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RESEARCH ARTICLE

TECHNIQUES AND RESOURCES

Interspecific *in vitro* assay for the chimera-forming ability of human pluripotent stem cells

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ABSTRACT

Functional assay limitations are an emerging issue in characterizing human pluripotent stem cells (PSCs). With rodent PSCs, chimera formation using pre-implantation embryos is the gold-standard assay of pluripotency (competence of progeny to differentiate into all three germ layers). In human PSCs (hPSCs), however, this can only be monitored via teratoma formation or *in vitro* differentiation, as ethical concerns preclude generation of human-human or human-animal chimeras. To circumvent this issue, we developed a functional assay utilizing interspecific blastocyst injection and *in vitro* culture (interspecies *in vitro* chimera assay) that enables the development and observation of embryos up to headfold stage. The assay uses mouse pre-implantation embryos and rat, monkey and human PSCs to create interspecies chimeras cultured *in vitro* to the early egg-cylinder stage. Intra- and interspecific chimera assays with rodent PSC lines were performed to confirm the consistency of results *in vitro* and *in vivo*. The behavior of chimeras developed *in vitro* appeared to recapitulate that of chimeras developed *in vivo*; that is, PSC-derived cells survived and were integrated into the epiblast of egg-cylinder-stage embryos. This indicates that the interspecific *in vitro* chimera assay is useful in evaluating the chimera-forming ability of rodent PSCs. However, when human induced PSCs (both conventional and naïve-like types) were injected into mouse embryos and cultured, some human cells survived but were segregated; unlike epiblast-stage rodent PSCs, they never integrated into the epiblast of egg-cylinder-stage embryos. These data suggest that the mouse-human interspecies *in vitro* chimera assay does not accurately reflect the early developmental potential/process of hPSCs. The use of evolutionarily more closely related species as host embryos might be necessary to evaluate the developmental potency of hPSCs.

KEY WORDS: Chimera, Pluripotency, ESC, iPSC, EpiSC

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INTRODUCTION

Criteria exist for assessing pluripotency, a category of biological plasticity, in a hierarchy from full pluripotency downward. A gold-standard assay of chimera-forming ability routinely involves injection of mouse embryonic stem cells (ESCs) and of induced pluripotent stem cells (iPSCs) into pre-implantation embryos (Bradley et al., 1984; Okita et al., 2007; Wernig et al., 2007). Germline transmission is generally considered the most rigorous evidence of pluripotency. Chimeric embryo assays as well as tetraploid complementation have demonstrated germline transmission for mouse ESCs/iPSC progeny (Nagy et al., 1990, 1993; Zhao et al., 2009; Kang et al., 2009). Germline transmission has also been tested for rat ESCs/iPSCs using chimera formation assays (Buehr et al., 2008). A less-stringent criterion for PSC pluripotency is to demonstrate contribution to all three germ layers of the embryo, as assayed by teratoma formation or *in vitro* differentiation (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998).

Even though most ESC lines tested are derived from blastocyst inner cell mass (ICM), only rodent ESCs generate chimeric offspring or contribute to germline transmission. Likewise, only rodent iPSCs are capable of chimera formation. Conventional human ESCs (hESCs) and iPSCs (hiPSCs) share more characteristics with mouse epiblast-derived stem cells (EpiSCs) than with mouse ESCs/iPSCs. Mouse EpiSCs can generate teratomas but cannot contribute to chimera formation with pre-implantation embryos (Tesar et al., 2007; Brons et al., 2007). Perhaps hESCs/hiPSCs are also non-chimera-forming cells. However, this cannot be tested by human-human chimera assay. The lack of chimera formation obstructs detailed developmental studies to determine the full extent of pluripotency of human PSCs. Although human PSCs and rodent PSCs are similarly termed 'pluripotent', this does not assign similar biological plasticity.

At present, pluripotency of human PSCs is assayed via teratoma formation or *in vitro* differentiation. Such assays test capacity to differentiate into three germ layers. However, developmental plasticity (ability within an embryo to co-operate with host cells in normal differentiation into various tissues) is difficult to assess. Generation of human-animal admixed embryos might allow characterizing the pluripotency of hPSCs more fully than teratoma formation or *in vitro* differentiation. Conventional hESCs and newly established naïve-like human PSCs have been injected into mouse embryos to assay chimera-forming ability *in utero* (James et al., 2006; Gafni et al., 2013). However, in some countries, including Japan, ethical concerns make it difficult to perform such assays; for example, governmental guidelines prohibit transfer of human PSC-injected interspecies chimeras into animal bodies and ban culture of such chimeras beyond 14 days or gastrulation stage.

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For these reasons, we established hiPSC lines with donor permission and developed interspecies chimeras *in vitro* to study the developmental plasticity of human PSCs. We observed injected conventional hiPSC lines and naïve-like iPSCs in developing mouse embryos, and periodically compared their behavior with that of PSCs from other species. Our results led us to conclude that even under highly optimized culture conditions it is difficult to predict the chimera-forming activity of naïve-like-iPSCs or even to discriminate it from that of hiPSCs. At the same time, control experiments using rodent ESCs and EpiSCs demonstrated that the *in vitro* interspecies chimera assay is a highly reliable system representing the behavior of chimeras *in vivo*. Overall, our study demonstrates limitations to the use of mouse embryos in evaluating the developmental state of human PSCs. Our results led us to conclude that even under highly optimized culture conditions it is difficult to predict or even to discriminate developmental potency of human PSCs when using mouse embryos.

RESULTS

Development of *in vitro* chimera assay

We designed an *in vitro* chimera assay that avoids ethical problems to clarify hPSC chimera-forming ability. As shown in Fig. 1A, fluorescence-labeled hPSCs were injected into mouse pre-implantation embryos that were allowed to develop *in vitro* as chimeras with monitoring of the distribution of the PSCs and their progeny. To establish this assay, we initially attempted, but failed, to duplicate studies reporting *ex vivo* development of mouse pre-implantation embryos from blastocyst stage to limb-bud stage (Chen and Hsu, 1982). Embryos planted onto dishes exhibited outgrowth but gradually degenerated after human cord serum replaced FBS; many batches of human cord serum were tested, but none supported mouse-embryo development *in vitro* (data not shown). We therefore sought culture conditions fit for our purposes.

