Stem cells and interspecies chimaeras

Stem Cells and Interspecies Chimeras: Past, Present, Future

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PREFACE

Chimeras are both an ancient imagination and a long-established research tool. Recent advances, particularly those dealing with the identification and capture of various kinds of stem cells, have broadened the repertoire and utility of mammalian interspecies chimeras and carved out new paths towards understanding fundamental biology as well as potential clinical applications.

MAIN TEXT

In Greek mythology, a chimera is a fearsome fire-breathing beast composed of parts of more than one animal, vividly depicted in Homer's *Iliad* as a lion-headed creature with a goat body and a serpent's tail. In modern bioscience, chimeras are entities made up of cells from two different organisms, extremely valuable basic biology research tools, with the potential for clinical applications. Experimental chimeras generated from cells of more than one individual of the same species, particularly in the mouse, have been instrumental and widely used for many biomedical studies. Here, however, we focus on mammalian chimeras generated from different species, also known as interspecies chimeras (See Box 1 for related terminologies), which has recently garnered attention among researchers and the pubic due to its potential for providing replacement human organs.

This review will provide a historical perspective, followed by a summary of recent advances in stem cell-derived interspecies chimeras and their potential applications. Next, we will discuss ethical guidelines, current policies, and impacts on society. Finally, we will conclude with an outlook for research in this and related areas. Interspecies chimera-related research covers a broad range of topics. Due to limited space, here we will focus on studies that deliver donor stem cells to pre-natal host embryos and fetuses. Research on conventional xenotransplantation approaches involving the introduction of donor cells into the postnatal animal has been reviewed elsewhere[1].

EARLIER STUDIES ON INTERSPECIES CHIMERAS

Interspecies chimeras in mammals were first developed in the 1970s as tools to aid the study of cell lineage and cell fate during embryonic development. Prior to the age of transgenic reporter

lines there was a need to explore other ways of identifying the origin of the different cell types brought together in a chimera. Building on the pioneering work of Le Douarin and colleagues using chick-quail chimeras[2,3], Gardner and Johnson were the first to test the species boundaries in mammals with rat-mouse chimeras[4]. When rat inner cell masses (ICMs) were injected into mouse blastocysts and transferred to the mouse uterus, the detailed distribution of the rat-derived cells could be observed in sections of the fetuses using species-specific antibodies. However, when the chimeras were left to go to term, the resulting offspring had very few remaining rat cells detectable. The reason for this loss is still not clear, but it was suggested that different developmental rates between two species that are evolutionarily separated about 17.9 million years ago (MYA) might lead to selection against the rat cells.

Rossant and co-workers went on to generate interspecies chimeras between two more closely related murine species, *Mus musculus* and *Mus caroli*. Live chimeras with contributions from both species were produced by injection of *M.caroli* ICMs into *M. musculus* blastocysts[5]. They then used satellite DNA differences between the two species and DNA-DNA *in situ* hybridization to provide a detailed description of the tissue types derived from the ICM and the trophectoderm of the blastocyst[6]. Following this success, other viable interspecies chimeras were also generated, including sheep-goat[7] and *Bos taurus-Bos indicus*[8].

Successful production of interspecies chimeras is highly dependent on matching the species origin of the trophectoderm derivatives and the maternal uterus. For example, *M.caroli* blastocysts cannot survive beyond early postimplantation in the *M.musculus* uterus, but it is actually possible to produce viable *M.caroli* offspring from a *M.musculus* mother when a blastocyst is reconstituted with M.*musculus* trophectoderm and *M.caroli* ICM[9,10]. This suggests that species boundaries can be extended considerably, provided that the interspecies combination is confined to the ICM derivatives.

These earlier studies opened the door for understanding evolutionarily conserved and divergent developmental processes in an interspecies setting *in vivo*. The derivation of pluripotent stem cells (PSCs) from early embryos as well as from differentiated cells through cellular reprograming has renewed the interest in generating interspecies chimeras[11]

STEM CELLS AND INTERSPECIES CHIMERAS

The first PSC-derived interspecies chimeras were generated by Lahn and co-workers by injecting *Apodemus sylvaticus* embryonic stem cells (ESCs) into mouse blastocysts. Despite *A.sylvaticus* diverged from mouse about 11.4 MYA, viable chimeras were obtained that contained significant contribution (up to 40% in some tissues) of *A.sylvaticus* cells to all major organs, including germ cells in mice[12]. Intriguingly, *A.sylvaticus*-mouse chimeras displayed features intermediate between *A.sylvaticus* and mouse including eye-size and jumpiness, albeit these phenotypes were not statistically quantified[12]. An intriguing question is whether interspecies chimeras can retain behavioral traits characterized of the donor species. A study by Goldman and colleagues has suggested that extensive contribution of human glial progenitors after grafting into the postnatal mouse forebrain, could alter the cognitive capability of the host[13].

Improvement in mouse (m) ESC culture eventually enabled the derivation of germline competent rat (r) ESCs[14-16], rekindling the interest in generating rat-mouse chimeras. A milestone study by Nakauchi and colleagues generated viable adult rat-mouse chimeras by injection of rPSCs into m-blastocysts or by injection of mPSCs into r-blastocysts. Chimerism in interspecies fetuses varied among individuals and tissues but was lower than that in mouse-mouse or rat-rat intraspecies chimeras. rPSC-derived viable rat-mouse chimeras have been confirmed by the Okabe[17] and Izpisua Belmonte laboratories (Wu et al., Unpublished data). The higher chimerism rate of rPSCs versus rICMs suggest that rPSCs may have growth advantages over rICMs, perhaps as a result of

in vitro culture . The phenotype of rat-mouse chimeras allows several conclusions: 1) The chimeras 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 PSCs, which were typically associated with morphological abnormalities and embryonic lethality[18]. 2) Chimeric contribution of xenogenic cells seems to display lineage bias. Kobayashi et al. observed that while a high percentage of mouse CD45+ cells were detected in r-blastocyst-derived chimeric livers, rat CD45+ blood cells were rarely detected in m-blastocyst-derived chimeric fetal livers. This observation was specific to interspecies chimeras as the same rPSC line efficiently contributed to CD45+ cells in intraspecies chimeric fetal livers[18]. 3) Rats do not have a gallbladder while mice do. Presence or absence of the gallbladders in rat-mouse chimeras appeared determined by the host species, suggesting that the donor PSC-derivatives are subject to regulation by the host programs that drive organogenesis. Whether high contributions of rPSCs to m-blastocyst-derived chimeras would interfere with the mouse gallbladder development remains an interesting and unresolved issue.

Unlike rodents, germline-competent ESCs have yet to be isolated from other species. Stable ESC lines have been derived from non-human primate (NHP) and human (h) blastocysts[19-24]. However, none of these lines were competent to generate intra- or inter-species blastocyst chimeras [25-27]. It was later realized that rodent and primate PSCs represent different phases of pluripotency, naïve versus primed respectively[28]. Mouse ESCs represent the naïve epiblast state[29] while epiblast stem cells (EpiSCs), another PSC type derived from the post-implantation epiblast of rodent embryos[30,31] are primed for differentiation. Many defining features of EpiSCs were frequently found in primate PSCs, suggesting that primate PSCs were stabilized at the primed pluripotent state. Consistent with their post-implantation identity, EpiSCs can engraft into post-implantation egg cylinders in culture and differentiate into all three embryonic germ lineages, including primordial germ cells (PGCs)[32-34]. Likewise, primed hPSCs grown in different culture conditions could also integrated into mouse gastrula stage embryos and formed *ex vivo* interspecies chimeras[34,35]. Izpisua Belmonte and colleagues further showed that primed hPSCs could chimerize gastrula stage chick embryos as well (Wu et al., unpublished data). Thus gastrula stage embryos, rather than pre-implantation blastocysts, are permissive for primed hPSCs to engraft and cross the xenobarrier[36,37].

With the recognition that primate ESCs correspond to the primed state, an unresolved question was whether hPSCs more similar to mESCs could be obtained. Derivation of genuine naïve hESCs, if achieved, could provide several practical advantages over primed cells, including improved single cell cloning efficiency, ease of transfection and the potential to generate interspecies chimeras. The first successful attempt of generating mESC-like hESCs came from a study by Jaenisch and colleagues where forced expression of Oct4, Klf4 and Klf2 factors was used to revert primed hESCs into a more immature state. Although converted cells share many salient features of mESCs, their long-term maintenance was still dependent on ectopic transgene expression[38]. Following this pioneering work, a flurry of recent studies reported conditions that can stabilize transgene-free hESCs with molecular features resembling mESCs[39-47]. Some of these cultures have also been used for *de novo* hESCs derivation from human blastocysts[41,43,46,47].

