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Blastocyst complementation: current progress and future directions in xenogeneic organogenesis

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Abstract

The generation of organs derived from pluripotent stem cells can be achieved in vivo through the blastocyst complementation technique. This method is based on the introduction of pluripotent stem cells into organogenesis-disabled pre-implantation embryos, where environmental signals instruct donor cells to colonize the vacant niche and to develop into the missing organ. When applied interspecies, this approach has the potential to produce human organs in genetically engineered livestock, offering a promising solution to the global transplants' shortage crisis. In this review, we summarize the current progress in blastocyst complementation research and highlight the key challenges that must be addressed to advance this field.

Keywords Blastocyst complementation, Pluripotent stem cells, Organ transplantation, Chimera, Interspecies organogenesis, Organ generation

Introduction

Organ transplantation is the ultimate treatment for several end-stage organ diseases. However, organ availability is limited. In 2023, approximately 52,000 patients were waiting for an organ transplant in Europe, but only 27,952 transplants were performed [1]. Most of the waitlisted patients face suboptimal health conditions with waiting times ranging from 10 months for a liver transplant to 45 months for a kidney transplant [2]. These patients often require medical procedures that not only compromise their quality of life but also imply a significant economic burden, with the average yearly cost per patient awaiting a kidney transplantation estimated at €40,000 [3]. Artificial organ support like dialysis, artificial liver support systems or left ventricular assist devices are commonly used as a bridge to transplant, in combination with pharmacological treatments.

Nevertheless, these approaches currently offer only partial or temporary alternatives to organ transplantation, and many of these patients often deteriorate further and die while waiting for a suitable organ. On one hand, destination therapy with artificial organs is under study, and for example, mechanical total artificial hearts have been implanted in some cases in patients affected by heart failure who were not eligible for transplant. However, the quality of life for these patients is compromised by the size, biocompatibility and durability of these devices and so far, they only represent a temporary solution to support patient survival until organ transplantation is available [4]. Another approach under study to bridge the gap between organ supply and demand is the xenotransplantation of pig organs genetically modified to avoid graft rejection [5]. In a recent clinical trial of heart xenotransplantation under compassionate use, a pig heart with ten individual gene modifications

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supported a human patient's life for 60 days [6]. Despite being a significant step forward, further refinement is needed before this approach becomes a viable option for clinical transplantation. On the other hand, tissue engineering research is engaged in generating transplantable organs in the laboratory. The decellularization-recellularization technique aims to use animals´ organ's support structure, the extracellular matrix, as a 3D scaffold to be repopulated with human stromal and parenchymal cells. Although promising, this technique remains largely in the experimental phase, with several hurdles yet to be overcome, as reviewed in [7]. Moreover, 3D-printed scaffolds have been used to produce patches of bioartificial tissues. These have been tested for security and effectiveness in many preclinical studies [8]and also a few clinical studies [9], sometimes in combination with bioactive molecules. However, the complexity and size of the human organs have not been reproduced in vitro yet.

In contrast, with the blastocyst complementation (BC) approach, organ generation has been achieved in vivo, by taking advantage of the natural embryonic environment, allowing endogenous processes to guide organ development from exogenous PSCs. In BC, donor PSCs are introduced into an organogenesis-disabled embryo at the pre-implantation stage (early morula or blastocyst) so that during development they can colonize the empty organ niche, receive proper extrinsic cues and eventually develop into target tissues. So far, several organs have been generated intra- and interspecies in rodents and pigs, and the expectation is that in the long run, BC could be used to obtain human organs from patient-derived PSCs in livestock animals such as pigs (Fig. 1). These organs and tissues could be used for transplantation in humans, and the chimeric animals produced could also constitute an unprecedented preclinical model for testing new drugs and therapies.

In this review we first explore the different strategies currently employed to obtain genetically engineered embryos for use as hosts in BC experiments. Next, we provide a summary of studies on both intra- and interspecies BC aimed at generating different organs. Finally, we conclude with an analysis of the challenges to interspecies complementation and of the ethical issues related to this technology, along with a projection of future directions in the field.

Strategies to produce organ-deficient host embryos for blastocyst complementation

To ensure full contribution of donor cells to the desired organ, it is essential to disable the targeted organ niche in host embryos, so that host cells cannot contribute to its formation. To date, two main approximations

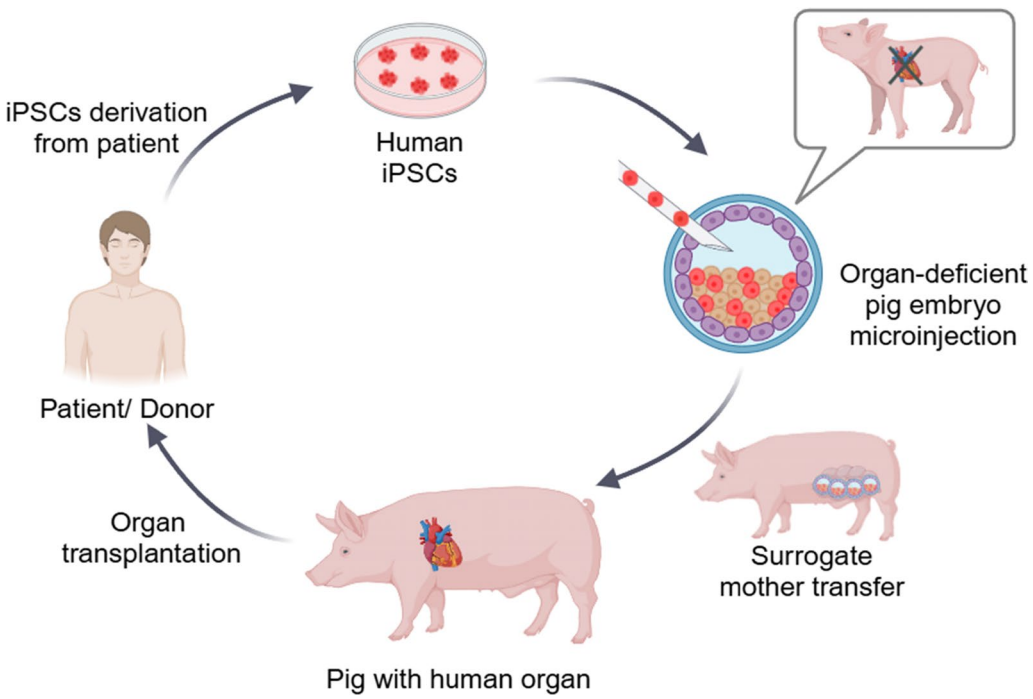

Fig. 1 Blastocyst complementation technique overview. Patient-derived PSCs are microinjected into genetically engineered pig embryos that are unable of developing the desired organ (heart in this example). After that, microinjected embryos are transferred to surrogate sows to let them develop to term. In the end, hPSC-derived organs from the resulting pig could be used for human transplantation

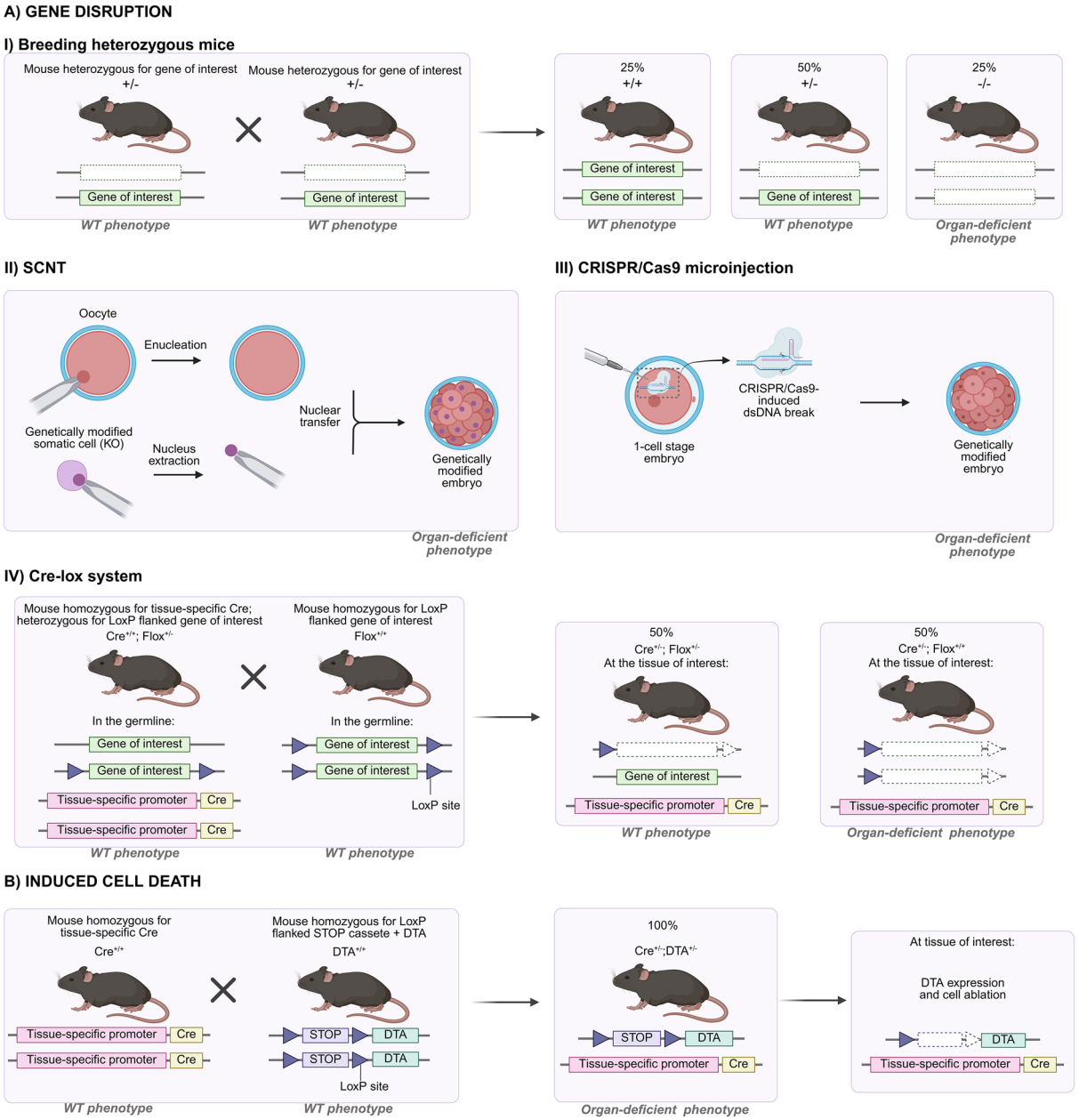

Fig. 2 Strategies to obtain organ-deficient embryos. **A** Gene disruption methods to obtain genetic KOs include I) Crossbreeding heterozygous mice carrying a mutation in a master regulator gene of organ development results in an organ-deficient phenotype in homozygous mutants, which accounts for 25% of the progeny. II) Nuclear transfer of a previously gene edited somatic cell into an enucleated oocyte followed by proper stimulation, allows the generation of a genetically modified homozygous cloned organism. III) Microinjection of the CRISPR/Cas9 system (Cas9 mRNA or protein along with guide RNAs) into 1-cell stage embryos results in the direct production of KO embryos. IV) Conditional KO by using the Cre-lox system (used if systemic deletion of the target gene interferes with normal development of multiple tissues). Mice expressing Cre recombinase under a tissue-specific promoter are crossbred with mice carrying the target gene flanked by LoxP sites. To obtain 50% organ-deficient progeny, mice homozygous for the tissue-specific Cre and heterozygous for the LoxP flanked target gene should be crossed with mice homozygous for the LoxP flanked target gene. **B** DTA-mediated cell ablation: engineered mice expressing the Cre-recombinase under a promoter specific for the tissue to be ablated are crossed with mice carrying the DTA gene preceded by a LoxP flanked STOP cassette. When homozygous mice are used, all resulting progeny will express the DTA in the target tissue, leading to an organ-deficient phenotype

have been employed to obtain organogenesis-disabled embryos for BC: gene disruption and induced cell death (Fig. 2).

