This reporting year, we have been investigating (A) in vitro and in vivo human:sheep chimerism and (B) biopsy methods to screen embryonic genotypes.

**Analysis of human:sheep chimerism:**

Our goal is to make a human:sheep chimera by injecting human iPSCs into sheep embryos. We previously discovered through our live-imaging platform that human iPSCs with forced expression of BCL2 survive and engraft sheep blastocysts (Figure 1A). We microinjected these cells into sheep preimplantation embryos and transferred into the uterus of surrogate ewes. A total of 83 embryos were transferred into 21 sheep. 22 (27%) embryos were recovered three weeks later and analyzed for the presence of human cells. The human cells could not be detected by fluorescence microscopy (Figure 1B). We therefore developed an ultra-sensitive digital PCR assay to detect at least 1 human cell in the presence of 100,000 sheep cells (Figure 1C, 1D). We detected human DNA in nearly half the embryos recovered (Figure 1E). Levels approached 1:10,000 (human:sheep cells). This is 10 times higher than previously reported in pig.
Figure 1: Human:Sheep chimerism in vitro and in vivo.

(A) Primed human iPSCs expressing BCL2 survive and engraft into the ICM of sheep blastocysts. (B) After transferring into a sheep uterus, the embryos were recovered 3 weeks later (top) and analyzed for fluorescence (bottom). Background fluorescence is detected primarily from the liver, but human cells cannot be seen. (C) A digital PCR assay was developed that can simultaneously quantify human cells relative to mammalian DNA over a large dynamic range, even if contaminated with 100,000-fold mammalian DNA. (D) This assay can quantify less than one human cell (~6 picograms) and is minimally influenced with the addition of 100 nanograms of sheep DNA. (E) DNA was extracted from the sheep embryos and the digital PCR assay was used to detect human cells. Red line indicates detection limit of 1 human cell in 100,000 sheep cells. Human DNA was found in 9 out 18 embryos analyzed.

Targeted chimerism:

To achieve our goal of generating human organs in large animals, we must first make human:animal chimeras. This can be achieved by injecting pluripotent stem cells (PSCs) into animal embryos, potentially resulting in systemic chimerism. Ethically, systemic chimerism is concerning due to brain or gamete contribution. In addition, high systemic chimerism leads to embryonic lethality in divergent species (data not shown). Therefore, we explored the practicality of generating chimeras by injecting lineage-restricted progenitor cells into animal blastocysts and post-implantation embryos (Figure 2A).

Human hematopoietic stem cells (HSCs) were injected into sheep fetal livers (ultrasound guided) around E40, which is before development of the adaptive immune system. Prior to injection, LNK was knocked out in the human HSCs by optimizing electroporation conditions with CRISPR/Cas9. Knocking out LNK in rodent HSCs leads to proliferation and takeover of the blood system. TIDE analysis showed 40% allelic knockout rate one day after electroporation (Figure 2B)—this was higher if analyzed after culturing for multiple days, suggesting continued cutting by Cas9 or a survival/proliferative advantage of the KO cells (data not shown). The electroporated
cells were transferred into six sheep fetuses, from which three lambs were born (Figure 2C). The lambs were sacrificed after 3 months. DNA was extracted from blood and multiple samples of bone marrow, liver, spleen and thymus. Droplet digital PCR was used to detect human DNA using an ultra-sensitive assay we previously developed (reported last year, manuscript in preparation). Unfortunately, no human cells were detected, while the positive controls containing 1:100,000 and 1:10,000 human:sheep blood mixtures were easily detected (Figure 2D).

Generating human organs in rats has translational and clinical value. No robust human:rat chimeras have been reported by injecting human PSCs into rat blastocysts. We therefore tried injecting human progenitors into post-implantation rat embryos. Human epithelial cells were able to engraft and survive two days after injecting into E11.5 rat embryos in utero (Figure 2E). Future experiments will explore embryo and/or cell genetic modifications that give the human cells a proliferative advantage, enabling an increase in chimerism over time. To see if we could detect chimeric changes in utero, we tested the sensitivity of a new bioluminescent reporter15 fused with tdTomato. Remarkably, we could detect labeled cells immediately after embryo transfer and in the days following (Figure 2F).

Injecting progenitor cells into post-implantation embryos is technically challenging. Controlling the exact location without removing or damaging the embryo is possible in late-stage fetuses, but injection is usually performed at early stages to prevent immune-rejection or complement an organ niche. Lineage-restricted progenitors can create chimeras following blastocyst injection if apoptosis is transiently inhibited by expressing Bcl2. However, this method is inefficient. We explored the rate at which progenitors die following injection into the blastocyst using our embryo live-imaging system (reported previous years, manuscript in preparation, Figure 2G). Bcl2 expression prolonged cell survival, however still the majority of progenitors died within 24 hours. Bcl2 in combination with ROCK inhibitor led to nearly all cells surviving, while supplementing with insulin enabled progenitor proliferation. The media supplements did not have a significant effect on blastocyst development rate (data not shown). This system will be used in combination with in utero imaging to create targeted chimeras in rodents, and, if successful, applied to sheep.
Figure 2: Targeted chimerism.