It has proven difficult to define the gold standard criteria for assigning the true naïve human pluripotent state[48]. Fan and colleagues took a systems biology approach and assessed the gene networks in PSCs from both mouse and human[49]. They found that gene networks of mESCs and EpiSCs were reproducible across all datasets examined, however, naïve hPSCs in different cultures exhibited high degrees of variation. Naïve gene networks appeared not well conserved between human and mouse PSCs, and both better resembled their respective blastocysts[49]. Thus, direct comparison of naïve hPSCs to pre-implantation human embryos can potentially serve as a molecular criterion for defining the human naïve pluripotent state. In this regard, a more recent study showed gene expression of the naïve 5i- and T2iLGÖ- hPSCs, but not of cells produced by other protocols, correspond to that of the human cleavage stage embryo[44].

Validating the naïve state in the mouse is achieved by demonstrating functional contributions in blastocyst chimeras. Due to ethical considerations, testing human putative naïve cells in such assays is limited to the use of animal host embryos, usually the mouse. Initial attempts at generation of human-mouse chimeric fetuses using naïve hPSCs have shown the difficulties that may lie ahead in establishing interspecies chimeric models. Hanna and colleagues reported the generation of E10.5 human-mouse chimeric embryos with hPSCs grown in NHSM medium[41]. In contrast, several other studies contradict those conclusions, with very inefficient and limited chimeric contribution of cells cultured in NHSM medium or other naïve conditions[42-44,50]. Intriguingly, however, NHP PSCs cultured in modified NHSM cultures were reported to contribute, albeit at a modest degree, to the formation of prenatal monkey-monkey and monkey-mouse chimeras[51,52].

The criteria used to evaluate chimerism were exclusively based on the presence of fluorescently labeled human cells in most studies. There are several potential problems associated with donor cell detection by fluorescence markers, especially when chimerism is low, including auto fluorescence and uptake of markers from dying cells. A recent study by Theunissen et al[44] used both fluorescence detection and a quantitative qPCR analysis of human mitochondrial DNA, a sensitive assay with a detection limit of 1 human cell in 10,000 mouse cells. They analyzed 1,400 putative chimeric fetuses and found only 7 chimeric fetuses containing 1 to at most 4 human cells per 10,000 mouse cells. While absence of human mitochondrial DNA is a strong negative result, a positive signal does not indicate functional integration of donor cells as the DNA can be derived from dead or lysed cells. Similarly, presence of GFP positive donor cells alone is not definitive proof of proper chimeric contribution as hPSC-derived cells often appear as a cluster and not fully integrated into animal tissues. Therefore definitive proof for human-animal chimera formation requires evidence of functional contributions

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 hPSCs. Interspecies chimera 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 to empirically test and evaluate the chimeric contribution of various hPSCs in large animal hosts are currently lacking. Izpisua Belmonte and colleagues have injected several existing hPSCs into pig morulae/blastocysts followed by embryo transfer to surrogate sows, however, preliminary results seem to resonate with the results from human-mouse chimeric studies (Wu et al., Manuscript in preparation). In parallel, Nakauchi and colleagues have injected hPSCs into sheep morulae/blastocysts and similarly observed limited chimeric contribution 28 days after transfer into surrogate ewes (Rashid et al. Unpublished data).

In addition to PSCs, developmentally more restricted stem cells or progenitors are also amenable for chimera formation Neural crest cells (NCCs), a multipotent embryonic progenitor cell population, are first specified in the neural plate border region between the neural epithelium and epidermis during neurulation stages[53]. Jaenisch and colleagues injected NCCs derived from E8.5 mouse embryos as well as from mESCs into the amniotic cavity of E8.5 mouse embryos in utero, where the NCCs could enter the neural tube through the open neuropore, thereby contributing to chimeric pigmentation formation in postnatal mice[54,55]. More recently, the authors also generated interspecies NC chimeras after transplanting PSC derived rat and human NCCs into E8.5 mouse embryos in utero. Importantly, both live rat-mouse and human-mouse NC chimeras were born and donor cells matured to functional melanocytes and contributed to visible hair pigmentation in the adult mouse[56].

Human neural stem cells (NSCs) have also been transplanted to rat fetuses for the generation of interspecies chimeric brains[57,58]. Transplanted human NSCs displayed wide chimera contribution to the rat CNS system and gave rise to neurons, astrocytes and oligodendrocytes. Human hematopoietic stem cells (HSCs) have been routinely transplanted into immunodeficient mice to assess their function and differentiation capacity[59]. However, these are mostly from post-natal injection. Human-sheep and human-pig hematopoietic chimeras generated via *in utero* transplantation of human CD34+ hematopoietic stem progenitor cells have been reported but the contribution of human blood cells was limited in these experiments probably because host HSCs competed with transplanted cells for the niche[60,61].

A summary of chimeras generated using different types of cultured stem cells is provided in Fig. 1.

FACTORS INVOLVED IN INTERSPECIES CHIMERISM

To improve the degree of chimerism from transplanted xenogeneic stem cells, especially PSCs, several important factors need to be considered (Fig. 2).

Matched developmental timing. Transplantation of donor cells into developmental stage matched (isochronic) host tissue may be critical for efficient cell engraftment into chimeras. mESCs could gain entry into host development following blastocyst injection but failed to thrive in the post-implantation epiblast[32]. Conversely, while EpiSCs are inefficient at generating blastocyst chimeras, they are capable of colonizing E7.5 but not E8.5 epiblast, a stage when pluripotency has been lost[32]. Similar observations have been made with hPSCs. Naïve hPSCs were readily incorporated into mouse[41,42,50] pig and cow ICMs but later contributions to the developing fetuses were rare and inefficient[42-44,50](Wu et al., Manuscript in preparation). On the other hand, primed hPSCs efficiently engrafted into E6.5 or E7.5 mouse epiblasts *in vitro*, but not pre-implantation mouse blastocysts[27,34,35]. A number of studies reported heterochronic chimeras in which cells of early developmental age were able to respond to a later tissue environment. Gage and colleagues injected hESCs into the lateral ventricle of E14 mice. Unlike post-natal brain where hESCs generate teratomas, the E14 mouse brain allowed some injected hESCs to properly differentiate and generate mature and active human neurons in the adult mouse forebrain[62]. Shinohara and colleagues generated functional spermatozoa after transplanting E6.5 epiblast or E8.5-16.5 fetal germ cells into the seminiferous tubules of postnatal mouse testis[63]. The reverse experiments, in which more advanced cells were returned to earlier stage embryos, seems less successful. PGCs isolated from post-implantation E8.5-11.5 embryos did not contribute to chimera formation following blastocyst injection[64]. Several groups have reported transplanting various differentiated lineages, such as neural precursor cells, HSCs or mesenchymal stem cells, into blastocysts claiming that the injected cells contributed to the respective lineages[65-68]. However, the validity of most of these findings has not been confirmed and remains controversial.

Providing selective advantage for donor cells. Several strategies may confer selective growth advantage to donor cells: 1) **Empty host niche.** If the host is genetically modified so that development of certain cell lineage(s) are compromised or eliminated from embryogenesis, the recipient niche can be exclusively utilized by donor cells for their differentiation, proliferation and function. This strategy has been widely used for assaying HSC functions *in vivo* after ablation of the donor's bone marrow niche, by myeloablative irradiation[69]. Spermatogonial stem cells (SSCs) or PGCs can be injected into seminiferous tubules of the recipient W/W^{V} mice testis lacking endogenous germ cells for proper spermatogenesis[63,70,71]. c-Kit mutant W^{sh}/W^{sh} mice that lack melanoblasts are more permissive for chimeric contribution of NCCs derived from rodent and human PSCs[56]. Importantly, this concept has also been adapted for the generation of xenogenic organs via interspecies blastocyst complementation[17,18]. 2) **Cell competition**. Cell competition was first studied in *Drosophila* where cells carrying a *Minute* mutation were outcompeted by wild-type cells with metabolic advantages[72]. Later studies in mammalian systems revealed that this process