The most used strategy is based on the biallelic disruption of master regulator genes of organ development through genetic knock-out (KO), by breeding heterozygous mutant mice (Fig. 2A-I) [10–31]. Genes targeted in these models are usually associated with key processes in target organ development such as cell proliferation, differentiation or migration in a cell-autonomous fashion. Although widely used, this method yields only a 25% of KO progeny, contributing to the low efficiency of the BC process. To date, this method has only been used to target a single cell lineage within the organ, as different cell lineages rely on different master genes and crossing double-heterozygous mice results in only a 6.25% occurrence of double KOs, which is insufficient for practical use. Parenchymal cells are usually targeted with this approach. Organs however, are composed of multiple cell types, which means that non-parenchymal cells in the complemented organ are a mix of donor and host cells. This represents a significant technical barrier when using this approach for producing transplantable xenogeneic organs.

Somatic cell nuclear transfer (SCNT) has also been used to directly produce KO embryos for BC [24, 32–37] (Fig. 2A-II), especially in species where the production of transgenic strains is challenging due to the lack of chimera-competent PSCs or a prolonged gestational period, as seen in pig. This technique allows for the induction of one or more gene disruptions in fibroblasts in vitro, whose nucleus are then transferred into enucleated oocytes. Egg's cytoplasmic factors induce the diploid cell to reprogram into a zygote, which upon electrical or chemical stimulation begins cell division and develops into a genetically modified clonedembryo [38], which can then be cultured in vitro to the morula/blastocyst stage and subsequently used for BC experiments. Nevertheless, the disadvantages of this approach are its technical complexity and the limited number of viable clones obtained (1–5%) [39].

Alternatively, direct production of homozygous KO embryos can also be achieved by introducing the *CRISPR/Cas9 system* into zygotes (Fig. 2A-III) [34, 36, 40–44]. This technology shows great promise for the direct production of KO embryos, with reported efficiencies up to 95% [43]. However, gene editing efficiency can vary significantly, and the process often impacts embryo viability, ultimately reducing the number of viable offspring. Finally, the generation of uncontrolled mosaic embryos, particularly when multiple genes are disrupted, remains one of the main drawbacks of this technology [45].

Less used strategies include conditional KO approaches based on the *Cre-lox system* (Fig. 2A-IV) [46–50]. In this method, floxed mice, which carry the target gene flanked by LoxP sites, are crossed with mice expressing the Cre recombinase under a specific promoter. This approach is particularly useful when the systemic deletion of the target gene interferes with normal development of multiple tissues, as the tissue specific Cre-lox system restricts gene deletion to specific cell populations. Additionally, it allows the production of KO embryos with an efficiency up to 50% ($Flox^{+/+}$ *x* $Flox^{+/-}$; $Cre^{+/+}$ progeny: 50% $Flox^{+/+}$; $Cre^{+/-}$), which can be increased to 100% if an inducible system is used ($Flox^{+/+}$ *x* $Flox^{+/+}$; $CreERT^{+/+}$ progeny: 100% $Flox^{+/+}$; $CreERT^{+/-}$).

In recent years**,** conditional cell ablation (Fig. 2B) [51–55] emerges as an alternative to KOs, enabling the production of dysorganogenetic embryos with an efficiency up to 100%. Selective cell ablation is achieved by controlling the expression of the diphtheria toxin subunit A (*DTA*) suicide gene through the Cre-lox system. Hence, when mice carrying the *DTA* gene preceded by a floxed-STOP cassette (usually in the *Rosa26 (R26)* locus: *R26-DTA*) are crossed with a promoter-specific Cre recombinase strain, DTA-mediated cell death occurs in all Cre-expressing cells [56]. By using tissue-specific *Cre* drivers, it becomes feasible to ablate any cell lineage with an efficiency up to 100%, because homozygous *Cre* knock-in (KI) and homozygous *R26-DTA* mice can be viable and fertile. Moreover, with this method, multiple cell types can be eliminated simultaneously. Furthermore, this system ensures the complete elimination of a particular progenitor pool, as it induces cell death of all cells expressing the Cre recombinase. This is particularly useful for BC of organs where no described KOs result in a complete agenesis phenotype. Cardiogenesis provides a clear example of this phenomenon: neither the disruption or deletion of *Gata4* [57, 58], *Nkx2.5* [59], *Tbx5* [60], *Mesp1* [61] or the double KO of *Id1* and *Id3* [13] have resulted in cardiac agenesis. Therefore, cell ablation strategies like *-Cre; DTA* may constitute a feasible approach for overcoming the limitations associated with the use of KO host embryos.

Organ generation through blastocyst complementation

The BC approach has been extensively explored in intraspecies and interspecies settings by several groups. On one hand, intraspecies experiments are useful to test different models to create an organ-free niche, and assess the feasibility of target organ complementation, while on the other hand, interspecies approximations allow the exploration of xenogeneic barriers. Unravelling the mechanisms underlying interspecies barriers is necessary

to achieve human organ generation using this technology, as livestock animals such as pigs are being considered as potential hosts.

Intraspecies blastocyst complementation

The first BC complementation study was performed in 1993 by Chen et al. [10], who injected wild-type (WT) mouse embryonic stem cells (mESCs) into *Rag2* (recombination-activating gene 2)- deficient ($Rag2^{-/-}$) mouse embryos, which cannot develop mature B and T lymphocytes, due to their inability to initiate VDJ recombination. They obtained adult chimeras whose mature B and T lymphocytes were entirely derived from injected cells. In this study they also unraveled the specific role of *IgJh* gene in B lymphocyte generation, by injecting $IgJh^{-/-}$ mESCs into $Rag2^{-/-}$ mouse embryos. BC indeed arose as a method to investigate gene function during mouse development. After this first work that established the technical foundation, researchers have applied intraspecies BC to generate several organs and tissues that we summarize in Table 1 and we describe in detail below.

Eye

In 1996, Liégeois et al. [11] performed ocular lens complementation in *Pitx3*-mutated mouse embryos (*aphakia* homozygous *ak/ak* blastocysts), obtained from the mating of *ak/ak* males and females. By injecting WT mESCs they reconstituted normal lenses, while using Rb-KO mESCs they obtained aberrant lenses presenting pathophysiological characteristics typical of Retinoblastoma (*Rb*) KO models.

Pancreas

In 2007 Stanger et al. [15], included a pancreas BC experiment in their study on the determinants of pancreas size, generating pancreata fully derived from injected mESCs in *Pdx1*-KO mouse embryos. Nevertheless, it was not until 2010 that Kobayashi et al. [16] pioneered the use of BC to produce functional donor cells-derived organs in vivo. In this study, *Pdx1*-KO mouse blastocysts obtained by the intercross of heterozygote mice were complemented by injecting WT mouse PSCs (mPSCs). (*Note: since crossing heterozygous mice is the most commonly used strategy to obtain host embryos, in subsequent studies we will only specify when alternative methods are applied*). In complemented adult mice, all pancreatic cell lineages were derived from mESCs or mouse induced PSCs (miPSCs) but pancreatic stroma (comprising nerves, vessels and fibroblasts) was composed of both donor and host cells. Remarkably, they demonstrated that mPSCs-derived pancreases were functional, by performing glucose tolerance tests on *Pdx1*-KO complemented mice. They also tested islets functionality upon transplantation into a diabetic mouse model. Recipient mice exhibited corrected hyperglycemia, and normal glucose levels were sustained during 60 days, indicating no rejection of the transplanted tissue. Later, Hashimoto et al. [22] also reported the generation of mESC-derived pancreata in *Pdx1*-KO mice, but with donor mESCs deficient for *Prdm14* and *Otx2* genes, to avoid germline and brain contribution. In 2017, Yamaguchi et al. [20] generated rat pancreata by injecting rat ESCs (rESCs) into rat blastocysts carrying a homozygous mutation in the *Pdx1* gene ($Pdx1^{mut/mut}$). The first BC study in large livestock animals was reported by Matsunari et al. in 2013 [32], using pancreatogenesis-disabled pig embryos overexpressing the *Pdx1-Hes1* mouse transgene, cloned by SCNT. They injected allogeneic pig blastomeres into pancreas-deficient embryos, and the resulting complemented chimeras exhibited reconstituted pancreas. Complemented pigs developed to adulthood and were fertile. Later on, in 2020, the same group complemented the pancreas using allogeneic pig blastomeres and *PDX*-KO cloned embryos obtained by SCNT. Moreover, they combined the disruption of both the pancreas parenchyma and the endothelial niche through *PDX1/KDR* (*FLK1* ortholog) double-KO pig embryos. Pig blastomeres microinjection in these embryos allowed to obtain one full term chimera whose endothelial, hematopoietic, and pancreatic tissues were derived from donor cells [33].

Liver

In 2010, Espejel et al. [17] generated chimeras to postnatally repopulate the liver in mice lacking fumarylacetoacetate hydrolase (*Fah*). *Fah*-deficient mice develop severe hepatic damage and die neonatally unless treated with 2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC). They injected miPSC into $Fah^{-/-}$ mouse embryos, which were then transferred into foster mothers treated with NTBC to prevent liver damage in chimeras before birth. They observed that postnatal NTBC withdrawal induced the expansion of iPSCs-derived hepatocytes to compensate for endogenous hepatocyte loss in chimeras. While this approach differs from conventional BC as it does not achieve colonization of the empty organ niche during embryonic development, it provides a method to generate a liver entirely derived from exogenous cells through chimera formation.

