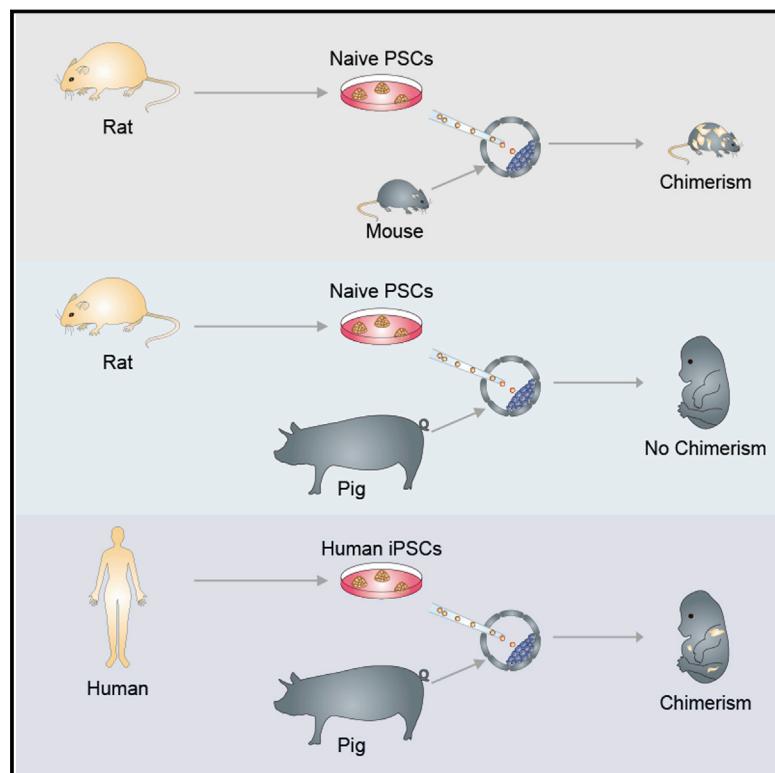


Interspecies Chimerism with Mammalian Pluripotent Stem Cells

Graphical Abstract



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In Brief

Human pluripotent stem cells robustly engraft into both cattle and pig pre-implantation blastocysts, but show limited chimeric contribution to post-implantation pig embryos.

Highlights

- Naive rat PSCs robustly contribute to live rat-mouse chimeras
- A versatile CRISPR-Cas9 mediated interspecies blastocyst complementation system
- Naive rodent PSCs show no chimeric contribution to post-implantation pig embryos
- Chimerism is observed with some human iPSCs in post-implantation pig embryos

Interspecies Chimerism with Mammalian Pluripotent Stem Cells

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SUMMARY

Interspecies blastocyst complementation enables organ-specific enrichment of xenogenic pluripotent stem cell (PSC) derivatives. Here, we establish a versatile blastocyst complementation platform based on CRISPR-Cas9-mediated zygote genome editing and show enrichment of rat PSC-derivatives in several tissues of gene-edited organogenesis-disabled mice. Besides gaining insights into species evolution, embryogenesis, and human disease, interspecies blastocyst complementation might allow human organ generation in animals whose organ size, anatomy, and physiology are closer to humans. To date, however, whether human PSCs (hPSCs) can contribute to chimera formation in non-rodent species remains unknown. We systematically evaluate the chimeric competency of several types of hPSCs using a more diversified clade of mammals, the ungulates. We find that naïve hPSCs robustly engraft in both pig and cattle pre-implantation blastocysts but show limited contribution to post-implantation pig embryos. Instead, an intermediate hPSC type exhibits higher degree of chimerism and is able to generate differentiated progenies in post-implantation pig embryos.

INTRODUCTION

Embryonic pluripotency has been captured in vitro at a spectrum of different states, ranging from the naïve state, which reflects

unbiased developmental potential, to the primed state, in which cells are poised for lineage differentiation (Weinberger et al., 2016; Wu and Izpisua Belmonte, 2016). When attempting to introduce cultured pluripotent stem cells (PSCs) into a developing embryo of the same species, recent studies demonstrated that matching developmental timing is critical for successful chimera formation. For example, naïve mouse embryonic stem cells (mESCs) contribute to chimera formation when injected into a blastocyst, whereas primed mouse epiblast stem cells (mEpiSCs) efficiently engraft into mouse gastrula-stage embryos, but not vice versa (Huang et al., 2012; Wu et al., 2015). Live rodent interspecies chimeras have also been generated using naïve PSCs (Isotani et al., 2011; Kobayashi et al., 2010; Xiang et al., 2008). However, it remains unclear whether naïve PSCs can be used to generate chimeras between more distantly related species.

The successful derivation of human PSCs (hPSCs), including ESCs from pre-implantation human embryos (Reubinoff et al., 2000; Thomson et al., 1998), as well as the generation of induced pluripotent stem cells (iPSCs) from somatic cells through cellular reprogramming (Takahashi et al., 2007; Park et al., 2008; Wernig et al., 2007; Yu et al., 2007; Aasen et al., 2008), has revolutionized the way we study human development and is heralding a new age of regenerative medicine. Several lines of evidence indicate that conventional hPSCs are in the primed pluripotent state, similar to mEpiSCs (Tesar et al., 2007; Wu et al., 2015). A number of recent studies have also reported the generation of putative naïve hPSCs that molecularly resemble mESCs (Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014). These naïve hPSCs have already provided practical and experimental advantages, including high single-cell cloning efficiency and facile genome editing (Gafni et al., 2013). Despite these advances, it remains unclear how the putative higher developmental potential of naïve hPSCs can be used to better

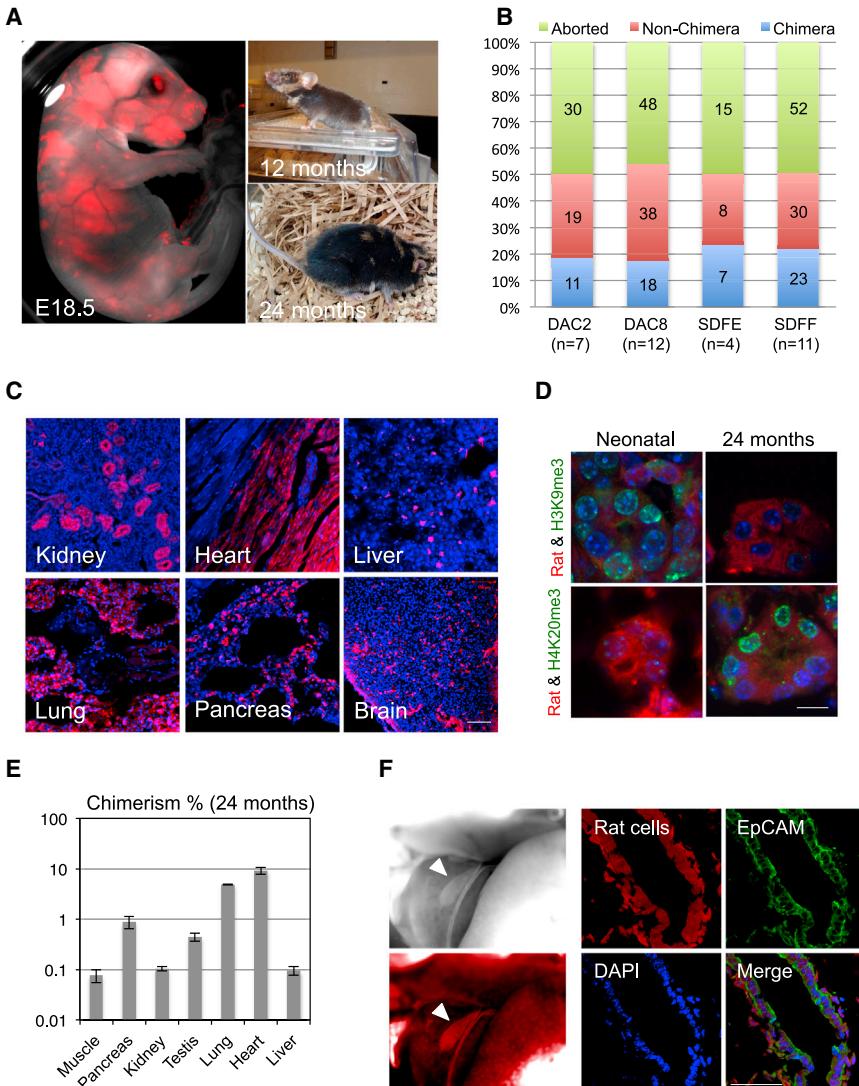


Figure 1. Interspecies Rat-Mouse Chimeras Derived from Rat PSCs

(A) Rat-mouse chimeras generated by rat ESCs (DAC2). Left, an E18.5 rat-mouse chimeric fetus. Red, hKO-labeled rat cells. Right, a 12-month-old (top) and 24-month-old (bottom) rat-mouse chimera.

