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Targeted Organ Generation Using *Mixl1*-Inducible Mouse Pluripotent Stem Cells in Blastocyst Complementation

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Generation of functional organs from patients' own cells is one of the ultimate goals of regenerative medicine. As a novel approach to creation of organs from pluripotent stem cells (PSCs), we employed blastocyst complementation in organogenesis-disabled animals and successfully generated PSC-derived pancreas and kidneys. Blastocyst complementation, which exploits the capacity of PSCs to participate in forming chimeras, does not, however, exclude contribution of PSCs to the development of tissues—including neural cells or germ cells—other than those specifically targeted by disabling of organogenesis. This fact provokes ethical controversy if human PSCs are to be used. In this study, we demonstrated that forced expression of Mix-like protein 1 (encoded by *Mixl1*) can be used to guide contribution of mouse embryonic stem cells to endodermal organs after blastocyst injection. We then succeeded in applying this method to generate functional pancreas in pancreatogenesis-disabled *Pdx1* knockout mice using a newly developed tetraploid-based organ-complementation method. These findings hold promise for targeted organ generation from patients' own PSCs in livestock animals.

Introduction

Recent developments in induced pluripotent stem cell (iPSC) technology permit establishment of individual patients' own PSCs [1,2]. However, current stem cell therapy mainly targets diseases that can be treated by cell transplantation. Faced with absolute deficiency of donor organs to treat patients with organ failure, regenerative medicine has the generation of organs as one of its ultimate goals. We propose that this be done using the patient's own PSCs, as represented by embryonic stem cells (ESCs), yielding organs that can be transplanted into the patient.

We recently demonstrated successful generation of PSC-derived pancreas and kidneys using blastocyst complementation in pancreatogenesis- or nephrogenesis-disabled mice [3,4]. We then succeeded in generating rat PSC-derived pancreas in mice by interspecific blastocyst complementation [3]. In an ancillary work, we developed pancreatogenesis-disabled pigs in which, through blastocyst complementation, we successfully generated exogenous-pig pancreata [5].

While these studies prepared us to examine the feasibility of generating human PSC-derived pancreata in pancreatogenesis-disabled pigs, some ethical issues on making such "admix chimeras" have yet to be solved. A part of the concern comes from the possibility that human iPSC-derived cells contribute to neural or germ cells in chimeric animals. To overcome this issue, in this study, we attempted to restrict differentiation of PSC-derived cells into endodermal organs by introducing a gene encoding the transcription factor Mixl1.

Mixl1 is a mouse homolog of a *Mix* family gene, originally discovered in *Xenopus* as a transcription factor inducing differentiation of pluripotent animal cap cells into the endoderm [6]. *Mixl1* also regulates endoderm and paraxial mesoderm formation, a potential reason for death early in development when *Mixl1* is defective [7]. Forced expression of *Mixl1* during mouse ESC differentiation in vitro represses mesodermal fate determination and promotes endodermal fate [8,9]. We speculated that this transcription factor can autonomously induce ESCs to form endodermal cells after blastocyst injection.

Materials and Methods

Animals

C57BL/6NCrSlc, BDF1, DBA/2CrSlc, 129/Sv, and ICR mice were purchased from SLC Japan (Shizuoka, Japan). *Pdx1-LacZ* heterozygous mice [10], kindly provided by Dr. Y. Kawaguchi (Kyoto University) and Dr. C. V. Wright (Vanderbilt University), were crossed with C57BL/6-, DBA2-, or BDF1-strain mice. In the Dox(+) setting, mice were given drinking water containing 2 mg/mL Dox (Clontech, Palo Alto, CA) and 3.5% sucrose (Wako, Tokyo,

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Japan). All experiments were performed in accordance with the animal care and use committee guidelines of the Institute of Medical Science, University of Tokyo.

Culture of mouse ESCs/iPSCs

Undifferentiated mouse ESCs/iPSCs were maintained on gelatin-coated dishes without feeder cells in Glasgow's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Nichirei Bioscience, Tokyo, Japan), 0.1 mM 2-mercaptoethanol (Invitrogen, San Diego, CA), 0.1 mM nonessential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 1% L-glutamine penicillin streptomycin (Sigma), 1,000 U/mL of mouse leukemia inhibitory factor (LIF) (Millipore, Bedford, MA) with or without 2 inhibitors [2i; 1 μM MEK inhibitor PD0325901 (Wako), and 3 μM GSK3 inhibitor CHIR99021 (Axon, Groeningen, The Netherlands)]. In the Dox(+) setting, Dox (2 μg/mL) was added to the culture medium. For differentiation of ESCs, retinoic acid (Sigma) was added to a concentration of 1 μM in the culture medium without LIF and 2i. DsRed-expressing mouse ESCs (EB3DR), kindly provided by Dr. H. Niwa (Center for Developmental Biology, RIKEN, Hyogo, Japan), were derived from EB3-ESCs [11] and carried *CAG* promoter-driven *DsRed*. Wild-type (WT) mouse ESCs (K3) were generated from hybrid blastocysts obtained after intercrossing of 129/Sv and C57BL6 mice (data not shown). *Pdx1* knock-out (KO) iPSCs were generated from *Pdx1* KO mouse-derived neonatal fibroblasts by introducing three mouse factors (*Oct3/4*, *Klf4*, and *Sox2*) in one retroviral vector (data not shown).

