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Fumarylacetoacetate hydrolase gene as a knockout target for hepatic chimerism and donor liver production

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SUMMARY

A reliable source of human hepatocytes and transplantable livers is needed. Interspecies embryo complementation, which involves implanting donor human stem cells into early morula/blastocyst stage animal embryos, is an emerging solution to the shortage of transplantable livers. We review proposed mutations in the recipient embryo to disable hepatogenesis, and discuss the advantages of using fumarylacetoacetate hydrolase knockouts and other genetic modifications to disable hepatogenesis. Interspecies blastocyst complementation using porcine recipients for primate donors has been achieved, although percentages of chimerism remain persistently low. Recent investigation into the dynamic transcriptomes of pigs and primates have created new opportunities to intimately match the stage of developing animal embryos with one of the many varieties of human induced pluripotent stem cell. We discuss techniques for decreasing donor cell apoptosis, targeting donor tissue to endodermal structures to avoid neural or germline chimerism, and decreasing the immunogenicity of chimeric organs by generating donor endothelium.

INTRODUCTION

The demand for viable human hepatocytes and transplantable human livers far outweighs the supply. Researchers rely on imperfect substitutes: animal hepatocytes, oncogenic cell lines, and induced pluripotent stem cell (iPSC)-derived hepatocyte-like cells (Yao et al., 2020). Concurrently, clinicians have investigated hepatocyte-seeded scaffolds (Minami et al., 2019; Shaheen et al., 2020; Uygun et al., 2010), bioartificial hepatocyte reservoirs (Chen et al., 2019; Sauer et al., 2002), and intravenous hepatocyte transplantation in humans (Dhawan et al., 2010; Jorns et al., 2016; Muraca et al., 2002; Soltys et al., 2017). Alternatively, the supply of transplantable livers can be increased through *in vivo* generation of whole livers through blastocyst complementation. Blastocyst complementation entails a recipient blastocyst with a genetic mutation disabling organogenesis, transplanted with pluripotent cells from a wild-type (WT) donor. As the chimeric embryo develops, the donor cells fill the induced competitive niche and form the missing organ. This method has been successfully used for interspecies organogenesis in rat-mouse chimeras, generating rat pancreas and thymus in mice, and mouse islets in rats (Isotani et al., 2011; Kobayashi et al., 2010; Yamaguchi et al., 2017).

Generation of a liver via blastocyst complementation has been explored. *Hhex*, a homeobox gene that regulates liver bud and hepatocyte development, has been disabled in recipient embryos in intraspecies blastocyst complementation, resulting in WT-predominant livers in these chimeras (Aravalli, 2021; Keng et al., 2000; Matsunari et al., 2020). However, *Hhex* mutations affect many organs and developmental processes, posing ethical issues for interspecies experiments. Another hepatogenesis target is the *FAH* gene, which encodes the fumarylacetoacetate hydrolase (FAH) enzyme, responsible for the final step in tyrosine metabolism (Grompe, 2001). FAH deficiency causes the human disease hereditary tyrosinemia type 1 (HT1): toxic metabolite fumarylacetoacetate (FAA) accumulates in hepatocytes and renal proximal tubule cells, leading to mutagenesis and apoptosis (Kubo et al., 1998). Preliminary data demonstrate the ability of $Fah^{-/-}$ recipient porcine blastocysts to successfully support the development of porcine donor-cell-derived livers. With any proposed gene intended to create a niche favoring donor-derived hepatocytes or transplantable livers, there are a variety of questions to consider: other than hepatocytes, what liver cell types are affected, leading to the potential for replacement by donor cells? What off-target or systemic effects does the mutation have? Does it exert selective pressure on the chimeric animals through *in utero* lethality? We discuss the potential of *FAH*, *Hhex*, and others herein.

