CELL BIOLOGY

Flexible adaptation of male germ cells from female iPSCs of endangered *Tokudaia osimensis*

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In mammals, the Y chromosome strictly influences the maintenance of male germ cells. Almost all mammalian species require genetic contributors to generate testes. An endangered species, *Tokudaia osimensis*, has a unique sex chromosome composition XO/XO, and genetic differences between males and females have not been confirmed. Although a distinctive sex-determining mechanism may exist in *T. osimensis*, it has been difficult to examine thoroughly in this rare animal species. To elucidate the discriminative sex-determining mechanism in *T. osimensis* and to find a strategy to prevent its possible extinction, we have established induced pluripotent stem cells (iPSCs) and derived interspecific chimeras using mice as the hosts and recipients. Generated iPSCs are considered to be in the so-called "true naïve" state, and *T. osimensis* iPSCs may contribute as interspecific chimeras to several different tissues and cells in live animals. Surprisingly, female *T. osimensis* iPSCs not only contributed to the female germ line in the interspecific mouse ovary but also differentiated into spermatocytes and spermatids that survived in the adult interspecific mouse testes. Thus, *T. osimensis* cells have high sexual plasticity through which female somatic cells can be converted to male germline cells. These findings suggest flexibility in *T. osimensis* cells, which can adapt their germ cell sex to the gonadal niche. The probable reduction of the extinction risk of an endangered species through the use of iPSCs is indicated by this study.

INTRODUCTION

Animals have evolved a diversity of mechanisms to determine two sexes—male and female. In mammals, the determination of sex is chromosomal and is not usually influenced by the environment. Almost all mammalian species strictly require genetic contributions to develop testes. If the Y chromosome is absent, the gonadal primordia develop into ovaries. Yamauchi *et al.* demonstrated that mice lacking the Y chromosome can procreate male germ cells (round spermatids) if they overexpress Y-related genes or spermatogenic non–Y-related genes (*1*, *2*). In mice, female (XX) primordial germ cells (PGCs) cannot be maintained as male germ cells for more than 2 days after birth even if they contribute to the testicular environment (*3*–*6*). These reports show the critical contribution of genetic contributors to the generation and maintenance of male germ cells.

A few mammalian species seem to not have such genetic constraints (*7*, *8*). An endangered species, the Amami spiny rat *Tokudaia osimensis*, has a specific chromosome constitution ($2n = 25$) with a male/female sex chromosome configuration of XO/XO. *T. osimensis*, a small rodent from the Amami-Oshima Island in Japan, is seriously endangered by the destruction of its natural environments and by its natural enemies (2007 International Union for Conservation of Nature Red List of Threatened Species; www.redlist.org/). *Tokudaia* has been molecularly and phylogenetically determined to be a distinct lineage that is positioned most closely to *Apodemus* (*9*). The divergence time of the *Tokudaia-Apodemus* clade is estimated to be 6.5 to 8.0 million years ago (Ma) (*10*), although the evolutionary distance of *Tokudaia* from *Mus musculus* and *Rattus* is 17.9 and 11.9 Ma, respectively (Time Tree; http://timetree.org).

T. osimensis lost both its Y chromosome and the master sex-determining gene *SRY* (sex-determining region Y), which means that it has acquired a discriminating sex-determining mechanism for somatic cell sex during its evolution. No sex distortion and no individuals with a sex chromosome constitution of XX or OO have been reported. However, most euchromatic regions of the Y chromosome include genes for spermatogenesis that are translocated to a single X chromosome shared by males and females (*7*). This finding raises the possibility that the genetic contributors to germ cell sex are equal in both sexes of this species. Some teleosts show high sexual plasticity in the determination of their germ cell sex (*11*, *12*). However, it is unrealistic to use the embryos, gonads, and germ cells to elucidate the sex determination mechanism of this endangered species. To overcome this restriction, we have generated induced pluripotent stem cells (iPSCs) as a source of germ cells and produced interspecific chimera using mice as the host and recipient. Our approach and results raise some interesting questions about mammalian evolution, epigenetic regulation, sex determination, and gamete genesis and suggest a possible strategy for conserving endangered species from probable extinction.

RESULTS

Derivation of iPSCs from *T. osimensis*

To establish iPSCs from a wild individual *T. osimensis* (Fig. 1A), we obtained a small tip of the tail from a live female and used it for fibroblast cell culture. Despite the male/female sex chromosome constitution (XO/XO), sex can be clearly distinguished by the position and morphology