Construction of vectors and gene introduction

For construction of *pRosa26-tTA-Mixl1*, a targeting vector for insertion of the single cassette Tet-Off regulatory system into the *Rosa26* locus (a kind gift from Dr. J. Miyazaki; Osaka University, Osaka, Japan) was modified to insert *Mixl1* as described [12]. For construction of *pRosa26-TIN-TRE-Mixl1* in Fig. 2B, a splice-acceptor sequence amplified from a *pSAβ-geo* vector (a kind gift from Dr. P. Soriano; Fred Hutchinson Cancer Research Center, Seattle, WA); *tdTomato* amplified from *ptdTomatoN1* (Clontech); *IRES* amplified from a *GCDNsam* vector (a kind gift from Dr. M. Onodera; National Research Institute for Child Health and Development, Tokyo, Japan); *Neo^r-SV40pA* amplified from a *pcDNA3* vector (Invitrogen); *TRE-Mixl1* amplified from *pRosa26-tTA-Mixl1*; and an insulator sequence [13] were inserted into the *Xba*I site of *pRosa26-SwaI,* a *pRosa26-1* vector (a kind gift from Dr. P. Soriano), by addition of an *Swa*I site upstream of the 5′ homology arm. For construction of *pCAG-tTA-IP* in Supplementary Fig. S2A (Supplementary Data are available online at www.liebertpub.com/scd), a *CAG* promoter [14], *HA*-tagged *tTA* amplified from *pTet-Off Advanced* (Clontech), and *IRES-Puro^r* amplified from *pCAG-Cre-IRES-Puro^r-pA* (a kind gift from Dr. J. Miyazaki) were inserted into the multicloning site of *pBluescript-SwaI,* that is, into *pBluescript KS(+)* (Stratagene, La Jolla, CA) modified by addition of an *Swa*I site into the start and end positions of the multicloning site. For construction of *pOct3/4-BAC-tTA-Venus*, bacterial artificial chromosome recombineering technology was used [15]. Homology arms for recombineering were amplified from genomic DNA of mouse ESCs by polymerase chain reaction (PCR) using PrimeSTAR or PrimeSTAR GXL DNA polymerase (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. The arms and *tTA-IRES-Venus* were subcloned into the multi-cloning site of the *pBT-loxP2-Zeo* vector, a *pBT-loxP2* vector (a kind gift from Dr. R. Kaneko; Gunma University, Gunma, Japan) modified by replacing a gene conferring neomycin resistance with one conferring zeocin resistance.

Electroporation for gene targeting and gene introduction was carried out as described [16,17]. In brief, $1–5 \times 10^6$ mouse ESCs suspended in PBS were mixed with vectors linearized by restriction-enzyme digestion and were transferred to a Gene Pulser cuvette (Bio-Rad, Richmond, CA). Electroporation was carried out at 230 V, 250 μF in Gene Pulser equipment (Bio-Rad). After electroporation, ESCs were seeded onto gelatin-coated dishes and 24–48 h later, drugs for selection were added to the culture medium.

Embryo culture and manipulation

Preparation of WT and *Pdx1* heterozygous intercrossing diploid embryos and of WT and EGFP-Tg mouse-derived tetraploid embryos was carried out according to published protocols [18]. In brief, eight-cell/morula-stage diploid embryos were collected in Medium 2 (M2; Millipore) from oviduct and uterus of mice at 2.5 days postcoitum (dpc). These embryos were transferred into a potassium simplex optimized medium with amino acids (KSOM-AA; Millipore) and were cultured for 24 h for blastocyst injection. For production of tetraploid embryos, two-cell stage diploid embryos were collected in M2 from oviduct of mice 1.5 dpc. These embryos were washed thrice with medium containing 0.01% polyvinyl alcohol (Sigma), 280 mM Mannitol (Sigma), 0.5 mM Hepes (Wako), and 0.15 mM $MgSO_4$ (Invitrogen). Electrofusion of blastomeres to produce tetraploid embryos was carried out using a DC pulse (100 V/mm, 30 μs, 1 time) followed by application of AC pulses (5 V/mm, 10 s) using ECM 2001 (BTX, Holliston, MA). These tetraploid embryos were transferred into KSOM-AA and were cultured for 24 h for four-cell/morula injection.

For micro-manipulation, ESCs were trypsinized and suspended in ESC culture medium. A piezo-driven micromanipulator (Prime Tech, Tokyo, Japan) was used to drill zona pellucida and trophectoderm under the microscope, and 5–10 ESCs were introduced into blastocyst cavities near the inner cell mass of diploid blastocysts or the perivitelline space of four-cell/morula stage tetraploid embryos. After the injection, diploid blastocyst embryos underwent follow-up culture for 1–2 h. Four-cell/morula stage tetraploid embryos underwent follow-up culture for 24 h to achieve blastocyst stage. Blastocysts of either origin were then transferred into the uteri of pseudopregnant recipient ICR female mice (2.5 dpc).