To obtain human organoids for clinical applications such as liver transplantation or enough human hepatocytes for extracorporeal liver support, we must also tackle the problem of interspecies blastocyst complementation between humans and evolutionarily divergent large animals such as swine. As the media and scientific frenzy over recent publication of human-primate blastocyst complementation has demonstrated, forays into this territory bring a new set of technical, ethical, and translational issues to the forefront (Tan et al., 2021). From a technical standpoint, the engraftment and proliferation of primate embryonic stem cells (ESCs) within pig blastocysts has been persistently low (Das et al., 2020; Fu et al., 2020; Nowak-Imialek et al., 2020; Wu et al., 2017). We discuss the role of naive and

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primed human pluripotent cells in blastocyst complementation and how human cell choice can be optimized based on differences between human, pig, and monkey embryonic transcriptomes during development (Liu et al., 2021). From an ethical standpoint, human-derived cells in the blastocyst must be prevented from differentiating into germline cells and neural tissue to maintain the recipient porcine phenotype. We summarize a variety of promising methods for limiting human cell contributions to the desired endodermal-only structures. From a translational standpoint, the immunogenicity of a chimeric liver must be addressed before transplantation. We address new research that disables host endothelial development via *Flk1*, *Kdr*, and *Etv2* mutations, making way for humanized donor-derived endothelium.

PROPOSED GENETIC TARGETS TO IMPAIR RECIPIENT BLASTOCYST HEPATOGENESIS

The liver is composed of multiple cell types derived from both endoderm (parenchymal cells such a hepatocytes) and mesoderm (Kupffer cells, lymphocytes, and cells comprising intrahepatic vasculature). Hepatic stellate cells (HSCs) possess features of all three primitive germ layers (Friedman, 2008). Complex interaction between the foregut anterior endoderm, cardiac mesoderm, and septum transversum mesenchyme (STM) leads to the development and maturation of the liver bud (Aravalli, 2021; Arterbery and Bogue, 2014; Saito et al., 2012; Si-Tayeb et al., 2010) The major pathways in this interaction are regulated by GATA4, inductive signals from fibroblast growth factors (FGFs), and transforming growth factor beta (TGFβ), working in parallel to regulate the early gene induction that leads to the formation of the liver and pancreas (Wandzoich and Zaret, 2009).

Gene-knockout mice have implicated several transcription factors in controlling different aspects of the liver development, including *Hhex*, *FoxA*, *Sox17*, *HNF1β*, *Prox1*, *OC1*, and *OC2*. In *Hhex*-deficient mouse embryos, liver bud is specified but proliferation is reduced and the migration of hepatoblasts into the STM does not occur (Bort et al., 2004; Martinez Barbera et al., 2000), and mice lacking FoxA1 and FoxA2 proteins fail to initiate development of the hepatic lineage (Lee et al., 2005). *Sox17* mediates segregation of liver, ventral pancreas, and the biliary system (Spence et al., 2009). Early in hepatopancreatic specification, *Sox17* is co-expressed with *Hhex* and *Pdx1* in the ventral foregut endoderm. However, at E8.5, its expression is downregulated in the liver primordium, and at E9.5 the co-expressing domain of *Sox17* and *Pdx1* segregates from the *Hhex*-expressing domain (Kanai-Azuma et al., 2002). The *Sox17* part then forms the extrahepatic biliary system, while the *Pdx1* section develops into ventral pancreas (Spence et al., 2009; Uemura et al., 2010). Additional studies have corroborated that *HNF1β* is essential for hepatic specification (Lokmane et al., 2008) and *Prox1* for hepatocyte specification (Seth et al., 2014) and migration (Sosa-Pineda and Oliver, 2000). Embryos lacking *HNF1β* have defects in liver bud formation and hepatic endoderm thickening, and none of the known markers of hepatic parenchymal cell progenitors are expressed in them (Lokmane et al., 2008). OC1 and OC2 stimulate the degradation of the basal lamina that surrounds the liver bud and regulate early liver expansion by controlling hepatoblast migration in the STM (Margagliotti et al., 2007). Thus, a tightly regulated network of intertwined signaling mechanisms regulate liver bud morphogenesis. A number of these genes have been proposed as targets for arrest of hepatogenesis in blastocyst complementation (Table 1).

When choosing a candidate for blastocyst complementation, it is important to consider the liver cell types affected by each gene. If the liver is intended for transplantation, hepatocytes, cholangiocytes, endothelial cells, HSCs, natural killer (NK), and natural killer T (NKT) cells are of special importance because they are not replaced by recipient cells following transplant. Donor Kupffer cells, dendritic cells, and T and B cells play important roles in graft tolerance, but are replaced by recipient cells within months (Jiang et al., 2020). Many of the genes described in Table 1 affect cell types beyond hepatocytes: *UbC*, *TBX3*, *Mab21l2*, and *OC1/2* affect the common cholangiocyte and hepatocyte precursor, hepatoblasts (Kropp and Gannon, 2016; Ryu et al., 2007, 2012; Saito et al., 2012; Suzuki et al., 2008). Hematopoietic lines are affected by *Hhex* and *UbC*, although to what extent this would impair the generation of hepatic lymphoid, dendritic, NK, NKT, or Kupffer populations has yet to be characterized (Paz et al., 2010; Ryu et al., 2007, 2012). A variety of strategies for generating endothelium via blastocyst complementation have been studied, and these are discussed more in detail below.