Mouse embryos were harvested at eight-cell to morula stages, cultured in higher K^+ concentration simplex optimization medium (KSOM) for one day *in vitro* (1 DIV) to yield blastocysts, and planted on dishes coated with mouse embryonic fibroblast-feeders or Matrigel to support their adhesion, in which they were fed with medium supplemented with rat serum. Rat serum was prepared by immediate centrifugation of rat blood (IC serum) as described for *ex vivo* culture of mouse post-implantation embryos (Takahashi and Osumi, 2010). In our experiments, freshly isolated IC serum from overnight-fasted rats or commercially available rat IC serum improved survival of cultured embryos. Survival of dish-planted embryos varied substantially, depending on the batch of rat serum, as reflected in longer survival-curve data-point error bars after planting (Fig. 1F, later than 5 DIV). Under this modified-stepwise culture condition (supplementary material Fig. S1), mouse embryos attached to culture dishes at 4 DIV and achieved early egg-cylinder stage at 6-7 DIV (Fig. 1B), advanced egg-cylinder stage around 9 DIV (Fig. 1C) and headfold stage around 11 DIV (Fig. 1D). Most cultured embryos that successfully attached to dishes, however, were malformed and stopped developing. On average, 24% of cultured embryos achieved egg-cylinder stage (Fig. 1F). To clarify whether embryos that had developed *in vitro* were biologically normal and able to form chimeras, we assayed chimerism *in vitro* by injecting fluorescence-labeled mESCs into mouse eight-cell-stage embryos or blastocysts and by periodically observing distributions of progeny of injected mESCs under fluorescence microscopy. As with chimeric embryos that developed *in utero*, fluorescence signals from injected mESCs were found in epiblast but not in extra-embryonic regions in every surviving chimeric embryo (Fig. 1E-E″). These results indicate that mouse embryos that develop *in vitro* until headfold stage are biologically similar in respect of chimera formation to those that develop *in vivo*.

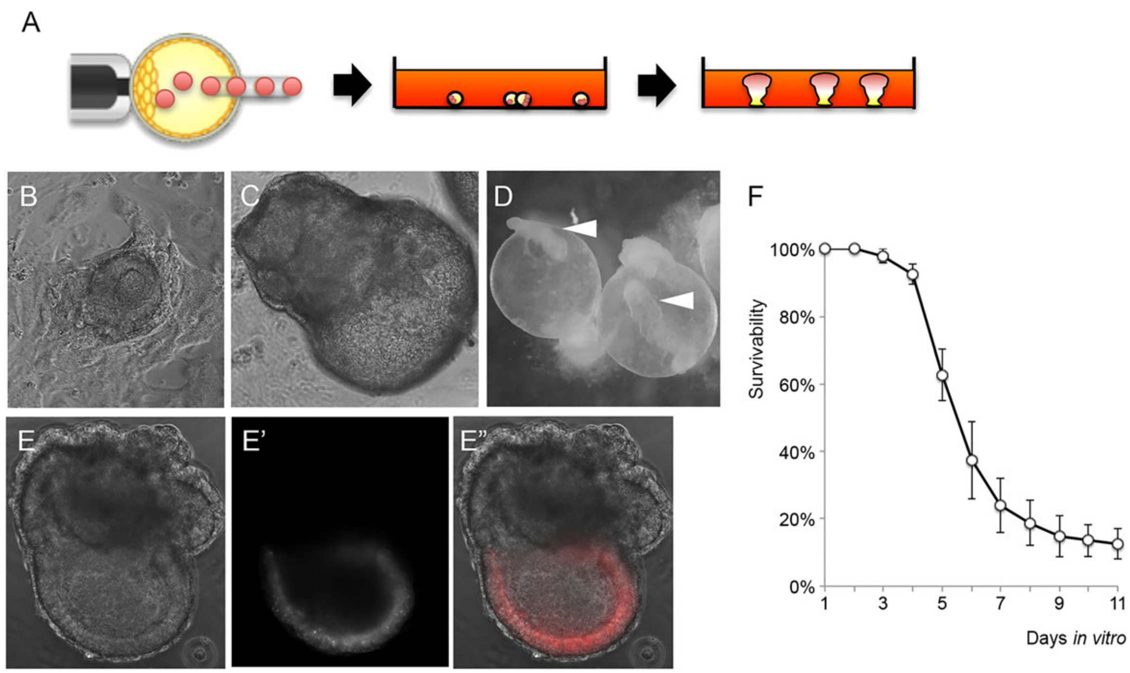

Fig. 1. Development of *in vitro* chimera assay. (A) An *in vitro* chimera assay was developed to determine chimera-forming ability of PSCs. Fluorescence-labeled cells were injected into mouse pre-implantation embryos that developed *in vitro*. (B-D) Embryos developed to early egg-cylinder stage at 6 DIV (B), late egg-cylinder stage at 8 DIV (C) and headfold stage at 10 DIV (D). Arrowhead indicates embryo body formed *in vitro*. (E-E″) Brightfield (E), fluorescence (E′) and overlaid (E″) images of embryo injected with EB3DR mouse ESCs at 7 DIV. EB3DR-derived cells express DsRed. (F) Plots indicate survivability of mouse embryos developed *in vitro* at each day.

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Definition of criteria for chimera-forming ability

We next tried to define criteria for chimera formation. As the survival of cultured embryos dramatically declined from attachment to egg-cylinder formation (5-7 DIV; Fig. 1F), we tried to define criteria for chimera-forming ability by comparing in our *in vitro* chimera assay the behavior of mESCs (chimera-forming PSCs) and mouse EpiSCs (non-chimera-forming PSCs). The progeny of injected mESCs proliferated and were located in blastocyst ICM at 2 DIV, following one day of culture after injection (Fig. 2A,B). Mouse ESC-derived cells contributed to ICM outgrowth but were not observed in extra-embryonic regions after attachment (Fig. 2C, dashed circle). By contrast, injected mouse EpiSCs failed substantially to proliferate and were located in the trophectoderm (Fig. 2D) as well as in the ICM (Fig. 2E) at 2 DIV. After attachment, mouse EpiSC-derived cells were completely absent or, rarely, survived in extra-embryonic regions (Fig. 2F). Mouse EpiSC-derived chimerism was observed in no embryo at 4 DIV, when most cultured embryos showed attachment (Fig. 2G; supplementary material Table S1).