Genotyping for targeting

DNA was extracted from picked-up PSCs using QIAamp DNA Mini Kits (Qiagen, Germantown, MD). For genotyping, PCR primers for amplification of the *Rosa26-tTA-Mixl1* knock-in locus were Fw, 5′-CCTCGGCTAGGTAGGGG ATCGGGACTCT-3′, and Rv, 5′-CGGAGAACCTGCGT

GCAATCCATCTTGTTC-3′ for 5′ upstream and Fw, 5′-GGATCACTCTCGGCATGGACGAGCTGTAC-3′, and Rv, 5′-AGCCTTAAACAAGCACTGTCCTGTCCTCAAG-3′ for 3′ downstream. PCR primers for amplification of the *Rosa26-TIN-TRE-Mixl1* knock-in locus were Fw, 5′-CCTCGGCTAGGTAGGGGATCGGGACTCT-3′, and Rv, 5′-GGGCCCTCACATTGCCAAAAGACGG-3′ for 5′ upstream.

Western blot analysis

Whole-cell extracts were prepared by resuspending ESCs in lysis buffer (NACALAI TESQUE, Kyoto, Japan). After electrophoresis, gels were transferred to nitrocellulose membranes. Membranes were blocked in Tris-buffered saline with Tween-20 containing 5% BSA and were treated with antibodies. Primary antibodies against FLAG (mouse IgG, Sigma; rat IgG, Novus Biologicals, Littleton, CO), Oct3/4 (mouse IgG; Santa Cruz Biotechnology, Dallas, TX), β-actin (mouse IgG; Cell Signaling Technology, Boston, MA), and Mixl1 (a kind gift from Dr. A. Elefanty; Monash University, Australia) were used. Horseradish-peroxidase–conjugated secondary antibodies used were directed against mouse or rat IgG. After antibody treatment, blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Pittsburgh, PA).

Immunostaining of cells, fetuses, and sections

ESCs, embryos, fetuses, and organs were fixed in 4% paraformaldehyde. Fetuses and organs were embedded in Optimal Cutting Temperature compound (Sakura Finetek, Tokyo, Japan) for frozen sections. Each sample was incubated with primary antibody for 1–2 h at room temperature (RT) and with secondary antibody for 1 h at RT. Primary antibodies against EGFP (rabbit IgG, Invitrogen; rat IgG, NACALAI TESQUE; goat IgG, abcam, Cambridge, United Kingdom), FLAG, DsRed (rabbit IgG; Clontech), Foxa2 (goat IgG; Santa Cruz), EpCAM (rat IgG, Developmental Studies Hybridoma Bank at the University of Iowa), HA (rabbit IgG; Cell Signaling Technology), PECAM1(rat IgG; BD, San Diego, CA), Insulin (rabbit IgG; Cell Signaling Technology), and Mixl1were used. Secondary antibodies used were Alexa488-, Alexa 568-, and Alexa 647-conjugated and were directed against rabbit, rat, and goat IgG (Invitrogen). After antibody treatment, samples were stained with 4′,6-diamidino-2-phenylindole (DAPI) to mark nuclei and were observed under fluorescence microscopy or confocal laser scanning microscopy. Sections of embryos were also scanned for chimerism analysis with Cellomics ArrayScan VTI HCS Reader technology (Thermo Scientific), and the data were analyzed by FlowJo software (Tree Star, Ashland, OR).

Flow cytometry analysis

To analyze chimerism at E9.5, fetuses were trypsinized at 37°C for 10 min. The digestion products were stained with Alexa-647-conjugated anti-EpCAM antibody (rat IgG; Santa Cruz Biotechnology) and subjected to FACSCanto II flow cytometry (BD Bioscience).

Glucose tolerance testing

Blood was sampled via tail vein at intraperitoneal glucose administration (1 g/kg; 0 min) and at 15, 30, 60, and 120 min thereafter. Blood glucose values were determined using a Medisafe-Mini glucometer (Terumo, Tokyo, Japan).

Results

Contribution of Mixl1-inducible ESCs was limited to gut endoderm in chimeras

To control the in vivo expression of *Mixl1*, we introduced a single-cassette Tet-Off regulatory system [12] into the *Rosa26* locus of mouse ESCs ubiquitously expressing *DsRed* (Supplementary Fig. S1A). Targeting was confirmed by PCR of genomic DNA using the primer pair described in the ‘‘Materials and Methods’’ section (Supplementary Fig. S1B). After excision of a *Neo* element flanked by loxP sites, FLAG-tagged *Mixl1* expression and IRES-mediated *EGFP* expression were induced by removal of doxycycline (Dox) from culture medium (Supplementary Fig. S1C, D). We used these *Mixl1*-inducible ESCs (RT5-ESCs) for experiments.

To check the effect of exogenous *Mixl1* expression in early development, we cultured RT5-ESCs in the presence (+) of Dox [Dox(+)] and injected them into WT mouse blastocysts. The foster mothers were given drinking water with or without Dox during pregnancy (Fig. 1A). The transferred embryos were analyzed at one week after embryo transfer (ET). In Dox(+) settings, DsRed-expressing RT5-ESC–derived cells were ubiquitously present throughout the body, as in normal chimeric embryos (Fig. 1B). However, in the absence of Dox, most DsRed-expressing cells accompanied gut endoderm (Fig. 1B). Distributions of DsRed-expressing cells were also confirmed by immunostaining with antibodies against the endoderm markers Foxa2 and EpCAM [19,20].