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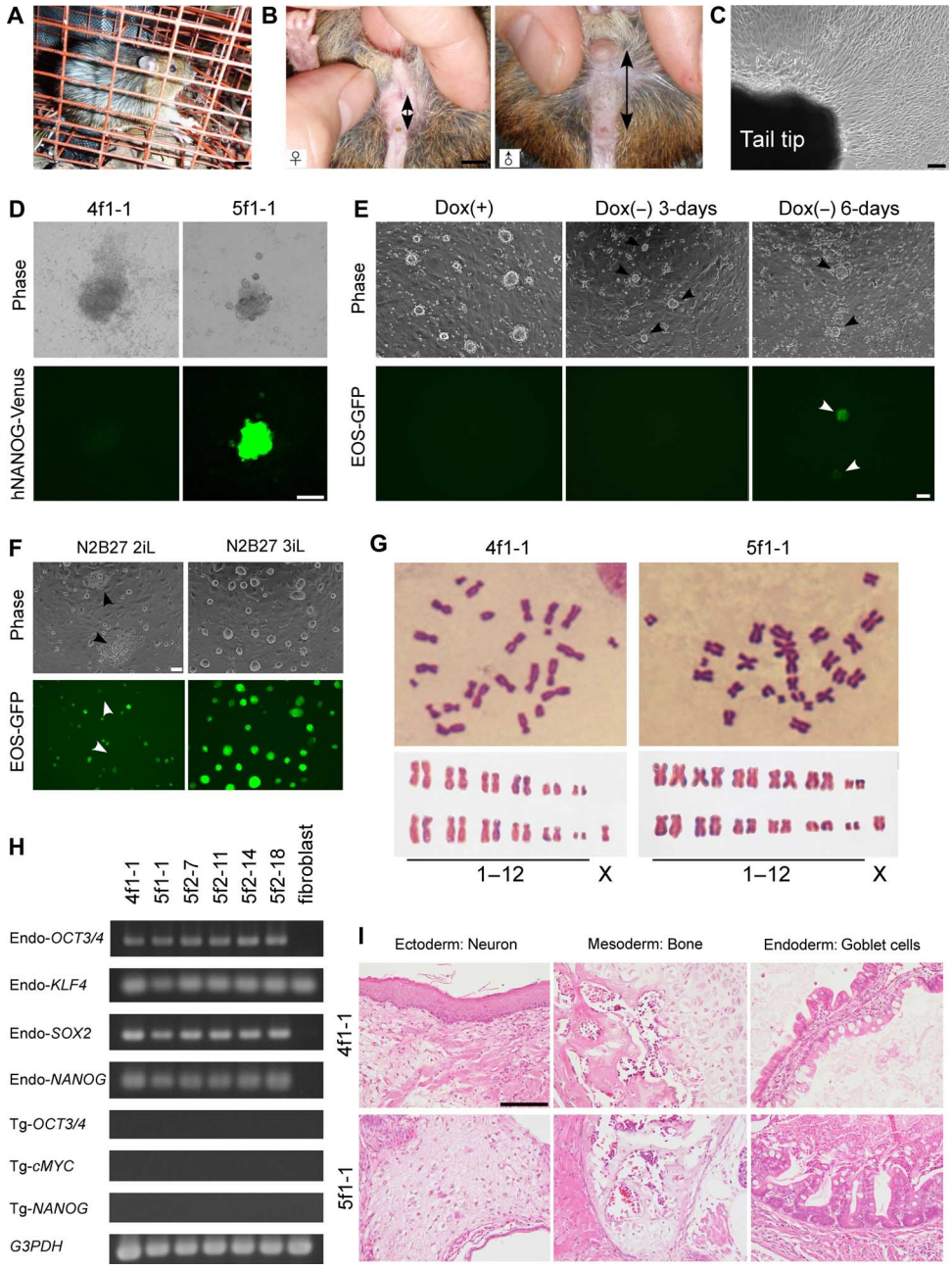

Fig. 1. Generation of iPSCs from *T. osimensis*. (**A**) The female *T. Osimensis* found in the Amami-Oshima Island. Scale bar, 1 cm. (**B**) Sex is clearly distinguished by the distance between the anus and genitals. Scale bar, 1 cm. (**C**) Fibroblast cells were propagated from a tail tip in culture. Scale bar, 100 μm. (**D**) Phase-contrast images of primary colonies and tet-inducible hNANOG-Venus fluorescent images. A strong Venus fluorescent signal was observed in 5f1-1. Scale bar, 100 μm. (**E**) Dox-independent appearance of iPSC (4f1-1) colonies and their EOS-GFP fluorescence. Arrowheads indicate colonies observed after the withdrawal of Dox. Scale bar, 100 μm. (**F**) N2B27 3iL contained a B-Raf inhibitor, SB590885, which prevented iPSC (4f1-1) differentiation. Arrowheads indicate the differentiated cells that appeared in the N2B27 2iL (without SB590885) culture condition. Scale bar, 100 μm. (**G**) Karyotype analysis confirmed the normal chromosome number ($2n = 25$) in 4f1-1 and 5f1-1. (**H**) RT-PCR of endogenous expression of undifferentiated marker genes and exogenous genes in all iPSC lines established. (**I**) Teratoma with three germ layers was confirmed after transplantation of *T. osimensis* iPSCs (4f1-1 and 5f1-1) into SCID mice. Scale bar, 50 μm.

of the genitals (Fig. 1B). Fibroblastic cells appeared and grew rapidly when cultured in somatic cell medium (Fig. 1C). To establish iPSCs, we used PiggyBac (PB) transposase vectors encoding mouse *Oct3/4*, *Klf4*, *Sox2*, and *c-Myc* (this four-gene set is termed "4f"), whose expression could be induced by doxycycline (Dox) treatment (*13*). Because *Nanog* is an essential factor for the induction of pluripotency in somatic cells from endangered felids (*14*), PB-tet-*hNANOG*-Venus (*15*) was also transfected (the transfection of 4f with *hNANOG* is termed "5f"). Additionally, PB-*EOS-GFP-puroR* was transfected to confirm the acquisition of pluripotency (*13*, *16*). Ten days after transfection, some dome-shaped colonies appeared (Fig. 1D). Primary colonies observed in the 5f-transfected dishes were detected by Venus fluorescence, which reflected

the overexpression of *hNANOG* (*15*). In the presence of Dox, primary colonies could be maintained for more than 15 passages over 2 months; however, the green fluorescent protein (GFP) signal from the EOS-GFP reporter did not appear in the 4f-derived cell lines (Fig. 1E). Moreover, immediately (1 day) after the withdrawal of Dox, almost all colonies disappeared. At the first examination, six of eight primary cell lines had disappeared after Dox withdrawal. Cell lines maintained in the presence of Dox could not be cryopreserved for a long time and were unable to form teratomas when transplanted into severe combined immunodeficient (SCID) mice (zero of six).

We noticed that a few of the remaining cells grew slowly after withdrawal of Dox. These cells grew without the appearance of the GFP signal from the EOS vector, which suggested the incomplete acquisition of pluripotency. Six days after the withdrawal of Dox, the number of remaining cells increased, and these cells formed colonies and gradually expressed the EOS-GFP signal (Fig. 1E and fig. S1B). Only one iPSC line (4f1-1) could be generated from 4f transfectants, but five lines (5f1-1, 5f2-7, 5f2-11, 5f2-14, and 5f2-18) were obtained from 5f transfectants (table S1). These results suggested that *NANOG* is an effective factor for reprogramming *T. osimensis* somatic cells as endangered felids (*14*). Almost all (seven of eight) mouse iPSC induction attempts, which were compared at each time point with controls, succeeded in the reprogramming of mouse fibroblasts; by contrast, when *T. osimensis* fibroblasts were used, we could obtain primary colonies from only two of nine attempts. An efficient method to derive iPSCs from *T. osimensis* needs to be established. At the first examination using fresh fibroblast cells, two iPSC lines (4f1-1 from 4f-transfected cells, and 5f1-1 from 5f-transfected cells) were successfully established. However, four iPSC lines (5f2-7, 5f2-11, 5f2-14, and 5f2-18) could be obtained in another experiment using cells that were cryopreserved from primary-cultured tail tip cells.