These results suggest that, in this assay, incorporation of engrafted cells into ICM is not diagnostic of successful chimera formation; instead, the chimera-forming ability in donor cells can be recognized by incorporation of their progeny into non-extra-embryonic tissue of attached embryos. These findings also support the results from this *in vitro* chimera assay as reflecting the *in vivo* chimera-forming ability.

We confirmed the reproducibility of this *in vitro* chimera assay in many mouse ESC, iPSC and EpiSC lines. All mouse ESC and iPSC lines behaved as described above and were considered to represent chimera-forming cells, as did one mouse EpiSC-subcloned line (EpiSC-sub) that was also judged to be chimera-forming (Fig. 3). Mouse EpiSC-sub cells proliferated more strongly than other mouse EpiSC line cells at all time points (Fig. 3A,E) and contributed to ICM outgrowth at 4 DIV (Fig. 3B). Although their frequency was lower than that of mouse ESC progeny, mouse EpiSC-sub-derived cells survived in chimeric embryos and were incorporated into non-extra-embryonic regions through development to egg-cylinder stage (Fig. 3E; supplementary material Table S1). Chimera-forming ability in mouse EpiSC-sub cells was confirmed by *in vivo* chimera assays, in which mouse EpiSC-sub-cell progeny were found to have contributed to multiple tissues (Fig. 3C,D; supplementary material Fig. S3), including germ cells (supplementary material Fig. S3C-E). Germline transmission in their offspring, however, has not yet been observed (data not shown). Although a subpopulation of short-term-cultured mouse EpiSCs reportedly can contribute to chimeras (Han et al., 2010), the EpiSC-sub line that we used had been cultured for more than 40 passages (supplementary material Table S2). The colonies that these EpiSC-sub cells formed (supplementary material Fig. S2C) resembled those of parental, non-chimera-forming EpiSCs (supplementary material Fig. S2B), but not those of mouse ESCs (supplementary material Fig. S2A). For further clarification, we performed flow cytometry analysis (supplementary material Fig. S4). As in parental EpiSCs (supplementary material Fig. S4E), EpiSC-sub cells did not stain with anti-CD31 (Pecam1) antibody (supplementary material Fig. S4H), which is known to mark mouse naïve-state PSCs (supplementary material Fig. S4B). Partial expression of SSEA1 was also consistent (supplementary material Fig. S4F,I). We concluded that EpiSC sub cells contributed to chimeras without having undergone conversion to the naïve pluripotent state.

Interspecies *in vitro* chimera assay for rat cells

Once this *in vitro* chimera assay was confirmed as accurate, we tested whether it yielded valid results for xenogeneic cells. Besides mouse ESCs/iPSCs, rat ESCs/iPSCs also are known to contribute to mouse chimera formation, and we thus used rat ESCs/iPSCs and rat EpiSCs for interspecies *in vitro* chimera assays. We established germline transmission-competent rat ESC and iPSC lines (Hamanaka et al., 2011; Kobayashi et al., 2012) and, as in allogeneic-cell studies, injected fluorescence-labeled rat ESCs (supplementary material Fig. S2D), iPSCs and EpiSCs (supplementary material Fig. S2E) into mouse pre-implantation embryos, with monitoring until egg-cylinder stage embryo formation. Engrafted rat ESCs or iPSCs proliferated in mouse embryos. Their progeny were incorporated into ICM at 2 DIV (supplementary material Fig. S5A), into ICM outgrowth at 4 DIV (supplementary material Fig. S5B) and into the epiblast of egg-cylinder stage embryos at 7 DIV (supplementary material Fig. S5C), like the progeny of engrafted mouse ESCs. In these respects, rat

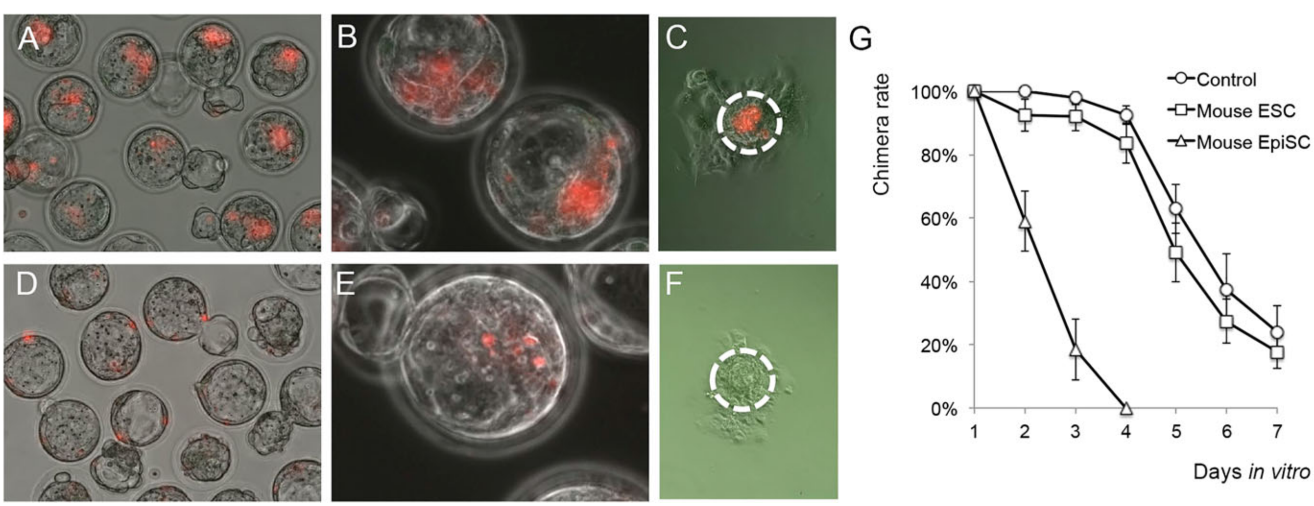

Fig. 2. Criteria for chimera formation competence in cells. To define the criteria for chimera formation, behavior of mouse ESCs and EpiSCs was compared by an *in vitro* chimera assay. (A-F) Overlaid images of chimeric embryos *in vitro*. Distributions of mouse ESC- (A-C) or EpiSC- (D-F) derived cells were observed at 2 DIV (A,D; shown at higher magnification in B,E) and after implantation (4 DIV; C,F). Dashed circles indicate the location of ICM outgrowth. (G) Plots indicate proportion of mouse ESC- or EpiSC-derived chimeric embryos per started embryos at each culture day compared with survival of non-injected controls.