As we more fully understand the sequelae of null mutations of master regulators of hepatogenesis, it may be necessary to create double or triple knockout animals to ensure that critical cell populations within the liver are replaced with donor-derived tissue. It is not known whether any of the proposed genes affect HSCs; if not, the *Hlx* homeobox gene may be necessary for HSC development and would warrant further investigation for possible knockout (Hentsch et al., 1996). A mutation in *FAH*, which does not affect cholangiocytes, could be paired with a mutation such as *GPC1*, whose knockdown results in biliary defects, although not complete agenesis (Cui et al., 2013).

Table 1. Potential gene targets for liver blastocyst complementation

Gene	Role	Homozygous knockout lethality	Other organs/systems affected
Hhex (Aravalli et al., 2020)	Homeobox gene, master regulator of hepatobiliary development, including liver bud development and hepatocyte genesis (Aravalli, 2021; Keng et al., 2000; Matsunari et al., 2020)' (Hunter et al., 2007)	*in utero*	hematopoiesis and lymphopoiesis (Paz et al., 2010), vasculogenesis (Cong et al., 2006), multiple processes in the anterior endoderm including organ development and chirality (Aravalli, 2021; Bort et al., 2006; Rankin et al., 2011)
Mab21l2	cell fate gene expressed in the septum transversum mesenchyme, without which hepatoblast proliferation in late hepatic bud development is impaired (Saito et al., 2012)	*in utero* E11.5–14.5 (embryonic day 11.5–14.5) in mice (Saito et al., 2012)	heart, eye, body wall (Saito et al., 2012)
Tbx3	transcription factor with early activating and lineage regulation role in hepatoblasts (hepatocyte/cholangiocyte precursor) (Suzuki et al., 2008)	*in utero* prior to E16.5 in mice (Davenport et al., 2003; Hoogaars et al., 2007)	sinoatrial node (Hoogaars et al., 2007), limbs, mammary system, yolk sac (Davenport et al., 2003)
OC1 and *OC2* (Crane et al., 2019)	transcription factors that initiate hepatic gene expression and migration in early hepatoblasts in the liver bud. *OC1/2*$^{-/-}$ livers fail to expand and regulate glycogen, and *OC1*$^{-/-}$ fail to form intrahepatic and extrahepatic biliary systems (Kropp and Gannon, 2016; Margagliotti et al., 2007)	unknown	pancreas, retina, central nervous system (Kropp and Gannon, 2016)
UbC	stress-inducible polyubiquitin gene. Many cell types in the liver undergo increased apoptosis (hepatocyte, erythroid, epithelial, pan-hematopoietic markers affected) (Ryu et al., 2007, 2012)	*in utero* 12.5–14.5 d in mice (Ryu et al., 2007)	impaired fibroblast proliferation (Ryu et al., 2007)
FAH	enzyme required for breakdown of toxic FAA in hepatocytes (Grompe, 2001)	lethal *in utero* for pigs, but not humans or mice. Juvenile liver disease and HCC risk (Hickey et al., 2014; Kvittingen, 1986; Russo and O'Regan, 1990)	renal proximal tubule

FAH$^{-/-}$ ANIMALS AS RECIPIENTS FOR BLASTOCYST COMPLEMENTATION

Although *Hhex* knockouts have successfully hosted donor-generated livers in porcine intraspecies blastocyst complementation (Matsunari et al., 2020), no interspecies *Hhex* studies exist. A main concern with *Hhex* is that its effects on organogenesis are not limited to the liver and could result in off-target donor cell growth (Aravalli, 2021; Bort et al., 2006; Cong et al., 2006; Jackson et al., 2015; Paz et al., 2010; Rankin et al., 2011). *Tbx3*, *OC1/2*, *Mab21l2*, and *UbC* have never been tested as targets for blastocyst complementation. *Fah* is an appealing target for blastocyst complementation for several reasons, including the well-described ability of *Fah*$^{-/-}$ livers to host the development of WT hepatocytes, whether the WT cells are introduced through genetic reversion or via deliberate transplantation (Nicolas et al., 2020; Overturf et al., 1996; Zhang et al., 2016).