(B) Chimera forming efficiencies with rat ESC lines (DAC2 and DAC8) and rat iPSC lines (SDFE and SDFF). n, number of embryo transfers.

(C) Representative fluorescence images showing hKO-labeled rat ESCs (DAC2) contributed to different tissues in the 24-month-old rat-mouse chimera. Red, hKO-labeled rat cells. Blue, DAPI. Scale bar, 100 μm.

(D) Representative immunofluorescence images showing the expression of aging-related histone marks, including H3K9me3 and H4K20me3, in the kidney tissue of neonatal and 24-month-old rat-mouse chimeras. Scale bar, 10 μm.

(E) Levels of chimerism of rat ESCs (DAC2) in different tissues of the 24-month-old rat-mouse chimera. Error bars indicate SD.

(F) Rat iPSCs (SDFE) contributed to the neonatal mouse gall bladder. Left, bright-field (top) and fluorescence (bottom) images showing a neonatal mouse gallbladder contained cells derived from rat iPSCs. White arrowheads indicate the gallbladder. Right, representative immunofluorescence images showing the expression of a gallbladder epithelium marker (EpCAM) by rat cells. Red, hKO-labeled rat cells; blue, DAPI. Scale bar, 50 μm.

See also Figure S1 and Table S2.

understand human embryogenesis and to develop regenerative therapies for treating patients.

Like naive rodent PSCs, naive hPSCs can potentially be used to generate interspecies chimeras for studying human development and disease, and producing functional human tissues via interspecies blastocyst complementation. To date, however, all reported attempts on generating hPSC-derived interspecies chimeras have used the mouse as the host animal, and the results obtained suggest that this process is rather inefficient (Gafni et al., 2013; Theunissen et al., 2014, 2016). Although the mouse is one of the most important experimental models for stem cell research, there are considerable differences between humans and mice (e.g., early post-implantation development, embryo size, gestational length, and developmental speed), which may hinder not only the efficiency but also the usefulness of human-mouse chimeric studies. Thus, expanding the repertoire of host species may complement this incipient but promising area of research in the field of regenerative medicine. In particular, interspecies chimera research of

hPSCs using ungulates, e.g., pigs, cattle, and sheep, could lead to improved research models, as well as novel *in vivo* strategies for (1) generating human organs and tissues, (2) designing new drug screening methodologies, and (3) developing new human disease models (Wu and Izpisua Belmonte, 2015). Experiments to empirically test and evaluate the

chimeric contribution of various types of hPSCs in the ungulates are thus imperative, but currently lacking. To start filling this void, we tested different types of hPSCs for their chimeric contribution potential in two ungulate species, pigs and cattle.

RESULTS

Naive Rat PSCs Robustly Contribute to Rat-Mouse Interspecies Chimera Formation

We first used rodent models to gain a better understanding of the factors and caveats underlying interspecies chimerism with PSCs. To this end, we used two chimeric-competent rat ESC lines, DAC2 and DAC8 (Li et al., 2008). We labeled both lines with a fluorescent marker, humanized kusabira orange (hKO), for cell tracking and injected them into mouse blastocysts. Following embryo transfer (ET) into surrogate mouse mothers, both DAC2 and DAC8 lines gave rise to live rat-mouse chimeras (Figures 1A and S1A). Many of the chimeras developed into

adulthood, and one chimera reached 2 years of age (Figure 1A), indicating that the xenogeneic rat cells sustained the physiological requirements of the mouse host without compromising its life span. We also generated two rat iPSC lines (SDFE and SDFF) from tail tip fibroblasts (TTFs) isolated from a neonatal Sprague-Dawley rat and used them to generate rat-mouse chimeras. Similar to rat ESCs, rat iPSCs could also robustly contribute to chimera formation in mice (Figure S1B). Overall, the chimera forming efficiencies of all rat PSC lines tested were ~20%, consistent with a previous report (Figure 1B) (Kobayashi et al., 2010).

We observed contribution of rat cells to a wide range of tissues and organs in both neonatal and aged rat-mouse chimeras (Figures 1C, S1A, and S1B). We examined aging-related histone marks in both neonatal and aged chimeras and found that the 2-year-old chimera exhibited histone signatures characteristic of aging (Figure 1D). We quantified the degree of chimerism in different organs of the aged chimera via quantitative qPCR analysis of genomic DNA using a rat-specific primer (Table S2). We found that different tissues contained different percentages of rat cells, with the highest contribution observed in the heart (~10%) (Figure 1E).

One anatomical difference between mice and rats is that rats lack a gallbladder. In agreement with a previous report (Kobayashi et al., 2010), we also observed the presence of gallbladders in rat-mouse chimeras (chimeras derived from injecting rat PSCs into a mouse blastocyst). Interestingly, rat cells contributed to the chimeric gallbladder and expressed the gallbladder epithelium marker EpCAM (Figures 1F and S1C), which suggests that the mouse embryonic microenvironment was able to unlock a gallbladder developmental program in rat PSCs that is normally suppressed during rat development.

A Versatile CRISPR-Cas9-Mediated Interspecies Blastocyst Complementation System

Chimeric contribution of PSCs is random and varies among different host blastocysts and donor cell lines used. To selectively enrich chimerism in a specific organ, a strategy called blastocyst complementation has been developed where the host blastocysts are obtained from a mutant mouse strain in which a gene critical for the development of a particular lineage is disabled (Chen et al., 1993; Kobayashi et al., 2010; Wu and Izpisua Belmonte, 2015). Mutant blastocysts used for complementation experiments were previously obtained from existing lines of knockout mice, which were generated by gene targeting in germ-line-competent mouse ESCs—a time-consuming process. To relieve the dependence on existing mutant strains, we developed a blastocyst complementation platform based on targeted genome editing in zygotes. We chose to use the CRISPR-Cas9 system, which has been harnessed for the efficient generation of knockout mouse models (Wang et al., 2013) (Figure 2A).

For proof-of-concept, we knocked out the *Pdx1* gene in mouse by co-injecting Cas9 mRNA and *Pdx1* single-guide RNA (sgRNA) into mouse zygotes. During mouse development, *Pdx1* expression is restricted to the developing pancreatic anlagen and is a key player in pancreatic development. Mice homozygous for a targeted mutation in *Pdx1* lack a pancreas and

die within a few days after birth (Jonsson et al., 1994; Offield et al., 1996). Similarly, *Pdx1*^{-/-} mice generated by the zygotic co-injection of Cas9 mRNA and *Pdx1* sgRNA were anaplastic, whereas other internal organs appeared normal (Figure S2A). These mice survived only a few days after birth. We observed the efficiency for obtaining *Pdx1*^{-/-} mouse via CRISPR-Cas9 zygote genome editing was ~60% (Figure S2F). Next, we combined zygotic co-injection of Cas9/sgRNA with blastocyst injection of rat PSCs, and found that rat PSC-derivatives were enriched in the neonatal pancreas of *Pdx1*^{-/-} mice and expressed α-AMYLASE, a pancreatic enzyme that helps digest carbohydrates (Figures 2B and S2B). Of note is that in these animals the pancreatic endothelial cells were still mostly of mouse origin, as revealed by staining with an anti-CD31 antibody (Figure 2B). Importantly, pancreas enriched with rat cells supported the successful development of *Pdx1*^{-/-} mouse host into adulthood (>7 months), and maintained normal serum glucose levels in response to glucose loading, as determined using the glucose tolerance test (GTT) (Figure S2C).