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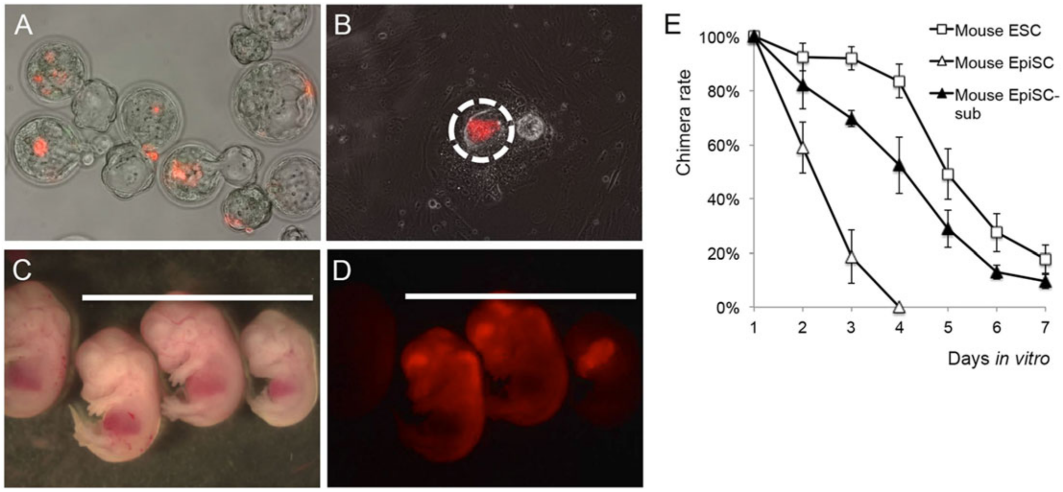

Fig. 3. *In vitro* chimera assay recapitulated chimera-forming ability of EpiSCs. One mouse EpiSC sub-clone (EpiSC-sub) was found by *in vitro* chimera assay to be competent to form chimeras. (A,B) Overlaid images indicate the distribution of donor EpiSC-derived cells at 2 DIV (A) and 4 DIV (B). Dashed circle indicates the location of ICM outgrowth. (C,D) EpiSC-sub-derived chimeric embryos dissected at E13.5. Brightfield (C) and fluorescence (D) microscopy images indicate the distribution of EpiSC-sub-derived cells. Bars indicate EpiSC-sub cell-derived chimeras. (E) Plots indicate proportion of EpiSC-sub-derived chimeric embryos among started embryos at each culture day.

ESCs/iPSCs fulfilled *in vitro* chimera-assay criteria for chimera-forming cells. By contrast, injected rat EpiSCs barely proliferated and completely disappeared from embryos by dish-attachment stage, similar to mouse EpiSCs (supplementary material Fig. S5D,E). Survival curves of rat ESC-/iPSC-engrafted interspecies chimeras lay midway between those of mouse ESC- and mouse EpiSC-engrafted chimeras (supplementary material Fig. S5G, Table S1). Progeny of rat cells survived less frequently than those of mouse ESCs, but kept forming chimeras until egg-cylinder stage, whereas progeny of mouse EpiSCs were entirely excluded from embryonic tissues (supplementary material Fig. S5G; later than 5 DIV). Chimera rates (number of chimeric embryos:total number of embryos) and chimerism (proportion of extrinsic cell progeny within chimeric embryo) *in vivo* are lower for rat-mouse interspecies chimeras than for intraspecific chimeras (Kobayashi et al., 2010). *In vivo*, the rat ESC line BLK-RT2 also formed interspecific chimeras with mouse embryos at lower rates than did mouse ESC lines (supplementary material Fig. S5F, Table S2). Low chimera rates observed for rat PSCs in this interspecies *in vitro* chimera assay were thus consistent with those *in vivo*. These results indicated that this *in vitro* chimera assay applied to the chimera-forming ability of rat cells as well as to that of mouse cells, and that the same criteria held for both.

Interspecies *in vitro* chimera assay for monkey cells

Primate PSCs have characteristics similar to human PSCs: they do not form chimeras in allogeneic settings (Tachibana et al., 2012) or xenogeneic settings (Simerly et al., 2011). We applied this *in vitro* chimera assay to cynomolgus monkey (*Macaca fascicularis*) ESCs (Suemori et al., 2001) (line CMK6; supplementary material Fig. S2F) to test whether the criteria that define chimera-forming ability in mouse and rat cells also held for primate cells. CMK6 cells labeled with *tdTomato*-encoded fluorescent protein were injected into mouse blastocysts. At one day after injection (2 DIV), more CMK6-derived cells than mouse EpiSC-derived cells survived (supplementary material Fig. S6A,D; compare with Fig. 2D). However, most of these cells disappeared from ICM outgrowth or lay in extra-embryonic regions at attachment (4 DIV) (supplementary material Fig. S6B,D; Table S1). A few embryos showed a focus of fluorescent signal in ICM outgrowth immediately after attachment; this was not observed with mouse EpiSCs. None of these, however, continued to express tdTomato until egg-cylinder stage embryo formation (supplementary material Fig. S6C,D). Using mouse embryos *in vivo*, we also confirmed interspecies-chimera CMK6 cell behavior. As *in vitro*, no fluorescent signal was observed later than at egg-cylinder stage embryo except for autofluorescence (supplementary material Fig. S6E,F, arrowheads). Primate ESC-derived interspecies chimeras *in vitro* thus recapitulated similar chimera behavior *in vivo*, indicating that criteria defined in rodents for assessing chimera-forming ability also held for primates.