Unlike many of the other proposed genes for liver blastocyst complementation, the absence of *FAH* genes is not lethal *in utero* for humans, allowing us to study the natural history of the deficiency through HT1 research. As a result of FAH deficiency, toxic precursors MAA and FAA accumulate; FAA possesses both mutagenic and carcinogenic properties (Jorquera and Tanguay, 2001). MAA and FAA have short half-lives and do not leave the cells where they are produced (Grompe, 2017). The only cell types that express all the necessary enzymes for tyrosine metabolism, and therefore are affected by the toxic accumulation, are hepatocytes and renal proximal tubule cells (Grompe, 2001). The clinical presentation of HT1 includes Fanconi renal tubular acidosis, and either fulminant liver failure in infancy or chronic childhood liver disease marked by mixed micro- and macro-nodular cirrhosis. In chronic disease, the risk of developing juvenile hepatocellular carcinoma (HCC) is high (Kvittingen, 1986; Russo and O'Regan, 1990). Importantly, the HT1 phenotype has successfully been recapitulated and studied in $Fah^{-/-}$ pigs (Hickey et al., 2014). Unlike some other liver master regulators, $FAH^{-/-}$ children and piglets lack other syndromic features; FAH deficiency is a precise method for targeting the liver without high risk of congenital abnormalities affecting other organs (Hickey et al., 2014).

Also contrary to other mutations proposed, $FAH^{-/-}$ individuals have preserved architecture and non-hepatocyte cells of the liver (Kelsey et al., 1993). The intact microenvironment has been demonstrated to support liver generation *in vivo*. In the 1990s, it was observed that children with HT1 commonly had spontaneous reversion of the *FAH* mutation in hepatocytes, causing overgrown nodules of normal tissue in their livers as the reverted hepatocytes out-competed those damaged by FAA accumulation (Kvittingen et al., 1994). Subsequent experiments demonstrated that exogenously introduced WT hepatocytes can repopulate $Fah^{-/-}$ livers in mice, rats, and pigs (Hickey et al., 2016; Nicolas et al., 2020; Overturf et al., 1996; Zhang et al., 2016). Human hepatocytes and hepatocyte-like cells can be infused into immunodeficient $Fah^{-/-}$ mice, repopulating the liver with human hepatocytes (Azuma et al., 2007; Bissig et al., 2010; Bissig-Choisat et al., 2016; Yuan et al., 2018).

In addition to the preserved hepatic architecture and the regenerative milieu, there is another feature that sets FAH deficiency apart from other proposed gene candidates for blastocyst complementation: pharmacologic treatment. The drug 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) blocks tyrosine catabolism at the second step, before the production of toxic FAA (Ellis et al., 1995). When given to patients with HT1, NTBC results in improvement in liver and kidney disease, and reduces the incidence of HCC when started in infancy (Al-Dhalimy et al., 2002; Joshi and Venugopalan, 2004; Maiorana and Dionisi-Vici, 2017; Maiorana et al., 2014). More importantly for blastocyst complementation, NTBC can be used to manipulate the selective pressure on a liver that has both $FAH^{-/-}$ and WT hepatocytes. While FAH deficiency is not lethal *in utero* in humans, it is lethal *in utero* in pigs; NTBC administered to pregnant sows rescues $Fah^{-/-}$ fetuses, making this drug a potent selective tool (Hickey et al., 2014).

PRELIMINARY DATA SUGGEST THAT PORCINE $FAH^{-/-}$ AND WT CHIMERAS ARE FEASIBLE

In a murine model, WT/$Fah^{-/-}$ chimeras have been created via blastocyst complementation (Espejel et al., 2010). In this study, NTBC was administered to dams throughout pregnancy, then weaned from pup diets after birth. Pups with high levels of chimerism in a biopsied digit gained weight normally and maintained normal markers of hepatic synthetic and metabolic function after NTBC was withdrawn, whereas animals with no WT chimerism failed to thrive and had abnormal liver function markers. The percentage of WT cells in the WT/$Fah^{-/-}$ chimeric liver increased, from below 50% 22 days after NTBC withdrawal to 100% 64 days after withdrawal (Espejel et al., 2010).