Determination of appropriate culture condition for *T. osimensis* iPSCs

Dox-independent cell lines could be cultured in N2B27 medium supplemented with the glycogen synthase kinase 3β inhibitor CHIR99021, the mitogen-activated protein kinase kinase inhibitor PD0325901, and mouse leukemia inhibitory factor (LIF; N2B27 2iL) medium, which can keep mouse pluripotent stem cells in the ground state (*17*). However, cobblestone-like cells that lacked the EOS-GFP signal appeared, and their numbers increased during five passages in N2B27 2iL medium (Fig. 1F and fig. S1, C and D). These results suggested that the N2B27 2iL culture condition is insufficient for maintaining *T. osimensis* iPSCs without differentiating. To find the appropriate culture condition for *T. osimensis* iPSCs, we evaluated several chemicals (kenpaullone, forskolin, SB590885, Gö 6983, IM-12, and WH-4-023) and a cytokine (activin A), which are able to keep pluripotent stem cells undifferentiated (fig. S1, C to E) (*15*, *18*, *19*). The B-Raf inhibitor SB590885, which can maintain naïve pluripotency in human embryonic stem cells (ESCs) even after withdrawal of Dox (absence of transgenic overexpression of *hNANOG* and *hKLF2*) (*19*), was the most effective in preventing differentiation of *T. osimensis* iPSCs (Fig. 1F and fig. S1, C to E). Henceforth, *T. osimensis* iPSCs were maintained in N2B27 2iL medium with SB590885 (termed "N2B27 3iL").

The EOS-GFP vector can express the puromycin resistance gene, which is controlled by the EOS promoter (*13*). Differentiated fibroblastic (EOS-GFP–negative) cells appeared when iPSCs were cultured in the feeder-free condition. However, these differentiated EOS-GFP–negative cells could be removed by puromycin treatment in the feeder-free condition (fig. S1F). The normal karyotype number ($2n = 25$; XO) was confirmed (4f1-1, 91.7%; 5f1-1, 86.4%) after 10 passages in the N2B27 3iL culture medium (Fig. 1G). Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) showed up-regulation of mRNA expression of the endogenous pluripotency-related genes (Fig. 1H). The LIF withdrawal assay showed that *T. osimensis* iPSCs could maintain their undifferentiated status in LIF medium (fig. S2); it appeared that these iPSCs were not pluripotent stem cells in the primed state but had naïve pluripotency. As noted above, although Dox-dependent cell lines did not form teratomas after transplantation into SCID mice, all Dox-independent iPSC lines could form teratoma with three germ layers (Fig. 1I). These results suggested that we successfully established *T. osimensis* iPSC lines and found the appropriate culture condition to maintain their naïve pluripotency.

Generation of interspecific chimeras using mouse embryos and recipients

If these iPSCs have naïve pluripotency, it would be possible to contribute to chimeric embryos after introduction into preimplantation embryos (*17*, *20*). However, it is not practical to prepare allogeneic preimplantation embryos from endangered species as a host and recipient. We have attempted to prepare preimplantation embryos and to generate interspecific chimeras using mice as the host and recipient for embryo transplantation (*21*). To evaluate the chimeric contribution to the embryos and several tissues in pups, we transfected a PB plasmid vector, PB-*CAG-Su9DsRed-IRES-NeoR*, which ubiquitously expresses DsRed protein into iPSCs, and we monitored the Su9 signal sequence it induced to identify mitochondria (Fig. 2A). Ten iPSCs were injected into mouse blastocysts, which were then transplanted into recipient mouse uterus. *T. osimensis* iPSCs contributed to the mouse embryos as interspecific chimeras at 12.5 days postcoitum (dpc) (Fig. 2B and fig. S3). The production ratio of interspecific chimeric embryos, as confirmed by the DsRed signal, was 25.0% ($n = 7$ of 28) (Table 1), which was unexpectedly high for xenogeneic chimeras when compared with the allogeneic mouse control experiment (30.8%; $n = 4$ of 13) (Fig. 2B). However, the chimeric distribution of *T. osimensis* iPSCs was lower than that of mouse allogeneic chimeras, which contributed to almost all embryonic bodies (Fig. 2B). A high ratio (25.5%; $n = 13$ of 51) of interspecific chimeric pups with dark brown skin and/or the DsRed signal was obtained (Table 1 and Fig. 2, C to E). Brown hair, which was derived from *T. osimensis* iPSCs, was retained on the adult interspecific chimeras (Fig. 2E).

Next, we determined the distribution of *T. osimensis*–derived cells in the adult (7 weeks after birth) interspecific chimeras by measuring the DsRed signal (Fig. 2, F and G). In sections immunostained with anti-DsRed antibody, representative images suggested that Su9-DsRed–positive cells were located in various types of tissues in varying proportions (Fig. 2G). The chimeric contribution of female-derived *T. osimensis* iPSCs into ovaries was also examined. $DsRed^+$ somatic cells were rarely observed in ovaries (Fig. 2G). Granular $DsRed^+$ signals were observed in an oocyte in a secondary follicle (Fig. 2H). These results suggest that female-derived *T. osimensis* iPSCs contributed to the female germ line in interspecific chimera.