is universal and highly conserved[73]. ,Myc-induced super-competition constitutes another mode of cell competition whereby cells with higher MYC expression outcompete neighboring wild-type cells[73]. Both types of cell competition have thus far only been examined in the intraspecies setting and their roles in interspecies chimera formation await exploration. Overexpression of c-MYC in human PSC-derived NCCs did not seem to give a more competitive edge for human cells, suggesting super-competition conferred by c-MYC may not work across species[56]. 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[74]. It remains to be seen whether expression of these pro-competition genes would promote cross-species contributions. 3) **Enhancement of donor cell survival**. Masaki *et al.* serendipitously found cells from a subclone of EpiSCs could generate blastocyst chimeras, in part due to their resistance to cell death after injection into blastocysts. This led to further examination of whether forced expression of an anti-apoptotic protein BCL2 might allow primed PSCs to engraft in blastocysts, survive and contribute to chimeras. Indeed, BCL-2-overexpressing EpiSCs also survived in mouse blastocysts and contributed to chimeric mice (Masaki *et al*., in revision). Even more surprisingly, when BCL2-expressing Sox17+ endoderm progenitors were injected into blastocysts, they also contributed to chimera formation but only to gut tissues (Masaki *et al.*, in revision). These results suggest that prevention of apoptosis supports survival of grafted progenitors in pre-implantation embryos and can extend the window of heterochrony that can be tolerated in blastocyst chimeras. Once reaching the appropriate differentiation stage, survived progenitors will take part in embryogenesis and follow their ordained developmental fate. Prevention of apoptosis also works for interspecies chimera formation between mouse and rat, *e.g.,* BCL2-expressing rat primed PSCs can contribute to chimeras upon injection into mouse blastocysts. BCL2 overexpression also promoted hESC survival in mouse embryos *in vitro*. Similar results were obtained with BCL2-overexpressing monkey ESC lines in mouse embryos *in vivo* (Masaki *et al.*, in revision). While the data are promising, it is worth noting that the progeny of primate PSC-derived cells was diverted towards the extraembryonic lineage. Thus, more experiments and optimizations are warranted before drawing any definitive conclusion.

Evolutionary considerations Overall, generation of interspecies chimeras between mouse and rat is less efficient than generation of intraspecies chimeras. The cause of this "xenobarrier" is not clear. Several possibilities exist, including incompatibilities in ligand-receptor combinations, differences in the affinity of adhesion molecules as a result of genetic evolution resulting from genetic diversification. To gain evolutionary insights into this "xenobarrier", Nakauchi and colleagues generated iPSCs from prairie vole, a rodent species that diverged from *M. musculus* about 30.4 MYA. When these prairie vole-derived iPSCs were injected into mouse embryos interspecies chimeras could be generated but embryonic development rate, degree of chimerism and survival to adulthood were lower than those in mouse-rat chimeras (Sato and Yamaguchi, manuscript in preparation). These data imply that genetic diversification or evolutionary distance is at least in part responsible for the "xenobarrier." In line with this, Jaenisch and colleagues observed more chimeric contribution of mouse NCCs to the mouse embryo than rat NCCs, and rat NCCs showed more efficient engraftment than human NCCs[56].

POTENTIAL APPLICATIONS

Interspecies chimeras are excellent experimental models for studying development, organismal homeostasis, stem cell potential and disease. More recently, progress in several technological frontiers has opened new possibilities for interspecies chimera research. Through genetic manipulation, the developmental niche(s) of the host animal could be brought to service exclusively for the donor cells, thereby generating organ/tissue-enriched chimeras, which bodes hope for solving the severe shortage of organ donors. Interspecies chimeras with human stem cell contributions could also serve as a novel platform for disease modeling and drug screening, providing *in vivo* readouts of disease onset and progression, drug efficacy and toxicity, with

relevant clinical value (Fig. 3)[11].

Organ generation: This can be potentially approached with blastocyst complementation or targeted organ complementation.

1) Interspecies blastocyst complementation. A holy grail for regenerative medicine is to grow entire body parts for patients in the laboratory as replacements for damaged or failing organs. This quest took a significant step toward fulfillment ten years ago with the discovery of iPSCs[75]. While efforts have been made to generate functional mature cells from iPSCs targeting diseases that are potentially amenable to treatment by cell transplantation, other diseases such as heart, kidney, liver and lung failure require whole organ replacement. Generating such complex, 3-dimensional tissues from iPSCs once seemed impossible. In 2010, Nakauchi's team reported the generation of a rat pancreas in mouse by complementing with rat iPSCs the pancreatic "organ niche" in mouse embryos lacking the ability to develop a pancreas[18]. More recently, his group succeeded in generating mouse pancreas in *Pdx1*-/- rats (Yamaguchi et al. submitted). They isolated mouse islets formed in rats and transplanted them into mice with streptozotocin-induced diabetes. Despite the isolated islets contained some rat endothelial and other non-parenchymal cells, 100 islets transplanted under the mouse renal capsule normalized blood glucose levels over 370 days without immunosuppression (Yamaguchi et al. submitted). This proof-of-concept study clearly indicates that the organs generated in this manner from iPSCs are just as functional as those from wild-type animals. Similar observations were made by Isotani *et al*[17], who complemented blastocysts from nude mice that lacked a thymus with rESCs, thereby generating a functional xenogenic rat thymus. Blastocyst complementation has also been tested for the generation of kidney in mouse with a *Sall1* knockout background. When mPSCs were used, a kidney was successfully generated, however, riPSCs 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[76].

These proof-of-concept studies could perhaps be expanded beyond rodents to larger animals whose organs are very 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 been achieved by Nakauchi and colleagues[77]. Pig fibroblasts overexpressing HES1 under the *Pdx1* promoter were cloned to give rise to embryos carrying a *Pdx1-Hes1* transgene. *Pdx1-Hes1* expression suppresses the pancreatic program, thus leading to the creation of pancreatogenesis-disabled pig embryos. Due to the lack of chimera-competent pig PSCs, cloned blastomeres expressing the fluorescent protein huKO were used to complement the *Pdx1-Hes1* embryos. These huKO-expressing blastomeres were able to contribute to chimera formation and generated an entire huKO+ pancreatic epithelium. Moreover, the chimeric pigs generated by complementation were able to grow into adulthood with a functional pancreas. Whether hPSCs can generate xenogeneic organs in pig remains an open question. Although similar in physiology and organ size, the evolutionary distance between human and pig (95 million years) is even greater than that between human and mouse (90 million years). Thus, the chimeric contribution of hPSCs is expected to be extremely low in pigs consistent with preliminary results from the Izpisua Belmonte group (Wu et al., Manuscript in preparation). Choosing a species closer to human, such as non-human primates, may work. However, the law in many countries prohibits such experiments. Developing strategies to improve human chimerism in a distant animal host, as discussed above, could help turn this ambitious goal into reality in the distant future.

2) Interspecies targeted organ complementation. One of the issues regarding *in vivo* organ generation in animals is ethical concern over potential gamete and neural contribution from PSCs. This issue might be addressed by developing methods of "targeted organ generation"[78]. One

approach is to modify PSCs so that their differentiation is restricted to the organ of interest. Conditional expression of the transcription factor *Mixl1* made it possible to induce differentiation of PSC-derived cells toward the endodermal lineage, thereby reducing the number of non-endodermal PSC-derived cells[79]. Introducing constructs containing suicide genes under neural- or germ-cell-specific promoters to completely eliminate formation of human iPSC-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 PSCs. In organ-deficient embryos, the organ niche is available for complementation throughout development of the organ. Therefore, to inject progenitor cells, instead of PSCs, 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 progenitor cells likely can colonize and give rise to the entire organ. Although this is a theoretically sound strategy and potentially reduces the ethical concern, there are several caveats: 1) the developmental stages need to be matched between human progenitors and the host animal fetuses. 2) Delivering human cells before the host organ becomes atretic is likely critical and challenging; and 3) human progenitor cells delivery need to be performed before the host immune system formed (less than 50 days in pigs).

***In vivo* disease modeling.** An important goal of experimental biology is to set up 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 a disease-appropriate phenotype for monogenic diseases such as Lesch-Nyhan syndrome[80] or of complex diseases such as Parkinson's or Alzheimer's. Given that disease specific iPSCs carry all genetic alterations that contributed to the ailment of the patient, the functional integration of patient derived cells into the tissues of the developing mouse embryo would allow study of the initiation, progression and manifestation of the respective disease. Host-specific developmental and physiological programs may alter the behaviors of donor human cells in a non-human host. Nevertheless, the power of studying the autonomous versus non-autonomous effects of the multiple genetic alterations that contribute to a human disease under *in vivo* conditions cannot be dismissed.

***In vivo* drug screening.** 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, interspecies chimeras with human stem cells can potentially offer a superior *in vivo* drug-screening platform. Interspecies chimeric formation with human iPSCs or iPSC-derived progenitors offers an attractive platform for 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.

ETHICAL, LEGAL, AND SOCIAL ISSUES

In his January 2006 State of the Union address, President George W. Bush asked Congress to pass a "Human Chimera Prohibition Act" that would have made felonies of many activities that would mix genes, cells, or tissues from human and non-human animals[81]. Ten years later political, ethical, and social concerns about chimeras remain.