Preliminary studies in our group have resulted in the successful birth of piglets after recipient porcine $Fah^{-/-}$ embryos at the morula stage were complemented with WT porcine morula cells. Sows who were not given NTBC and immunosuppression during pregnancy gave birth to animals that were ubiquitously composed of WT cells in derivatives of all three embryonic germ layers. WT cells had a competitive advantage in every tissue, not just those affected by toxic FAA accumulation. However, sows who were given NTBC daily gave birth to piglets possessing entirely $Fah^{-/-}$ cells, without evidence of any retained WT tissue. This is different from the results obtained by Espejel et al. in their mouse study; the reasons for the difference between species is unclear and may be related to the low number of piglet studies (6 WT/$Fah^{-/-}$ piglets in six separate gestations) that have been analyzed. Studies to investigate the percentage of chimerism in intraspecies chimeras generated using this method are ongoing.

EMBRYONIC STAGE MATCHING

Despite the promises of blastocyst complementation for liver regeneration, the results have been strikingly inconsistent. Pinpointing the causes of success and failure is complicated by ambiguous stem cell terminology, lack of standard assays, and unclear significance of *in vitro* and early *in vivo* endpoints. Here, we will focus on the details of a single aspect in blastocyst complementation research

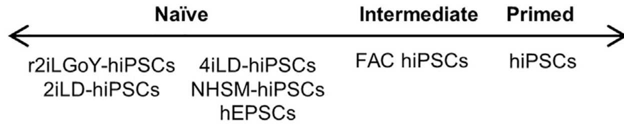

Figure 1. Protocols have been developed to create hiPSCs with a spectrum of naive to primed features

The relationships between some types of naive-like hiPSCs, and exactly where they might fall on this spectrum, are still unknown.

that we believe holds the key for further research: the importance of selecting donor and recipient cells that are developmentally synchronized (Huang et al., 2012; Mascetti and Pedersen, 2016; Wu and Izpisua Belmonte, 2015), a process that becomes more difficult and nuanced with evolutionarily divergent species.

Early investigations into murine ESCs (mESCs) revealed that pluripotent stem cells form a spectrum of distinct populations, ranging from naive progenitor cells to primed pluripotent cells. Naive cells were isolated from pre-implantation mouse embryos, and primed cells from post-implantation epiblasts (Kumari, 2016). While both naive and primed lines can differentiate into all three germline layers *in vitro*, the cell types have distinct behaviors *in vitro* and *in vivo*: importantly, naive mESCs are able to form chimeras through blastocyst complementation, while primed cells such as mEPI-SCs (mouse epiblast stem cells) cannot form germline chimeras (Huang et al., 2012). The first attempts to isolate human ESCs (hESCs) showed that, unlike mice, hESCs exist in a primed state even in pre-implantation embryos (Tesar et al., 2007). Thus commenced the search for a method to artificially manufacture naive hESCs that possessed chimeric capabilities *in vivo*.

Because true hESCs of any stage are not widely available for research, methods to imitate them via environmental and genetic manipulation of somatic cells have been discovered. These somatic-derived cells are called human iPSCs (hiPSCs). Methods have now been developed to generate a variety of hiPSCS to recapitulate the spectrum of naive and primed cells found in native ESCs (Figure 1). Debate continues surrounding the equivalence of hESCs and hiPSCs; while by some measures, such as transcriptome analysis and teratoma assays, these cell types can be very similar, hiPSCs can retain methylation patterns from the somatic line they originate from, which may be especially detrimental to the authenticity of naive-like hiPSCs (Ware, 2017).

Recent investigations aimed at recapitulating the chimeric capabilities of naive mouse ESCs in hiPSCs have generated cell types such as naïve human stem cell media hiPSCs (NHSM-hiPSCs) and human extended pluripotent stem cells (hEPSCs) (Chan et al., 2013; Gafni et al., 2013; Gao et al., 2019; Theunissen et al., 2014; Ware et al., 2014). These human lines share metabolic, epigenetic, transcriptomic, and colony morphology features with naive mESCs. While primed hiPSCs do not show successful chimerism with mouse embryos even at the late E7.5 stage, promising results have shown that naive hiPSCs can integrate into mouse blastocysts and persist in organ tissue after implantation (Gafni et al., 2013; Wu et al., 2015; Yang et al., 2017).

