of a post-natal mouse testis. The reverse experiments, in which more advanced cells were returned to earlier-stage embryos, were less successful. Primordial germ cells isolated from post-implantation E8.5–E11.5 embryos did not contribute to chimaera formation following blastocyst injection. Several groups have reported the transplantation of various differentiated lineages—such as neural precursor cells, HSCs or mesenchymal stem cells—into blastocysts and have claimed that the injected cells contributed to the respective lineages. The validity of these findings has, however, not been confirmed and remains controversial.

Several strategies may confer a selective growth advantage to donor cells. First, an empty host niche strategy. If the host is genetically modified, compromising or eliminating the development of certain cell lineage(s) in embryogenesis, the recipient niche can be used exclusively by donor cells for their differentiation, proliferation and function. This strategy has been widely used for assaying HSC functions in vivo after the myeloablative irradiation of a donor’s bone-marrow stem-cell niche. Spermatogonial stem cells or primordial germ cells can be injected into the seminiferous tubules of a recipient W/WV mouse testis lacking endogenous germ cells for proper spermatogenesis. c-Kit-mutant Wsh/Wsh mice that lack melanoblasts are more permissive for the chimeric contribution of NCCs derived from rodent and human PS cells. Notably, this concept has also been adapted for the generation of xenogenic organs via interspecies blastocyst complementation.

Second, a cell-competition strategy. Cell competition was first studied in Drosophila in which cells carrying a Minute (also known as RpS17) mutation

were outcompeted by wild-type cells with metabolic advantages. Later studies in mammalian systems revealed that this process is universal and highly conserved. MYC-induced super competition constitutes another mode of cell competition, in which cells with higher MYC expression out-compete neighboring wildtype cells. Both types of cell competition have, thus far, only been examined in the intraspecies setting and their roles in interspecies chimaera formation await exploration. Overexpression of c-MYC in human-PS-cell-derived NCCs did not seem to give a more competitive edge for human cells, suggesting that super-competition conferred by c-MYC may not work across species. Zwaka and colleagues identified a network of genes whose downregulation confers embryonic cells with the ability to out-compete wild-type cells in development, a feature reminiscent of MYC-driven super-competition. Whether or not expression of these pro-competition genes would promote cross-species contributions remains to be determined.

Third, an enhancement of donor cell survival approach. Nakauchi and colleagues found cells from a subclone of EpiS cells could generate blastocyst chimaeras, in part owing to their resistance to cell death after injection into blastocysts. This led to further examination of whether the forced expression of the anti-apoptotic protein BCL2 might allow primed PS cells to engraft in blastocysts, survive and contribute to chimaeras. Indeed, BCL2-overexpressing EpiS cells also survived in mouse blastocysts and contributed to chimeric mice. Furthermore, when BCL2-expressing SOX17+ endoderm progenitors were injected into blastocysts, they also contributed to chimaera formation, but only to gut tissues. These results suggest that the prevention of apoptosis supports the survival of grafted progenitors in pre-implantation embryos and can extend the degree of heterochrony that can be tolerated in blastocyst chimaeras. Once the appropriate differentiation stage is reached, surviving progenitors will then take part in embryogenesis and follow their ordained developmental fate. The prevention of apoptosis is also a valid strategy for mouse–rat interspecies chimaera formation. For example, BCL2-expressing rat primed PS cells can contribute to chimaeras after injection into mouse blastocysts. BCL2 overexpression also promoted human ES cell survival in mouse embryos in vitro. Similar results were obtained with BCL2-overexpressing monkey ES cell lines in mouse embryos in vivo. Although the data are promising, it should be noted that the progeny of primate PS-cell derived cells were diverted towards the extraembryonic lineage. Thus, more experiments and optimizations are warranted before any definitive conclusions can be drawn.

Overall, the generation of interspecies chimaeras between mouse and rat is less efficient than the generation of intraspecies chimaeras. The cause of this ‘xenobarrier’ is not clear; however, there are several potential reasons, including ligand–receptor incompatibilities and differences in the affinity of adhesion molecules as a result of genetic diversification. To gain evolutionary insights into the xenobarrier, Nakauchi and colleagues generated induced pluripotent stem (iPS) cells from the prairie vole (Microtus ochrogaster), a rodent species that

diverged from M. musculus about 32.7 million years ago. When prairie vole iPS cells were injected into mouse embryos, interspecies chimaeras could be generated, but the embryonic development rate, degree of chimerism and survival to adulthood were lower than in mouse–rat chimaeras. These data imply that genetic diversification or evolutionary distance is at least partly responsible for the xenobarrier. In line with this, Jaenisch and colleagues observed a greater chimeric contribution of mouse NCCs to the mouse embryo than that made by rat NCCs, and rat NCCs showed more efficient engraftment than human NCCs.