Taking advantage of the flexibility of the CRISPR-Cas9 zygotic genome editing, we next sought to enrich xenogeneic rat cells toward other lineages. *Nkx2.5* plays a critical role in early stages of cardiogenesis, and its deficiency leads to severe growth retardation with abnormal cardiac looping morphogenesis, an important process that leads to chamber and valve formation (Lyons et al., 1995; Tanaka et al., 1999). Mice lacking *Nkx2.5* typically die around E10.5 (Lyons et al., 1995; Tanaka et al., 1999). Consistent with previous observations, CRISPR-Cas9 mediated inactivation of *Nkx2.5* resulted in marked growth-retardation and severe malformation of the heart at E10.5 (Figure S2D). In contrast, when complemented with rat PSCs, the resultant *Nkx2.5*^{-/-} mouse hearts were enriched with rat cells and displayed a normal morphology, and the embryo size was restored to normal (Figures 2C and S2D). Of note is that although rat PSCs rescued embryo growth and cardiac formation in E10.5 *Nkx2.5*^{-/-} mouse embryos, to date we still have not obtained a live rescued chimera ($n = 12$, where n is the number of ETs). *Pax6* is a transcription factor that plays key roles in development of the eye, nose and brain. Mice homozygous for a *Pax6* loss-of-function mutation lack eyes, nasal cavities, and olfactory bulbs, and exhibit abnormal cortical plate formation, among other phenotypes (Gehring and Ikeo, 1999). *Pax6* is best known for its conserved function in eye development across all species examined (Gehring and Ikeo, 1999). In agreement with the published work, CRISPR-Cas9 mediated *Pax6* inactivation disrupted eye formation in the E15.5 mouse embryo (Figure S2E). When complemented with rat PSCs, we observed the formation of chimeric eyes enriched with rat cells in *Pax6*^{-/-} mouse neonate (Figures 2D and S2E). Similar to *Pdx1*^{-/-}, we observed efficient generation of homozygous *Nkx2.5*^{-/-} and *Pax6*^{-/-} mouse embryos via zygotic co-injection of Cas9 mRNA and sgRNAs (Figure S2F). All DNA sequencing results of CRISPR-Cas9 mediated gene knockouts and gRNA sequences are summarized in Tables S1 and S2, respectively.

In sum, for the pancreas, heart, and eye, as well as several other organs (data not shown), we successfully generated chimerized organs that were enriched with rat cells, demonstrating

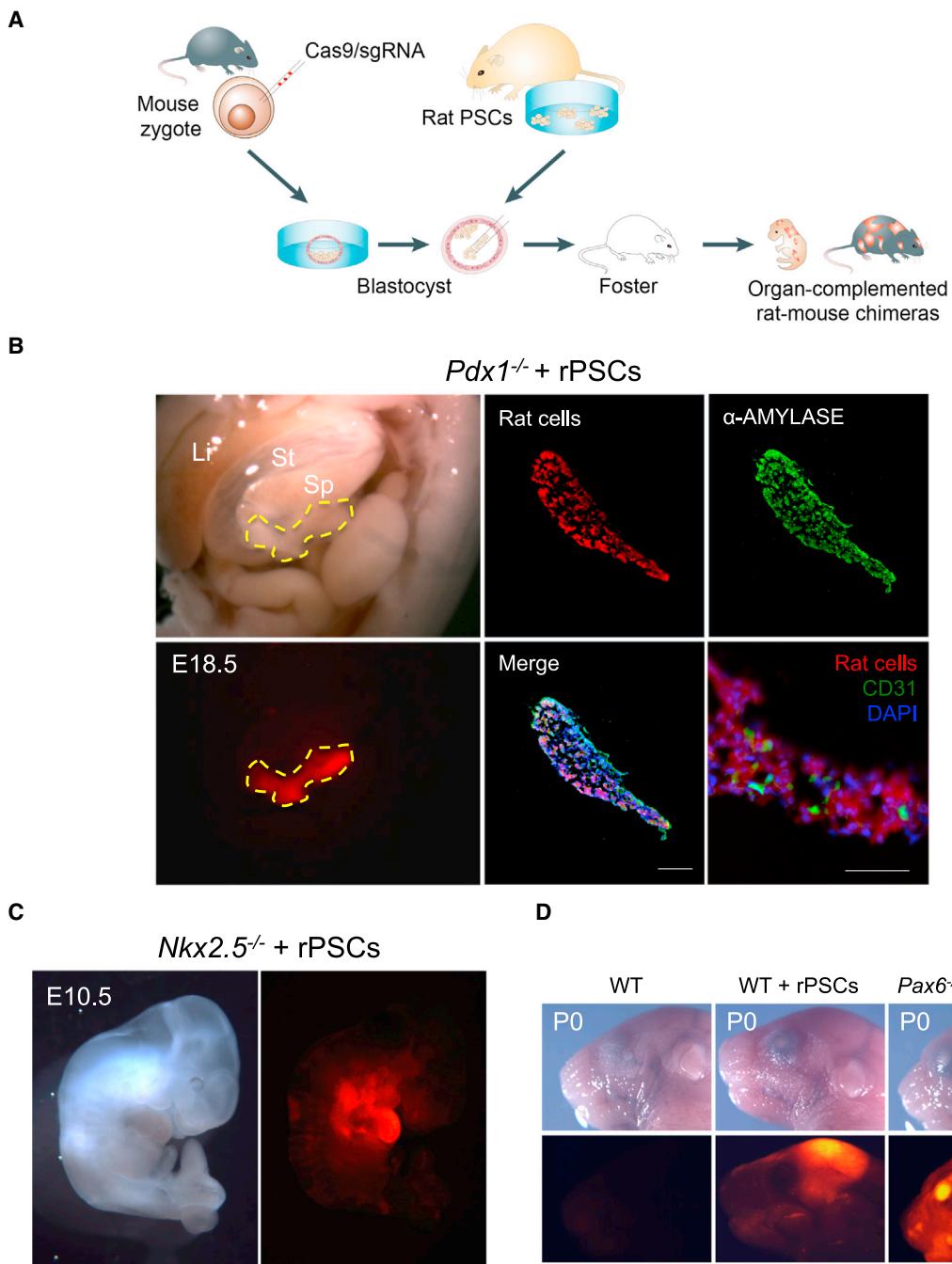


Figure 2. Interspecies Blastocyst Complementation via CRISPR-Cas9-Mediated Zygote Genome Editing

(A) Schematic of the CRISPR-Cas9 mediated rat-mouse blastocyst complementation strategy.

(B) Left, bright-field (top) and fluorescence (bottom) images showing the enrichment of rat cells in the pancreas of an E18.5 *Pdx1^{-/-}* mouse. Li, liver; St, stomach; Sp, spleen. Yellow-dotted line encircles the pancreas. Red, hKO-labeled rat cells. Middle and right (top), representative immunofluorescence images showing rat cells expressed α -amylase in the *Pdx1^{-/-}* mouse pancreas. Blue, DAPI. Right (bottom), a representative immunofluorescence image showing that some pancreatic endothelial cells, as marked by a CD31 antibody, were not derived from rat PSCs. Scale bar, 100 μ m.

(C) Bright field (left) and fluorescence (right) images showing the enrichment of rat cells in the heart of an E10.5 *Nkx2.5^{-/-}* mouse. Red, hKO-labeled rat cells.

(D) Bright field (top) and fluorescence (bottom) images showing the enrichment of rat cells in the eye of a neonatal *Pax6^{-/-}* mouse. Red, hKO-labeled rat cells. WT, mouse control; WT+rPSCs, control rat-mouse chimera without Cas9/sgRNA injection.

See also Figure S2 and Tables S1 and S2.