The leap from integrating naive hiPSCs into mice to integrating human/primate cells into porcine embryos has been challenging, partially due to the lack of data about embryonic stages in these organisms. Understanding of stage matching in primate and porcine embryos was recently bolstered by the identification of transcriptome evolution patterns during embryological development in pigs, humans, and cynomolgus monkeys (Liu et al., 2021). Based on the progression of pluripotency transcriptomes, r2iLGoY-hiPSCs (Liu et al., 2017) were correlated with the earliest pig ICM cells (E5–6), NHSM-hiPSCs fell between the spherical and filamentous stages (E11–12), and primed hiPSCs corresponded to filamentous stages (E12–13). Transcriptome evolution and stage matching helps explain why even naive NHSM-hiPSCs had poor integration into pig blastocysts (Wu et al., 2017); the porcine cells were 5–6 days behind the developmental trajectory of the NHSM-hiPSCs. In order to form a successful chimera, it seems that hiPSCs must satisfy *two* requirements: they must be naive, and must be intimately matched to the stage of the recipient embryo.

Table 2 summarizes the four published attempts to make interspecies porcine and primate chimeras, as well as the recent landmark creation of human and monkey interspecies chimeras. All porcine hosts were E4–6 embryos that were complemented with either primed or naive donor hiPSCs. Based on the work of Liu et al. (2021), at least some of these hiPSCs represented an inappropriately late developmental trajectory compared with the pig embryo. The most successful attempt was the combination of naive hEPSCs and cynomolgus monkey recipient embryos, which have a closer developmental trajectory and closer evolutionary distance to humans than pigs do (Liu et al., 2021; Tan et al., 2021). Further attempts to more closely correlate the transcriptomic evolution of donors and recipients will be needed, perhaps using cells such as r2iLGoY-hiPSCs, which correspond to an early embryonic stage in pigs.

Although new naive hiPSC lines are a hopeful line of inquiry for stage matching and successful chimera formation, they have several drawbacks. First, as we will discuss next, restricting the lineage of human cells by using primed or differentiated cells may be important for ensuring that human cells do not end up in chimeric animal nervous systems or germ cells. Second, there are conflicting reports that naive hiPSCs may carry *in vivo* carcinogenic risks, and more research is needed to address this issue (Stirparo et al., 2021).

Table 2. Porcine recipient blastocyst stage, primate iPSC type, and outcome correlation

Paper	Pig blastocyst age	Human/primate ESC type		Outcome
Wu et al. (2017)	5–6 d (parthegenotes), 6 d (zygotes)	human	2iLD-hiPSCs 4i-hiPSCs NHSM-hiPSCs FAC-hiPSCs	FAC-hiPSCs had the highest integration into post-implantation embryos, but all cell types had low percentages of human-pig chimerism (exact percentage not given)
Das et al. (2020)	4 d		hiPSCS, cultured in mTeSR1 or TeSR-E8 according to previously published protocol (Chen et al., 2011)	12 out of 23 *in vivo*-developed embryos contained donor human cells, on average <0.01% human tissue
			same as above, with hiPSCs overexpressing BCL-2	51 out of 63 *in vivo*-developed embryos contained donor human cells, on average 0.05% human tissue
Zhong et al. (2019)	5–6 d		NHSM-hiPSCs FAC-hiPSCs	NA (methods paper)
Nowak-Imialek et al. (2020)	4d	primate	cyiPSCs	74.5% survival and expansion after 48 h *in vitro*
	6d			43% survival and expansion after 24 h *in vitro*
	Mix of 4-cell, 16-cell, and morulae stage			no *in vivo* chimeras had surviving cynomolgus cells in the embryonic disk
Fu et al. (2020)	5d		intermediate or naive cyiPSCs	on average, 27–30 cynomolgus cells after 48 h *in vitro*. No *in vivo* chimeras survived
			primed cyiPSCs	on average, 16 cynomolgus cells after 48 h *in vitro*. No *in vivo* chimeras survived
			DESC-cyiPSCs, which have both primed and naive features	2 out of 10 neonatal pigs were chimeras with cynomolgus tissues in a variety of organs
Paper	Primate blastocyst age	Human/primate ESC type		Outcome
Tan et al. (2021)	6 d	human	hEPSCs (Gao et al., 2019)	human cells comprising up to 7.08% of epiblast layer, 4.96% of hypoblast layer, and limited trophectoderm contributions

cyiPSCs, primed cynomolgus monkey PSCs; NA, not available.