Unlike in Dox+ settings, when Dox was lacking, DsRed expression colocalized with that of both Foxa2 and EpCAM in most cells (Fig. 1C). Quantitative data obtained using flow cytometry or image analysis also indicated preferential contribution of RT5-ESCs to gut endoderm (Figs. 1D and 2A and Supplementary Fig. S2).

We then examined chimerism in adulthood. In Dox(+) settings, chimeras showed clear chimerism of coat color between donor-derived cells (129ola-derived) and host-blastocyst–derived cells (BDF1xB6-derived; Fig. 1E). They also showed internal-organ chimerism as judged by DsRed expression (Fig. 1E). On the other hand, in the absence of Dox, coat-color chimerism was slight or unrecognizable (Fig. 1E), but internal-organ chimerism was apparent, especially in endoderm-derived organs such as the pancreas (Fig. 1E). These data strongly suggest that forced expression of *Mixl1* in ESC progeny during development directed the fate of ESC-derived cells toward endodermal lineages.

Forced expression of Mixl1 until postimplantation epiblast stage suffices for endodermal induction

While we found that the forced expression of *Mixl1* can guide ESC fate toward endodermal lineages after blastocyst injection, the period required for induction was still unknown. Especially for future applications, exogenous expression of the transgene should be minimized and tightly controlled by using optimal promoters. Furthermore, if a precise period is established, we might replace transgene use by other methods such as protein or RNAs delivery during a