In 2020 Matsunari et al. [33] rescued liver development by BC in *HHEX*-KO pig embryos generated by SCNT. The injection of allogeneic WT blastomeres in these embryos resulted in full-term chimeras with livers mostly derived from donor blastomeres. One year later, *Hhex*-KO embryos obtained by CRISPR/Cas9-mediated gene editing in zygotes or SCNT were used by Ruiz-Estevez et al. [34] to grow livers intraspecies in mice and

Table 1 Intraspecies blastocyst complementation studies

Host specie	Target gene	Tissue/organ	Empty niche strategy	Donor cells	Efficiency* (%)	Developmental stage	Functional outcome	References (year)
Mouse	*Rag2*	B and T lymphocytes	1	mESCs (WT and IgJh-deleted)	n = 2**	Adult	N/A	[10] (1993)
Mouse	*Pitx3*	Ocular lens	1	mESCs (WT and *Rb*-KO)	7/48 (14.6)	Adult	Lens-positive adult chimeras responded to visual stimuli	[11] (1996)
Mouse	*Mesp1, Mesp2*	Heart	1	Mouse blastomeres	5/72	E8.5 and E9.5	N/A	[12] (2000)
Mouse	*Id1, Id2, Id3*	Heart	1	mESCs	n = 5**	Adult	Rescued hearts were functionally normal, as analyzed by echocardiography	[13] (2004)
Mouse	*Foxn1*	Thymus	1	mESCs (WT and *Vegfa*-KO	N/A	Adult	T cells were present in the peripheral blood, confirming the functionality of *Foxn1*$^{+/+}$ mESCs-derived thymic epithelial cells	[14] (2005)
Mouse	*Pdx1*	Pancreas	1	mESCs	N/A	Adult	N/A	[15] (2007)
Mouse	*Pdx1*	Pancreas	1	mESCs/miPSC	n = 19**	Adult	miPSC-derived pancreas secreted insulin in response to glucose tolerance test (GTT) and complemented mice maintained normal serum glucose levels miPSC-derived islet transplantation into diabetic mice resulted in insulin production and normal blood glucose levels after GTT. Normal glycemia was sustained after 60 days	[16] (2010)

Table 1 (continued)

Host specie	Target gene	Tissue/organ	Empty niche strategy	Donor cells	Efficiency* (%)	Developmental stage	Functional outcome	References (year)
Mouse	*Fah*	Liver	1	miPSCs	6/40 (15)	Adult	Liver function parameters (blood albumin, bilirubin, AP and ALT) in *Fah*-deficient mice with miPSC-derived hepatocytes similar to WT mice up to P300 *Fah*-deficient mice with 100% miPSC-derived hepatocytes tolerated 2/3 partial hepatectomy Transplantation of miPSC-derived hepatocytes into adult *Fah*-deficient mice repopulated their liver and made them NTBC-independent	[17] (2010)
Mouse	*Sall1*	Kidney	1	mESC/miPSCs	5/138 (3.6)	Neonatal	miPSC-derived kidneys functionality was inferred by the presence of urine accumulation in the bladders of complemented neonates	[19] (2012)
Pig	*PDX1*	Pancreas	2	Pig blastomeres	5/189 (2.6)	Adult	Complemented pigs had normal serum glucose levels and serum biochemical profiles at 6 and 12 months of age. GTT results were also normal	[32] (2013)
Mouse	*Nkx2.5*	Heart	3	mESCs	82/639 (12.8)	E10.5	N/A	[51] (2015)
Mouse	*Tsc22d3*	Spermatids	4	mESCs	N/A	Adult	Germline transmission demonstrated correct germ cell differentiation in complemented chimeras	[46] (2016)
Rat	*Pdx1*	Pancreas	1	rESCs	3/73 (4.1)	Adult	Complemented rats recovered normal glucose levels after GTT	[20] (2017)

Table 1 (continued)

Host specie	Target gene	Tissue/organ	Empty niche strategy	Donor cells	Efficiency* (%)	Developmental stage	Functional outcome	References (year)
Mouse	*Emx1*	Forebrain	3	mESCs	n = 16**	Adult	SHIRPA phenotypic assessment of basic autonomic, neurological, and sensory functions revealed no differences between adult complemented chimeras and controls Novel-object recognition and Morris water maze paradigms results in adult complemented chimeras were similar to controls	[52] (2018)
Mouse	*Flk1*	Hematoendothelial lineages	1	mESC	n = 11**	Adult	Bone marrow transplantation from complemented mice into irradiated recipients rescued hematopoiesis and generated T, B and myeloid lineages	[21] (2018)
Mouse	*Ctnnb1/Fgfr2*	Lung	4	mPSC	4/40 (10) (*Fgfr2*-KO), 5/130 (3.8) (*Ctnnb1*-KO)	Adult	Pulmonary function tests (Flexivent) showed comparable levels of airway resistance, lung elastance and compliance between adult complemented chimeras and WT	[47] (2019)
Rat	*Sall1*	Kidney	1	rESCs	3/80 (3.8)	Embryonic	N/A	[23] (2019)
Mouse	*Pdx1*	Pancreas	1	*Prdm14*-KO; *Otx2*-KO mESCs	20/220 (9.1)	Adult	GTT results were improved in complemented mice	[22] (2019)
Mouse	*Etv2*	Hematoendothelial lineages	1	mESC	N/A	Embryonic	N/A	[24] (2020)
Pig	*ETV2*	Hematoendothelial lineages	2	Pig blastomeres	n = 3**	Neonatal	N/A	[24] (2020)
Mouse	*Flk1*	Hematoendothelial lineages	1	mESCs	3/172 (1.7)	Adult	N/A	[25] (2020)
Mouse	*Fgf10*	Lung	1	mESC	5/638 (0.8)	Adult	N/A	[26] (2020)
Pig	*PDX1*	Pancreas	2	Pig blastomeres	*PDX1*-KO: 2/325 (0.6)	Neonatal	N/A	[33] (2020)
Pig	*PDX1, KDR*	Pancreas and hematoendothelial lineages	2	Pig blastomeres	*PDX1/KDR*-KO: 1/92 (1)	Neonatal	N/A	[33] (2020)

Table 1 (continued)

Host specie	Target gene	Tissue/organ	Empty niche strategy	Donor cells	Efficiency* (%)	Developmental stage	Functional outcome	References (year)
Pig	*HHEX*	Liver	2	Pig blastomeres	*HHEX*-KO: 3/95 (3.2)	Neonatal	N/A	[33] (2020)
Pig	*SALL1*	Kidney	2	Pig blastomeres	*SALL1*-KO: 1/97 (1)	E43	N/A	[33] (2020)
Mouse	*Nanos3*	Germ cells	5	WT mESCs/*Dnmt3b*-KO mESCs	n = 3**	Adult	Correct spermatogenesis was validated by the generation of mESC-derived offspring in complemented chimeras	[41] (2020)
Mouse	*Nkx2.1*	Lung and thyroid	1	mESCs	n = 9**	Neonatal	N/A	[27] (2020)
Mouse	*Hhex*	Liver	5	miPSCs	2/81 (2.5)	E12.5	N/A	[34] (2021)
Pig	*HHEX*	Liver	2	Pig blastomeres	2/94 (2.1)	E25	N/A	[34] (2021)
Rat	*Prdm14*	Germ cells	1	rESCs	7/143 (4.9)	Adult	Correct germ cell specification and development was demonstrated by germline transmission of donor rESCs	[28] (2021)
Pig	*MYF5, MYOD, MYF6*	Skeletal muscle	2	Pig blastomeres	N/A	Neonatal	Neurally evoked functional assessment of the anterior compartment musculature showed that contractile function and innervation were similar in WT and rescued piglets	[35] (2021)
Mouse	*Foxn1*	Thymus	1	mESCs	N/A	Adult	Thymus-derived T lymphocyte development was normal in complemented mice, as assessed by T cell proliferation and activation assays The tumor-inhibitory effect of T cells after anti-PDL1 administration was normal in thymus-complemented mice bearing implanted tumors	[29] (2022)
Mouse	*Tsc22d3*	Spermatozoa	4	mESC/miPSCs	10/45# (22.2)	Adult	Germline transmission of donor mESCs confirmed the generation of functional spermatozoa	[48] (2022)

Table 1 (continued)

Host specie	Target gene	Tissue/organ	Empty niche strategy	Donor cells	Efficiency* (%)	Developmental stage	Functional outcome	References (year)
Mouse	*Gcm2*	Parathyroid glands	5	mESCs	N/A	Adult	In complemented mice, plasma calcium and basal parathyroid hormone (PTH) levels were normal and PTH secretion in response to hypocalcemia was similar to controls Transplantation of mESC-derived PTGs beneath the renal capsule in mice following parathyroidectomy ameliorated the hypocalcemia mESCs-derived PTGs could rescue the *Gcm2*-KO phenotype in neonates after subcutaneous transplantation	[42] (2023)
Mouse	*Nkx2.5*	Heart	6	mESCs	N/A	E9.5	N/A	[63] (2023)
Mouse	*Fgfr2*	Lung Thymus	4	miPSCs	n = 5**	Adult	Respiratory function analysis showed no differences between complemented chimeras and controls	[49] (2023)
Mouse	*Nkx2.5*	Heart	3	mESCs/miPSCs	21/602# (3.5)	Adult	Echocardiographic analysis revealed no differences in heart function between complemented and control mice	[53] (2023)
Mouse	*Tie2*	Vascular system	3	miPSCs	5/191# (2.6)	E14.5	N/A	[53] (2023)
Mouse	*Nkx2.5, Tie2*	Heart and vascular system	3	mESCs/miPSCs	11/602# (1.8)	Adult	Echocardiographic analysis revealed no differences in heart function between complemented and control mice	[53] (2023)
Mouse	*Mesp1, Mesp2*	Heart	1	mESCs	4/625 (0.6)	Adult	Normal cardiac function of mice with mESCs-derived hearts was tested by electrocardiography, echocardiography, and treadmill testing	[30] (2023)

Table 1 (continued)

Host specie	Target gene	Tissue/organ	Empty niche strategy	Donor cells	Efficiency* (%)	Developmental stage	Functional outcome	References (year)
Mouse	*Pax7*	Satellite cells	3	mESCs/miPSCs	11/50 (22)	Adult	Intramuscular transplantation of donor-derived myoblasts restored dystrophin expression in DMD mice. Muscle force measurements showed a slower force decline in transplanted muscles compared to controls, although other force-related parameters displayed no significant differences between groups	[54] (2024)
Mouse	*Hesx1*	Forebrain	5	mESCs	66/445 (14.8)	Adult	Performance in the Morris water maze, open-field assay, and contextual fear conditioning tests showed no differences between complemented chimeras and controls	[43] (2024)
Mouse	*Omp*	Olfactory sensor neurons	3	mESCs	n = 16**	Adult	Buried cookie test confirmed that donor mESCs could restore OSN function in complemented chimeras	[55] (2024)
Mouse	*Fgfr2*	Salivary glands	4	miPSCs	5/78 (6.4)	Adult	miPSCs-derived salivary glands functionality was inferred by the formation of periodic-acid-Schiff mucin-producing acinar cells	[50] (2024)
Mouse	*Fgfr2b*	Lung	6	mESCs	3/64 (4.7)	Neonatal	N/A	[64] (2024)
Pig	*HHEX*	Liver	2	Pig blastomeres	2/120 (1.6)	E28	N/A	[37] (2024)
Mouse	*Mesp1*, *Mesp2*	Heart	1	mESCs	5/243 (2.1)	E14.5	N/A	[31] (2024)

1: Crossbreeding heterozygous mice; 2: SCNT of previously gene edited somatic cells; 3: DTA ablation; 4: Conditional KO by using the Cre-lox system; 5: CRISPR/Cas9-mediated gene editing in 1-cell stage embryos; 6: tetraploid complementation-based approach; * complemented / transferred embryos; ** total of transferred blastocysts not reported; # assuming Mendelian ratios; ALT: alanine aminotransferase; AP: alkaline phosphatase

pigs, respectively. In both cases, complemented embryos were analyzed prenatally (at E12.5 in mice and E25 in pig), showing high donor cell contribution to the restored liver, and presence of liver-specific protein expression. More recently, Simpson et al. [37]generated *FOXA3*-driven *HHEX* conditional KO pig embryos (*FOXA3*^{Cre}*; HHEX*$^{loxP/loxP}$) by SCNT to restrict *HHEX* deletion specifically to liver development. After injection of donor pig blastomeres, liver formation was rescued at E28, obtaining two fetuses whose hepatocytes originated from donor cells.