The Issues: Four ethical and social issues exist concerning research with, and potential human clinical use of chimeras: animal welfare, sources of donor cells, general public discomfort with chimeras, and the "humanization" of the host species.

With chimeras, as any new intervention with non-human animals the pain or disability the animals could suffer might be hard to predict. Researchers must be more sensitive to the possibility of pain

and suffering than with their usual laboratory animals[82,83]. If chimeras were ultimately developed to provide transplants for humans, carnivorous cultures should not ban their use, but humane treatment and slaughter would be essential.

Although widely legal for research use, some people consider the use of hESCs or of cells derived from aborted fetuses unethical; this would be true for chimeras as well.

Public discomfort, sometimes expressed as concerns about "unnaturalness," clearly exists about chimeras. These seem to boil down to a reaction that any species mixing is inherently wrong[84]. Whether public concerns without a good logical basis[85] should be considered "ethical" issues might be debated, but they might have substantial effects on research and its funding.

The most controversial aspect of human/non-human chimeras has been the fear that they may in some way confer ethically important human characteristics on non-human hosts. Substantial discussion of the humanization concern started around 2003[86] and continued for several years[82,83,87-89].

The precise concerns about "humanization" have remained somewhat unclear. One author argues that three specific characteristics of human/non-human chimeras should be carefully examined: human-like cognitive abilities, human gametes, or human-like appearances[90]. Most discussion has focused on the brain; some suggest that chimeras could lead to moral confusion of humans and non-humans[86] or diminution of human dignity[87,88,91,92]. One powerful, but unlikely, concern is that such chimeras would actually have sufficient human-like consciousness or intelligence to "deserve" but be denied treatment as persons[89]. Others have noted merely that the issue is controversial and argued that researchers should be very careful with research that could be seen as possibly giving the host some human-like cognitive abilities[82,83].

As the earlier parts of this article indicate, creation of a non-human animal with a substantially human brain, or with enough human characteristics to warrant human treatment[93], seems currently unlikely, especially given possible measures to limit unplanned spread of the stem cells. The possibility cannot be entirely dismissed – one study already found improvements in mouse memory after insertion of human glial progenitor cells[13] and the increasing use of naïve hPSCs may lead to wider differentiation.

Laws, Regulations, and Guidance: Different national laws and regulations concerning laboratory animal care and use exist, as well as regulation of proper treatment of food animals. These need only to be carefully applied to novel chimeric animals.

Similarly, varying national laws and regulations govern the use of fetal tissue or hESCs in research. Because chimera research might produce dramatic results, they could bring public attention to these issues. The case of the U.S. may show the ways some of these concerns may play out.

The U.S. banned federal funding of research with fetal tissue in the late 1980s[93]. The 1993 passage of the N.I.H. Revitalization Act[94] authorized federal funding for research involving appropriately obtained fetal tissue. Recent controversy over Planned Parenthood's acquisition of fetal tissue for transfer to researchers has revived controversy about this statute.

Similarly, federal funding for research that could create hESCs has been banned since 1995. The Clinton Administration in 2000 concluded that this allowed federal funding of research to study hESC lines that others had created using non-federal funding. The Bush Administration stringently limited the extent of such funding; the Obama Administration broadly permitted it in 2009. This

position was upheld by a federal appellate court[95]. The position, however, can be changed by legislation or by a policy decision by a new president. (Note, however, that, in U.S., this issue has mainly concerned federal funding for research, not limitation of non-federally funded research.)

Humanization has received the most attention, largely through guidance instead of law or regulation. Human/non-human chimera research has been addressed as part of general guidelines from the U.S. National Academy of Sciences in 2005[96]; the International Society for Stem Cell Research, first published in 2006[97] and updated in 2016[98] and the U.K. Academy of Medical Sciences in 2011[99]. Specific guidance on chimera research came from the ISSCR ethics committee in 2007[83] and a group based at Johns Hopkins provided ethical recommendations for research inserting human cells into the brains of non-human primates[100]. The guidelines urge that the insertion of human stem cells (or tissues) into non-human animals be reviewed by oversight committees. Under the NAS Guidelines, when a committee reviews a protocol "particular attention should be paid to the probable pattern and effects of differentiation and integration of the human cells into the nonhuman animal tissues" The NAS Guidelines also ensure that the animals that have received these human cells or tissues not be allowed to breed for fear that they may produce some human gametes[101]. These guidelines also contain some useful specific prohibitions, such as on placing any ESCs in human blastocysts or hESCs into the blastocysts of non-human primates.

If properly applied, these guidelines should go far to assuaging the humanization concerns. The overseeing committees should, after considering the likely patterns of differentiation and distribution, not approve research that has a significant chance of arguably conferring human cognitive characteristics or creating a human appearance.

No regulations or guidelines directly address unspecified public concerns about chimeras, One group urged that researchers react to these worries by being open and transparent about their research, while regularly making the case that such research is important[82].

The real consequences of ethical and social concerns about chimeras are not ancient history. In September 2015 the U.S. N.I.H. announced a moratorium on funding any research in which human pluripotent cells or human neural progenitor cells were placed into any non-human vertebrate embryo before gastrulation[102]. The reasons for the moratorium were unclear. In November 2015 N.I.H. held a workshop to discuss these issues and in August 2016 issued a draft policy that calls for an internal NIH committee to review applications for some kinds of human/non-human chimera funding[103, 104].

CONCLUSION AND OUTLOOK

Notwithstanding groundbreaking advances in generating animal interspecies chimeras, at present, when it comes to human-animal chimeras, science has more questions than answers. It remains unknown whether more extensive chimerism can be obtained between human and other closer species. Will human organs generated through interspecies complementation be suitable for transplantation with host blood vessels and nerve cells still present? In this regard strategies such as humanizing host animals and/or multi-lineage complementation may help. Fundamental questions remain such as how to resolve heterochronic developmental processes and inherent differences in gestation length between human and other species? Whether xeno-generated human cells, tissues and organs would be functionally compatible with human physiology? Will it possible to generate human cells able to compete equally with host cells?

Developing novel strategies such as those described in this essay towards enhancing the degree of human-animal chimerism 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 monitoring and controlling specific cell behaviors may help to overcome some of

the present human-animal interspecies barriers. Similarly, engineering approaches to humanize animal models may help lower the threshold of xenotransplantation. If successful, these new approaches, rooted in the fields of cell and developmental biology, may expand the breadth of chimera research from the laboratory into potential clinical applications, including 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 challenges– scientific, medical, ethical, political, financial, and others and not everything that can be done in the field of chimera 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.

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AUTHOR CONTRIBUTIONS

J.W., H.G., R.J., H.N., J.R. and J.C.I.B conceived the study and wrote the manuscript.

AUTHOR INFORMATION

The authors declare no competing financial interests.

FIGURE LEGENDS

Figure 1 | Chimeras generated from cultured stem cells. Stem cells with varying degree of developmental potency have been derived from different developmental stages. *In situ* transplantation allows cultured stem cells to generate intra- or inter-species chimeras. Rodent ESCs/iPSCs can contribute to post-natal chimeras in a host from the same or a different rodent species. Primate naïve PSCs can contribute, although very limitedly, to developing mouse embryos after blastocyst injection. Primed PSCs can engraft into mouse gastrula stage and generate *ex vivo* chimeric embryos. Lineage progenitors or adult stem cells can contribute to chimera formation in an organ of a developing fetus or post-natal animal.

Figure 2 | Strategies to improve interspecies chimerism with hPSCs. a, Matching developmental timing is critical for pluripotent and multipotent stem cells to engraft and generate intra- and inter-species chimeras. **b,** Choosing an evolutionary closer host may help increase the chimerism of donor hPSCs. **c,** Genetically modify host animals to disable organ development may enable donor hPSCs to populate the targeted organ with minimal competition from the host**. d,** Modify donor hPSCs to enhance their survival, proliferation and/or cell competition may also help increase the degree of chimerism.

Figure 3 | Potential application of interspecies chimera with hPSCs. Organ generation via interspecies blastocyst complementation or interspecies targeted organ complementation may help solve the severe shortage of organ donors worldwide. Human-animal chimeras will also be useful for a better understanding of the etiology, onset and progression of human diseases, as well as for testing candidate drugs' efficacy and safety *in vivo*.