Flexible adaptation of male germ cells in the xenogeneic testicular environment

The main objective of this research was to elucidate the germ cell sex determination and maintenance of *T. osimensis* using female-derived iPSCs. In the case of mouse chimeras, female (XX) pluripotent stem cells rarely contribute as prospermatogonia in male host embryos, whereas all of them disappear within the first few days after birth (*3*–*6*). The disappearance

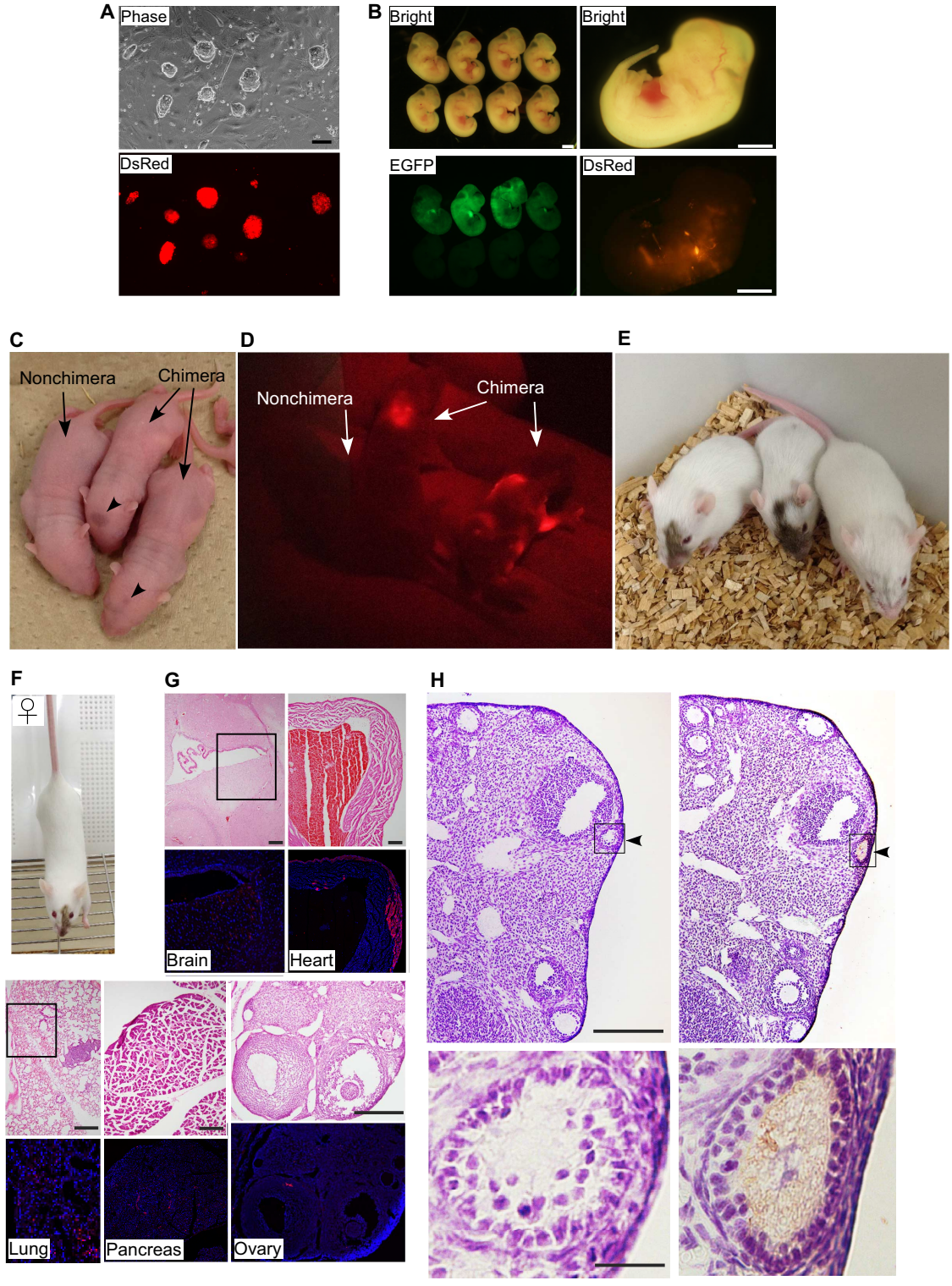

Fig. 2. Chimeric contribution of *T. osimensis* iPSCs in interspecific chimera. (**A**) A PB vector, pPB-CAG-Su9DsRed-ires-NeoR, was transfected into *T. osimensis* iPSCs (5f1-1). Scale bar, 100 μm. (**B**) Chimeric contribution of mouse ESCs (left, GFP) and *T. osimensis* iPSCs (right, Su9DsRed) in mouse embryos. Although mouse ESCs contributed to almost all of the bodies in 11.5-dpc embryos (top four embryos), *T. osimensis* iPSCs contributed partially in the embryonic bodies. Scale bars, 500 μm. (**C**) Photograph of newborn interspecific chimeras. Arrowheads indicate dark brown skin from the *T. osimensis* iPSC contribution. (**D**) Chimeric contribution of *T. osimensis* iPSCs in pups clearly visualized by DsRed signals. (**E**) Photograph of interspecific chimera at 3 weeks of age. (**F**) Photograph of an interspecific female chimera at 7 weeks of age. (**G**) Immunohistochemical detection of the chimeric contribution of *T. osimensis* iPSCs in several tissues. DsRed signals were sparsely distributed in several tissues. Scale bars, 200 μm. (**H**) Granular DsRed signal was detected in serial sections of an oocyte of a secondary follicle in a chimeric ovary (arrowhead). Left: Immunoreacted with normal immunoglobulin G (IgG) (negative control). Right: Immunoreacted with anti-DsRed antibody. Scale bar, 200 μm. The boxed regions are enlarged in the bottom images. Scale bar, 20 μm.

Table 1. Generation of interspecific chimeras using *T. osimensis* iPSCs. Chimera, DsRed$^+$ and/or brown-haired; f, female; m, male; GC, germline contribution.