Interspecies chimaeras are excellent experimental models for studying development, organismal homeostasis, stem-cell potential and disease. Recently, progress in several technological frontiers has opened new possibilities for interspecies chimaera research. Through genetic manipulation, the developmental niche(s) of the host animal can be made to serve donor cells exclusively, thereby generating organ- or tissue-enriched chimaeras, which at some stage could help provide much-needed organs could also serve as a platform for disease modeling and drug screening, providing in vivo readouts of disease onset and progression, drug efficacy and toxicity, with relevant clinical value.

This can be potentially approached with blastocyst complementation or targeted organ complementation. The ability to grow entire body parts for patients in the laboratory, as replacements for damaged or failing organs, is the holy grail of regenerative medicine. This quest took a considerable step forwards ten years ago with the discovery of iPS cells. Although efforts have been made to generate functional mature cells from iPS cells that target diseases that are potentially amenable to treatment by cell transplantation, other conditions, such as heart, kidney, liver and lung failure, require whole-organ replacement. Generating such complex, three-dimensional tissues from iPS cells once seemed impossible. In 2010, Nakauchi and colleagues reported the generation of a rat pancreas in mouse, using rat iPS cells to complement the pancreatic organ niche in mouse embryos that are unable to develop a pancreas. More recently, the same group succeeded in generating a mouse pancreas in Pdx1$^{-/-}$ rats. To evaluate functionality, mouse islets formed in rats were transplanted into mice with streptozotocin induced diabetes. Although the isolated islets contained some rat endothelial cells and other non-parenchymal cells, 100 islets transplanted under the mouse renal capsule normalized blood glucose levels over 370 days without immunosuppression. This proof-of-concept study indicates that organs generated from iPS cells in this manner can be just as functional as those from wild-type animals. In addition to pancreas, Isotani et al. complemented blastocysts from nude mice lacking a thymus with rat ES cells and generated a xenogenic rat thymus. Usui et al. tested the blastocyst complementation strategy for the generation of kidney tissue in Sall1-knockout mice. When mouse PS cells were used, a kidney was successfully generated. However, rat PS cells failed in this context, suggesting that key molecules involved in the interaction between mesenchyme and ureteric bud during kidney development might not be

conserved between mice and rats. Most recently, we broadened the utility of interspecies blastocyst complementation by combining CRISPR–Cas9-mediated zygote genome editing with the blastocyst injection of rat PS cells, thereby removing the need for existing mutant mouse strains. This strategy allowed successful complementation for several lineages, including pancreas, heart and the eye, among others.

These proof-of-concept studies could perhaps be expanded beyond rodents to larger animals, whose organs are much more similar in size to those of humans. That is, pig, sheep or monkey embryos may eventually be complemented with human stem cells to generate replacement organs for any part of the human body. Pig–pig blastocyst complementation has already been achieved by Nakauchi and colleagues. Pig fibroblasts overexpressing the transcriptional repressor HES1 under the pancreas specific PDX1 promoter were cloned to give rise to embryos carrying a PDX1-HES1 transgene. PDX1-HES1 expression suppresses the pancreatic development program, thus leading to the creation of pancreatogenesis-disabled pig embryos. Owing to the lack of chimaera competent pig PS cells, cloned blastomeres expressing the fluorescent protein huKO were used to complement the PDX1-HES1 embryos. These huKO+ blastomeres were able to contribute to chimaera formation, generating an entire huKO+ pancreatic epithelium. Moreover, the chimeric pigs generated by complementation were able to grow into adulthood with a functional pancreas. Whether or not human PS cells can generate xenogeneic organs in pigs remains unknown. Although similar in physiology and organ size, the evolutionary distance between human and pig (96 million years) is even greater than that between human and mouse (90 million years). Thus, the chimeric contribution made by human PS cells is expected to be low in pigs, consistent with recent findings. Choosing a species closer to humans, such as non-human primates, may be more likely to work, although the law in many countries prohibits such experiments. Developing strategies to improve human chimerism in a distant animal host as discussed above, could help to turn this goal into reality in the future.