Kidney

Intraspecies BC to generate mPSCs-derived kidneys was performed in 2012 by Usui et al. [19] using *Sall1*-KO mouse blastocysts as recipients. In complemented pups, nephron epithelium consisted entirety of donor cells, while collecting ducts epithelium and renal stroma was a mix of host and donor cells. However, none of the complemented pups reached adulthood as they all died shortly after birth without nursing properly, similar to non-complemented *Sall1*-KO mice [62]. As *Sall1* is also expressed in the brain, authors suggested that pup loss may be caused by insufficient correction of neurological functional abnormalities due to cell-intrinsic *Sall1* deficit, that in the brain does not produce cell loss. In 2019, Goto et al. [23] generated living fetuses with kidneys derived from allogeneic rESCs using *Sall1*$^{mut/mut}$ rats as recipients. In pigs, the anephrogenic phenotype of *SALL1*-KO was compensated by Matsunari et al. [33] through the microinjection of pig blastomeres into kidney-deficient pig embryos generated by SCNT. However, they only obtained one fetus with normal kidney development at E43, without obtaining any full-term complemented piglets.

Heart

Generating an empty heart niche has proven challenging, as existing gene KOs of cardiac master regulators such as *Nkx2.5* [59], *Gata4* [57, 58], *Tbx5* [60], *and Mesp1* [61] impair heart development but fail to fully disable cardiomyocytes (CM) progenitor's pool. In 2000, Kitajima et al. [12] performed morula aggregation experiments with *Mesp1/2* double KO (dKO) mouse embryos and WT mESCs, finding that dKO cells could contribute to the atrial appendage, while they were almost absent in the ventricles. Later on, Fraidenraich et al. [13] reported that WT ESCs injection in *Id1-Id3* KO mouse blastocysts could rescue cardiac defects and embryo lethality. However, the rescue seemed to result to a non-cell autonomous effect, as it was seen with as little as 20% contribution of injected cells to the heart. The first evidence that conditional cell ablation could completely empty the CM niche was provided by Sturzu et al. [51]. They injected *Nkx2.5-Cre; R26-DTA* mESCs expressing the inducible R26-LoxP-STOP-LoxP-DTA toxin (*R26-DTA*) upon *Nkx2.5* promoter activation (*Nkx2.5-Cre*) into WT embryos, to generate chimeras with quantifiable fractions of CM progenitor ablation, calculated based on extracardiac chimerism level. Using this method, they determined that up to 60% CM progenitor loss in the mammalian fetal heart still permits normal development. To corroborate their results, they also performed a BC experiment, evaluating the range of WT mESCs' contribution in *Nkx2.5-Cre; R26-DTA* embryos and obtaining E10.5 chimeras with hearts fully restored by donor-derived CMs, which were not further analyzed. In 2023, Founta et al. [63] used a tetraploid aggregation chimera formation method to empty the cardiac niche and generate hearts derived from donor mESCs. Four-cell stage tetraploid embryos were aggregated with "host" mESCs expressing the DTA under the control of the AR1 cardiac enhancer of the *Nkx2.5* gene and "donor" WT mESCs. As tetraploid cells cannot contribute to embryonic tissues, embryos were derived from both host and donor mESCs lines whereas heart tubes were formed by donor WT mESCs at E9.5.

Recently, we generated [53] adult mice with hearts whose CMs were entirely derived from donor mPSCs by using cardiac-disabled *Nkx2.5-Cre; R26-DTA* embryos as recipients. A detailed reporter gene analysis revealed *Nkx2.5*-driven expression in 100% of CMs, but also in 60-to 70% of cardiac ECs, fibroblasts and SMCs. Moreover, *Nkx2.5-Cre; R26-DTA* embryos were analyzed at different developmental stages, and CM ablation was confirmed beginning at E7.5 and being complete at E9.5. Upon mPSCs injection, complemented chimeras with normal heart morphology were recovered at E14.5 and adult stage. Histological analysis revealed that 100% of the CMs were derived from exogenous cells. Combining *Nkx2.5-Cre* with *Tie2-Cre* and *R26-DTA* we simultaneously complemented the heart parenchyma and the endothelial niche. Chimeras were recovered at E14.5 and at adult stage, showing normal vascular density and no histopathological alterations. Adult complemented chimeras presented normal cardiac function, as analyzed by echocardiography, proving that mPSCs-derived hearts were functional. Complemented mice survived more than 15 months.

Recently, two BC studies reported the generation of heart from donor mESCs in *Mesp1/2* double KO mouse embryos [30, 31]: Abe et al. [30]showed functionality of the complemented hearts at adult stage, and complementation of nearly all parenchymal and stromal cells in the heart, while Yuri et al. [31] reported the presence of complemented hearts composed prevalently by exogenous

mESCs at E14.5. However, in accordance with previous results from morula aggregation experiments from Kitajima et al. [12], *Mesp1/2* KO cells could colonize the atria of the complemented heart [31].

Brain

Chang and colleagues [52] utilized a cell ablation strategy to create an empty niche in the forebrain to perform intraspecies BC. Injection of donor mESCs into mouse blastocysts expressing the inducible DTA toxin upon *Emx1* promoter activation (*Emx1-Cre; R26-DTA* mice) enabled the generation of a reconstituted forebrain with nearly 100% donor cell contribution to hippocampus and cerebral cortex, while a small percentage (less than 10%) of host cells detected in those regions were assumed to be ECs or microglia, which are not derived from dorsal telencephalon. Adult complemented mice presented cerebral structures that were indistinguishable from those of control mice, and they maintained intact learning and memory skills. More recently, Huang et al. [43] rescued forebrain development by injecting mESCs into *Hesx1*-KO mouse embryos obtained by CRISPR/Cas9-driven gene editing. The majority of cerebral cortex and hippocampus was composed of donor-derived cells (100% in CA1, CA3 and cortex) and complemented chimeras's brain size was normal, allowing chimera's survival to adulthood. Finally, Throesch et al. [55] generated olfactory sensor neurons (OSN) from donor mESCs using OSN-deficient embryos as recipients, obtained by *Omp1*-mediated conditional DTA expression (*Omp1-Cre; R26-DTA*). Chimeric mice presented a restored olfactory epithelium and normal olfactory behavior.

Lung

In 2019, a conditional KO approach based on the Cre-lox system was used by Mori et al. [47] to selectively disable lung development. Early respiratory endoderm progenitors' specification or expansion were targeted by *Ctnnb1* or *Fgfr2* deletion, respectively, using the tissue specific *Shh-Cre* mice. Lung formation was rescued in both models by mESCs microinjection and complemented lungs were functional, allowing mice survival into adulthood. Donor contribution was high in lung epithelium (around 95%), while in lung stromal cells was variable (around 60%). One year later, Kitahara et al. [26] used CRISPR/Cas9-edited *Fgf10* $Ex1^{mut}/Ex3^{mut}$ compound heterozygous ($Fgf10^{mut/mut}$) mouse embryos to disable lung formation in mice and generate lungs derived from donor mESCs. *Fgf10* is a paracrine key regulator of lung development, orchestrating not only epithelial growth and branching but also the differentiation of lung mesenchymal progenitors into various stromal lineages. Consequently, the contribution of mESC-derived cells to lung non-epithelial tissues was significant. Nonetheless, the election of a soluble factor such as Fgf10 could have been counterproductive, as the Fgf10 produced by donor cells could partially rescue host cells contribution to the target tissue, which indeed was a mix of donor and host cells (60–90% donor contribution in lung epithelial cells). In parallel, Wen et al. [27] generated both lung and thyroid tissues by BC in *Nkx2.1*-KO mice. In the resulting complemented chimeras, nearly all lung epithelial cells were mESCs-derived (between 95 and 99%), whereas 20 to 50% of stromal cells derived from donor cells. Despite mESCs complementation induced thyroid morphogenesis, it was insufficient to correct the tracheoesophageal fusion present in *Nkx2.1*-KO mice, leading to their death at birth. In a more recent study, Miura and colleagues [49] used *Foxa2*-driven *Fgfr2* conditional KO embryos ($Foxa2^{Cre}$; $Fgfr2^{flox}$) as recipients for intraspecies BC of both lung epithelium and stroma, as well as thymic epithelium. In complemented embryos, lung and thymic epithelium were completely derived from donor cells, while the exogenous contribution to lung mesenchymal niche was high (50–80%), although not complete. Complemented mice survived to adulthood with normal lung function. In 2024, Yuri et al. [64] used a tetraploid-based organ complementation method to correct lung agenesis in *Fgfr2b-KO* mice with WT mESCs. For that, they injected *Fgfr2b*-KO mESCs into tetraploid embryos at E2.5 and one day later, GFP-mESCs. All epithelial lung cells were GFP^+ as analyzed at E14.5. Full-term E19.5 chimera fetuses retrieved by cesarean showed normal appearance and reconstituted lung formation, in contrast with cyanotic non-chimeras, which lacked lungs.

Thymus

The thymus is an organ fundamental for the maturation of T immune cells, and homozygous mutation of the transcription factor *Foxn1* ($Foxn1^{nu/nu}$) results in athymic animals, known as Nude mice, characterized by the lack of mature T immune cells. Müller et al. [14] utilized BC to reconstitute thymus formation in $Foxn1^{nu/nu}$ mice, obtaining thymus epithelial cells (TECs) exclusively derived from injected mESCs, and showing the restoration of mature T cells. Comparing thymic vascular structures formed upon injection of WT and *Vegfa*-KO ESCs, they demonstrated the essential role of TECs' VEGFA production in establishing thymus vascular patterning. Subsequently, Nakauchi's team [29] demonstrated the functionality of T cell matured in the complemented thymy of $Foxn1^{nu/nu}$ mice. Finally, as mentioned above, Miura et al. [49] complemented TECs together with lungs in $Foxa2^{Cre}$; $Fgfr2^{flox}$ mice.