Cell line	Number of injected blastocysts	Number of transplanted (recipients)	Implanted or born		
			12.5-dpc chimera/implanted (%)	Chimera/pups (%)	GC/chimera (adult)
4f1-1	86	70 (5)	2/15 (13.3)	4/26 (15.4)	1 (f/m, 0:1)/4 (f/m, 2:2)
5f1-1	205	185 (11)	5/13 (38.4)	9/25 (36.0)	2 (f/m, 1:1)/9 (f/m, 4:5)
Total	291	255 (16)	7/28 (25.0)	13/51 (25.5)	3/13 (f/m, 6:7)

of XX spermatogonia in neonatal mouse testis suggests the important contribution of Y-linked spermatogenesis genes in XY cells (*22*, *23*). We reasoned that if *T. osimensis* germ cell sex determination is affected by inherent genetic discriminations between male and female, as in the mouse, *T. osimensis* iPSCs would never be maintained as male germ cells in the adult interspecific testis. On the other hand, if female *T. osimensis*–derived cells are affected by the gonadal environment in chimeric embryos, they should differentiate into male germline cells and survive in the xenogeneic testicular environment.

To evaluate germ cell differentiation of iPSCs in vivo, gonads from adult male chimeras (7 weeks of age) were dissected. Although DsRed fluorescent signals were not detected in the microscopic observation of the whole testis, granular (mitochondrial) DsRed signals were occasionally detected in spermatocytes in testicular sections (Fig. 3B). DsRed$^+$ cells localized as clusters around the testicular basement membrane and distributed to the lumen of tubules in accordance with spermatogenesis. DsRed$^+$ spermatocytes were also positive for the germ cell–specific antigen, mouse vasa homolog (MVH), and a germ cell marker, TRA98 (Fig. 3, C and D). TRA98 antibody effectively recognizes almost all spermatogenic cells at varied staining intensity, except for late spermatids (later stages of XII) (*6*, *24*). *T. osimensis* iPSCs differentiated not only to spermatocytes but also to TRA98-negative elongating spermatids, which have an elongated nucleus (Fig. 3E). Moreover, the inter- and postmeiotic male germ cell–specific proteins ACRBP/sp32 (*25*) and IZUMO1 (*26*) were also detected on *T. osimensis*–derived testicular cells (Fig. 3, F and G). ACRBP/sp32 is a binding protein that is specific for the precursor (pro-ACR) and intermediate forms of the sperm serine protease ACR. It is localized specifically on acrosomes of meiotic male germ cells (round spermatids) (*25*). Moreover, a sperm-specific antigen, IZUMO1, which is a transmembrane protein that is responsible for sperm-egg fusion and is localized on acrosomes of spermatids and sperm (*26*), was also confirmed. These results demonstrated that female-derived *T. osimensis* iPSCs contributed and survived as male germ line with spermatogenesis in the xenogeneic testicular environment. However, germline contribution into xenogeneic gonads was severely restricted [DsRed$^+$ cells/TRA98$^+$ cells, 25 of 79,807 (0.031%)]. *T. osimensis* iPSCs contributed much less to the male germ cell lineage than did other somatic tissues. Possibly because of xenogeneic restriction, germline transmission of *T. osimensis* iPSCs into the female germ line in adult chimeric ovaries was also rarely observed: DsRed$^+$ oocyte/oocytes, 1 of 345 (0.29%).

DISCUSSION

Our study has four major findings. First, iPSCs were successfully established from an endangered species, *T. osimensis*. Second, the culture condition of the combination of N2B27 2iL with SB590885 (N2B27 3iL) medium induced a high naïve pluripotency. Third, these iPSCs effectively contributed to mouse embryos or pups as interspecific chimera with germline contribution. Finally, XO female iPSCs could differentiate into male germ cells in the interspecific mouse testis and survive for more than 7 weeks after birth. Derivation of iPSCs and production of live animals as chimeras using this unique species may provide new insights into the mechanisms of sex determination after disappearance of the Y chromosome during mammalian evolution. Moreover, derivation of naïve-state iPSCs from endangered species may be effective both for conserving genes and cells and as a source for germ cell generation.

Recently, iPSC generations of endangered species were achieved in orangutan, drill, and northern white rhinoceros (*27*, *28*). Because all exhibited prime pluripotency, it seems difficult to contribute to host embryos as a chimera. Putative naïve-state iPSCs have been established from an endangered felid species, *Panthera uncia* (*29*). These iPSCs formed dome-shaped colonies, maintained their pluripotency in LIF medium, and could form teratomas when transplanted into SCID mice. However, true naïve-state characteristics related to developmental plasticity have not been examined using chimeric animal production. To modify primed-state iPSCs so that they can contribute to host embryos as chimera seems to require naïve conversion or adjustment of stem cell status to have region specificity (*30*, *31*). If naïve-state or region-specific iPSCs can be generated successfully from endangered species, embryos and recipient animals of the other mammalian species should be considered. For these reasons, interspecific chimera production using an appropriate animal as the host and recipient species is the preferred strategy for generating tissues and germ cells of these species.

To our knowledge, no interspecific chimeric pups and germ cells have been generated using iPSCs from species other than the mouse and rat (*32*, *33*). The chimeric contribution in the xenogeneic environment other than the mouse and rat is very restricted (*34*). Although a cohort of about 40 PGCs is first identifiable at 6.25 to 7.25 dpc in the mouse (*35*), the germline contribution of *T. osimensis* iPSCs as spermatocytes (0.031%) and oocyte (0.29%) in a xenogeneic environment was severely restricted. Germline differentiation hardly occurs in the xenogeneic (mouse and rat) environment (*21*). For this reason, the in vitro differentiation into PGC-like cells and transplantation with gonadal somatic cells into immunodeficient mice should be examined (*36*, *37*).