Interspecies *in vitro* chimera assay for hPSCs

We further tested the chimera-forming ability of hiPSCs. Using SeVdp vectors carrying human *OCT4*, *SOX2*, *KLF4* and *MYC*, hiPSC lines were established from peripheral blood mononuclear cells donated from five different volunteers with informed consent. Transduced SeVdp vectors were eliminated from established hiPSC lines by transfecting with siRNA (Nishimura et al., 2011). More than two lines of transgene-free hiPSC were established from each donor, labeled with *tdTomato* and assayed. For the first one or two days after injection, more labeled cells survived in each chimeric embryo than in mouse EpiSC progeny (Fig. 4A), and some of these cells were found in the ICM (Fig. 4B). However, hiPSC-derived cells barely existed in ICM outgrowth after attachment (Fig. 4C) and were absent from the epiblast of egg-cylinder stage embryo (Fig. 4D). The fact that hiPSC progeny existed outside ICM or egg-cylinder stage embryos (Fig. 4D) indicates that culture conditions were not harmful for hiPSC-derived cell survival. Overall, injected hiPSCs behaved similarly to injected primate ESCs (Fig. 4H, hiPSC; supplementary material Table S1). The results were consistent within all tested hiPSC lines, suggesting that hiPSCs do not possess the developmental plasticity required to form interspecies chimeras with mouse embryos, as reported for *in utero* work (James et al., 2006).

We also conducted interspecies *in vitro* chimera assays of specifically treated hiPSC lines to screen for chimera-forming cell lines. Single-cell dissociation causes apoptosis in hESCs and hiPSCs via blebbing (Watanabe et al., 2007). Single-cell dissociation of hiPSCs using trypsin before intra-embryonic injection might impede

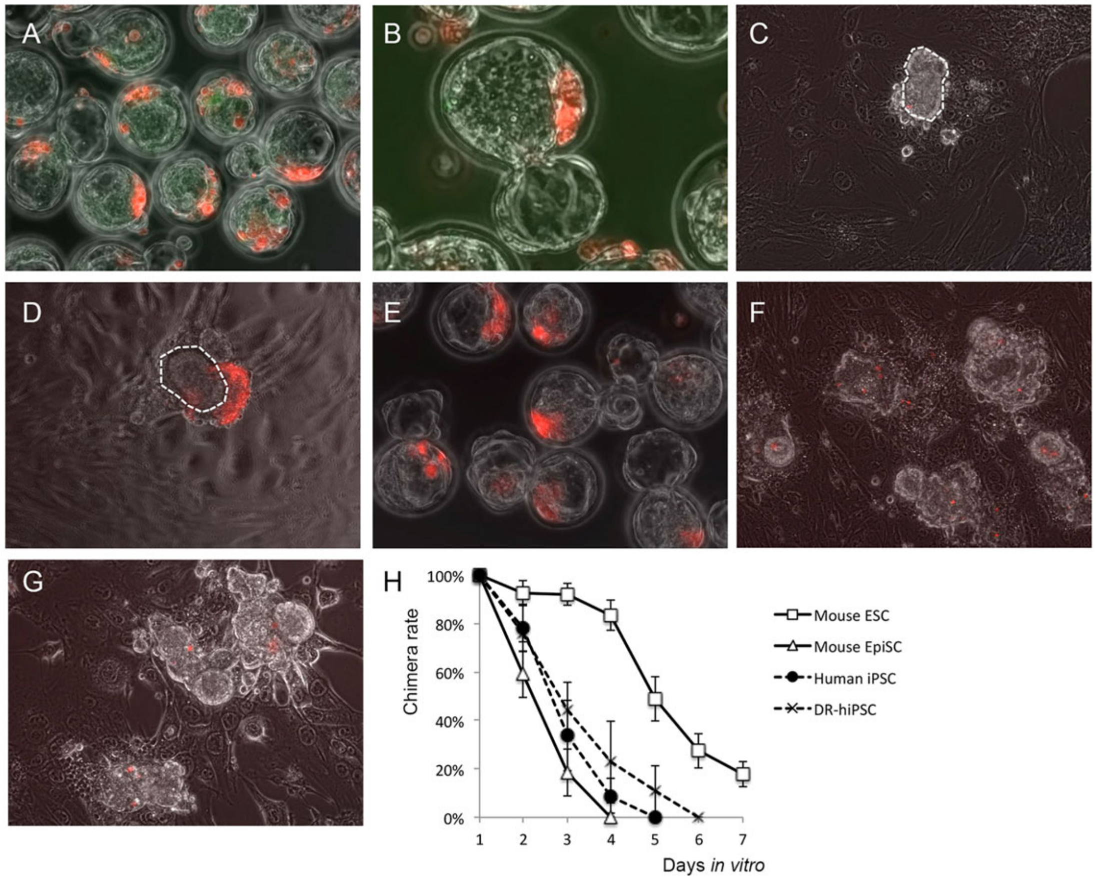

Fig. 4. Interspecies *in vitro* chimera assay: hiPSCs. Chimera-forming ability of hiPSCs was tested by interspecies *in vitro* chimera assay. (A-G) Overlaid images show the distribution of engrafted hiPSCs (A-D) or DR-hiPSCs (E-G) in red at 2 DIV (A,B,E), 4 DIV (C,F) and 6 DIV (D,G). Dashed circles indicate embryonic regions, such as ICM outgrowth (C) or epiblast (D). (J) Plots indicate proportion of hiPSC-derived chimeric embryos per started embryos at each culture day.

their competency to form chimeras. We therefore established hiPSC lines resistant to single-cell dissociation (DR-hiPSCs). Rho-associated protein kinase (ROCK) inhibitor is usually employed to prevent blebbing after single-cell dissociation. To establish DR-hiPSC lines, we trypsinized hiPSCs and plated the resulting dissociated cells without ROCK inhibitor. Most of the cells died via apoptosis but a few survived. After several repetitions of dissociation and plating without ROCK inhibitor, the surviving hiPSCs no longer underwent apoptotic cell death after plating. Crucially, established DR-hiPSC lines were morphologically similar to the parental hiPSC line (supplementary material Fig. S2G). DR-hiPSC lines assayed *in vitro* for chimera-forming ability behaved like normal hiPSC lines (Fig. 4H; supplementary material Table S1). Although no significant cell death was observed at 2 DIV (Fig. 4E), DR-hiPSC progeny were nearly all dead when the embryos attached (Fig. 4F), and no DR-hiPSC-derived cells were incorporated into the epiblast of egg-cylinder stage-embryos (Fig. 4G). It could be inferred that cell death via blebbing did not impede nor enhance competence of hiPSCs to form interspecies chimera with mouse embryos.