Germline

The generation of germ cells via BC has been studied as a strategy to enhance germline transmission in chimeric animals produced by embryo microinjection of genetically engineered ESCs. To achieve this, different genes have been targeted to produce host embryos lacking germline cells. Miura et al. [41] injected mESCs into *Nanos3*-KO mouse embryos produced by zygote injection of CRISPR-Cas9 and they obtained fertile chimeric male mice with mature spermatozoa entirely derived from donor cells. In another study [28], *Pdrm1*-KO rat embryos were complemented with rESCs, generating fertile adult chimeras with all germ cells derived from donor cells. Koentgen et al. [46] reported the generation by BC of gametes exclusively derived from mESCs in male mice deficient for the X-linked gene *Tsc22d3,* obtained crossing *Tsc22d3*$^{flox/flox}$ females and a R26$^{Cre/Cre}$ males. Similarly, Zvick et al. [48] complemented the male germline after mESCs injection into these *Tsc22d3*-KO mouse embryos. In both cases, complemented chimeras can be fertile and transmit exclusively the genome of donor mESCs.

Skeletal muscle

Skeletal muscle was first produced through BC using pig blastomeres as donors. Maeng et al. [35] generated pig embryos lacking skeletal muscle by SCNT through the simultaneous inactivation of *MYF5*, *MYOD* and *MYF6* genes. Complemented chimeras reached full term, were analyzed at 1 month of age and showed normal histology, morphology and function, with a skeletal muscle composed of 90% donor-derived cells. The remaining host cells were presumably ECs or nerve cells, which were not targeted by the triple-KO strategy. More recently, Bar-Nur's team [54] targeted the PAX7^{+} population to generate satellite cells derived from donor PSCs using *Pax7-Cre/ERT2; R26-DTA* host embryos. They also corrected Duchenne Muscular Dystrophy (DMD) mutation in iPSCs from DMD mice and used the BC approach to enable the exclusive generation of iPSC-derived gene-edited satellite cells. Interestingly, these satellite cells could efficiently produce new myoblasts, which restored dystrophin expression when transplanted into mice with dystrophic muscles.

Parathyroid glands

Functional parathyroid glands (PTGs) were generated from mESCs through BC in *Gcm2*-KO mice generated by CRISPR-Cas9 zygote injection [42]. In complemented mice, the parathyroid chief cells were 100% derived from donor cells while other cell lineages, such as endothelial or other stromal cells were chimeric (50–70%). mESCs-derived PTGs could rescue postoperative hypoparathyroidism after transplantation in mice.

Salivary glands

In 2024, Tanaka et al. [50] injected mPSCs in *Foxa2*-driven *Fgfr2* conditional KO embryos (*Foxa2*^{Cre}*; Fgfr2*^{flox}) to rescue salivary glands formation. In adult mice, complemented salivary glands were functional and of normal size, with the epithelial compartment entirely derived from donor cells, while stromal cells were chimeric.

Hematoendothelial lineages

In organs generated using BC, the endothelium and other stromal cells are usually a mixture of donor and host cells. This is especially critical when producing transplantable organs in animal models, as xenogeneic ECs can potentially trigger rejection after transplantation, particularly in discordant models such as human and pig [65]. Thus, efforts have been made to generate a vascular system entirely derived from donor cells by BC. Hamanaka et al. [21] emptied the vascular niche by using *Flk1* mutated (*Flk1*$^{mut/mut}$) mouse embryos. After injecting mESCs into these embryos, the lethal phenotype induced by *Flk1* homozygous mutation was rescued. Complemented chimeras reached adulthood, and their vascular and hematopoietic compartments were entirely derived from donor cells, while perivascular cells and SMCs were mosaic (39–95% donor cell contribution). Using a similar approach, Wang et al. [25] performed intraspecies BC in *Flk1*-KO mice. The injection of mESCs into these embryos rescued the lethal embryonic phenotype and resulted in the entire *CD31*$^{+}$ endothelial population being derived from donor cells, as analyzed at E10.5 and adult stage. Another master regulator of hematoendothelial lineages, *Etv2*, was targeted by Das et al. [24] to prove that mESCs could rescue the *Etv2*-KO phenotype by BC. They also performed the same experiment in pigs, injecting pig blastomeres into *ETV2*-KO pig embryos generated by SCNT. In complemented embryos at E18, endothelial and hematopoietic cells were exclusively derived from exogenous cells, while other tissues were chimeric. Similar results were obtained when embryos were analyzed at E24 and full-term stages. In 2023, we complemented [53] the vascular system intraspecies by injecting EC-ablated *Tie2-Cre; R26-DTA* mouse embryos with mPSCs. Characterization of the *Tie2-Cre* model showed that this transgene efficiently and specifically targets all ECs and approximately 90% of the hematopoietic cells. *Tie2-Cre; R26-DTA* embryos showed massive ECs ablation starting from E9.5, and died around E10.5. After mPSCs injection, embryos were analyzed at E14.5 and

presented 100% of ECs derived from donor mPSCs, both in major vessels and at capillary level.

In a subsequent set of experiments, we and others have tested the possibility to complement simultaneously the vasculature with the parenchymal compartment of an organ. Matsunari et al. [33] complemented *PDX1/KDR* double-KO pig embryos, which combine the disruption of both the pancreatic and endothelial niches, while we performed heart and vascular system complementation with a *Nkx2-5-Cre; Tie2-Cre; R26-DTA*-induced cell ablation mouse model [53].

Interspecies blastocyst complementation

The success of intraspecies BC has demonstrated the viability of this technique for growing organs from PSCs. Nevertheless, to make BC a feasible option for producing humanized organs, it is crucial to address the challenge of using host embryos from a species different from the donor PSCs. Table 2 summarizes the current state of interspecies BC research, which is detailed below.

Pancreas

The first proof-of-concept for interspecies BC was provided by Nakauchi's team in 2010 [16], who reported the generation of a functional mouse-sized pancreas derived from rat PSC (rPSCs) in apancreatic *Pdx1*-KO embryos generated by the intercross of heterozygous mice. Pancreata (endocrine and exocrine tissues) of rat-to-mouse complemented chimeras were formed by rat cells, while the stroma was of mosaic origin. Nonetheless, both embryonic and postnatal lethality was high and only two chimeras survived to adulthood, indicating potential incompatibilities in signaling pathways between mouse and rat. Some years later, Wu et al. [40] reproduced these results by combining the BC technique with the CRISPR-Cas9 system to generate pancreas-disabled *Pdx1*-KO embryos.

In 2017, Nakauchi's team performed the same experiment in reverse interspecies settings, injecting mPSCs into $Pdx1^{mut/mut}$ rat blastocysts [20]. mPSCs could occupy the vacant niche and gave rise to functional rat-sized pancreata in rats. EGFP (mPSCs reporter) was co-expressed with exocrine and endocrine markers in islets, acini and duct epithelium. However, two mouse-to-rat complemented rats eventually developed type I diabetes, which the authors hypothesized to be caused by a loss of immune tolerance. They also demonstrated that mPSCs-derived islet transplantation in diabetic mice could normalize blood glucose levels for over 370 days.

Thymus

The second report of in vivo organ formation through interspecies BC was the complementation of mouse thymus by rESCs using Nude mice as recipients, in 2011 [18]. Thymus formation was rescued by rESCs, and Nude mice presented mature $CD4^+$ and $CD8^+$ T lymphocytes of mouse origin. Moreover, complemented thymi could support rat $CD4^+$ and $CD8^+$ cells formation when transplanted under the kidney capsule of Nude rats.

Kidney

Usui et al. [19] attempted to complement the kidney interspecies after succeeding in obtaining kidneys by intraspecies BC. Nevertheless, they found that rPSCs were unable to generate rat kidneys in *Sall1*-KO mice. In these knockouts the metanephric mesenchyme is unfunctional, being unable to expand and to interact with the ureteric buds. The authors hypothesized that mouse-rat molecular incompatibilities in metanephric mesenchyme-ureteric bud interactions may explain the failure of interspecies kidney complementation.

However, subsequently, Goto et al. [23] proved that rPSCs poorly contributed to the metanephric mesenchyme, thus providing insights for the lack of kidney complementation in a rat-to-mouse setting. On the contrary, when mouse-to-rat kidney complementation in $Sall1^{mut/mut}$ rat blastocysts was performed, they observed that mPSCs enabled the generation of neonatal kidneys. In mouse-to-rat complemented mice, metanephric mesenchyme- derived cells were entirely of donor mPSCs origin, whereas the other compartments in the kidney were chimeric. Intriguingly, mPSC-derived kidneys closely resembled the size of normal mouse kidneys, suggesting a potential influence of donor cells on kidney size. Unfortunately, none of the mouse-to-rat complemented chimeras survived to adulthood due to defects in milk suckling, similarly to mouse-to-mouse complemented chimeras. Recently, Wang et al. [36] achieved a milestone by generating humanized mesonephros in nephric-defective *SIX1/SALL1*-dKO pig embryos generated by combining SCNT and CRISPR/Cas9 microinjection. For that, they employed human iPSCs (hiPSCs) with an enhanced chimeric potential by culture media optimization, utilizing 4 chemicals + LIF medium (4CL) and overexpression of two pro-survival genes: *MYCN* and *BCL2*. They showed an enrichment of hiPSC-derived cells in the mesonephros area (50–65%) at E25 and E28, whereas other cell lineages such as germ or neural cells had a minimal hiPSC contribution. Of note, the overall ratio of human to pig cells ranged from 1:10,000 to 1:50,000.

Heart

The first attempt to generate rPSCs-derived hearts in mice was reported by Wu et al [40]. For that, they injected rPSCs into *Nkx2.5*-KO mouse embryos generated by CRISPR/Cas9 zygotic genome editing.