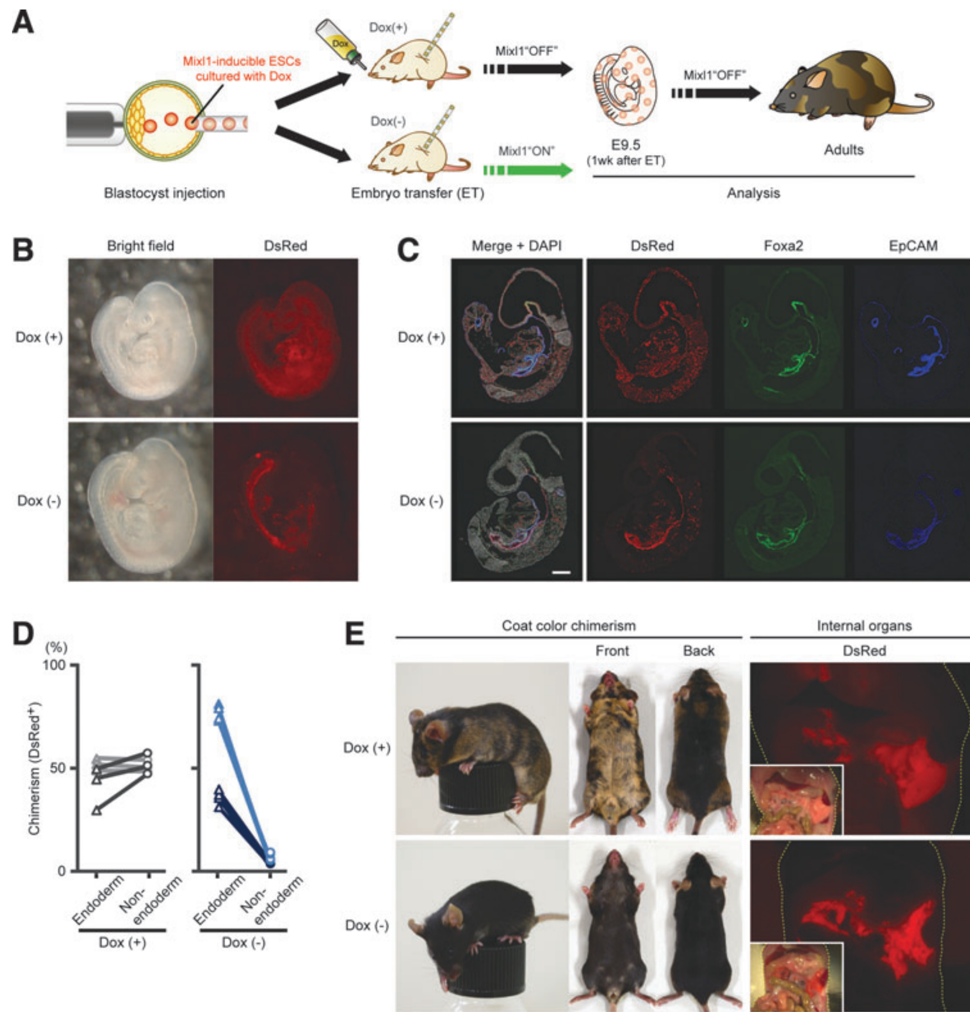

FIG. 1. Mixl1-inducible ESCs can preferentially contribute to gut endoderm after blastocyst injection. **(A)** Experimental schema for analysis of chimera generated by injection of Mixl1-inducible ESCs into wild-type blastocyst. **(B)** Chimeric fetuses at 1 week after transfer of RT5-ESC–injected wild-type blastocysts (developmental stage: E9.5 embryo) into foster-mother uteri. Foster mothers were given drinking water with (+) or without (−) Dox. **(C)** Distribution of RT5-ESC–derived cells in E9.5 chimeric fetus. In Dox(−) setting, to exclude the effect of EGFP expression on multi-color staining, Dox was given to foster mothers at 1 day before analysis. Sections were immunostained for DsRed (*red*) and for endodermal markers Foxa2 (*green*) and EpCAM (*blue*), with nuclear counterstaining (*white*) using DAPI. **(D)** Comparison of chimerism between endodermal and nonendodermal embryonic tissues in Dox(+) and Dox(−) settings. Sections immunostained in **(C)** were imaged by ArrayScan technology, with collected data analyzed by FloJo software (Supplementary Fig. S2). Both Foxa2- and EpCAM-expressing cells were defined as ''endoderm (*triangular dots*)''; cells expressing neither were defined as ''nonendoderm (*circular dots*).'' Three or four sections from each of a pair of embryos were evaluated. Values obtained from the same sections are connected by *lines*. **(E)** Adult chimeric mice generated by injection of RT5-ESCs into wild-type blastocysts. In the Dox(−) setting, foster mothers were given drinking water with Dox except during 1 week after embryo transfer. Coat-color chimerism differences between host-blastocyst progeny (BDF1xB6-derived) and donor-ESC progeny (129ola-derived) are shown, as are macroscopic views of contributions of RT5-ESC–derived cells to internal organs. *Yellow dashes* outline bodies. Sections in **(C)** were observed under confocal laser scanning microscopy. Scale bars, 100 μm. DAPI, 4′,6-diamidino-2-phenylindole; ESCs, embryonic stem cells.

short period, thereby preventing transgene integration into genomic DNA. Thus, to identify the period required for endodermal induction by forced in vivo expression of *Mixl1*, we administered Dox at various time points (days 0, 2, 3, 4, 5, and 7 after ET) and then analyzed individual embryos at day 7 after ET by flow cytometry, using antibodies against the endodermal marker EpCAM (Fig. 2A). Although the degree of chimerism for cells that expressed EpCAM or failed to express EpCAM did not differ significantly until day 3 after ET (Fig. 2A), chimerism for EpCAM-expressing cells rose significantly when Dox administration began on day 4 after ET or thereafter (Fig. 2A). These data suggest that for endodermal induction, forced expression of *Mixl1* is necessary until 4 days after ET, corresponding to the developmental stage of E6.5 embryos.

Based on these results, we attempted to regulate the expression of exogenous *Mixl1* under the control of an early development marker, Oct3/4, that should enable timely, autonomous expression of exogenous *Mixl1* without using Dox. For this purpose, we introduced an expression unit