BOX 1 | DEFINING CHIMERAS

A chimera is typically defined as an organism composed of a mixture of different cell populations that derive from more than one zygote. They can be formed by processes such as mixing of early embryos or grafting of tissues from different stages of development or the adult. Chimeras should be distinguished from hybrids, offspring resulting from breeding between different species, and from mosaics, animals carrying genetically different cells. Chimeras can be categorized as intraspecies or interspecies depending on whether cell derivatives from two zygotes are from the same or different species.

Creating interspecies chimeras requires a donor species and a host species. The donor species provides cells of embryonic, fetal, or adult origin, either from primary tissue or from cell cultures. The host species provides the physiological environment and life support for embryonic, fetal, or adult chimeric animals. In stem cell research, generation of an interspecific chimera often involves transplanting multi- or pluri-potent stem cells from the donor into an animal recipient at embryonic, fetal, or postnatal stages of development.

Tissue distribution and duration of chimerism often differ depending on the donor cell type and the developmental stage of the host. PSCs are less restricted in developmental potential than other stem cell types and thus can give rise to a high degree of chimerism with wide tissue distribution when transferred to pre-implantation host embryos. Chimeras generated by progenitors or adult stem cells are more confined to tissues specific to their cell type and may need to be grafted to appropriate sites and developmental stages. Chimeras can also be classified by the developmental age of the host at the time of analysis, e.g. chimeric embryos and chimeric fetuses, or at the time of donor cell injection, e.g. blastocyst chimeras.

Other important distinctions are between heterotopic versus orthotopic, and heterochronic versus isochronic chimeras. Orthotopic chimeras are generated by transplanting donor cells to their cognate location where they can participate in natural developmental processes or proper tissue organization of the host – for example, moving donor liver cells to the embryonic host's liver at the right stage of liver development. Heterotopic chimeras occur with differentiation or integration of donor cells in a different site within the host animal than that appropriate for their origins, for example transplanting donor pancreatic β cells to the liver. Heterochronic and isochronic chimeras are distinguished by the temporal properties between donor and host. If donor cells are delivered to the host at a time matching their *in vivo* origin, an isochronic chimera will be formed, otherwise the chimera formed will be heterochronic.

REFERENCES

1. Ito, R., Takahashi, T., Katano, I. & Ito, M. Current advances in humanized mouse models. *Cellular & Molecular Immunology* **9,** 208–214 (2012).
2. Le Douarin, N. M. The ontogeny of the neural crest in avian embryo chimaeras. *Nature* **286,** 663–669 (1980).
3. Douarin, N. L. & Kalcheim, C. *The Neural Crest*. (Cambridge University Press., 1982).
4. Gardner, R. L. & JOHNSON, M. H. Investigation of early mammalian development using interspecific chimaeras between rat and mouse. *Nature* **246,** 86–89 (1973).
5. Rossant, J. & Frels, W. Interspecific chimeras in mammals: successful production of live chimeras between Mus musculus and Mus caroli. *Science* **208,** 419–421 (1980).

A pioneering study reported the first viable interspecies chimeras generated by injection of *M.caroli* ICMs into *M. musculus* blastocysts.

6. Rossant, J., Vijh, M., Siracusa, L. D. & Chapman, V. M. Identification of embryonic cell lineages in histological sections of M. musculus in-equilibrium M. caroli chimaeras. *J Embryol Exp Morphol* **73,** 179–191 (1983).
7. Fehilly, C. B., Willadsen, S. M. & Tucker, E. M. Interspecific chimaerism between sheep and goat. *Nature* **307,** 634–636 (1984).
8. Williams, T. J., Munro, R. K. & Shelton, J. N. Production of interspecies chimeric calves by aggregation of Bos indicus and Bos taurus demi-embryos. *Reprod. Fertil. Dev.* **2,** 385–394 (1990).
9. Rossant, J., Croy, B. A., Chapman, V. M., Siracusa, L. & Clark, D. A. Interspecific chimeras in mammals: a new experimental system. *Journal of Animal Science* **55,** 1241–1248 (1982).
10. Rossant, J., Mauro, V. M. & Croy, B. A. *Importance of trophoblast genotype for survival of interspecific murine chimeras*. **69,** 141–149 (J. Embryol. exp. Morph., 1982).
11. Wu, J. & Izpisua Belmonte, J. C. Dynamic Pluripotent Stem Cell States and Their Applications. *Cell Stem Cell* **17,** 509–525 (2015).
12. Xiang, A. P. *et al.* Extensive contribution of embryonic stem cells to the development of an evolutionarily divergent host. *Human Molecular Genetics* **17,** 27–37 (2008).

The first study reported PSC-derived viable interspecies chimeras between two distant rodent species.

13. Han, X. *et al.* Forebrain engraftment by human glial progenitor cells enhances synaptic plasticity and learning in adult mice. *Cell Stem Cell* **12,** 342–353 (2013).
14. Ying, Q.-L. *et al.* The ground state of embryonic stem cell self-renewal. *Nature* **453,** 519–523 (2008).

The landmark paper describing the pluripotent ground state and the ground state culture ultimately enabled derivation of genuine ESCs from rat blastocysts (refs 15, 16)

15. Buehr, M. *et al.* Capture of authentic embryonic stem cells from rat blastocysts. *Cell* **135,** 1287–1298 (2008).
16. Li, P. *et al.* Germline competent embryonic stem cells derived from rat blastocysts. *Cell* **135,** 1299–1310 (2008).
17. Isotani, A., Hatayama, H., Kaseda, K., Ikawa, M. & Okabe, M. Formation of a thymus from rat ES cells in xenogeneic nude mouse↔rat ES chimeras. *Genes to Cells* **16,** 397–405 (2011).
18. Kobayashi, T. *et al.* Generation of rat pancreas in mouse by interspecific blastocyst injection of pluripotent stem cells. *Cell* **142,** 787–799 (2010).

The landmark paper reported PSC-derived viable mouse-rat interspecies chimeras and popularized the concept of interspecies blastocyst complementation

19. Thomson, J. A. *et al.* Isolation of a primate embryonic stem cell line. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 7844–7848 (1995).
20. Sasaki, E. E. *et al.* Establishment of novel embryonic stem cell lines derived from the common marmoset (Callithrix jacchus). *Stem Cells* **23,** 1304–1313 (2005).
21. Simerly, C. R. *et al.* Establishment and characterization of baboon embryonic stem cell lines: an Old World Primate model for regeneration and transplantation research. *Stem Cell Research* **2,** 178–187 (2009).
22. Thomson, J. A. *et al.* Pluripotent cell lines derived from common marmoset (Callithrix jacchus) blastocysts. *Biology of Reproduction* **55,** 254–259 (1996).
23. Thomson, J. A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science*

282, 1145–1147 (1998).
24. Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A. & Bongso, A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nature Biotechnology* **18,** 399–404 (2000).
25. Tachibana, M. *et al.* Generation of chimeric rhesus monkeys. *Cell* **148,** 285–295 (2012).
26. Simerly, C. *et al.* Interspecies chimera between primate embryonic stem cells and mouse embryos: Monkey ESCs engraft into mouse embryos, but not post-implantation fetuses. *Stem Cell Research* **7,** 28–40 (2011).
27. James, D., Noggle, S. A., Swigut, T. & Brivanlou, A. H. Contribution of human embryonic stem cells to mouse blastocysts. *Developmental Biology* **295,** 90–102 (2006).
28. Nichols, J. & Smith, A. Naive and primed pluripotent states. *Cell Stem Cell* **4,** 487–492 (2009).
29. Boroviak, T., Loos, R., Bertone, P., Smith, A. & Nichols, J. The ability of inner-cell-mass cells to self-renew as embryonic stem cells is acquired following epiblast specification. *Nature Cell Biology* **16,** 516–528 (2014).
30. Brons, I. G. M. *et al.* Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* **448,** 191–195 (2007).
31. Tesar, P. J. *et al.* New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* **448,** 196–199 (2007).
32. Huang, Y., Osorno, R., Tsakiridis, A. & Wilson, V. In Vivo differentiation potential of epiblast stem cells revealed by chimeric embryo formation. *Cell Reports* **2,** 1571–1578 (2012).
33. Kojima, Y. *et al.* The transcriptional and functional properties of mouse epiblast stem cells resemble the anterior primitive streak. *Cell Stem Cell* **14,** 107–120 (2014).
34. Wu, J. *et al.* An alternative pluripotent state confers interspecies chimaeric competency. *Nature* **521,** 316–321 (2015).

One of two pioneering studies that demonstrated functional engraftment of primed hPSCs into gastrula stage mouse embryos.

35. Mascetti, V. L. & Pedersen, R. A. Human-Mouse Chimerism Validates Human Stem Cell Pluripotency. *Cell Stem Cell* **18,** 67–72 (2016).