Table 2 Interspecies blastocyst complementation studies

Host specie	Target gene	Tissue/organ	Empty niche strategy	Donor cells	Efficiency* (%)	Developmental stage	Functional outcome	References (year)
Mouse	*Pdx1*	Pancreas	1	riPSCs	10/139 (7.2)	Neonatal (n = 10) and adult (n = 2**)	riPSC-derived islets secreted insulin in response to GTT and maintained normal serum glucose levels (analyzed in adult complemented mice)	[16] (2010)
Mouse	*Foxn1*	Thymus	1	rESCs	n = 4 **	Adult	Transplantation of rESC-derived thymi beneath the renal capsule of athymic *rnu/rnu* rats led to T lymphocyte generation	[18] (2011)
Mouse	*Sall1*	Kidney	1	riPSCs	0	–	N/A	[19] (2012)
Mouse	*Pdx1*	Pancreas	5	rPSCs	N/A	Adult	rPSC-enriched pancreas maintained normal serum glucose levels in response to GTT in chimeras	[40] (2017)
Mouse	*Pax6*	Eye	5	rPSCs	N/A	Neonatal	N/A	[40] (2017)
Mouse	*Nkx2.5*	Heart	5	rPSCs	N/A	E10.5	N/A	[40] (2017)
Rat	*Pdx1*	Pancreas	1	mESCs / miPSCs	9/365(2.5)	Adult	mPSC-derived pancreata allowed blood glucose level recovery after GTT in $Pdx^{mut/mut}$ rats Transplantation of mPSC-derived islets generated in $Pdx^{mut/mut}$ rats into diabetic mice maintained normal glucose levels for over 370 days, providing proof of concept for BC-mediated regenerative medicine. Accordingly, blood glucose levels rose when transplanted islets were removed	[20] (2017)
Mouse	*Flk1*	Hematoendothelial lineages	1	riPSCs	17/137 (12.4)	E9.5	N/A	[21] (2018)

Table 2 (continued)

Host specie	Target gene	Tissue/organ	Empty niche strategy	Donor cells	Efficiency* (%)	Developmental stage	Functional outcome	References (year)
Rat	*Sall1*	Kidney	1	mESCs	12/252 (4.8)	Neonatal	Intraurethral dye infusion in P0 complemented chimeras confirmed ureter-bladder patency, suggesting that mESC-derived kidneys could potentially excrete urine Measurement of blood urea nitrogen and creatinine levels in P0 complemented chimeras revealed no differences compared to non-chimera *Sall1*-KO rats	[23] (2019)
Pig	*ETV2*	Hematoendothelial lineages	2	*BCL2* hiPSCs	51/1321 (3.9)	E17 and E18	N/A	[24] (2020)
Mouse	*Flk1*	Hematoendothelial lineages	1	rESCs	14/342 (4.1)	E10.5	N/A	[25] (2020)
Rat	*Prdm14*	Germ cells	1	mESCs / miPSCs	4/76 = 5.3	Adult	mESC-derived round spermatids injected into mouse oocytes resulted in healthy offspring, proving germline transmission	[28] (2021)
Pig	*MYF5, MYOD, MYF6*	Skeletal muscle	2	*TP53*-KO hiPSCs	15/577 (2.6)	E20 and E27	N/A	[35] (2021)
Mouse	*Tsc22d3*	Spermatozoa	4	rESCs	8/549[#] (1.5)	Adult	Intracytoplasmic sperm injection (ICSI) of rESC-derived spermatozoa into rat oocytes did not produce live offspring ICSI of rESC-derived testicular germ cells failed to generate any live offspring, although several implantation sites with resorbed embryos were detected	[48] (2022)
Rat	*Gcm2*	Parathyroid glands	5	mESCs	3/100 (3)	Neonatal	N/A	[42] (2023)
Pig	*SIX1, SALL1*	Kidney	2 and 5	*MYC-BCL2* hiPSCs	5/1820 (0.3)	E25 (n = 2) and E28 (n = 3)	N/A	[36] (2023)
Mouse	*Nkx2.5*	Heart	3	rESCs	19/724[#] (2.6)	E10.5	Heartbeat was observed in rPSC-derived hearts	[53] (2023)
Mouse	*Nkx2.5, Tie2*	Heart and vascular system	3	rESCs	0/290[#]	–	N/A	[53] (2023)
Mouse	*Mesp1, Mesp2*	Heart	1	rESCs	1/153 (0.65)	E11.5	N/A	[30] (2023)
Mouse	*Hesx1*	Forebrain	5	rESCs	16/6836 (0.2)	Adult	Performance in the Morris water maze, open-field assay, and contextual fear conditioning tests showed no differences between complemented chimeras and controls	[43] (2024)

Table 2 (continued)

Host specie	Target gene	Tissue/organ	Empty niche strategy	Donor cells	Efficiency* (%)	Developmental stage	Functional outcome	References (year)
Mouse	*Omp*	Olfactory sensor neurons	3	rESCs	117 /1343 (8.7)	Adult	Donor rESCs were able to restore OSN function in complemented chimeras, as assessed by the buried cookie test, although the rescue was less robust than in mouse-to-mouse complemented chimeras	[55] (2024)
Mouse	*Fgfr2b*	Lung	6	rESCs	3/446 (0.7)	Neonatal	N/A	[64] (2024)
Rat	*Nkx2.1*	Lung	5	mESCs	n = 4 **	E20.5	N/A	[44] (2024)
Mouse	*Mesp1, Mesp2*	Heart	1	rESCs	4/261 (1.5)	E12.5	Complemented chimeras presented an observable heartbeat	[31] (2024)

1: Crossbreeding heterozygous mice; 2: SCNT of previously gene-edited somatic cells; 3: DTA ablation; 4: Conditional KO by using the Cre-lox system; 5: CRISPR/Cas9-mediated gene editing in 1-cell stage embryos; 6: tetraploid complementation-based approach. * complemented / transferred embryos; ** total blastocysts not reported; # assuming Mendelian ratios

Nkx2.5-KO embryos exhibit compromised heart formation, due to failure in heart tube looping, leading to embryonic death around E10 [40, 59]. The authors found that upon rPSCs blastocyst injection, heart morphology was rescued, with an enrichment of rat cells in the heart, as analyzed at E10.5, while they were unable to obtain adult complemented chimeras. More recently, we used [53] the selective cell ablation mouse model *Nkx2.5-Cre; R26-DTA* as hosts for BC experiments with rESCs, and obtained complemented hearts at E10.5. Remarkably, complemented chimeras' CMs were entirely of rat origin and correctly specified, as evidenced by single-cell RNA-sequencing (scRNA-seq) analysis, proving that mice and rat cardiogenesis is compatible. Moreover, the majority of sinoatrial, epicardial and ECs were also of rat origin and correlated with control rat cells. However, it was not possible to obtain complemented hearts at later timepoints. Transcriptomic analysis of the rPSC-derived hearts identified differential expression in genes involved in metabolism and response to oxygen levels compared to rat controls, suggesting a potential alteration in embryo oxygenation.

Utilizing a Mesp1/2 dKO mouse model, two independent groups achieved heart complementation with rESCs [30, 31]. In both studies full interspecies complementation of both atria and ventricles are shown, in contrast with previous results intraspecies [12, 31]. While Abe et al. [30] only analyzed embryos at E11.5, the complemented embryos obtained by Yuri et al. [31] were viable until E12.5, and they were resorbed afterwards. The authors identified structural abnormalities in the complemented hearts at E12.5, as well as in control chimera embryos with high rat cell contribution to the heart at E12.5 and E14.5, and hypothesized that embryo loss was due to molecular incompatibilities between mouse and rat cells during heart development.

Eye

Wu et al. [40] demonstrated that rat eye tissues could be generated by BC in *Pax6*-KO mouse embryos produced by CRISPR/Cas9-driven genome editing, obtaining chimeric eyes enriched with rat cells.

Germline

The rat *Prdm14*-KO germline-null model was used by Kobayashi et al. [28] to generate germ cells from mPSCs in an interspecies setting. Mouse progenitor germ cells successfully colonized *Prdm14*-KO rat gonads at E15.5 and testes from two out of three adult *Prdm14*-KO complemented chimeras displayed spermatogenesis. However, based on the abnormal motility observed in mPSCs-derived sperm, the authors suggested that spermiogenesis might be impaired in a xenogeneic environment. Because of that, sperm functionality was demonstrated through in vitro fertilization, producing normal mouse offspring. In the reverse interspecies setting, Zvick et al. [48] generated rat spermatozoa in *Tsc22d3*-KO mice obtained with the Cre-lox system. rESCs injection could restore normal testes development and gave rise to spermatozoa entirely derived from donor cells. Nevertheless, mature rat spermatozoa were immotile and moreover failed to produce any live offspring when used for in vitro fertilization of rat oocytes, although they gave rise to live embryos that were resorbed before birth.

Skeletal muscle

CRISPR/Cas9–mediated deletion of *MYF5*, *MYOD* and *MYF6* genes in porcine fetal fibroblasts combined with SCNT were used by Maeng et al. [35] to produce host pig embryos to generate humanized skeletal muscle. To increase human cells contribution, they employed *TP53*-KO hiPSCs. Chimeric embryos obtained at E20 and E27 presented chimerism levels ranging from 1:1,000 to 1:100,000 in the tail and head tip and displayed normal development. Immunohistochemical analysis showed that the chimerism was predominantly located in the skeletal muscles and that hiPSCs contributed to the entire $MYF5^+$ and $MYOD^+$ cell populations. More recently, Bar-Nur's team [54] generated muscle satellite cells ($PAX7^+$) derived from corrected DMD miPSCs in WT rats. Mouse-to-rat chimeras reached adulthood and exhibited skeletal muscles containing both miPSCs and host-derived (rat) $PAX7^+$ cells. After transplantation, miPSCs-derived $PAX7^+$ cells isolated from mouse-to-rat chimeras could restore dystrophin expression in the muscles of DMD mice. Although this study is not properly a BC work, as $PAX7^+$ cells in rat chimeras are a composite of host and donor cells, it underscores the clinical potential of combining genome editing, cellular reprogramming and interspecies chimera formation for cell transplantation purposes.

Parathyroid glands

Recently, Kano and colleagues [42] generated mouse PTGs by mESC injection into *Gcm2*-KO rats obtained by CRISPR/Cas9-mediated genome editing. Although they demonstrated that mESC-derived PTG were functional, complemented chimeras died soon after birth and presented large umbilical hernias. The reasons behind this remain unclear.

Brain

Two studies published in 2024 reported the use of interspecies BC to generate neuronal structures derived from rPSCs in mice. Huang et al. [43] demonstrated that

injecting rESCs into *Hesx1*-KO mouse embryos (obtained by CRISPR/Cas9-mediated genome editing) could rescue forebrain development, resulting in adult chimeras with reconstituted brain structures. However, the percentage of rat cells in these structures diminished as embryonic development progressed, decreasing from 90–100% at E9.5 to ~ 60% by E15.5. The authors suggested that this could be due to an interspecies barrier which induces a reduction of the chimeric rate during mid-to-late embryo development. Simultaneously, Throesch et al. [55] introduced rESCs into mouse embryos defective for olfactory sensor neurons (*Omp1-Cre; R26-DTA* mice) to rescue olfactory circuits in an interspecies setting. ESCs-derived neurons could rescue the olfactory behavior, albeit less efficiently than in mouse-to-mouse chimeras, and the resulting olfactory bulbs presented smaller and disorganized glomeruli.