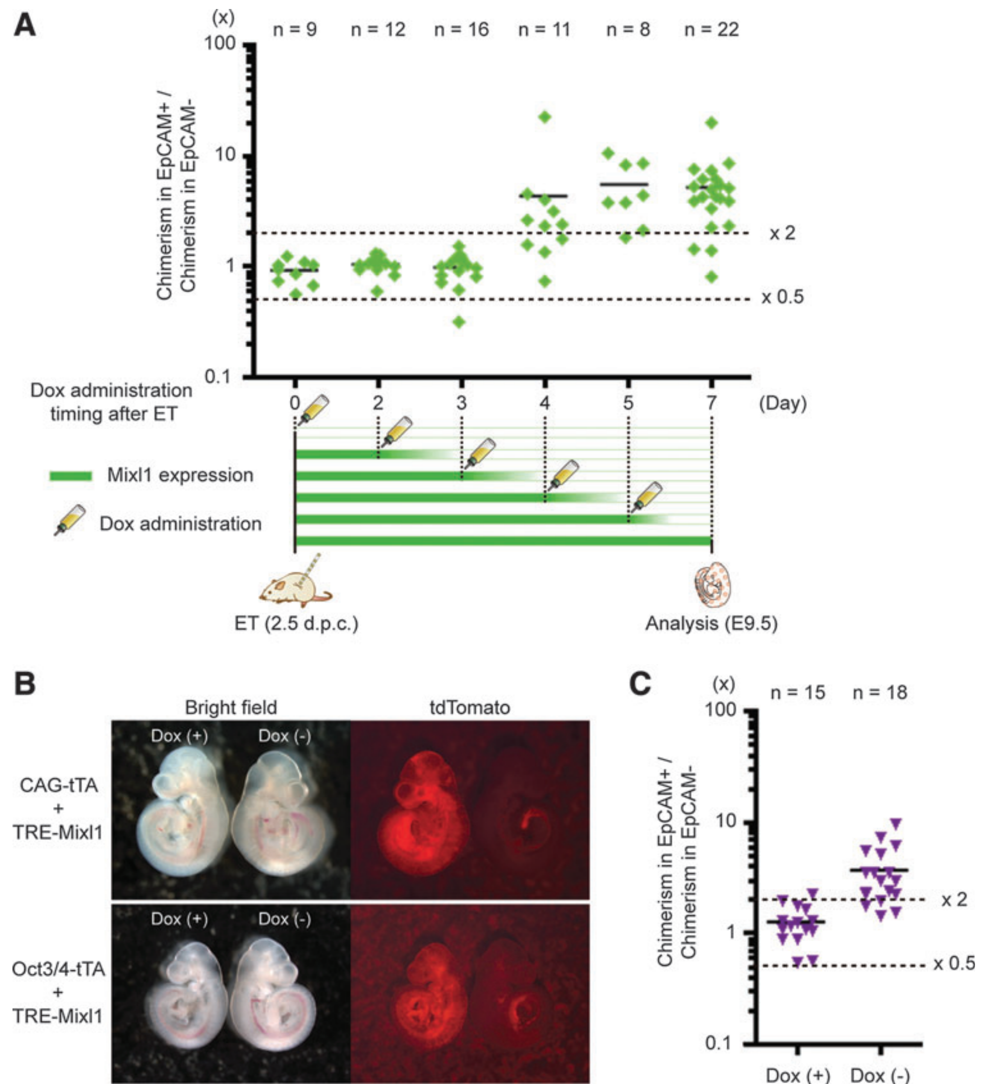

FIG. 2. Forced expression of *Mixl1* until the epiblast stage is necessary for endodermal induction. **(A)** Degrees of chimerism in embryonic endodermal tissues with varying periods of Dox administration. Chimerisms were analyzed at E9.5. After anti-EpCAM antibody staining, DsRed-expressing cells were sorted into EpCAM-expressing (EpCAM$^+$) and EpCAM-lacking (EpCAM$^-$) fractions by flow cytometry. *Diamonds* indicate values calculated by dividing percentage of chimerism in the EpCAM$^+$ fraction by percentage of chimerism in the EpCAM$^-$ fraction for individual chimeric embryos. **(B)** Chimeric fetuses at 1 week after embryo transfer of CHT5- or OTiV1-ESC–injected wild-type blastocysts. In each image, the fetus at the viewer's *left* grew in the presence (+) of Dox and the fetus at the viewer's *right* grew in the absence (−) of Dox. **(C)** Degrees of chimerism in embryonic endodermal tissues in Dox(+) and Dox(−) settings. *Triangles* indicate values for individual chimeric embryos.

composed of a tetracycline transactivator (*tTA*) under the control of an *Oct3/4* promoter (Oct3/4-tTA) and a tet-response element (TRE) with *Mixl1* (TRE-Mixl1) into ESCs, thereby yielding OTiV-ESCs (Supplementary Fig. S4A). Unlike ESCs carrying an expression unit composed of *tTA* under the control of a CAG promoter (CAG-tTA) instead of Oct3/4-tTA (hereafter, CHT5-ESCs, Supplementary Fig. S3), OTiV-ESCs express exogenous Mixl1 under the proper control of an Oct3/4 promoter in the absence of Dox (Supplementary Fig. S4B–D). We investigated the effect on chimerism after an injection of OTiV1-ESCs into blastocysts. In embryos, one week after ET, maternal ingestion of Dox permitted OTiV1-ESCs to differentiate into all embryonic lineages equally in the absence of exogenous expression of *Mixl1* (Left embryo in Fig. 2B). However, as expected, in the absence of Dox, OTiV1-ESCs preferentially differentiated into gut endoderm, and the same result was obtained when using RT5- or CHT5-ESCs (Fig. 2B, C). These data suggest that expression of exogenous *Mixl1* for endodermal induction is required until the epiblast stage of early development and permits the inference that the period of forced expression of *Mixl1* can be minimized during development.

Mixl1-inducible ESCs permit generation of functional pancreata via blastocyst complementation

Finally, we tried to confirm the generation of organs from these *Mixl1*-inducible ESCs through blastocyst complementation. For this purpose, we developed a novel ''tetraploid-based organ-complementation method.'' In conventional blastocyst complementation, *Mixl1*-inducible ESCs are injected into pancreatogenesis-disabled *Pdx1* KO mouse-derived blastocysts. Crossing heterozygous parental mice generates apancreatic *Pdx1* KO mouse embryos only at a rate of one in four. In this tetraploid-based organ-complementation method, instead of injecting *Mixl1*-inducible ESCs into *Pdx1* KO mouse-derived blastocysts, both *Mixl1*-inducible ESCs and *Pdx-1* KO mouse-derived iPSCs are injected into WT or EGFP-labeled tetraploid embryos (Fig. 3A). This enables us to generate apancreatic *Pdx1*-KO mice and to perform organ complementation more steadily and efficiently. We, therefore, used this technique for confirmation of *Mixl1*-inducible ESC-derived organ generation.