In general, genetic contributors play an indispensable role in determining and maintaining the male germ cell sex in most mammals. Although mouse PGCs are dimorphic, the fate of XX prospermatogonia in the testis after birth differs from that of XY prospermatogonia (*6*). Using mutant mice lacking a Y chromosome (XO) and transgenic approaches, researchers have demonstrated that overexpression by the gonadal promoter *WT1*-driven Y-related (*Sry* and *Eif2s3y*) or non–Y-related (*Sox9* and *Eif2s3x*) genes is necessary to develop and maintain male germline cells (*1*, *2*). *SRY* was lost in *T. osimensis*, whereas *SOX9* was expressed in the testes (*38*). Our previous report suggests

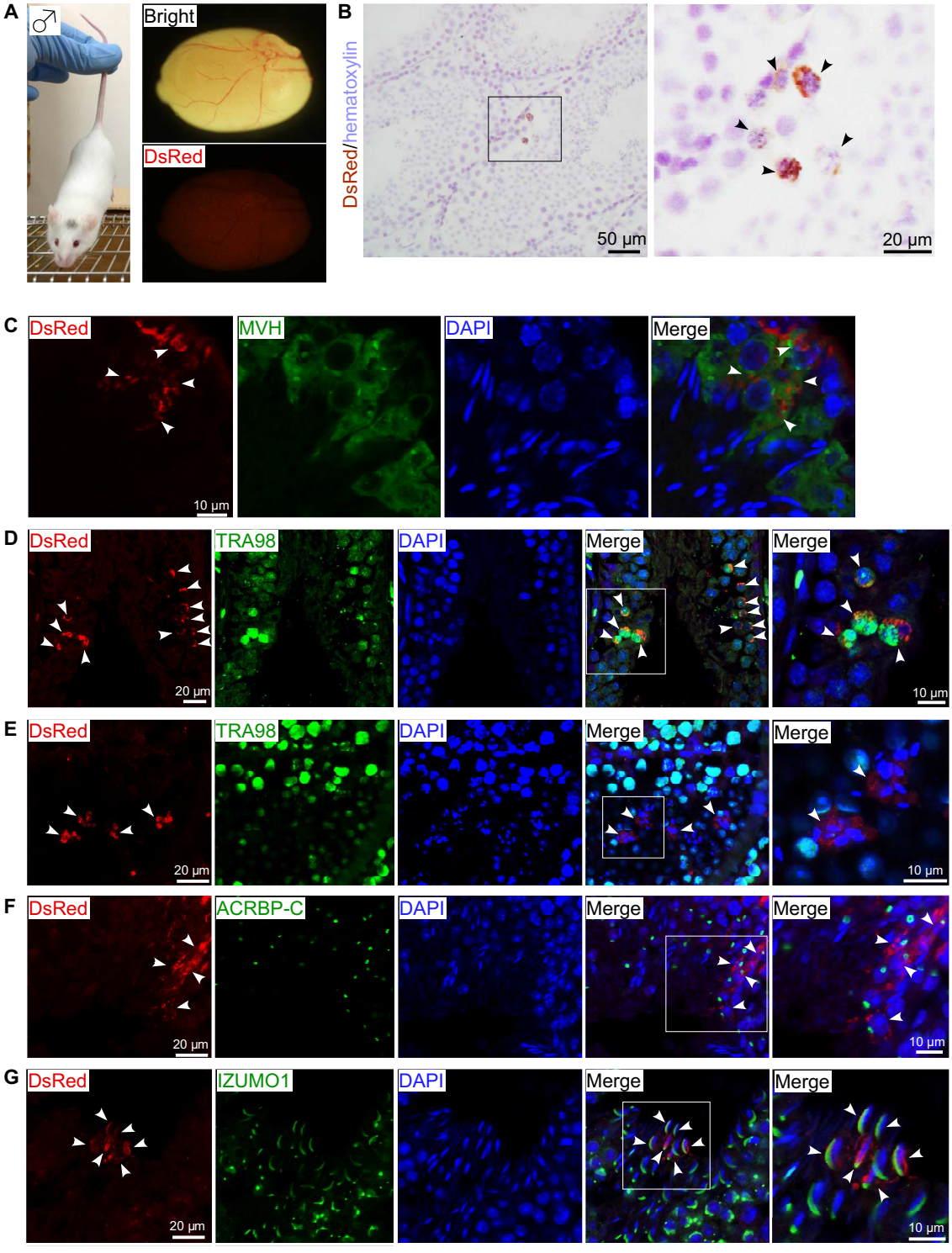

Fig. 3. Male germline contribution of *T. osimensis* iPSCs in an adult interspecific chimera. (**A**) Photograph of an interspecific male chimera at 7 weeks of age. Right panels indicate the testis from this chimeric male. Tubular contribution of *T. osimensis* iPSCs was not observed. (**B**) Testicular section of this interspecific chimera rarely detected *T. osimensis* iPSCs, which differentiated to putative germ cells as clustered signature (arrowheads). Dot-like signals, which indicate mitochondrial staining, are shown by anti-DsRed antibody (brown). The boxed region is enlarged at the right. Sections were counterstained with hematoxylin. (**C**) Immunofluorescence detection of DsRed and germ cell marker MVH. DsRed signals localized as a dot-like signature surrounding the cell nuclei (arrowheads) and indicate a mitochondrial localization. (**D**) Immunofluorescence detection of DsRed and a germ cell marker TRA98. DsRed signals localized at mitochondria. Almost all spermatocytes were stained by anti-TRA98 antibody at various staining intensities. (**E**) DsRed signal (arrowheads) detection in TRA98-negative elongating spermatids, which have sharply curved nuclei. (**F**) DsRed signal (arrowheads) detection of ACRBP/sp32 in acrosomes of round spermatids and (**G**) IZUMO1 in acrosomes of elongating spermatids. Merged images are shown on the right. The boxed region is enlarged at the far right. Sections were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI).

that *SOX9* expression in the testes must be regulated by a new sex-determining gene. *EIF2S3X* as well as *EIF2S3Y* were located on a single X chromosome caused by a Y-to-X translocation (*7*). *EIF2S3Y* expression was observed in male and female gonads. Moreover, comparative genomic hybridization has shown that there is almost no difference (except for the heterochromatic and telomeric regions) between male and female in *T. osimensis* (*39*). Therefore, we supposed that these genetic contributors functioned in female somatic cells for differentiation into male germ cells and their maintenance. We have elucidated the high sexual plasticity in *T. osimensis* germ cells, but the question about a master regulator that is critical for individual sex determination remains to be answered.

In conclusion, this study demonstrated that *T. osimensis* cells have high sexual plasticity, which can cause reversible differentiation and adaptation from female somatic cells to male germline cells in the male reproductive niche. True naïve iPSCs of this species may be useful in future studies of in vitro germ cell derivation, X-chromosome inactivation, gonadal development, and developmental potential of XX, XO, and OO embryos. Although challenging, the derivation of naïve iPSCs to prevent the probable extinction of rare animal species seems to be feasible.