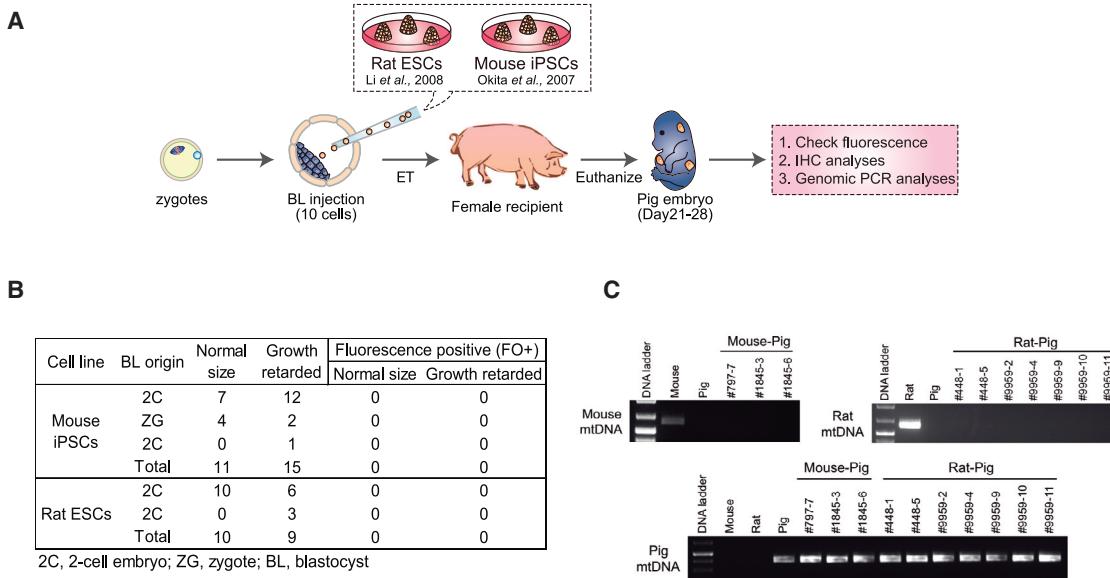


Figure 3. Naive Rodent PSCs Fail to Contribute to Chimera Formation in Pigs

(A) Schematic of the generation and analyses of post-implantation pig embryos derived from blastocyst injection of naive rodent PSCs.

(B) Summary of the pig embryos recovered between day 21–28 of pregnancy.

(C) Genomic PCR analyses of pig embryos derived from blastocyst injection of mouse iPSCs or rat ESCs. Mouse- and rat-specific mtDNA primers were used for the detection of chimeric contribution from mouse iPSCs and rat ESCs, respectively. Pig-specific mtDNA primers were used for the control.

See also Tables S2 and S3.

the efficacy and versatility of the CRISPR-Cas9 mediated interspecies blastocyst complementation platform.

Naive Rodent PSCs Do Not Contribute to Chimera Formation in Pigs

It is commonly accepted that the key functional feature of naive PSCs is their ability to generate intraspecies germline chimeras (Nichols and Smith, 2009). Studies in rodents also support the notion that attaining the naive pluripotent state is the key step in enabling chimera formation across species boundaries (Xiang et al., 2008; Isotani et al., 2011; Kobayashi et al., 2010). However, it has not yet been tested whether naive rodent PSCs can contribute to chimera formation when using a non-rodent host. To further examine the relationship between naive PSCs and interspecies chimerism, we injected rat ESCs into pig blastocysts followed by ET to recipient sows. In addition to rat ESCs, we also used a germline competent mouse iPSC line (Okita et al., 2007). Several criteria were used to determine the chimeric contribution of rodent cells in pig embryos, namely, (1) detection of fluorescence (hKO) signal, (2) immunohistochemical (IHC) labeling of embryo sections with an anti-hKO antibody, and (3) genomic PCR with mouse- or rat-specific primers targeting mitochondrial DNA (mtDNA) (Figure 3A). We terminated the pregnancy between day 21–28 of pig development and collected embryos derived from the injection of mouse iPSCs or rat ESCs into pig blastocyst (26 and 19 embryos, respectively) (Figure 3B; Table S3). We failed to detect any hKO signal in both normal size and growth retarded embryos (Figure 3B). We next sectioned the pig embryos and stained them with an antibody against hKO. Similarly, we did not detect any hKO-positive cells

in the embryonic sections examined (data not shown). Finally, we employed a more sensitive test, using genomic PCR to amplify rat- or mouse-specific mtDNA sequences (pig-specific mtDNA primers served as the loading control) (Table S2). Consistently, genomic PCR analyses did not detect any rodent contribution to the pig embryos (Figure 3C). Taken together, although naive rodent PSCs can robustly contribute to rodent-specific interspecies chimeras, our results show that these cells are incapable of contributing to normal embryonic development in pigs.

Generation of Naive, Intermediate, and Primed hiPSCs

Next, we sought to systematically evaluate the chimeric competency of hPSCs in ungulate embryos. We generated hiPSCs using several reported naive PSC culture methods, a culture protocol supporting a putative intermediate pluripotent state between naive mESCs and primed mEpiSCs (Tsukiyama and Ohnata, 2014), and a primed culture condition (Figure 4A). Mouse ground state culture condition (2iL) induces the differentiation of primed hPSCs. However, when combined with the forced expression of NANOG and KLF2 (NK2), transcription factors that help to maintain murine naive pluripotency, 2iL culture can stabilize hPSCs in an immature state (Takashima et al., 2014; Theunissen et al., 2014). We generated doxycycline (DOX)-inducible NK2-expressing naive hiPSCs cultured in 2iL medium from primed hiPSCs (2iLD-hiPSCs). Transgene-free primed hiPSCs were reprogrammed from human foreskin fibroblasts (HFFs) using episomal vectors (Okita et al., 2011). For comparison, we also generated naive hiPSCs from HFFs using the NNSM culture condition (Gafni et al., 2013) (NNSM-hiPSCs). It has been shown that cells grown in 4i medium, a

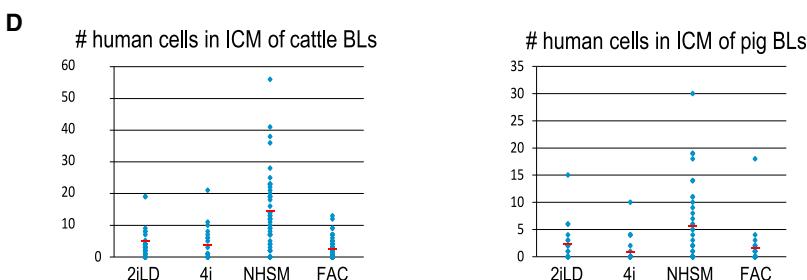
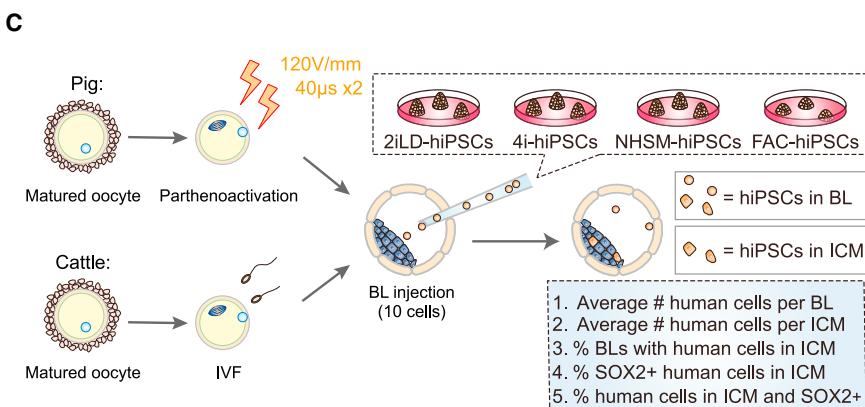
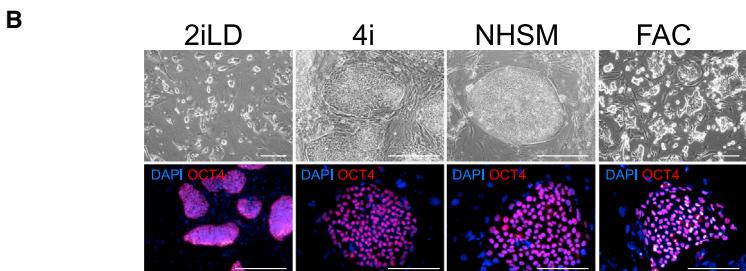
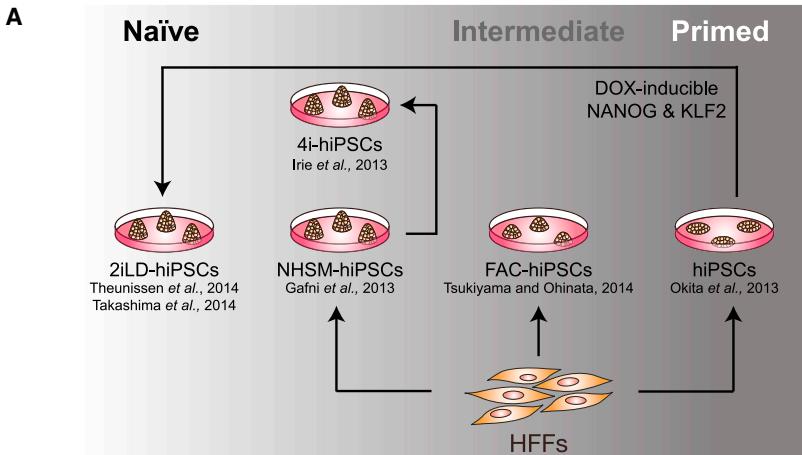


Figure 4. Generation and Interspecies ICM Incorporation of Different Types of hiPSCs

(A) Schematic of the strategy for generating naïve, intermediate, and primed hiPSCs.