Takashima and colleagues recently demonstrated that newly established human naïve-like PSCs, called reset cells, can form chimeras in ICM outgrowth (Takashima et al., 2014). We decided to apply our *in vitro* chimera assay to reset cells. As transient overexpression of NANOG and KLF2 converted conventional hiPSCs to reset cells, we introduced a tet-on NANOG/KLF2 inducible system into hiPSC lines via Ai-LV vectors (Yamaguchi et al., 2012). The converted naïve-like PSCs had compact cytoplasm and exhibited mESC-like colony formation when cultured with MEK inhibitor, GSK3β inhibitor and leukemia inhibitory factor (LIF) (2iL) plus doxycycline (Dox) (2iL+Dox), or with 2i plus protein kinase C inhibitor (PKCi) instead (2iL+PKCi) (Fig. 5A-C; supplementary material Fig. S2I,J), as reported. The cells also showed naïve-like gene expression profiles, with upregulation of *TBX3*, *STELLA* and *TFCP2L1*, and downregulation of the meso-endodermal markers *T*, *SOX17* and *AFP* (Fig. 5D). These features led us to conclude that we had successfully reproduced induction of reset cells. Reset cells were maintained with Dox; Dox was withdrawn and PKCi treatment begun 48 h before the cells were injected. Injected cells located in both ICM and trophectoderm before attachment (Fig. 5E). However, most of them disappeared or failed to contribute to ICM outgrowth. The rare injected-cell progeny that survived were located in ICM outgrowth (Fig. 5F) or showed abnormal development; e.g. formation of secondary ICM-like structures, with progeny cells surrounded by host cells (Fig. 5G). Most injected reset cells disappeared when the embryo developed to egg-cylinder stage (Fig. 5I, 6-7 DIV; supplementary material Table S1). The surviving cells rarely proliferated, however. Instead, they were segregated from host tissue and aggregated in extra-embryonic regions (Fig. 5H), never contributing to the epiblast. These results suggest the difficulty of contributing to the formation of chimeras between human cells and mouse

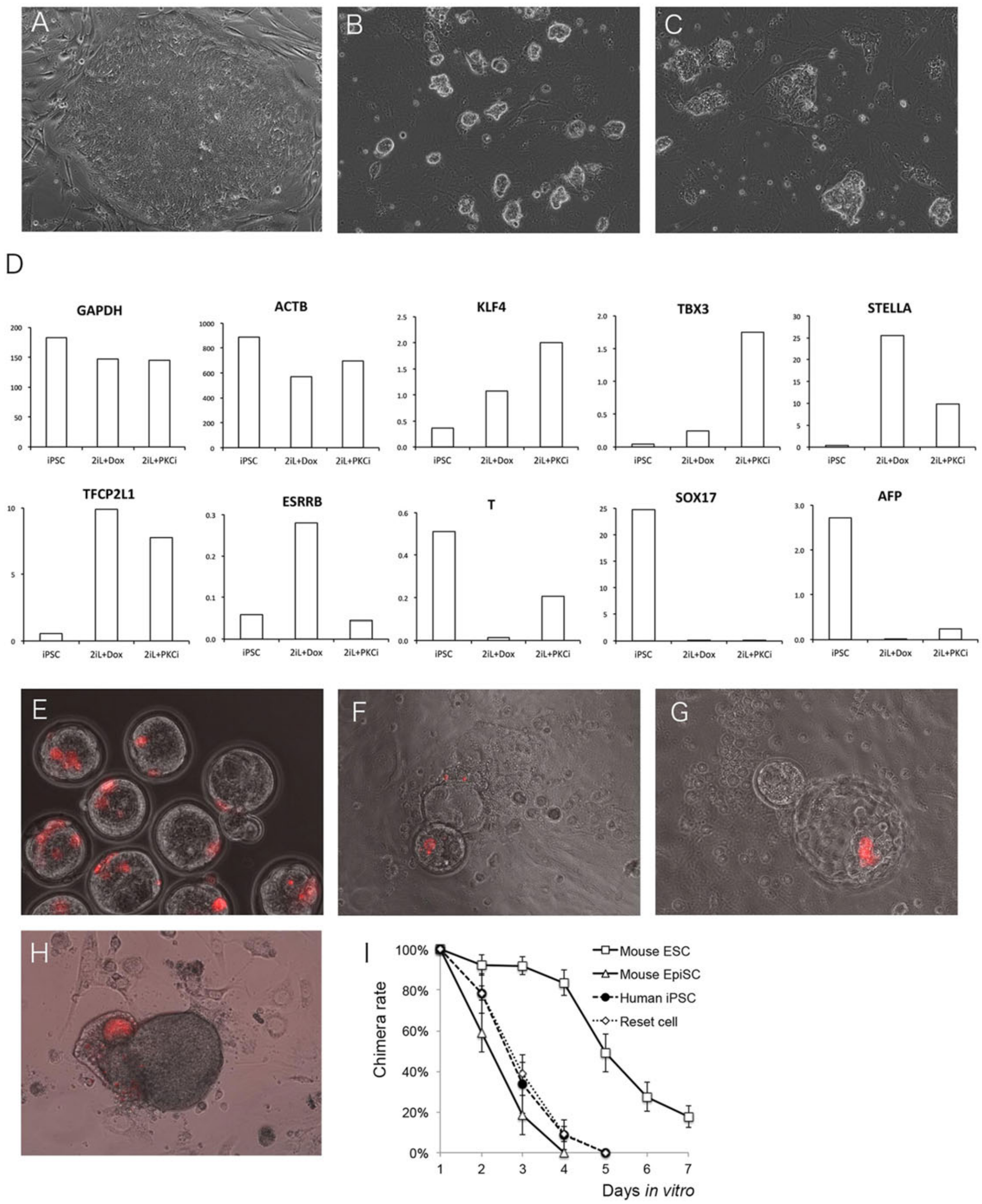

Fig. 5. Interspecies *in vitro* chimera assay: human reset cells. (A-C) Appearances of conventional hiPSCs (A), naïve-like PSCs with 2iL+Dox (B) and naïve-like PSCs with 2iL+PKCi (C). (D) Relative expression levels of selected genes from normalized microarray data. (E-H) Overlaid images show the distribution of engrafted human reset cells at 2 DIV (E), 4 DIV (F,G) and 7 DIV (H). (I) Plots indicate proportion of naïve-like hiPSC-derived chimeric embryos per started embryos at each culture day.

pre-implantation embryos even when the human cells are in a naïve-like pluripotent state.