MATERIALS AND METHODS

Experimental design

Animals

Animal care and handling conformed to the National Institutes of Health guidelines. All animal experiments were conducted according to the Guidelines for Animal Experiments of the Miyazaki University and the Okayama University of Science. A wild individual of *T. osimensis* was originally captured on Amami-Oshima Island in February 2013 with permission from the Agency for Cultural Affairs and the Ministry of the Environment in Japan. We obtained a tail tip from a female *T. osimensis* that had been accidentally torn by a field trap. Sex was clearly distinguished by the position and morphology of the genitals. We released the captured *T. osimensis* into the field after recording the individual characteristics and giving first aid to the injured tail. The obtained tail tip was soaked in AmnioMax (Invitrogen Life Sciences) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) (P/S) and was sent to Miyazaki University on ice.

Culture of T. osimensis fibroblast cells

Three days after it was obtained, the tail tip of the female *T. osimensis* was washed with Hanks' balanced salt solution containing P/S and then dissected into small pieces. To obtain fibroblast cells, the dissected tail tip was placed in a gelatin-coated tissue culture dish and cultured in Glasgow modified Eagle's medium (GMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), insulin (10 μg/ml), transferrin (5.5 μg/ml), selenium (6.7 ng/ml), 1% nonessential amino acids, 0.1 mM β-mercaptoethanol, and P/S (Invitrogen Life Sciences) at 37°C under 6% CO_2 in air. The culture medium (somatic cell medium 1) was changed daily. Fibroblast cells were passaged at a dilution of 1:3 to 1:5 until the cells grew to 90 to 100% confluency. To generate iPSCs, fibroblast cells were used within five passages.

iPSC derivation and culture

Cultured fibroblast cells were trypsinized to disperse them into single cells and plated on a six-well tissue culture plate 1 day before transfection. Plasmid vectors pCAG-PBase, PB-TRE3G-OKS, PB–TRE3G–c-Myc, and PB-[CAG-Tet3G; EOS-C (3+)-EGFP-IRES-puro], which have been described previously (*13*), were transfected to derive 4f iPSCs. *PB-tet-hNANOG-Venus* was introduced into fibroblast cells to derive 5f iPSCs (*15*). Transfection was achieved using Lipofectamine 3000 according to the manufacturer's protocol (Invitrogen Life Sciences). On the day after transfection, Dox (1.5 μg/ml; Takara Bio Inc.) was added to the culture medium (somatic cell medium 2) comprising GMEM, 10% FBS, and P/S. Four days after transfection, 3×10^5 to 5×10^5 cells were seeded onto mitomycin C–treated mouse embryonic fibroblasts (MEFs) and cultured at 37°C under 6% CO_2 in air in medium [iPSC medium (iPSM)] comprising GMEM, 15% knockout serum replacement (KSR; Invitrogen Life Sciences), 0.3% FBS, P/S, and mouse ESGRO (10^3 U/ml; Invitrogen Life Sciences) in the presence of Dox (1.5 μg/ml). Ten days after transfection, dome-shaped colonies were selected, dissociated with trypsin-EDTA, and cultured on feeder cells in the same culture medium in the presence of Dox (1.5 μg/ml). To generate Dox-independent cell lines, Dox was withdrawn from iPSM when cells had grown to confluence. To prevent autonomous differentiation, iPSM was replaced with N2B27 3iL comprising 48% DMEM/F12, 48% Neurobasal medium, 1% N2 supplement, 2% B27 supplement, 0.5% KSR, 2 mM L-glutamine, P/S (Invitrogen Life Sciences), mouse ESGRO (10^3 U/ml), 3 μM CHIR99021, 1 μM PD0325901, and 0.5 μM SB980885 (Wako).

Additional chemicals and growth factors used in this research included kenpaullone (5 μM; Stemgent Inc.), forskolin (10 μM; Wako, Pure Chemical Industries), Gö 6983 (5 μM; Wako), IM-12 (1 μM; ReproCell), WH-4-023 (1 μM; ReproCELL), and Activin A (10 ng/ml; Wako). Fresh medium was changed daily, and cells were passaged every 3 to 4 days on MEFs. To culture cells in the feeder-free condition, the culture dish was coated with human plasma fibronectin (15 μg/ml; Millipore). To remove differentiated cells, puromycin (4 μg/ml) was added to the culture medium. The fluorescent signals of the cells were detected using a BZ-9000 Series All-in One Fluorescence Microscope using BZII image analysis software (Keyence).

RT-PCR analysis

To determine the mRNA sequences of *T. osimensis* genes (*OCT3/4*, *KLF4*, *SOX2*, *c-MYC*, *NANOG*, and *G3PDH*), degenerate primers were designed from complementary DNA (cDNA) sequences of the mouse, rat, *Macaca mulatta*, and human. Total RNAs were isolated using ISOGEN (Nippon Gene) from the *T. osimensis* iPSC line (4f1-1). After deoxyribonuclease treatment to prevent contamination by genomic DNA, first-strand cDNA was synthesized using a TaKaRa RNA PCR kit (TaKaRa Bio Inc.). The synthesized cDNA was amplified by PCR using the degenerate primers listed in table S2, with a cycling program of 94°C for 3 min and 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. RT-PCR products were sequenced, and the sequences of *T. osimensis* genes were determined. After determination of each sequence, primers (Endo primers) that specifically recognized *T. osimensis* cDNAs were designed (table S2). Endo primers were used to evaluate mRNA expression after iPSC induction. To evaluate the leaky expression of exogenous PB vectors after withdrawal of Dox, transgenic primers were designed to detect transgenic-specific sequences (table S2). For quantitative RT-PCR (qRT-PCR), a LightCycler 96 (Roche Applied Science) was used to measure mRNA expression levels using the FastStart Essential DNA Green Master (Roche Applied Science), with a program of 94°C for 10 min, 40 cycles of 94°C for 10 s, 60°C for 10 s, and 72°C for 10 s. The expression levels of each gene were normalized to *G3PDH* and calculated using the comparative C_t method. For statistical analysis of the qRT-PCR results, all data were expressed as means ± SD. Values were compared using *t* tests. $P < 0.05$ was considered to be significant.