One of two pioneering studies that demonstrated functional engraftment of primed hPSCs into gastrula stage mouse embryos.

36. Wu, J. & Belmonte, J. C. I. Stem Cells: A Renaissance in Human Biology Research. *Cell* **165,** 1572–1585 (2016).
37. Mascetti, V. L. & Pedersen, R. A. Contributions of Mammalian Chimeras to Pluripotent Stem Cell Research. *Cell Stem Cell* **19,** 163–175 (2016).
38. Hanna, J. *et al.* Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. *Proceedings of the National Academy of Sciences* **107,** 9222–9227 (2010).

A pioneering study demonstrating naïve human pluripotent state resembling mESCs could be stabilized in culture.

39. Chan, Y.-S. *et al.* Induction of a human pluripotent state with distinct regulatory circuitry that resembles preimplantation epiblast. *Cell Stem Cell* **13,** 663–675 (2013).
40. Duggal, G. *et al.* Alternative Routes to Induce Naïve Pluripotency in Human Embryonic Stem Cells. *Stem Cells* **33,** 2686–2698 (2015).
41. Gafni, O. *et al.* Derivation of novel human ground state naive pluripotent stem cells. *Nature* **504,** 282–286 (2013).

Refs 39, 41-43, 46, first studies describing different culture conditions for naive hESCs.

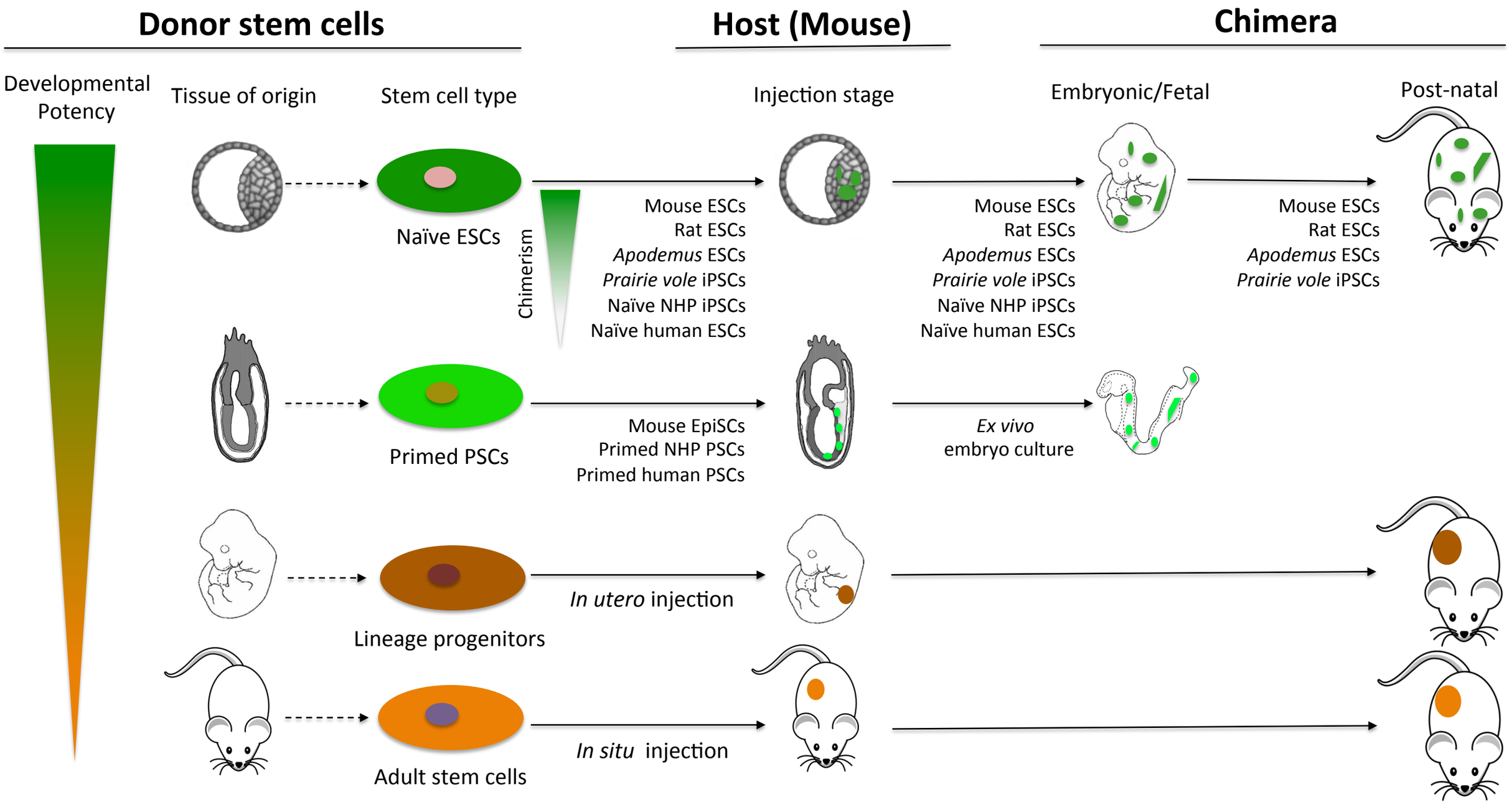
42. Takashima, Y. *et al.* Resetting transcription factor control circuitry toward ground-state pluripotency in human. *Cell* **158,** 1254–1269 (2014).
43. Theunissen, T. W. *et al.* Systematic identification of culture conditions for induction and maintenance of naive human pluripotency. *Cell Stem Cell* **15,** 471–487 (2014).
44. Theunissen, T. W. *et al.* Molecular Criteria for Defining the Naive Human Pluripotent State. *Cell Stem Cell* **19,** 1–14 (2016).
45. Wang, J. *et al.* Primate-specific endogenous retrovirus-driven transcription defines naive-like stem cells. *Nature* **516,** 405–409 (2014).
46. Ware, C. B. *et al.* Derivation of naive human embryonic stem cells. *Proceedings of the National Academy of Sciences* **111,** 4484–4489 (2014).
47. Guo, G., Meyenn, von, F., Santos, F., Chen, Y. & Reik, W. Naive Pluripotent Stem Cells Derived Directly from Isolated Cells of the Human Inner Cell Mass. *Stem cell reports* (2016). doi:10.1016/j.stemcr.2016.02.005
48. Pera, M. F. In search of naivety. *Cell Stem Cell* **15,** 543–545 (2014).
49. Huang, K., Maruyama, T. & Fan, G. The naive state of human pluripotent stem cells: a synthesis of stem cell and preimplantation embryo transcriptome analyses. *Cell Stem Cell* **15,** 410–415 (2014).
50. Masaki, H. *et al.* Interspecific in vitro assay for the chimera-forming ability of human pluripotent stem cells. *Development* **142,** 3222–3230 (2015).
51. Chen, Y. *et al.* Generation of Cynomolgus Monkey Chimeric Fetuses using Embryonic Stem Cells. *Cell Stem Cell* **17,** 116–124 (2015).
52. Fang, R. *et al.* Generation of naive induced pluripotent stem cells from rhesus monkey fibroblasts. *Cell Stem Cell* **15,** 488–496 (2014).
53. Dupin, E. & Sommer, L. Neural crest progenitors and stem cells: from early development to adulthood. *Developmental Biology* **366,** 83–95 (2012).
54. Jaenisch, R. Mammalian neural crest cells participate in normal embryonic development on microinjection into post-implantation mouse embryos. *Nature* **318,** 181–183 (1985).
55. Huszar, D., Sharpe, A. & Jaenisch, R. Migration and proliferation of cultured neural crest cells in W mutant neural crest chimeras. *Development* **112,** 131–141 (1991).
56. Cohen, M. A. *et al.* Human neural crest cells contribute to coat pigmentation in interspecies chimeras after in utero injection into mouse embryos. *Proceedings of the National Academy of Sciences* **113,** 1570–1575 (2016).
57. Brüstle, O. *et al.* Chimeric brains generated by intraventricular transplantation of fetal human brain cells into embryonic rats. *Nature Biotechnology* **16,** 1040–1044 (1998).
58. Keyoung, H. M. *et al.* High-yield selection and extraction of two promoter-defined phenotypes of neural stem cells from the fetal human brain. *Nature Biotechnology* **19,** 843–850 (2001).
59. Goyama, S., Wunderlich, M. & Mulloy, J. C. Xenograft models for normal and malignant stem cells. *Blood* **125,** 2630–2640 (2015).
60. Zanjani, E. D., Almeida-Porada, G., Livingston, A. G., Flake, A. W. & Ogawa, M. Human bone marrow CD34- cells engraft in vivo and undergo multilineage expression that includes giving rise to CD34+ cells. *Exp. Hematol.* **26,** 353–360 (1998).
61. Fujiki, Y. *et al.* Successful multilineage engraftment of human cord blood cells in pigs after in utero transplantation. *Transplantation* **75,** 916 (2003).
62. Muotri, A. R., Nakashima, K., Toni, N., Sandler, V. M. & Gage, F. H. Development of functional human embryonic stem cell-derived neurons in mouse brain. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 18644–18648 (2005).
63. Chuma, S. *et al.* Spermatogenesis from epiblast and primordial germ cells following transplantation into postnatal mouse testis. *Development* **132,** 117–122 (2005).