(B) (Top) Representative bright-field images showing the colony morphologies of naïve (2iLD-, 4i-, and NHSM-hiPSCs) and intermediate (FAC-hiPSCs) hiPSCs. Bottom, representative immunofluorescence images of naïve and intermediate hiPSCs stained with an anti-OCT4 antibody. Red, OCT4; blue, DAPI. Scale bar, 100 μm.

(C) Schematic of the experimental procedures for producing cattle and pig blastocysts obtained from in vitro fertilization (IVF) and parthenoactivation, respectively. Blastocysts were subsequently used for laser-assisted blastocyst injection of hiPSCs. After hiPSC injection, blastocysts were cultured in vitro for 2 days before fixation and analyzed by immunostaining with an anti-HuNu and an anti-SOX2 antibodies. Criteria to evaluate the survival of human cells, as well as the degree and efficiency of ICM incorporation are shown in the blue box.

(D) Number of hiPSCs that integrated into the cattle (left) and pig (right) ICMs after ten hiPSCs were injected into the blastocyst followed by 2 days of in vitro culture. Red line, the average number of ICM-incorporated hiPSCs. Blue dot, the number of ICM-incorporated hiPSCs in each blastocyst.

See also Figure S3 and Table S4.

simplified version of NHSM, have a significant potential for germ cell induction, a distinguishing feature between naive mESCs and primed mEpiSCs (Irie et al., 2015). Thus, we also culture-adapted NHSM-hiPSCs in 4i medium (4i-hiPSCs), resulting in stable 4i-hiPSCs with similar morphological and molecular characteristics to parental NHSM-hiPSCs (Figure 4B). In addition, we generated another type of hiPSC by direct reprogramming of HFFs in a modified mEpiSC medium containing bFGF, Activin-A, and CHIR99021 (FAC; Figure 4A). mEpiSCs cultured in FAC medium exhibited features characteristic of both naive mESCs and primed mEpiSCs, supporting an intermediate pluripotent state (Tsukiyama and Ohnata, 2014). hiPSCs generated and cultured in FAC medium (FAC-hiPSCs) displayed a colony morphology intermediate between that of 2iLD- and primed hiPSCs, with less defined borders (Figure 4B). 2iLD-hiPSCs, NHSM-hiPSCs, 4i-hiPSCs, and FAC-hiPSCs could all be stably maintained long term in culture, preserving normal karyotypes and the homogeneous, nuclear localization of OCT4 protein (Figure 4B; data not shown). Notably, similar to hiPSCs grown in naive cultures (2iLD-hiPSCs, NHSM-hiPSCs, 4i-hiPSCs), FAC-hiPSCs could also be efficiently propagated by single-cell dissociation without using a ROCK kinase inhibitor. After injecting cells into the kidney capsule of immunodeficient NSG mice, all of these hiPSCs formed teratomas that consisted of tissues from all three germ layers: endoderm, mesoderm, and ectoderm (Figure S3A). To facilitate the identification of human cells in subsequent chimera experiments, we labeled hiPSCs with either green fluorescence protein (GFP) or hKO fluorescence markers.

Chimeric Contribution of hiPSCs to Pig and Cattle Blastocysts

The ability to integrate into the inner cell mass (ICM) of a blastocyst is informative for evaluating whether hiPSCs are compatible with pre-implantation epiblasts of the ungulate species. This is also one of the earliest indicators of chimeric capability. We therefore evaluated interspecies chimeric ICM formation by injecting hiPSCs into blastocysts from two ungulate species, pig and cattle.

Cattle-assisted reproductive technologies, such as in vitro embryo production, are well established given the commercial benefits of improving the genetics of these animals. Cattle also serve as a research model because of several similarities to human pre-implantation development (Hansen, 2014; Hasler, 2014). Using techniques for producing cattle embryos in vitro, we developed a system for testing the ability and efficiency of hiPSCs to survive in the blastocyst environment and to integrate into the cattle ICM (Figure 4C). Cattle embryos were obtained by in vitro fertilization (IVF) using in vitro matured oocytes collected from ovaries obtained from a local slaughterhouse. The tightly connected cells of the blastocyst trophectoderm from large livestock species, such as pig and cattle, form a barrier that complicates cell microinjection into the blastocoel. Thus, microinjection often results in embryo collapse and the inability to deposit the cells into the embryo. To facilitate cell injection we employed a laser-assisted approach, using the laser to perforate the zona pellucida and to induce damage to a limited number of trophectoderm cells. This allowed for easy access into the blastocyst cavity for transferring the human cells (Figure S3B). Furthermore,

the zona ablation and trophectoderm access allowed use a blunt-end pipette for cell transfer, thus minimizing further embryo damage. This method resulted in a nearly 100% injection effectiveness and >90% embryo survival.

To determine whether hiPSCs could engraft into the cattle ICM, we injected ten cells from each condition into cattle blastocysts collected 7 days after fertilization. After injection, we cultured these blastocysts for additional 2 days before analysis. We used several criteria to evaluate the chimeric contribution of hiPSCs to cattle blastocysts: (1) average number of human cells in each blastocyst, (2) average number of human cells in each ICM, (3) percentage of blastocysts with the presence of human cells in the ICM, (4) percentage of SOX2+ human cells in the ICM, and (5) percentage of human cells in the ICM that are SOX2+ (Figure 4C). Our results indicated that both naive and intermediate (but not primed) hiPSCs could survive and integrate into cattle ICMs, albeit with variable efficiencies (Figures 4D and S3C–S3E; Table S4). Compared with other cell types, 4i-hiPSCs exhibited the best survival (22/23 blastocysts contained human cells), but the majority of these cells lost SOX2 expression (only 13.6% of human cells remained SOX2+). On average, 3.64 4i-hiPSCs were incorporated into the ICM. NHSM-hiPSCs were detected in 46 of 59 injected blastocysts, with 14.41 cells per ICM. Of these, 89.7% remained SOX2+. For 2iLD-hiPSCs, 40 of 52 injected blastocysts contained human cells, with 5.11 cells per ICM, and 69.9% of the ICM-incorporated human cells remained SOX2+. FAC-hiPSCs exhibited moderate survival rate (65/101) and ICM incorporation efficiency (39/101), with an average of 2.31 cells incorporated into the ICM, and 89.3% remaining SOX2+.

We also performed ICM incorporation assays by injecting hiPSCs into pig blastocysts. Because certain complications are frequently associated with pig IVF (Abeydeera, 2002; Grupen, 2014) (e.g., high levels of polyspermic fertilization), we used a parthenogenetic activation model, which enabled us to efficiently produce embryos that developed into blastocysts (King et al., 2002). Pig oocytes were obtained from ovaries collected at a local slaughterhouse. Once the oocytes were matured in vitro, we removed the cumulus cells and artificially activated the oocytes using electrical stimulation. They were then cultured to blastocyst stage (Figure 4C). We injected ten hiPSCs into each pig parthenogenetic blastocyst and evaluated their chimeric contribution after 2 days of in vitro culture (Figures 4C and S3C–S3E; Table S4). Similar to the results in cattle, we found that hiPSCs cultured in 4i and NHSM media survived better and yielded a higher percentage of blastocysts harboring human cells (28/35 and 37/44, respectively). Also, among all blastocysts containing human cells, we observed an average of 9.5 cells per blastocyst for 4i-hiPSCs and 9.97 cells for NHSM-hiPSCs. For NHSM-hiPSCs, 19/44 blastocysts had human cells incorporated into the ICM. In contrast, only 6/35 blastocysts had 4i-hiPSCs localized to the ICM. For 2iLD-hiPSCs, we observed an average of 5.7 cells per blastocyst, with 2.25 human cells localized to the ICM. For FAC-hiPSCs, an average of 3.96 and 1.62 human cells were found in the blastocyst and ICM, respectively. Once incorporated into the ICM, 82.2%, 72%, 60.9%, and 40% of 2iLD-, 4i-, NHSM-, and FAC-hiPSCs, respectively, stained positive for the pluripotency

marker SOX2. These results indicate that both naive and intermediate hiPSCs seem to perform better when injected into cattle than pig blastocysts. This suggests a different *in vivo* blastocyst environment in pig and cattle, with the cattle blastocysts providing an environment that is more permissive for hiPSC integration and survival.