Lung

Yuri et al. [64] applied the tetraploid-based organ complementation method to generate rESCs-derived lung epithelial cells in *Fgfr2b*-KO mouse embryos. Although rat-to-mouse complemented chimeras were obtained, lungs were not functional after birth and chimeras showed cyanotic skin and died postnatally. Authors hypothesized that this was due to developmental timing incompatibilities between rat and mouse, being the rat cells-derived lungs development delayed compared to mouse. In another study of 2024, Wen et al. [44] combined CRISPR-Cas9 genome editing with BC to generate mESCs-derived lungs and thyroid tissues in *Nkx2.1-KO* rats. Complemented mouse-to-rat chimeras recovered at E20.5 presented reconstituted lung lobes and thyroid tissue, however they were smaller in body and lung size compared to WT rats. Histological analysis showed full contribution of donor cells in lung epithelial cells and thyroid, while about 30% of the complemented embryos showed nearly 100% also in the mesenchymal, endothelial, SMC and immune compartments of the lungs at E20.5. scRNAseq analysis revealed correct specification of the mouse-derived lung cells in *Nkx2.1-KO* rats, nevertheless, similarly to what observed in intraspecies *Nkx2.1-KO* complemented chimeras [27], mESCs failed to correct the tracheoesophageal fusion associated with the KO phenotype.

Hematoendothelial lineages

Complementation of hematoendothelial lineages has encountered some difficulties with the transition towards an interspecies setting. When Hamanaka et al. [21] tried to generate rat ECs in *Flk1*$^{mut/mut}$ mice by rPSCs injection, no live fetuses were obtained at E13.5 and beyond. Although they recovered *Flk1*$^{mut/mut}$ chimeric fetuses at Mendelian ratio at E9.5 (*Flk1*-KO mice are viable until E8.5–9.5) [66], they reported that most of them were delayed or small (10/17), probably a sign of incomplete complementation. Two years later, Wang et al. [25] reported similar results: after injecting rESCs into *Flk1*-KO mice blastocysts, *Flk1*-KO chimeric fetuses were recovered up to E10.5. In this case, although average rat cells contribution to the embryo was 2–3%, all hematoendothelial cells were rESCs-derived in recovered embryos. Since a developmental arrest was observed at E10.5, the authors examined the cell composition of the vascular network in *Flk1*-KO rat-to-mouse chimeras and found that neither rat pericytes were generated from rESCs nor mouse pericytes were recruited. Therefore, they hypothesize interspecies incompatibility in the EC-pericyte recruitment pathways between mouse and rat, and an inability of rat cells to differentiate to pericytes in the *Flk1*-KO mouse model. In 2023, we attempted [53] interspecies vascular complementation in combination with heart complementation using the *Nkx2.5-Cre; Tie2-Cre; R26-DTA* approach. Although we could obtain few *Nkx2.5-Cre; Tie2-Cre; R26-DTA* chimeras at E10.5, we couldn't rescue the lethal phenotype. In summary, vascular complementation has been elusive in rat-to-mouse models, suggesting incompatibilities in vascular development pathways between these species.

In 2020, Das et al. [24] attempted to generate human ECs in pigs. After noticing that hiPSCs experienced cell death when injected into *ETV2*-KO pig embryos (generated by SCNT), they overexpressed an antiapoptotic factor, *BCL2*, in hiPSCs to increase their chimera formation efficiency, obtaining a fivefold increase in human-to-pig cell contribution, although chimerism levels remained relatively low (1:2,000 cells). Remarkably, in ETV2-KO chimeric embryos at E17, concomitant expression of human nuclear antigen and the EC marker *TIE2* was detected, and essentially all TIE2^{+} cells were derived from hiPSCs.

Barriers and challenges to interspecies complementation

In BC, donor cells need to robustly contribute to the host embryo to be able to successfully complement the desired organ. Indeed, a minimum amount of donor cells within the vacant niche is required to ensure the threshold of progenitors needed for organ development.

Different studies have shown that donor chimerism is reduced when PSCs from a species different from that of the host embryo are employed [16, 67, 68]. Donor cell contribution in interspecies settings may be limited by various barriers, including mismatched developmental stages and rates, incompatible ligand-receptor interactions or divergent signaling pathways, and cell competition, all of which can contribute to the elimination of

Table 3 Summary of interspecies barriers and ethical challenges for BC clinical application

Category	Key barriers	Potential solutions & future directions
Developmental mismatched stages	Different developmental stages between donor PSCs and recipient embryo	Optimization of naïve hPSCs culture media to achieve isochronic injection of donor PSCs Inhibition of apoptosis in donor PSCs
Cell competition	Elimination of xenogeneic cells by their recognition as "loser" cells	Modulation of cell competition processes by engineering donor PSCs or host embryos to increase donor PSCs engraftment
Ligand-receptor incompatibility and/or divergent signaling pathways	Species-specific variations in key signaling molecules affecting organ formation	Engineering donor PSCs or host embryos to express compatible receptors and signaling molecules
Immunological rejection	Host immune response against residual xenogeneic cells in the PSC-derived organ	Immunosuppressive strategies Donor cell replacement of non-parenchymal tissues by targeting multiple cell types in host embryos (e.g. ECs)
Ethical challenges	Moral status of human-animal chimeras, widespread human chimerism in animals, use of non-human primate surrogates of embryos	Application of national laws and oversight by a specialized committee Development of international regulations Continuously updated regulatory frameworks Chimerism confinement to target tissues

donor cells [69–72] (Table 3). Tackling these barriers could increase donor cell chimeric contribution and, thus, interspecies BC efficiency.

hPSCs, in particular, contribute poorly to human-animal chimeras. A critical aspect for chimera generation is that donor cells and the host embryo need to match their developmental stage [69, 73–75]. For the engraftment of donor cells in preimplantation embryos (morula or blastocyst stage), these cells need to be in an equivalent condition, referred to as the "naïve" state. Both mouse and rat PSCs can be maintained in vitro under standardized naïve conditions and thus these cells can efficiently produce chimeras [16, 67]. However, for hPSCs, which have traditionally been derived and maintained in a standardized medium that induces the expression of post-implantation PSCs markers (known as "primed" state), several naïve culture conditions have been tested, but none have produced cells with robust contribution to interspecies chimeras (chimerism levels obtained with human cells varied from 0,001% to 1%) [40, 68, 76–80]. In this regard, Aksoy et al. [81] observed that, in contrast to mESCs, only a few naïve hPSCs retained the ability to proliferate after injection into rabbit or macaque morulae, regardless of the naïve conversion method used. In fact, most hPSCs either died or stalled in the G1 phase, losing pluripotency markers.

In addition to developmental stage matching, the evolutionary distance between donor and host species might play a key role in the efficiency of chimera generation. For instance, while mouse and rat species diverged approximately 20 million years ago, human diverged from both mouse and pig around 90 million years ago (http://timetree.org). Species divergence implies intrinsic differences in embryo development, for example, while rodent embryos proliferate to form an elongated structure known as "egg cylinder", primate and pig embryos develop into a flattened structure called "embryonic disc" [82]. This might hinder exogenous cells capacity of adapting to the xenogeneic context. Moreover, gestation periods also vary among species: 19 days in mice, 21 days in rats, 115 days in pigs, and 266 days in humans, approximately. Differences in developmental timing between species might affect the ability of xenogeneic cells to synchronize with the developmental pace of the host. Accordingly, a recent study reported that rPSC-derived lungs generated in mice by BC remained immature [64], reflecting the slower developmental rate of rat cells. Nevertheless, despite inherent differences in developmental timing between species, rPSC-derived brain tissues synchronized with mouse host development in forebrain complemented chimeras, suggesting that non-cell autonomous mechanisms may influence donor cell developmental timing [43]. Therefore, future studies will clarify the determinants of developmental synchronization of exogenous cells with the host embryo. Moreover, studies on rat↔mouse organ complementation have demonstrated that donor cells are guided by extrinsic developmental cues to determine the size of the rescued pancreas [16, 20], as rPSCs- derived pancreas in mouse had the size of a mouse pancreas and vice-versa. However, the size of mPSC-derived kidneys generated in rats was similar to that of mouse kidneys [23], suggesting the existence of species-specific intrinsic and extrinsic developmental cues that differ among organs.

A common feature to all xenogeneic barriers with human cells and animal host embryos is the elimination

of hPSCs. Therefore, one of the first approaches proposed to prevent xenogeneic cell elimination during early embryonic development is the inhibition of apoptosis in donor cells. As a proof of concept, Masaki et al. [83] tested this strategy by overexpressing the antiapoptotic factor *Bcl2* in mouse epiblast stem cells (mEpiSCs, isolated from the post-implantation epiblast) to enable their engraftment in the mouse blastocyst upon heterochronic injection. They observed that, when apoptosis was prevented, surviving donor cells maintained their developmental fate until the host was synchronized with their developmental stage and thereafter they could colonize the host embryo. In the same way, rat EpiSC (rEpiSCs) overexpressing *Bcl2* were able to undergo interspecies chimeric colonization of the mouse embryo. These blastocyst injections of mEpiSCs and rEpiSCs produced live born chimeras which survived into adulthood. *BCL2* overexpression was also successfully applied to naïve hiPSCs to boost their integration in pre-implantation rabbit embryos [81]. Nevertheless, this approach had no effect on proliferation, and it was insufficient to prevent hiPSCs loss of pluripotency. Using another anti-apoptotic strategy, Huang et al. [84] achieved primed hPSCs engraftment into late morulas/early blastocysts from various species (mouse, rabbit and pig), by overexpressing BMI1, a polycomb factor that suppresses apoptosis. Moreover, BMI1-overexpressing primed hPSCs could contribute to mouse embryos at E10.5, although with low chimerism levels. Apoptosis blockage via P53 depletion has been proposed as another strategy to promote hPSCs integration into animal host embryos. Using *P53*-KO naïve hPSCs, Bayerl et al. [79] increased both the number of human-to-mouse chimeras and the level of chimerism. Zhu et al. [85] overexpressed the prosurvival gene *MYCN* in combination with *BCL2* in donor primed hiPSCs, obtaining an increase in hiPSCs-derived contribution in preimplantation mouse, rat and pig embryos, as well as in mouse mid-gestation chimeras, producing functional human hematopoietic/endothelial progenitor cells. Complementation experiments between human and pig have also taken advantage of apoptosis inhibition in donor cells. *P53-KO* primed hiPSCs were used to generate human-derived skeletal muscle cells in pig embryos [35], while *BCL2*-overexpressing primed hiPSCs were utilized to generate human-derived ECs in pig embryos [24]. Later, *MYCN-BCL2* overexpression in 4CL naïve hPSCs was used by Wang et al. [36] to produce humanized mesonephros in pig embryos.