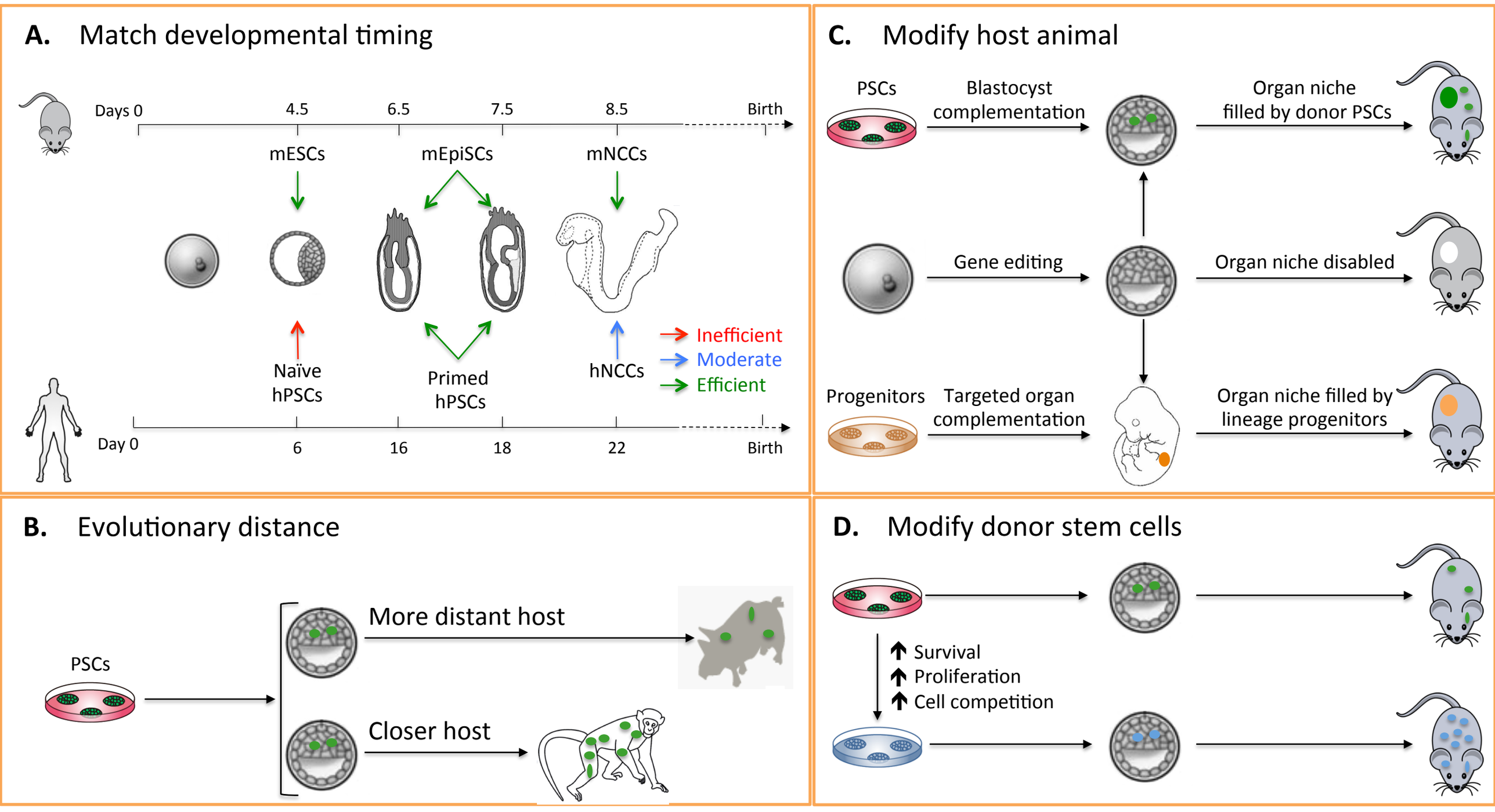
64. Leitch, H. G. *et al.* On the fate of primordial germ cells injected into early mouse embryos. *Developmental Biology* **385,** 155–159 (2014).
65. Clarke, D. L. *et al.* Generalized potential of adult neural stem cells. *Science* **288,** 1660–1663 (2000).
66. Jiang, Y. *et al.* Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418,** 41–49 (2002).
67. Geiger, H., Sick, S., Bonifer, C. & Müller, A. M. Globin Gene Expression Is Reprogrammed in Chimeras Generated by Injecting Adult Hematopoietic Stem Cells into Mouse Blastocysts. *Cell* **93,** 1055–1065 (1998).
68. Harder, F., Henschler, R., Junghahn, I. & Lamers, M. C. Human hematopoiesis in murine embryos after injecting human cord blood–derived hematopoietic stem cells into murine blastocysts. *Blood* (2002).
69. Wilson, A. & Trumpp, A. Bone-marrow haematopoietic-stem-cell niches. *Nat. Rev. Immunol.* **6,** 93–106 (2006).
70. Brinster, R. L. & Zimmermann, J. W. Spermatogenesis following male germ-cell transplantation. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 11298–11302 (1994).
71. Hayashi, K., Ohta, H., Kurimoto, K., Aramaki, S. & Saitou, M. Reconstitution of the Mouse Germ Cell Specification Pathway in Culture by Pluripotent Stem Cells. *Cell* **146,** 519–532 (2011).
72. Morata, G. & Ripoll, P. Minutes: Mutants of Drosophila autonomously affecting cell division rate. *Developmental Biology* **42,** 211–221 (1975).
73. Amoyel, M. & Bach, E. A. Cell competition: how to eliminate your neighbours. *Development* **141,** 988–1000 (2014).
74. Dejosez, M., Ura, H., Brandt, V. L. & Zwaka, T. P. Safeguards for cell cooperation in mouse embryogenesis shown by genome-wide cheater screen. *Science* **341,** 1511–1514 (2013).
75. Takahashi, K. & Yamanaka, S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell* **126,** 663–676 (2006).