Chimeric Contribution of hiPSCs to Post-implantation Pig Embryos

Although ICM incorporation of hiPSCs is the necessary first step to contribute to the embryo proper of host animals, it has limited predictive value for post-implantation chimera formation, as other factors are involved. Next, we investigated if any of the naive and intermediate hiPSCs that we generated, which showed robust ICM incorporation in pre-implantation blastocysts, could contribute to post-implantation development following ET. The pig has certain advantages over cattle for experiments involving post-implantation embryos, as they are a polytocous species, and are commonly used as a translational model given their similarities to humans concerning organ physiology, size, and anatomy. We thus chose the pig for these experiments. Since there was little to no contribution of primed hiPSCs, even at the pre-implantation blastocyst stage, we excluded these cells from the ET experiments. Pig embryos were derived *in vivo* or through parthenogenesis. A total of 167 embryo donors were used in this study, from which we collected 1,298 zygotes, 1,004 two-cell embryos and 91 morulae (**Table S5**). Embryos were cultured *in vitro* until they reached the blastocyst stage (**Figures S4AA** and **S4B**). Overall, 2,181 good quality blastocysts with a well-defined ICM were selected for subsequent blastocyst injections, of which 1,052 were derived from zygotes, 897 from two-cell embryos, 91 from morulae, and 141 from parthenogenetic activation (**Table S5**). We injected 3–10 hiPSCs into the blastocoel of each of these blastocysts (**Figures 5A, S4A, and S4C; Table S6**). After *in vitro* embryo culture, a total of 2,075 embryos (1,466 for hiPSCs; **Table S6**; 477 for rodent PSCs; **Table S3**) that retained good quality were transferred to surrogate sows. A total of 41 surrogate sows received 30–50 embryos each, resulting in 18 pregnancies (**Table S6**). Collection of embryos between day 21–28 of development resulted in the harvesting of 186 embryos: 43 from 2iLD-hiPSCs, 64 from FAC-hiPSCs, 39 from 4i-hiPSCs, and 40 from NHSM-hiPSCs (**Figures 5B, S4A, S4D, and S4F**). In addition, 17 control embryos were collected from an artificially inseminated sow (**Figure 5B**).

Following evaluating the developmental status of the obtained embryos, more than half showed retarded growth and were smaller than control embryos (**Figures 5B** and **S4B**), as was seen when pig blastocysts were injected with rodent PSCs (**Figure 3B**). Among different hiPSCs, embryos injected with FAC-hiPSCs were more frequently found to be normal size (**Figure 5C**). From the recovered embryos, and based on fluorescence imaging (GFP for 2iLD-hiPSCs and FAC-hiPSCs; hKO for 4i-hiPSCs and NHSM-hiPSCs), we observed positive fluorescence signal (FO+) in 67 embryos among which 17 showed a normal size and morphology, whereas the rest were morphologically underdeveloped (**Figures 5B**). In contrast, among fluorescence negative embryos we found the majority (82/119) appeared normal size (**Figure 5E**), suggesting contribution of hiPSCs might have

interfered with normal pig development. Closer examination of the underdeveloped embryos revealed that 50 out of 87 were FO+ (**Figures 5B**). Among all the FO+ embryos the distribution of normal size versus growth retarded embryos for each cell lines was: 3:19 for 2iLD-hiPSCs, 7:14 for FAC-hiPSCs, 2:12 for 4i-hiPSCs, and 5:5 for NHSM-hiPSCs (**Figure 5D**). Among normal size embryos we found 3/13 from 2iLD-hiPSCs, 7/47 from FAC-hiPSCs, 2/14 from 4i-hiPSCs, and 5/25 from NHSM-hiPSCs that were FO+ (**Figure 5B**). All normal size FO+ embryos derived from 2iLD-hiPSCs, 4i-hiPSCs, or NHSM-hiPSCs showed a very limited fluorescence signal (**Figure S5A**). In contrast, normal size FO+ FAC-hiPSC-derived embryos typically exhibited a more robust fluorescence signal (**Figures 6A** and **S5A**).

Detecting fluorescence signal alone is insufficient to claim chimeric contribution of donor hiPSCs to these embryos, as auto-fluorescence from certain tissues and apoptotic cells can yield false positives, especially when chimerism is low. We thus sectioned all normal size embryos deemed positive based on the presence of fluorescence signal and subjected them to IHC analyses with antibodies detecting GFP or hKO. For 2iLD-hiPSC-, 4i-hiPSC-, and NHSM-hiPSC-derived embryos, in agreement with fluorescence signals observed in whole-embryo analysis, we detected only a few hKO- or GFP-positive cells in limited number of sections (**Figure S5A**). This precluded us from conducting further IHC analysis using lineage markers. For FAC-hiPSC-derived embryos, we confirmed via IHC analysis (using an anti-GFP antibody) that they contained more human cells (**Figures 6A, S5A, and S5B**). We then stained additional sections using antibodies against TUJ1, EPCAM, SMA, CK8, and HNF3 β (**Figures 6B and S5C**) and observed differentiation of FAC-hiPSCs into different cell lineages. In addition, these cells were found negative for OCT4, a pluripotency marker (data not shown). Moreover, the presence of human cells was further verified with a human-specific HuNu antibody staining (**Figure 6B**) and a sensitive genomic PCR assay using a human specific *Alu* sequence primer (**Figure 6C; Table S2**). Together, these results indicate that naive hiPSCs injected into pig blastocysts inefficiently contribute to chimera formation, and are only rarely detected in post-implantation pig embryos. An intermediate hPSC type (FAC-hiPSCs) showed better chimeric contribution and differentiated to several cell types in post-implantation human-pig chimeric embryos. It should be noted that the levels of chimerism from all hiPSCs, including the FAC-hiPSCs, in pig embryos were much lower when compare to rat-mouse chimeras (**Figures 1C, 1E, S1A, and 1B**), which may reflect the larger evolutionary distance between human-pig than between rat-mouse.

DISCUSSION

Our study confirms that live rat-mouse chimeras with extensive contribution from naive rat PSCs can be generated. This is in contrast to earlier work in which rat ICMs were injected into mouse blastocysts ([Gardner and Johnson, 1973](#)). One possible explanation for this discrepancy is that cultured PSCs acquire artificial features that make them more proliferative and/or better able to survive than embryonic ICM cells, which in turn leads to their more robust xeno-engraftment capability in a mouse host.