Cell competition is an evolutionarily conserved quality control mechanism that removes suboptimal or potentially harmful cells. In the early mouse embryo, this process ensures the selection of optimal cells by enabling neighboring cells to identify less fit, though otherwise viable, cells and trigger their elimination through apoptosis [86]. Modulating cell competition has been proposed as a strategy to bypass early developmental barriers and to mitigate the elimination of donor cells in a xenogeneic environment. Zheng et al. [70] reported the role of the MYD88-P53-P65 axis in cell competition between human cells and mouse host embryos. Genetic inhibition of this pathway allowed the generation of interspecies chimeras between primed hPSCs and mouse embryos as analyzed up to E8-9 days of gestation. Two recent preprint studies have explored the mechanisms behind interspecies cell competition by using mouse and human PSCs co-cultures. Hu et al. [71] described the RNA sensing system retinoic acid-inducible gene (RIG-I)-like receptor (RLR) signaling pathway as a key mechanism behind the "winner" status of mPSCs when co-cultured with hPSCs. Genetic suppression of RLR signaling in mouse embryos improved the survival and chimerism of unmodified donor hPSCs in vivo. More recently, Tanaka et al. [87]identified Ephrin-EphA interactions as a mechanism by which mPSCs outcompete hPSCs and induce their apoptosis, by using a cell–cell contact-dependent reporter system. Moreover, they observed that suppressing Ephrin signaling enhanced the survival of donor hPSCs, both in co-cultures and in vivo, upon injection into mouse embryos.

Besides protecting donor cells from induced cell death, another approach to enhancing interspecies chimerism is addressing ligand-receptor incompatibilities that may arise during early developmental stages. Recently, Ballard et al. [72] observed lack of cell adhesion between rodent and primate PSCs, which could be attributed to species-specific mismatches in cell adhesion molecules. To overcome this barrier, they used synthetic antigens and nanobodies on hiPSCs and mouse embryos, adding up a further increase of interspecies chimerism of *MYD88*-KO, 4CL naïve hiPSCs. Future research will help identify other critical ligand-receptor incompatibilities, potentially leading to targeted modifications that could enable more successful interactions between divergent species.

Although chimerism is lower in mouse ↔ rat interspecies chimeras than in intraspecies chimeras from both species, rat PSCs contribute substantially to mouse embryos and vice versa. In interspecies rodent chimeras, however, variation in chimerism among organs/tissues is more pronounced than in intraspecies chimeras [67], suggesting that ligand-receptor incompatibilities and/or divergent signaling pathways appear during organogenesis and are tissue-specific. This may be the cause of the failure of interspecies complementation of certain organs: for example, while intra-species kidney complementation has been achieved successfully [19, 23], it was not possible to rescue kidney development in rat-to-mouse

chimeras [19]. In this case, poor contribution of rPSCs to the metanephric mesenchyme in the mouse host has been proposed as a barrier [23]. Interestingly, however, mouse kidneys can be produced in Sall1$^{mut/mut}$ rats [23], suggesting that these molecular incompatibilities arise only when rat cells integrate into mouse embryos, rather than when mouse cells integrate into rat embryos.

Moreover, in interspecies rodent chimeras, it has been observed that a delicate balance must be achieved (Fig. 3): on the one hand, a certain chimerism threshold needs to be reached to allow BC, while on the other hand, high contribution of donor cells is related with embryonic lethality and morphological malformations. Therefore, it has been proposed that xenogeneic barriers related to high chimerism levels impede normal chimeras' development [16, 67].

It is believed that these xenogeneic barriers are especially important around mid-gestation, as high xenogeneic cell contribution is associated with embryonic lethality just before E11.5 [67]. Accordingly, several studies have shown that the chimerism level in live fetuses at E11.5 and later stages is lower than the average chimerism measured in live chimeras at E9.5 [16, 43, 67]. In line with this data, currently reported adult rat-to-mouse complemented chimeras present chimerism levels below 25% when analyzed in non-targeted tissues [16, 40, 43, 88].

Nishimura et al. [89] developed a strategy to overcome this barrier by selectively enhancing donor chimerism from E11.5 onward, creating a 'cell competitive niche' through the deletion of *Igf1r* in host embryos, as demonstrated in a mouse intraspecies setting. At adult stage, they found that chimerism in kidney, brain and lung approached 100%, while chimerism was lower in other organs. In a rat-to-mouse interspecies setting, they analyzed the effect of *Igf1r* deletion at E18.5 and found a significant increase in contribution of donor cells to certain organs compared to WT hosts (10–40% compared to 1–18% on average, respectively). However, all the interspecies chimeras died perinatally, presumably due to lung problems. The authors propose the depletion of *Igf1r* in an organ specific way to bypass interspecies chimeras' mortality and aid full replacement of a target organ cell population by exogenous cells. At present, xenogeneic organ generation with this approach remains to be tested.

If the challenges outlined above are eventually overcome and human organs are successfully generated through interspecies BC, a major remaining hurdle will be the potential for immunological rejection upon transplantation. This risk arises from the likely presence of xenogeneic cells in non-parenchymal tissues, such as nerves or blood vessels, which depends on the global level of chimerism. However, when mouse-derived pancreatic islets generated in rats were transplanted into diabetic mice [20], immunosuppression was required only during the first five days following transplantation. The authors proposed that, during this period, residual donor (rat-derived) cells present in the graft were eliminated by the host (mouse) immune system, and no signs of hyperacute rejection were observed. Nevertheless, it remains to be tested whether immunosuppression can be discontinued after transplantation of whole organs. To further decrease xenogeneic immunogenicity of organs generated by interspecies BC, host ECs could be eliminated along with parenchymal cells from recipient embryos, so that they are replaced with donor-derived cells [33, 53].

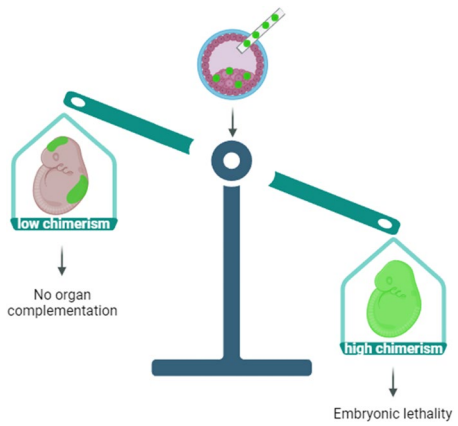

Fig. 3 The intricate balance to achieve interspecies organ complementation. Low chimerism levels may not be sufficient to complement an empty organ niche, resulting in embryonic lethality. However, if chimerism levels are too high, embryonic development is impaired and complemented embryos ultimately die

Ethical issues

Nowadays BC is in its experimentation phase, being its application for the production of humanized organs in farm animals a long-term goal. As with all other fields of biomedical research, the implementation of robust scientific and ethical review and oversight ensures best practices in both science and ethics.

However, when it comes to the experimentation with human-animal chimeras significant ethical concerns arise. Three primary concerns have been identified: (1) tampering with the natural order, (2) violation of human dignity and (3) the potential humanization of the chimeric animals, with acquisition of human-like consciousness [90].

The International Society for Stem Cell Research (ISSCR), between other academic societies and national academies of science, has provided widely recognized guidelines that many countries and research institutions

follow to decide whether to approve proposed research and to oversee it [91, 92].

Following the ISSCR guidelines all research involving chimera experimentation using human cells and embryo transfer in recipient animals must be approved and overseen by an ethical committee and a governmental body through a specialized review process. The Key ISSCR Guidelines responding to the three fundamental ethical concerns on BC with hPSCs are: (1) Incremental Research Approach: the length of chimeric embryo gestation must be well justified and limited to the minimum necessary to achieve the scientific aim. The chimerism rate and scope must be assessed at different stages of prenatal development before advancing to experimentation at full gestation stage. This approach helps prevent the generation of organisms with unintended widespread integration of donor cells. (2) Prohibited practices: transferring chimeric embryos containing human and animal cells into the uterus of a human or any ape. Breeding animals that have human cells with the potential to form human gametes. (3) Targeted Chimerism: to avoid unpredictable distribution of donor cells, scientists are encouraged to limit chimerism to specific organs or tissues. This strategy minimizes ethical concerns related to donor cell contributions in sensitive areas like the brain or reproductive organs.

In this context, initial studies in mouse intraspecies settings have established a foundation for various approaches (Table 3).

One strategy consists in directing exogenous cells differentiation through the forced expression of a tissue-specific master gene: Kobayashi et al. [93] restricted the differentiation of injected mPSCs to endodermal tissues by introducing an inducible cassette encoding the transcription factor *Mixl1*. They reported that after exogenous *Mixl1* induction, most of the microinjected cells preferentially contributed to endodermal tissues at E9.5 and adult stage, although mild chimerism in other cell lineages was still observed.

As an alternative strategy to confine chimerism in the desired organ niche, committed progenitor cells could be injected into post-implantational embryos instead of injecting PSCs into blastocysts. Nevertheless, these cells need to be delivered in a specific location to provide them the proper environment for their engraftment and in utero injection is technically challenging. To overcome that, Masaki et al. [83] showed that overexpression of *Bcl2* in $SOX17^{+}$ mouse endodermal progenitors enabled their successful engraftment into mouse blastocysts, resulting in E9.5 chimeras with exogenous cells contribution confined to endodermal tissues.

Alternatively, differentiation of injected PSCs into non-targeted tissues could be prevented by gene KOs. Hashimoto et al. [22] used CRISPR/Cas9 gene targeting to impede donor mESCs contribution to gametes and brain, through *Prdm14* and *Otx2* genes disruption, respectively. After injecting edited mESCs into apancreatic *Pdx1*-KO mouse embryos, adult mice with mESCs-derived pancreas were obtained, without donor contribution to gametes and brain, as analyzed by immunohistochemistry and absence of germline transmission.

Finally, another approach that has been proposed is the introduction of an inducible suicide cassette to eliminate donor cells differentiated to undesired cell types. This strategy has yet to be tested and the potential effect of massive cell death on embryo viability remains unknown [88].

All in all, the safe advancement of BC technology depends on prioritizing the development of technical strategies to address its ethical implications, enforcing appropriate national and international regulations, ensuring robust oversight, and maintaining clear, accurate communication about the current state of the technology and experimentation to the public.

Conclusions and future directions

Though currently in the experimental stage, BC is a promising approach that could one day enable the generation of humanized organs in livestock, potentially addressing the current shortage of organs available for transplantation. However, to make the generation of these humanized organs a reality, several scientific and ethical challenges will need to be addressed. Understanding the molecular incompatibilities between divergent species will be crucial, along with developing strategies to significantly increase the contribution of hPSCs to host embryos. Additionally, new strategies to replace all the cell types that form an organ will be both challenging and critically important. Overcoming these technical barriers must go hand in hand with addressing the ethical implications of this technique. Guidelines for BC research emphasize the importance of preventing unpredictable and widespread chimerism in human-animal chimeras. Therefore, it will be critical to confine human chimerism to the target organs to prevent human cell contribution to neural or germ tissues, and to maintain overall chimerism low. Overcoming these challenges, alongside the continuous update of institutional regulations, will move us closer to a future where growing human-compatible organs in animals becomes a reality, potentially revolutionizing transplantation medicine.

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