One of the issues regarding in vivo organ generation in animals is ethical concern over potential gamete and neural contribution from PS cells. This issue could be addressed by developing methods of targeted organ generation, minimizing the likelihood of any undesired gameto- or neurogenesis. One approach is to modify PS cells, restricting their differentiation to the organ of interest. Conditional expression of the transcription factor gene Mixl1 made it possible to induce differentiation of PS-cell-derived cells towards the endodermal lineage, thereby reducing the number of non-endodermal PS-cell-derived cells. The introduction of constructs containing suicide genes under neural- or germ-cell-specific promoters to eliminate completely the formation of human iPS-cell-derived neural and germ cells in the host animal could be explored. Another approach is the use of committed progenitors or organ buds instead of PS cells. As discussed, the organ niche of organ-deficient embryos is available for

complementation throughout development of the organ. Therefore, injecting lineage committed progenitor cells, (such as cultured nephron progenitor cells, rather than PS cells, into the embryo at the right place and time may allow complementation of organ deficiency with little chance of generating 'off-target' humanized tissues. If the host organ niche is emptied by genetic manipulations, donor human progenitor cells will probably be able to colonize it, giving rise to a human-cell-enriched organ. Although theoretically this is a sound strategy and potentially reduces the ethical concern, there are caveats: the developmental stages need to be matched between human progenitors and the host animal fetus; delivering human cells before the host organ becomes atretic is likely to be critical and challenging; and delivery of human progenitor cells needs to be performed before the host immune system is formed (less than 50 days in pigs).

An important goal of experimental biology is the development of model systems that allow for the study of human diseases under in vivo conditions. Transgenic animals have been successful in modeling a variety of human diseases but have failed to provide disease-appropriate phenotypes for monogenic diseases such as Lesch–Nyhan syndrome or of complex diseases such as Parkinson's or Alzheimer's. Given that disease-specific iPS cells carry all genetic alterations that contributed to disease pathology, the functional integration of patient-derived cells into the tissues of the developing mouse embryo would allow for study of the initiation, progression and manifestation of the disease in question. Host-specific developmental and physiological programs may alter the behavior of donor human cells in a non-human host. Nevertheless, the ability to study the autonomous versus non-autonomous effects of the multiple genetic alterations that contribute to a human disease under in vivo conditions cannot be dismissed.

Compared to existing drug screening platforms, which often involve the use of patient samples and immortalized cell lines and/or in vivo transgenic mouse models, the use of interspecies chimaeras with human stem cells could potentially offer a superior in vivo drug-screening platform. Interspecies chimeric formation using human iPS cells or iPS-cell derived progenitors offers an attractive platform for the personalized in vivo testing for drug efficacy and toxicity. This approach holds the potential to be a robust preclinical testing platform for more accurate predictions of clinical outcomes.

Notwithstanding any ground-breaking advances in generating animal interspecies chimaeras, there are currently more questions than answers concerning human–non-human chimaeras. It is not known whether more extensive chimerism can be obtained between humans and other more related species, nor is it known whether human organs generated in chimaeras, with host nerves and vasculature, will be suitable for transplantation. In this regard, strategies such as the humanization of host animals and/or multi-lineage complementation may help. Fundamental problems remain, such as how best to resolve heterochronic developmental processes and inherent differences in gestation length between humans and other species; whether or not xeno-

generated human cells, tissues and organs would be functionally compatible with human physiology; and whether it is possible to generate human cells that are able to compete equally with host cells.

The development of strategies to enhance the degree of human–animal chimerism, such as those described here, will be necessary if interspecies chimeras are to reach their full biological and clinical potential. Engineering approaches to confer human stem cells with novel genetic circuits that monitor and control specific cell behaviors may help to overcome some of the present human–animal interspecies barriers. Similarly, engineering approaches to humanize animal models may help to lower the threshold of xenotransplantation. If successful, these new approaches, rooted in the fields of cell and developmental biology, may expand the breadth of chimaera research from the laboratory into potential clinical applications. This could aid the development of new drug screening, efficacy and toxicity methodologies, as well as the creation of disease models that may ultimately enhance diagnosis and improve the treatment of numerous pathologies.

Although the way ahead has many scientific, medical, ethical, political, financial, and other challenges and not everything that can be done in the field of chimaera research should be done, we owe it to future generations of patients and scientists to think about these challenges and experimentally proceed forward with consensual ethical, legal and social guidelines.