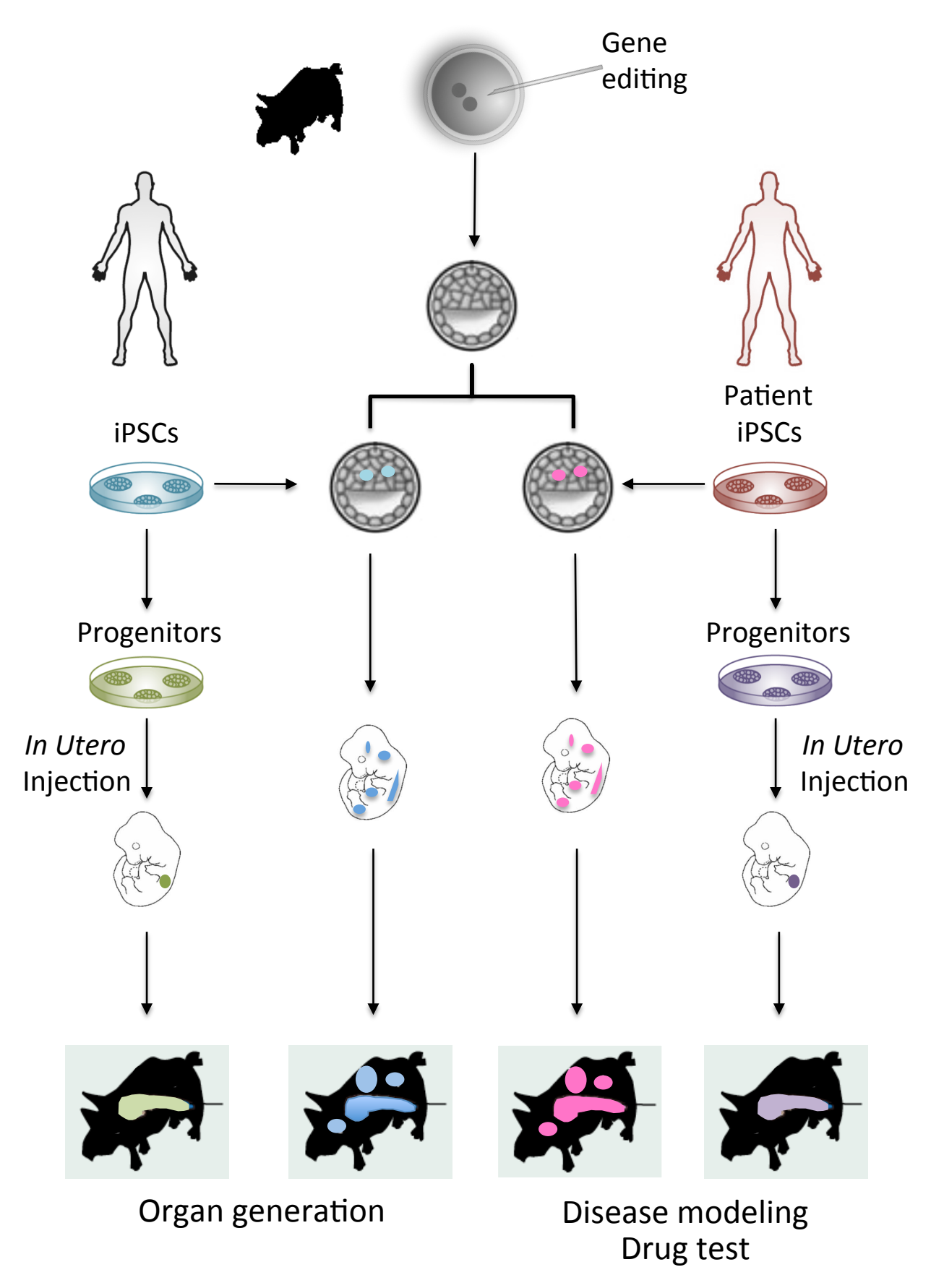
The landmark study established that somatic cells could be induced to PSCs by defined transcription factors.

76. Usui, J.-I. *et al.* Generation of Kidney from Pluripotent Stem Cells via Blastocyst Complementation. *The American Journal of Pathology* **180,** 2417–2426 (2012).
77. Matsunari, H. H. *et al.* Blastocyst complementation generates exogenic pancreas in vivo in apancreatic cloned pigs. *Proc. Natl. Acad. Sci. U.S.A.* **110,** 4557–4562 (2013).
78. Rashid, T., Kobayashi, T. & Nakauchi, H. Revisiting the flight of Icarus: making human organs from PSCs with large animal chimeras. *Cell Stem Cell* **15,** 406–409 (2014).
79. Kobayashi, T., Kobayashi, T., Kato-Itoh, M., Kato-Itoh, M. & Nakauchi, H. Targeted organ generation using Mixl1-inducible mouse pluripotent stem cells in blastocyst complementation. *Stem Cells and Development* **24,** 182–189 (2015).
80. Kuehn, M. R., Bradley, A., Robertson, E. J. & Evans, M. J. A potential animal model for Lesch–Nyhan syndrome through introduction of HPRT mutations into mice. *Nature* **326,** 295–298 (1987).
81. The Human Chimera Prohibition Act of 2005, S 659.
82. Greely, H.T., Cho, M.K., Hogel, L.F., & Satz, D.M. Thinking about the human neuron mouse. *Am. J. Bioethics* **7**, no. 5, 27–40 (2007).
83. Hyun, I. *et al.* Ethical standards for human-to-animal chimera experiments in stem cell research. *Cell Stem Cell* **1**,159-163 (2007).
84. Hermerén, G. Ethical considerations in chimera research. *Development* **142**, 3-5 (2015).
85 Hyun, I. From naïve pluripotency to chimeras: A new ethical challenge? *Development* **142**, 6-8 (2015).
86. Robert, J.S. & Baylis, F. Crossing species boundaries. *Am. J. Bioethics* **3**, no. 3, 1–13

(2003).
87. Karpowicz, P., Cohen C.B., & van der Kooy, D. Is it ethical to transplant human stem cells into nonhuman embryos. *Nat Med* **10**, 331–35 (2004).
88. Karpowicz, P., Cohen C.B., & van der Kooy, D. Developing human-nonhuman chimeras in human stem cell research: ethical issues and boundaries. *Kennedy Inst. Ethics J*. **15**, 107–34 (2005).
89. Streiffer, R. Human/non-human chimeras. In The Stanford Encyclopedia of Philosophy, ed. Edward N. Zalta, http://plato.stanford.edu/entries/chimeras/ (2009).
90. Greely, H.T. Human/nonhuman chimeras: Assessing the issues. In Oxford Handbook of Animal Ethics (ed. Beauchamp, T. & Frey, R.G., Oxford Univ. Press, 2011).
91. Johnston, J. & Eliot, C. Chimeras and human dignity. *Am J Bioethics* **3**, no. 3, 6–7 (2003).
92. Palacios-González, C. Human dignity and the creation of human–nonhuman chimeras. *Med Health Care Philos*. **18**, 487–499 (2015).
93. United States National Institutes of Health. Special Notice: Moratorium on Certain Fetal Tissue Research. Bethesda, MD. (1988).
94. N.I.H. Revitalization Act of 1993, Pub. L. 103-43.
95. *Sherley v. Sibelius*, 689 F.3d 776 (D.C. Circuit 2012).
96. Committee on Guidelines for Human Embryonic Stem Cell Research. Guidelines for Human Embryonic Stem Cell Research (Washington, D.C.: National Academies Press, 2005).
97. International Society for Stem Cell Research. Guidelines for the Conduct of Human Embryonic Stem Cell Research, (2006) retrieved from http://www.isscr.org/home/publications/guide-clintrans.
98. International Society for Stem Cell Research. Guidelines for Stem Cell Science and Clinical Translation (2016), retrieved from http://www.isscr.org/home/publications/2016-guidelines.
99. Academy of Medical Sciences. *Animals containing human materials*. (2011) retrieved from https://issuu.com/acmedsci/docs/animals_containing_human_material.
100. Greene, M., et al. Moral issues of human–nonhuman primate neural grafting. *Science* **309**, 385–86 (2005).
101. Palacios-González, C. Ethical aspects of creating human-nonhuman chimeras capable of human gamete production and human pregnancy. *Monash Bioeth Rev* **33**, 181–202 (2015).
102. United States National Institutes of Health. NIH research Involving introduction of human pluripotent cells into non-human vertebrate animal pre-gastrulation embryos. (2015) retrieved from https://grants.nih.gov/grants/guide/notice-files/NOT-OD-15-158.html.
103. U.S. National Institutes of Health. Next steps on research using animal embryos containing human cells. (2016) retrieved from http://osp.od.nih.gov/under-the-poliscope/2016/08/next-steps-research-using-animal-embryos-containing-human-cells.
104. Reardon, S. U.S. agency to lift ban on funding human-animal hybrids, *Nature* **536**, 135 (2016).


Donor stem cells
Host (Mouse)
Chimera
Developmental Potency
Tissue of origin
Stem cell type
Injection stage
Embryonic/Fetal
Post-natal
Naïve ESCs
Chimerism
Mouse ESCs
Rat ESCs
Apodemus ESCs
Prairie vole iPSCs
Naïve NHP iPSCs
Naïve human ESCs
Mouse ESCs
Rat ESCs
Apodemus ESCs
Prairie vole iPSCs
Naïve NHP iPSCs
Naïve human ESCs
Mouse ESCs
Rat ESCs
Apodemus ESCs
Prairie vole iPSCs
Primed PSCs
Mouse EpiSCs
Primed NHP PSCs
Primed human PSCs
Ex vivo
embryo culture
Lineage progenitors
In utero injection
Adult stem cells
In situ injection


A. Match developmental timing
Days 0
4.5
6.5
7.5
8.5
Birth
mESCs
mEpiSCs
mNCCs
Naïve hPSCs
Primed hPSCs
hNCCs
Inefficient
Moderate
Efficient
Day 0
6
16
18
22
Birth
B. Evolutionary distance
PSCs
More distant host
Closer host
C. Modify host animal
PSCs
Blastocyst complementation
Organ niche filled by donor PSCs
Gene editing
Organ niche disabled
Progenitors
Targeted organ complementation
Organ niche filled by lineage progenitors
D. Modify donor stem cells
Survival
Proliferation
Cell competition


Gene
editing
iPSCs
Patient
iPSCs
Progenitors
Progenitors
In Utero
Injection
In Utero
Injection
Organ generation
Disease modeling
Drug test