CURRENT STANDINGS IN PORCINE AND PRIMATE INTERSPECIES BLASTOCYST COMPLEMENTATION

In 2017, Wu et al. (2017) reported the first successful generation of human-pig chimeras via blastocyst complementation. Donor hiPSCs at naive and intermediate developmental stages resulted in chimeric embryos that survived *in vivo* for 3–4 weeks. The percentage of chimerism was low (although unquantified), and chimeric fetuses were growth restricted or malformed, consistent with studies in rodent intraspecies chimeras (Yamaguchi et al., 2018). Das et al. (2020) made the second reported attempt in 2020, disabling porcine *ETV2*, a hematoendothelial regulator, in recipient blastocysts. Human and porcine cells exchanged calcein in a chimeric embryo, indicating formation of gap junctions between species cells. Chimeric embryos again rarely survived *in vivo*, although surviving fetuses had increased percentages of hiPSCs.

To avoid the ethical dilemmas of human research while working toward technical success, some groups have turned to cynomolgus monkey cells, which are a closer match for human embryonic behavior and human liver cell function (Aravalli and Steer, 2020; Liu et al., 2021). Nowak-Imialek et al. (2020) first used parthenogenetic pig blastocyst recipients with cynomolgus monkey iPSC (cy-iPSC) donors. After 11 days of *in vivo* development, cynomolgus cells were found in the trophectoderm but not in the epiblast. Repeating the experiment with an adjustment in cy-iPSC stage by Fu et al. (2020) yielded much more promising results: differentiated cynomolgus cells were found in all three germ layers of porcine embryo. Furthermore, *in vivo* studies yielded the birth of chimeric animals, with functional cynomolgus cells in the liver and kidney. However, similar to other pig-primate chimeras, the primate contribution to chimerism was very small: 0.1% to 0.01% in different neonatal tissues. Overall, these results support the importance of iPSC stage in future porcine-primate chimerism success.

The landmark research by Tan et al. (2021) in 2021described the first hiPSC donors and primate blastocyst recipient chimeras. This research is notable for the high percentage of donor cells incorporated into the chimera, up to 7%, and the enrichment in cell-cell interaction pathway gene expression in both cell types. The use of an early naive stage of hiPSC, called hEPSC, is the most prominent methodological change from prior studies and therefore may have played a role in this success. The similarities in human and cynomolgus developmental trajectory and transcriptome patterning may also have been a factor, as this study found increasing correlation between chimeric human and chimeric cynomolgus cell transcriptomes over time.

If the lessons from these recent advances are to be applied to human liver development for hepatocytes and transplantation, further increases in chimerism percentage, attention to ethics, and translational concerns will need to be addressed.

IMPROVING IN PRIMATE BLASTOCYST COMPLEMENTATION FOR HEPATOGENESIS

Improving the yield of human tissue

The low percentage of human cells retained in porcine blastocysts may necessitate genetic modifications to hiPSCs to increase their competitiveness and resistance to apoptosis in an unfamiliar embryonic environment (Das et al., 2020; Fu et al., 2020; Nowak-Imialek et al., 2020; Wu et al., 2017). One candidate is anti-apoptotic factor BCL2 (Das et al., 2020; Masaki et al., 2016). hiPSC donors overexpressing BCL2 implanted into pig recipient embryos had a 5-fold increase in viable chimeric embryos *in vivo*, and also an increase in the percent chimerism seen in individual embryos (Das et al., 2020). Overexpression of BCL2 has also been shown to facilitate the survival and expansion of donor cell lines that were more terminally differentiated than the recipient embryo, potentially indicating a solution to the problem of interspecies stage matching (Masaki et al., 2016). Another candidate to improve the yield of donor cells is IGFr1. IGFr1 overexpression resulted in increased intraspecies murine chimerism and viable chimeras (Nishimura et al., 2021). However, these results were not seen until after E10.5, which may be too late to rescue early-stage interspecies chimeras (Ballard and Wu, 2021; Nishimura et al., 2021).