(A) Injection of xenogenic pluripotent stem cells leads to systemic chimerism, which raises ethical concerns and can cause embryonic lethality during development (top). Chimerism can be targeted by injecting lineage committed progenitor cells into the blastocyst or injecting cells directly into the developing organ niche. (B) To enhance proliferation of human HSCs following injection into sheep fetal livers, LNK was knocked with CRISPR/Cas9 and TIDE analysis performed to analyzed indels. 40% alleles knocked out rate. (C) Human HSCs were injected into six sheep fetal livers prior to the development of the immune system, from which three lambs were born. (D) The lambs were sacrificed three months after birth and various tissues were analyzed by ddPCR to detect human chimerism. No human DNA was found. Positive controls were prepared by mixing human and sheep blood at 1:100,000 and 1:10,000. (E) GFP-labelled human epithelial cells were injected into E11.5 rat embryos and analyzed at E13.5. Top: whole embryo; bottom: region of interest. (F) AkaLuc-tdTomato fusion reporter can be used to detect changes in chimerism in developing embryos. 40 mouse blastocysts were injected with 10 mouse ESCs each and transferred into two pseudopregnant recipients; IVIS imaging was performed on live mice 7 days later. One mouse (left) received embryos injected with cells that had no reporter; the other mouse (right) received embryos injected with cells expressing AkaLuc-tdTomato. (G) Lineage restricted progenitors can be injected into the blastocyst instead of the post-implantation embryos. Most of the cells die if left in just embryo media (KSOM, dashed line and gray bar). Bcl2 expression slightly enhances cell survival (blue) and can lead to targeted chimerism (Masaki et al., 2016). ROCK inhibitor greatly enhances cell survival at the preimplantation stage (purple), and addition of insulin enhances proliferation (green). n = 80; *p < 0.05; ***p < 0.001.

CRISPR KO sheep embryos:

In order to generate organs through interspecies blastocyst complementation we will need to generate knockout animals to serve as hosts. Furthermore, we may find that specific knock-ins must be made into host embryos in order to facilitate interspecies complementation. We have previously developed the use of the CRISPR-Cas9 system to generate specific knockouts in different mammal species. We have also developed the use of adeno associated virus serotype 6 (AAV6) vectors in combination with the CRISPR-Cas9 system to achieve robust gene knock-in, in rodents. One current limitation of gene editing technologies is the generation of genetically mosaic embryos. Often only a fraction of the cells of a developing embryo have a desired gene knocked in or out and there is currently no method to detect and select for non-mosaic edited embryos. We have recently demonstrated that we can detect site specific homologous directed recombination (HDR) using the CRISPR-Cas9 system and a donor for homologous directed recombination that expresses a reporter gene in mouse embryos17 (Figures 3A, 3B, 3C). Using this technology in combination with live imaging we are able to detect mosaic knock-in events in mouse embryos as they occur (Figure 3D). We are currently using this methodology to optimize different embryo editing conditions in order to consistently achieve non-mosaic embryo editing in mammalian embryos. Once we have optimized our methodology in mouse embryos, we will move to applying the same methods to sheep embryos, which we have recently optimized electroporation conditions for (Figures 3E, 3F).
Figure 3: Detectable gene editing with the CRISPR-Cas9 system and AAV6.

(A) Schematic outlining the process of delivering the CRISPR-Cas9 system into mouse embryos, followed by the delivery of an AAV6 donor with a marker to detect gene editing events as they occur in mouse embryos. (B) Representative images of embryos electroporated, demonstrating the ability to detect cells that have undergone HDR at the Rosa26 locus by an increase in fluorescence intensity versus controls. Mock embryos were electroporated but no Cas9 or AAV6 vector was added to embryos. (C) Graph showing the difference in mean fluorescence intensity when embryos are edited with Cas9 + AAV versus control, over time following electroporation. (D) Representative images of different embryos that have undergone HDR at the Rosa26 locus at different points in their development, resulting in different amounts of mosaicism in embryos. (E) Viability of sheep embryos versus untreated control when Cas9 was delivered with different electroporation conditions, compared to previously published work in our lab using microinjection (Villarino et al 2017). Viability was determined by the number of embryos that survived to the blastocyst stage of development and viability relative to control for each condition is shown below the x-axis. (F) Percentage of alleles with INDELs in seven different individual sheep embryos as assessed by TIDE, electroporated using electroporation condition one from 5D.

Vilarino et al. 2017. CRISPR/Cas9 microinjection in oocytes disables pancreas development in sheep. Sci Repor doi: 10.1038/s41598-017-17805-0
A new biopsy method to screen embryonic genotype:

Our goal is to efficiently generate organ-deficient sheep. We previously disrupted pancreatic development in sheep via CRISPR/Cas9 targeting of PDX1 in zygotes (Figure 4A, 4B). However, we found that only a fraction of sheep fetuses was apancreatic. We therefore developed a biopsy system to screen sheep blastocysts prior to embryo transfer (Figure 4C, 4D, 4E). Sequencing revealed that at least half the edited embryos are mosaics of cells with different genotypes. Regardless, the biopsy approach can enrich the population of edited embryos prior to transfer into a sheep uterus. This is important to increase efficiency in future interspecies organogenesis experiments.

Figure 4: Preimplantation biopsy to genotype embryos.
Disabling host organogenesis can be performed by injecting CRISPR/Cas9 into sheep oocytes or zygotes (A). A 208 bp CRISPR/Cas9 induced deletion was made in Pdx1 to disrupt pancreatogenesis in sheep embryos. This deletion could be detected by gel-electrophoresis (B). Embryos were biopsied with minimal damage (C). Embryos with PDX1 KO genotype were transferred to a surrogate uterus. After development, the fetal genotype did not always correspond with the biopsy genotype. Thus, some fetuses had a pancreas (D). Sequencing revealed a multitude of alleles (E), and at least half the embryos were mosaic.