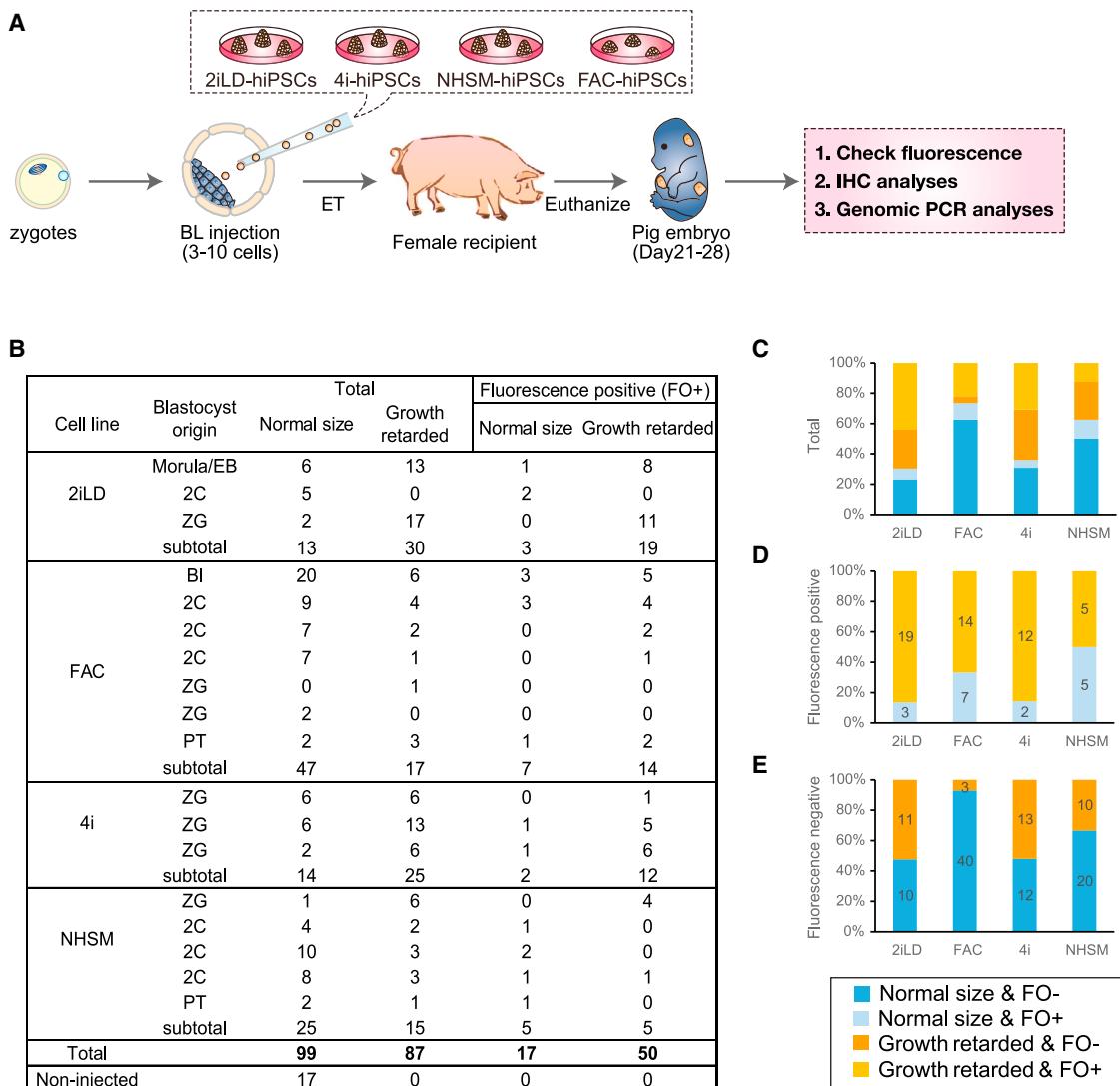


Figure 5. Generation of Post-implantation Human-Pig Chimeric Embryos

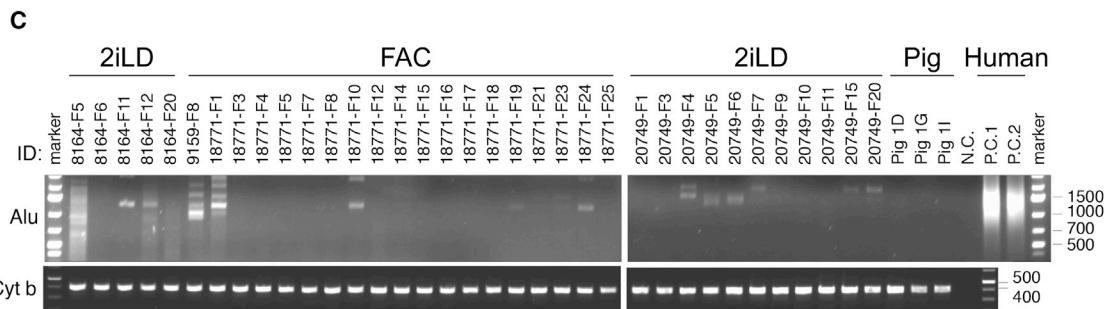
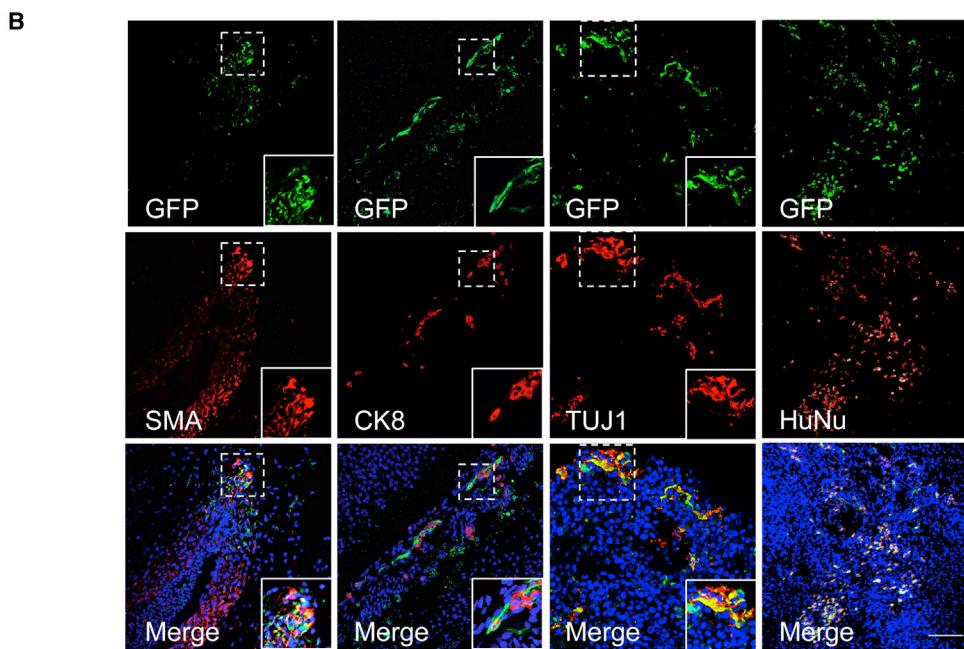
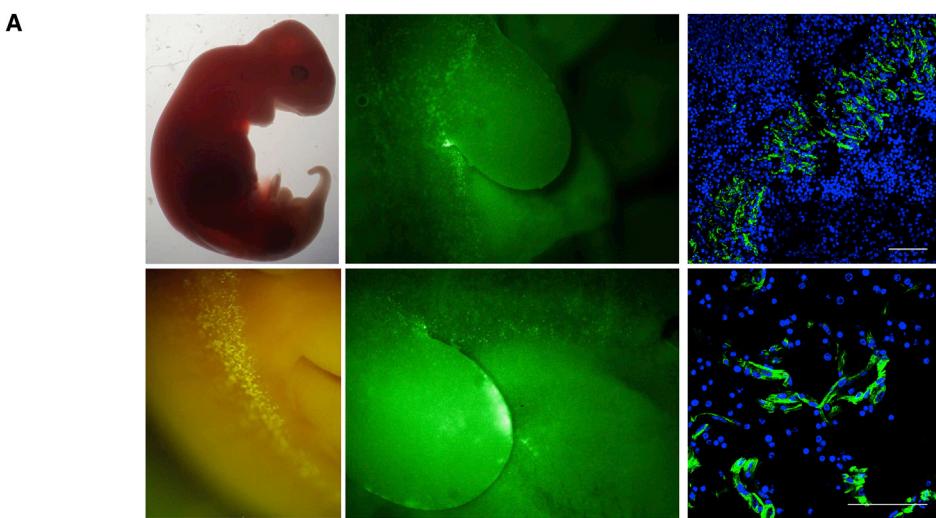
- (A) Schematic of the experimental procedures for the generation and analyses of post-implantation pig embryos derived from blastocyst injection of naive and intermediate hiPSCs.
- (B) Summary of the pig embryos recovered between day 21–28 of pregnancy.
- (C) Bar graph showing proportions of normal size and growth retarded embryos, as well as the proportion of fluorescence-positive and -negative embryos, generated from different types of hiPSCs.
- (D) Bar graph showing the proportion of normal size and growth-retarded embryos (among those exhibiting a fluorescence signal) generated from different types of hiPSCs.
- (E) Bar graph showing the proportion of normal-sized and growth-retarded embryos (among those without exhibiting a fluorescence signal) generated from different types of hiPSCs.

See also Figure S4 and Tables S5 and S6.

Rat-mouse chimeras generated by injecting donor rat PSCs into a mouse host were mouse-sized and developed into adulthood with apparently normal appearance and physiology. We further show in this study that a rat-mouse chimera could live a full mouse lifespan (about 2 years) and exhibit molecular signatures characteristic of aged cells. This demonstrates that cells from two different species, which diverged ~18 million years ago, can live in a symbiotic environment and are able to support normal organismal aging. The fact that rat PSCs were able to

contribute to the mouse gallbladder, an organ that is absent in the rat, highlights the importance of embryonic niches in orchestrating the specification, proliferation, and morphogenesis of tissues and organs during organismal development and evolutionary speciation (Izpisúa-Belmonte et al., 1992).