Improving the specificity of donor cell destinations: Targeting the endoderm

Arrest of organogenesis in one organ does not preclude donor cells from participating in other developmental processes. The largest ethical challenge facing liver blastocyst complementation is increasing the specificity of chimerism to ensure that human cells populate the liver and not neurological tissue. One strategy to improve endodermal specificity of donor cells is overexpression of Mixl1, which promotes endodermal fate (Lim et al., 2009). Intraspecies blastocyst complementation in mice found that donor cells overexpressing Mixl1 for a short period in early development resulted in very few donor cells outside endodermal organs (Kobayashi et al., 2015).

Another strategy to target donor cells to the endoderm uses extraembryonic endoderm stem (XEN) cells as donor cells instead of iPSCs. XEN cells are stem cells derived from the primitive endoderm in pigs and rodents, which contribute to both extraembryonic and gut endoderm in chimeras (Niakan et al., 2013; Park et al., 2021). Intraspecies pig chimeras generated using pig XEN donor cells resulted in donor cells restricted exclusively to the endoderm and extraembryonic

structures. However, no peer-reviewed reports of human XEN cells are yet available.

A final strategy that has successfully been employed is the use of Sox17+ stem cells, which are fated to become endodermal progenitor lines (Niakan et al., 2010). Although Sox17+ donor cells are too developmentally advanced to be synchronized with a recipient blastocyst, by simultaneously overexpressing BCL2 in the donor, the stage requirement can be circumvented. The resulting intraspecies mouse chimera has donor cells limited to the gut endoderm or yolk sac (Masaki et al., 2016).

Targeting the vascular endothelium to reduce immunogenicity

Although human hepatocytes from a liver with porcine endothelium would be applicable in a number of research and therapeutic settings, transplantation of a human organ with porcine endothelium would risk hyperacute rejection, acute humoral rejection, and coagulopathy; so far, no therapeutic is available to blunt all of these responses (Cooper et al., 2014, 2015). If the goal is to develop transplantable livers, the recipient porcine embryo should have a null mutation in master regulators of both liver and endothelial development. The first successful blastocyst complementation of endothelial cells used an $FLK\text{-}1^{1173/1173}$ mutation in mouse intraspecies complementation, resulting in completely donor-derived endothelium (Hamanaka et al., 2018). Intraspecies porcine chimeras with $KDR^{-/-}$ recipients also demonstrated full-term fetuses with donor-derived vasculature (Matsunari et al., 2020). More recently, Das et al. (2020) complemented donor hiPSCs to recipient porcine embryos with an $ETV2^{-/-}$ mutation, resulting in an endothelium that was 100% human.

CONCLUSIONS

Early embryonic complementation is a promising method that could yield high quantities of human hepatocytes or fully transplantable livers from porcine hosts. While many hepatogenesis-disabling genetic knockouts have been proposed, most of these genes have off-target effects; further study is needed to determine the effects on non-hepatocyte liver cells and other organ systems. We propose FAH as a target for disabling hepatogenesis, based on its specificity to the liver, well-understood natural history, known ability to preserve the regenerative milieu of the liver, and potential for selective manipulation using NTBC pharmacotherapy.

Before this goal is realized, the study of blastocyst complementation must focus on exploiting promising recent advancements in experimental technique, ethics, and translation. From a technical perspective, the proliferation of primate ESCs within pig blastocysts has been persistently low. New research into stage synchronization between porcine and primate blastocysts has provided insight into the disappointing results of previous porcine-primate chimerism experiments. By more closely synchronizing donor and recipient embryo stages, or by manipulating donor cells to overexpress anti-apoptotic genes until the appropriate developmental stage, donor contributions to chimeras can be increased. Recent landmark results in primate-human chimerism provide proof of concept for the importance of embryonic stage and developmental synchronization. From an ethical standpoint, human-derived cells in the blastocyst must be prevented from differentiating into neural tissue. New methods to target donor cells to the endoderm layer via overexpression of Mixl1 or development of lineage-restricted stem cells have succeeded in restricting donor contributions to the endoderm. From a translational standpoint, the immunogenicity of a chimeric liver is being addressed with techniques to generate donor-derived endothelium.

AUTHOR CONTRIBUTIONS

E.L.L., D.J.J., E.D.N., and S.L.N. conceptualized the manuscript. D.J.J., E.D.N., B.A., and S.L.N. performed preliminary experiments. E.L.L., R.N.A., and S.L.N. wrote the manuscript. E.L.L, D.J.J., E.D.N., B.A., R.N.A., and S.L.N. critically revised the manuscript.

CONFLICTS OF INTERESTS

The authors declare no competing interests.

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