DISCUSSION

Here, we optimized the culture conditions for an *in vitro* chimera assay to evaluate the pluripotency of hiPSCs with regard to chimera formation. Despite our efforts, preliminary studies of *in vitro* development of potential pre-implantation host embryos of various species (rat, pig, rabbit) found that only mouse embryos could develop beyond the egg-cylinder stage (data not shown). Therefore, we chose mouse pre-implantation embryos as hosts for an interspecies chimera assay. Results of our *in vitro* chimera assay reliably recapitulated those of conventional *in vivo* chimera assays, including a mouse-monkey interspecies chimera assay. In non-rodent species, incorporation of progeny into ICM has often been used as an indication of chimera-forming ability (James et al., 2006; Tachibana et al., 2012). However, in our study, injected hiPSCs survived better than mouse EpiSC in pre-implantation embryos; indeed, some of them seemed to be incorporated into ICM. However, once they reached post-implantation stage, neither hiPSC- nor mouse EpiSC-derived cells contributed to embryo development at all. These data suggest that the incorporation of progeny into ICM does not suffice, especially for interspecies chimeras, to assess chimera-forming ability, and that incorporation of progeny into embryonic regions after dish attachment is a more reliable indication. This criterion permitted the recognition of the chimera-forming ability of cells introduced into mouse embryos in allogeneic settings or xenogeneic settings, with results paralleling those obtained *in vivo*. We therefore assessed hiPSCs as non-chimera-forming cells. We also showed how to find requirements for chimera formation by an *in vitro* chimera assay of DR-hiPSC lines, with results suggesting that apoptosis caused by single-cell dissociation did not underlie a lack of chimera-forming competency.

Several research groups have studied interspecies chimera formation using human PSCs *in vivo* (James et al., 2006; Gafni et al., 2013). Interspecies *in vitro* chimera assays might be ethically more acceptable, even when species more closely related to humans than mouse are used as host embryos. *In vitro* chimera assays also allow observation of donor-cell behavior within the embryo at multiple time points, something impossible *in vivo*. Such observations led to the discovery that ICM localization of donor-cell progeny does not assure chimera formation when interspecies chimeras are created with mouse hosts and human or monkey PSCs.

However, our *in vitro* chimera assay also highlighted significant limitations. One is the low yield for embryo development. The most stringent criterion in our *in vitro* chimera assay is donor-cell contribution to the epiblast of egg-cylinder stage embryos. In our studies, ∼20% of cultured embryos attained egg-cylinder stage, whereas with embryos transferred *in vivo* the yield of development to egg-cylinder stage was ∼80%. Furthermore, some developed embryos were malformed. Although every embryo developing *in vitro* has some abnormality, as such embryos lack interaction with maternal tissues, severely malformed embryos stop developing. Another limitation is the use of mouse embryos as hosts. The assay identified all tested hiPSC lines and reset cells as non-chimera-forming cells with mouse embryos; however, this does not mean that such cells cannot form chimeras with embryos of monkey, pig or other species. Takashima and colleagues reported that reset cells aggregated with mouse embryos were lost during embryo development to egg-cylinder stage (Takashima et al., 2014). We also found that those progeny of reset cells that survived were excluded from the epiblast of mouse embryos and autonomously formed an anlage of undefined type. The incapacity of even human naïve PSCs to act in concert with mouse embryos may be caused by various reasons. These include differences in gastrulation mechanism and in ligands or adhesion molecules: rodent gastrulae uniquely form cylinders, whereas gastrulae of most other species form disks, and many growth factors vary between human and mouse. To exclude these problems, use of species evolutionarily closer to humans as host embryos is desirable. *In vitro* development systems for monkeys or other large animals must be elaborated accordingly. If this proves difficult, assays must be conducted *in vivo*.

Transcriptome analysis revealed that transcription networks in the reset cells strongly resembled those in human pre-implantation embryo and differed greatly from those in conventional human ESCs/iPSCs (Huang et al., 2014). However, in our studies, conventional hiPSCs and reset cells behaved similarly in developing mouse embryos. This suggests that those cells were excluded from mouse embryo development, regardless of their developmental state.

In the course of setting up the assay, we unexpectedly found that even long-term-cultured mouse EpiSCs still can contribute to chimeras, albeit at a lower frequency than mouse ESCs. Chimera-forming EpiSC-sub lines showed typical EpiSC morphology and no signs of conversion to the naïve pluripotent state (the cells should express Pecam1 upon such conversion, which they did not). Differences in pluripotency-regulating circuits and/or gene expression profiles have been thought to define chimera formation in pre-implantation embryos. However, Han et al. (2010) reported the generation of chimeras using short-term cultured mouse EpiSC subpopulation cells with gene-expression profiles entirely similar to those of main-population cells. Furthermore, mouse iPSC lines established in medium supplemented with basic fibroblast growth factor (bFGF, or FGF2) rather than LIF, theretofore considered crucial in maintaining pluripotency of mouse ESCs/iPSCs, formed chimeras with germline transmission (Di Stefano et al., 2010). Biomarkers of chimera-forming ability accordingly remain to be identified. Consequently, our findings also emphasize the importance of functional assays of chimera-forming ability. Of interest is that EpiSC-sub cells harbor a karyotypic abnormality that is now under investigation with respect to its relationship with chimera-forming ability.

In vitro culture will probably also be useful for observation of mouse embryos that harbor embryonic-lethal traits, as the stepwise culture conditions employed allow mouse embryos to attain the headfold stage and permit tracing of changes at multiple developmental stages.

In conclusion, the present assay for conventional hiPSCs, modified hiPSCs or reset iPSCs revealed limitations on the use of mouse embryos as hosts for such cells. We propose that the use of embryos of other species evolutionarily closer to humans will be important to assess the developmental state of hPSCs.