Karyotype analysis

All iPSC lines were incubated with colcemid (final concentration, 100 ng/ml) for 4 hours at 37°C in 6% CO_2 in air. Cells were trypsinized

and pelleted at 120*g* for 3 min, resuspended in 6 ml of 75 mM KCl, and incubated at 37°C for 15 min. Cells were centrifuged at 120*g* for 3 min and then fixed using a 50% Carnoy's solution (acetic acid to methanol ratio, 1:3). The centrifugation and fixation steps were repeated three times. During the last repeat, cells were kept in Carnoy's solution and then dropped onto glass slides. Chromosome spreads were stained with Giemsa solution. At least 20 metaphase spreads were counted for each iPSC line.

Alkaline phosphatase staining

T. osimensis iPSCs were stained using an alkaline phosphatase detection kit (Sigma) according to the manufacturer's protocol.

Teratoma formation

To generate teratomas, 1×10^6 to 2×10^6 iPSCs were injected under the kidney or testis of 5- to 8-week-old SCID mice. At 4 to 8 weeks after transplantation, the teratomas were dissected and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). Paraffin wax sections were stained with hematoxylin and eosin.

Plasmid preparation

To prepare pPB-CAG-Su9DsRed-IRES-NeoR, the Su9DsRed2 fragment was excised from pCXN-Su9DsRed2 (*40*) using the restriction endonuclease *Eco* RI. The Su9DsRed2 fragment was introduced into the *Eco* RI site of pPB-CAG-cHA-IN.

Production of interspecific chimeras

To evaluate whether the iPSCs from *T. osimensis* could contribute to mouse embryos and pups as an interspecific chimera, iPSC lines 4f1-1 and 5f1-1 were transfected with pPB-CAG-Su9DsRed-IRES-NeoR with Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen Life Sciences). After transfection, G418 (400 μg/ml) was added to the selection. Recipient embryos were obtained by in vitro fertilization using superovulated Institute of Cancer Research (ICR) mouse oocytes and ICR sperm in HTF medium and cultured in KSOM (ARK Resource) to develop into blastocysts. Transfected iPSCs were trypsinized to disperse them into single cells, and 10 iPSCs were injected into the blastocoel of mouse blastocysts using a Piezo-driven micromanipulator (PrimeTech Ltd.). Interspecific blastocysts were transplanted into the uterus of a pseudopregnant ICR mouse. On day 12.5 of gestation, the embryos were recovered, and the DsRed signal was observed using an MVX10 fluorescence microscope (Olympus). As a control experiment, C57BL6 mouse ESCs with a CAG-EGFP transgenic line (*41*) were injected into mouse blastocysts. Chimeric embryos were transplanted into the uterus of pseudopregnant ICR mice, and embryos were recovered at 11.5 dpc. The chimeric contribution was determined by GFP fluorescence. On day 19.5, interspecific chimeric pups were delivered by cesarean section or vaginal delivery, and then the live ones were nursed by lactating ICR strain female mice. Chimerism of newborn pups was determined by the presence of the DsRed signal, brown skin, and hair color. To assess the chimeric contribution in tissues, including germ cells, 7 weeks after birth, chimeric mice were anesthetized or sacrificed, and dissected tissues including gonads were removed. After observation under an MVX10 fluorescence microscope, the tissue samples were fixed in 4% paraformaldehyde in PBS at room temperature overnight.

Immunohistochemical analysis

Tissues of adult mice were fixed in 4% paraformaldehyde in PBS (pH 7.4) at room temperature overnight. Tissues were washed in a sucrose gradient in PBS and then embedded in optimal cutting temperature compound. Frozen sections were cut to 10-μm thickness and placed onto silane-coated glass slides. The frozen sections were washed with PBS, microwaved at 95°C for 10 min in citrate buffer (pH 6.0), or treated with HistoVT One according to manufactures protocol (Nacalai Tesque) and preincubated with normal goat IgG (500 μg/ml) and 1% BSA in PBS for 1 hour to block nonspecific binding of antibodies. Then, the sections were reacted overnight with the primary antibodies. Primary antibodies were DsRed [ab62341 (dilution 1:100; Abcam) or sc-390909 (dilution, 1:20; Santa Cruz Biotechnology], DDX4/MVH [ab13840 (dilution, 1:200; Abcam)], TRA98 [ab82527 (dilution, 1:250; Abcam)], ACRBP/sp32 (ACRBP-C) [a gift from T. Baba (dilution, 1:200)] (*25*), and IZUMO1 (#125) [a gift from M. Ikawa (dilution, 1:200)] (*26*). After washing with 0.075% Brij L23 in PBS, some slides were reacted with Alexa-conjugated fluorescent antibodies for 1 hour. After washing in 0.075% Brij 35 in PBS, the nuclei were counterstained with DAPI (Molecular Probes). Immunofluorescent signals were captured with Zeiss Laser Scanning microscopy LSM 700 (Zeiss). Some slides were reacted with horseradish peroxidase–conjugated antibody and visualized with 3,3′-diaminobenzidine and H_2O_2 and then counterstained with hematoxylin. As a negative control, normal rat or rabbit IgG was used instead of the primary antibodies at the same concentration in every experiment. The $DsRed^+$ and $DsRed^-$ oocytes and the $DsRed^+$ spermatocytes in gonadal sections were counted under a microscope. Primary antibody–positive germ cells in the gonadal sections were counted on a BZ-9000 Series All-in One Fluorescence Microscope using the BZII image analysis software.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/5/e1602179/DC1

fig. S1. Determining appropriate culture conditions for *T. osimensis* iPSCs.
fig. S2. LIF-dependent maintenance of naïve pluripotency of *T. osimensis* iPSCs.
fig. S3. Chimeric contribution of *T. osimensis* iPSCs in interspecific embryos.
table S1. Characterization of *T. osimensis* iPSC lines.
table S2. PCR primers.

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