Reproductive rates are shown in Table 1. In neonates, EGFP expression was seen only in placentas of tetraploid

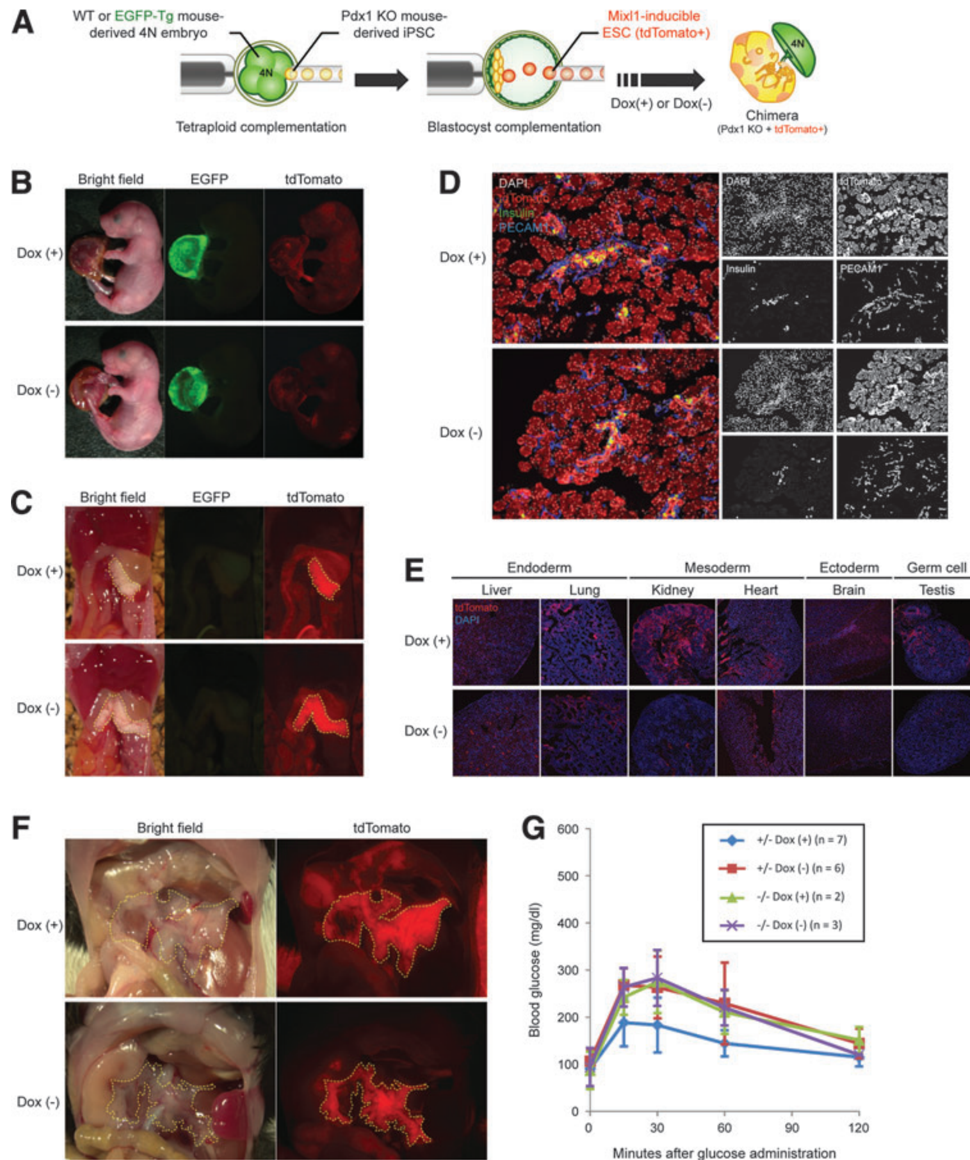

FIG. 3. Blastocyst complementation using *Mixl1*-inducible cells enables generation of functional pancreata in vivo. **(A)** Schema of novel blastocyst complementation system using tetraploid complementation and *Mixl1*-inducible ESCs. **(B)** Neonates generated via method shown in **(A)**. To make tetraploid embryos, two-cell stage embryos derived from transgenic mice that ubiquitously express EGFP (EGFP-Tg) were electrofused. In the Dox(−) setting, foster mothers were given drinking water without Dox until 4 days after embryo transfer and were then given water with Dox. **(C)** Macroscopic views of internal organs of neonates shown in **(B)**. *Yellow dashes* outline pancreata entirely composed of CHT5-ESC–derived cells. **(D)** Microscopic views of distribution of CHT5-ESC–derived cells in pancreata of newborn pups. Sections were immunostained for DsRed (*red*), insulin (*green*), and PECAM-1 (*blue*), with nuclear counterstaining using DAPI (*white*). **(E)** Microscopic views of distribution of CHT5-ESC–derived cells in various tissues of newborn pups. Sections were immunostained for DsRed (*red*), with nuclear counterstaining using DAPI (*blue*). **(F)** Macroscopic views of adult pancreata generated by an injection of CHT5-ESCs into *Pdx1*$^{-/-}$ blastocysts. In both Dox(+) and Dox(−) settings, mice have pancreata completely derived from CHT5-ESCs expressing *tdTomato*. **(G)** Results of glucose tolerance testing in *Pdx1*$^{-/-}$ and *Pdx1*$^{+/-}$ mice complemented with CHT5-ESCs in Dox(+) or Dox(−) settings (see *insert*). Blood was sampled via tail vein at intraperitoneal glucose administration (1 g/kg; 0 min) and at 15, 30, 60, and 120 min thereafter. Sections in **(D, E)** were observed under confocal laser scanning microscopy.