Previous interspecies blastocyst complementation experiments generated host embryos by crossing heterozygous mutant mouse strains, which were themselves generated through targeted gene disruption in germline competent ESCs.



(legend on next page)

These experiments are labor intensive and time consuming. Moreover, only ~25% of blastocysts derived from genetic crosses are homozygous mutants, posing a limitation for efficient complementation. CRISPR-Cas9 mediated zygote genome editing offers a faster and more efficient one-step process for generating mice carrying homozygous mutations, thereby providing a robust interspecies blastocyst complementation platform. Additionally, the multiplexing capability of CRISPR-Cas9 (Cong et al., 2013; Yang et al., 2015) could potentially be harnessed for multi-lineage complementation. For example, in the case of the pancreas, one might hope to eliminate both the pancreatic parenchyma and vasculature of the host to generate a more complete xenogeneic pancreas. Despite the advantages, there are several technical limitations of the CRISPR-Cas9 blastocyst complementation system that need to be overcome before unlocking its full potential. First, gene inactivation relies on the error-prone, non-homologous end joining (NHEJ) pathway, which is often unpredictable. In-frame mutations and mosaicism are among the factors that may affect outcomes. A more predictable targeted gene inactivation strategy that utilizes homologous recombination (HR) is still inefficient in the zygote. Second, each embryo must be injected twice when using this system and embryos must be cultured *in vitro* for several days before ET, thereby compromising embryo quality. Technical advancements that include a more robust gene-disruption strategy (e.g., targeted generation of frameshift mutations via homology independent targeted integration [Suzuki et al., 2016]), alternative CRISPR/Cas9 delivery methods, and improved culture conditions for manipulated embryos will likely help improve and optimize the generation of organogenesis-disabled hosts.

We observed a slower clearance of an intraperitoneally injected glucose load for *Pdx1*^{-/-} than *Pdx1*^{+/+} rat-mouse chimeras, while both were slower than wild-type mouse controls (Figure S2C). While this result may seem to contradict a previous report (Kobayashi et al., 2010), the discrepancy is likely due to the development of autoimmune type inflammation that is often observed in adult rat-mouse (chimeras made by injection of rat PSCs into mouse blastocyst, data not shown) (>7 months, this study) and mouse-rat chimeras (chimeras made by injection of mouse PSCs into rat blastocyst; H. Nakauchi, personal communication), which is less evident in young chimeras (~8 weeks; Kobayashi et al. 2010). Interestingly though, we did observe a similarly slower clearance of glucose load in wild-type rats, although the initial spike was much lower in rats compared to mice or chimeras (Figure S2C). Thus, the rat cellular origin might also have played a role in the different GTT responses observed.

Rodent ESCs/iPSCs, considered as the gold standard cells for defining naive pluripotency, can robustly contribute to intra- and inter-species chimeras within rodent species. These and other results have led to the assumption that naive PSCs are the cells of choice when attempting to generate interspecies chimeras involving more disparate species. Here, we show that rodent PSCs fail to contribute to chimera formation when injected into pig blastocysts. This highlights the importance of other contributing factors underlying interspecies chimerism that may include, but not limited to, species-specific differences in epiblast and trophectoderm development, developmental kinetics, and maternal microenvironment.

To date, and taking into consideration all published studies that have used the mouse as the host species, it is probably appropriate to conclude that interspecies chimera formation involving hPSCs is inefficient (De Los Angeles et al., 2015). It has been argued that this apparent inefficiency results from species-specific differences between human and mouse embryogenesis. Therefore, studies utilizing other animal hosts would help address this important question. Here we focused on two species, pig and cattle, from a more diverse clade of mammals and found that naive and intermediate, but not primed, hiPSCs could robustly incorporate into pre-implantation host ICMs. Following ET, we observed, in general and similar to the mouse studies, low chimera forming efficiencies for all hiPSCs tested. Interestingly, injected hiPSCs seemed to negatively affect normal pig development as evidenced by the high proportion of growth retarded embryos. Nonetheless, we observed that FAC-hiPSCs, a putative intermediate PSC type between naive and primed pluripotent states, displayed a higher level of chimerism in post-implantation pig embryos. IHC analyses revealed that FAC-hiPSCs integrated and subsequently differentiated in host pig embryos (as shown by the expression of different lineage markers, and the lack of expression of the pluripotency marker OCT4). Whether the degree of chimerism conferred by FAC-hiPSCs could be sufficient for eliciting a successful interspecies human-pig blastocyst complementation, as demonstrated herein between rats and mice, remains to be demonstrated. Studies and approaches to improve the efficiency and level of hPSC interspecies chimerism (Wu et al., 2016), such as matching developmental timing, providing a selective advantage for donor hPSCs, generating diverse hPSCs with a higher chimeric potential and selecting a species evolutionarily closer to humans, among others parameters, will be needed.

The procedures and observations reported here on the capability of human pluripotent stem cells to integrate and differentiate in a ungulate embryo, albeit at a low level and efficiency, when

Figure 6. Chimeric Contribution of hiPSCs to Post-implantation Pig Embryos

- (A) Representative bright field (left top) fluorescence (left bottom and middle) and immunofluorescence (right) images of GFP-labeled FAC-hiPSCs derivatives in a normal size day 28 pig embryo (FAC #1). Scale bar, 100 μ m.
 - (B) Representative immunofluorescence images showing chimeric contribution and differentiation of FAC-hiPSCs in a normal size, day 28 pig embryo (FAC #1). FAC-hiPSC derivatives are visualized by antibodies against GFP (top), TUJ1, SMA, CK8 and HuNu (middle). (Bottom) Merged images with DAPI. Insets are higher magnification images of boxed regions. Scale bar, 100 μ m.
 - (C) Representative gel images showing genomic PCR analyses of pig embryos derived from blastocyst injection of 2iLD-iPSCs (surrogates #8164 and #20749) and FAC-hiPSCs (surrogates #9159 and #18771) using a human specific Alu primer. A pig specific primer Cyt b was used for loading control. nc, negative control with no genomic DNA loaded. pc, positive controls with human cells. Pig 1D, 1G, and 1I, pig controls. ID, surrogate and pig embryos.
- See also Figure S5 and Table S2.

optimized, may constitute a first step towards realizing the potential of interspecies blastocyst complementation with hPSCs. In particular, they may provide a better understanding of human embryogenesis, facilitate the development and implementation of humanized animal drug test platforms, as well as offer new insights on the onset and progression of human diseases in an *in vivo* setting. Ultimately, these observations also raise the possibility of xeno-generating transplantable human tissues and organs towards addressing the worldwide shortage of organ donors.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and six tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2016.12.036>.

AUTHOR CONTRIBUTIONS

J.W. and J.C.I.B. conceived the study. J.W. generated and characterized all naïve and intermediate hiPSC lines. K.S. generated and characterized primed

hiPSCs. J.W. and T.H. generated rat iPSCs. J.W., A.P.-L., T.Y., M.M.V., D.O., A.O., P.R., C.R.E., J.W., and P.M.R. performed immunohistochemistry analyses of mouse and pig embryos. K.S., T.Y., E.S., A.P.-L., and M.M.V. performed genotyping, genomic PCR, and genomic qPCR analyses. A.S., M.S., and J.P.L. performed mouse Cas9/sgRNA injection, blastocyst injection, and embryo transfer. Y.S.B., M.S., and M.V. prepared hiPSCs, performed morulae and blastocyst injections, and analyzed hiPSC contribution to cattle and ppig ICMs. H.W. produced parthenogenetic pig embryos. D.A.S., Y.S.B., and M.V. produced cattle embryos. Work at UC Davis and University of Murcia was performed under the supervision of P.J.R. and E.A.M., respectively. E.A.M., M.A.G., C.C., I.P., C.A.M., S.S.B., A.N., and J.R. designed, coordinated, performed, and analyzed data related to pig embryo collection, embryo culture, blastocyst injection, embryo transfer, and embryo recovery. E.N.D., J.L., I.G., P.G., T.B., M.L.M.-M., and J.M.C. coordinated work between Salk, and University of Murcia. J.W., P.J.R., and J.C.I.B. wrote the manuscript.

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