embryos and not in embryos proper (Fig. 3B). When Dox had been given, contributions of CHT5-ESC progeny were observed throughout the body, except the pancreas; only the pancreas was completely composed of CHT5-ESC derivatives (Fig. 3B–D), as shown earlier [3,4]. On histologic study, contributions of CHT5-ESCs were observed not only in endodermal organs but also in the other germ-layer–derived organs such as kidney, heart, brain, and testis (Fig. 3E). In contrast, a few progeny of CHT5-ESCs were observed throughout the body in embryos not exposed to Dox until 7 days after ET (Fig. 3B). However, pancreata were completely composed of CHT5-ESC derivatives (Fig. 3C, D). These findings were also supported by a histologic study with the exception of the heart, where significant numbers of

TABLE 1. RESULTS OF TETRAPLOID-BASED ORGAN-COMPLEMENTATION METHOD

1st cell	*2nd cell*	*Tetraploid embryo*	*Dox*	*TE*	*Pups (% TE)*	*Analysis as neonates*	*Chimeras*	*With PSC pancreas*
Pdxl KO iPSC	—	WT	—	147	24 (16)	24	—	0
	EB3DR	WT	—	201	32 (16)	9	9	9
	RT5	WT	(+)	60	16 (27)	16	12	11
	RT5	WT	(−)	78	12 (15)	12	7	7
	CHT5	WT or EGFP Tg	(+)	122	8 (7)	7	3	3
	CHT5	WT or EGFP Tg	(−)	248	19 (8)	18	6	5
K3	—	WT	—	82	14 (17)	5	—	—

iPSC, induced pluripotent stem cell; PSC, pluripotent stem cell; TE, transferred embyros; WT, wild type.

CHT5-ESC–derived cells were found (Fig. 3E). The reason for this unexpected contribution to heart tissues is not clear.

Finally, to assess the function of pancreata composed of CHT5-ESC progeny, we conducted glucose tolerance testing in adult mice generated by an injection of CHT5-ESCs into blastocysts obtained by crossing of *Pdx1*$^{+/-}$ mice. In adults, after glucose administration, blood glucose levels of mice with *tdTomato*-expressing pancreata were normally maintained whether or not Dox had been supplied during development (Fig. 3F, G). These data suggest that pancreata derived from *Mixl1*-inducible ESCs can normally function in vivo.

Discussion

While generation of a functional organ via blastocyst complementation in pigs is an important step toward generation of human organs in large animals, several issues remain. First, whether this approach can be considered for humans and pigs is still a big challenge. These two species are evolutionarily far wider diverged from one another than are mouse and rat. Second, most human ESCs or PSCs are believed to be epiblast-stage PSCs with chimerism competency that is extremely limited [21,22], because their stage is developmentally more advanced (''primed'' status) than is that of rodent PSCs (''naïve'' status) [23]. However, recent reports provide crucial data on long-term maintenance of naïve-status human PSCs [24–26]. These findings hold promise for generation of organs derived from patients' own PSCs. Third, the generation of human-pig chimeras arouses ethical concerns, of which one is the possibility that a chimeric pig may have human PSC-derived cells in tissues other than the target organ. It is, therefore, desirable to guide differentiation of human PSC-derived cells toward the target organ. There are several ways to do this. One approach is genetically to manipulate human PSCs to induce apoptosis on differentiation toward neurons or germ cells by introducing a construct containing an apoptosis gene under control of a tissue-specific promoter. Another approach is to transplant committed progenitor cells into postimplantation embryos instead of injecting PSCs into blastocysts. In this study, we exogenously induced expression of *Mixl1* in order to restrict differentiation of ESC-derived cells. The results showed that most of the cells exogenously expressing Mixl1 preferentially contributed to endoderm derivatives. In particular, almost no contribution of ESC-derived cells to coat color was found, while internal organs included ESC contributions. However, contributions to tissues in other lineages also were observed in some cases. This might occur because the expression pattern of exogenous Mixl1 is not completely homogeneous among developing ESCs after blastocyst injection. Indeed, even when undifferentiated ESCs were used, immunostaining revealed that exogenous Mixl1 and EGFP are expressed at different levels after removal of Dox (Supplementary Fig. S1B, D). This could also be caused by silencing of TRE inserted into the *Rosa26* locus. Consequently, exogenous Mixl1 expression below a certain level might fail to shift the fate of ESC progeny toward the endoderm. Nonetheless, the system described here should significantly decrease the contribution of PSC-derived cells to nonendodermal organs and help reduce ethical concerns associated with the generation of human-pig chimeras.

We demonstrated that when directing lineage contributions, expression of *Mixl1* is required only during the first 4 days after embryo transfer. If a drug-inducible promoter is used to drive expression of *Mixl1*, drug administration is required only during those first 4 days. Alternatively, as shown in this study, incorporation of a cell-intrinsic system in which *Milx1* expression is controlled by an *Oct4* promoter yields expression only during the critical time period.

In conclusion, we present a novel way to limit and to control PSC fate in vivo. Inducible expression of a lineage-specifier gene such as *Mixl1* for the endoderm, in early PSC derivatives, might also assist studies of specific germ layers or cell lineages in vivo. Furthermore, as discussed earlier, this approach may find application in targeted generation of organs derived from patients' own PSCs.

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Author Disclosure Statement

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