MATERIALS AND METHODS

In vitro development of mouse pre-implantation embryos

Embryos collected 2.5 days post-coitum in Medium 2 (Millipore) from the oviduct and uterus of C57BL/6 female mice mated with DBA/2 male mice were cultured in KSOM (Millipore) for 1 DIV, permitting the embryos to develop into blastocysts. Cells to be assayed were injected into morula or blastocyst embryos; differences in results between the two groups were not appreciated. Blastocyst embryos were cultured in Connaught Medical Research Laboratories (CMRL) 1066-based medium supplemented with sera. Detailed procedures are described in the supplementary material methods.

Preparation of hPSC lines

Consenting volunteers donated peripheral blood used to establish hiPSCs by transducing human *OCT4*, *SOX2*, *KLF4* and *MYC* via SeVdp vectors (Nishimura et al., 2011). Established hiPSCs were maintained on mitomycin C-treated MEFs in hESC medium [knockout Dulbecco's modified Eagle medium containing 15% knockout serum replacement, 2 mM glutamax, 1% NEAA (all Life Technologies) and 5 ng/ml bFGF (Peprotech)]. Cells were routinely passaged every 4-6 days. To convert hiPSCs to reset cells, the cells were infected with a tet-on AiLV vector that carries NANOG and KLF2 separately. Tet-on NANOG/KLF2-carrying hiPSCs were treated with Dox for 24 h, then placed into medium containing 2 μg/ml Dox, 20 ng/ml human LIF (Peprotech), 1 μM of GSK3β inhibitor CHIR99021 (Axon Medchem) and 1 μM of MAPK/ERK kinase-inhibitor PD0325901 (Wako Pure Chemicals) (2iL) as described (Takashima et al., 2014). Once the cells converted to naïve-like morphology, the cells could be maintained without Dox by adding PKC inhibitor Go6983 (Sigma-Aldrich) at 5 μM to 2iL condition instead. To fluorescence-label the cells, human PSCs were infected with a lentiviral vector carrying a CAG promoter-driven *tdTomato* construct, with *tdTomato*-expressing cells purified by cell sorting.

Maintenance of mouse ESC lines

A *DsRed*-expressing mouse ESC line (EB3DR) was cultured with mouse LIF (Millipore), 1 μM of CHIR99021 and 1 μM of PD0325901. Detailed procedures are described in the supplementary material methods.

Preparation of mouse EpiSC lines

The EpiSC line principally assayed was established from an EB3DR-injected chimeric E6.5 embryo as described (Murayama et al., 2015). EpiSC sub lines were subcloned by single-cell sorting from an established EpiSC line (Tesar et al., 2007). EpiSC sub cells were labeled with *tdTomato*, as were hiPSCs (described above). These EpiSC lines were maintained in hESC medium on mitomycin C-treated MEF feeder cells.

Chimera formation

Chimeric embryos were generated by microinjection of PSCs into eight-cell- or blastomere-stage embryos. BDF1 or ICR mouse embryos were collected in Medium 2 at eight-cell- or morula stage and were transferred into KSOM and cultured for several hours (for eight-cell-stage injection) or for 24 h (for blastocyst injection). Injected PSCs were trypsinized into single cells and suspended in culture medium. A piezo-driven micro-manipulator (Primetech) was used to drill zona pellucida under the microscope, and ten PSCs were introduced into the subzonal space of each individual embryo. After injection, embryos underwent follow-up culture in KSOM until blastomere stage. They were then transferred into the uteri of pseudopregnant recipient ICR mice for *in vivo* chimera assays. For *in vitro* chimera assays, chimeric embryos were transferred into CMRL medium as described.

Microarray analysis

Total RNA was extracted from conventional hiPSCs, reset cells cultured with L2i+Dox and reset cells cultured with L2i+PKCi after withdrawal of Dox for 14 days. Harvested RNA was processed into cRNA and hybridized against a SurePrint G3 Hyman GE microarray 8×60K chip (Agilent Technologies), following the manufacturer's instructions. Microarray data are available in GEO under the accession number GSE66657.

Ethics statement

Animal experiments were performed under guidelines of the Institutional Animal Care and Use Committee of the Institute of Medical Science, University of Tokyo, Japan. *In vitro* chimera studies, including those employing human iPSC-injected chimeras, were reviewed and approved by the ethics committee of the Institute of Medical Science, University of Tokyo, Japan (H. Kiyono et al.; institutional approval number 21-68-0409) before the study began. Human iPSC-derived interspecies chimera studies were also approved by the Ministry of Education, Culture, Sports, Science and Technology (Japan). Signed, informed consent was obtained before human peripheral blood samples were obtained from volunteers.

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Competing interests
H.N. is a founder and shareholder of iCELL and ChimERA, and a founder, shareholder and scientific advisor for Megakaryon and ReproCELL.

Author contributions
H.M. developed the concepts and performed experiments and data analysis; M.K.-I., A.U. and H.S. performed experiments; T.Y., S.H., T.K., K.N., M.O. and M.N. prepared the materials required for the experiments; H.N. supervised all studies.

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Supplementary material
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http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.124016/-/DC1

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Supplemental Methods

In vitro development of mouse pre-implantation embryos

Embryos collected 2.5 days post-coitum in Medium 2 [Millipore] from oviduct and uterus of C57BL/6 female mice mated with DBA/2 male mice were cultured in KSOM [Millipore] for 1 DIV, permitting the embryos to develop into blastocysts. The cells to be assayed were injected into morula or blastocyst embryos; differences in results between the two were not appreciated. Blastocyst embryos were plated onto mitomycin C-treated mouse embryonic fibroblast (MEF)-coated dishes or Matrigel [Becton Dickinson]-coated dishes and were cultured in Connaught Medical Research Laboratories (CMRL) 1066-based medium supplemented with 1% non-essential amino acids (NEAA), 0.1mM β-mercaptoethanol, and 2mM Glutamax [all Life Technologies] (CMRL-based medium) and with sera. CMRL-based medium supplemented with 10% fetal bovine serum (FBS) was used from 1 DIV to 3 DIV. An equal volume of CMRL-based medium containing 30% FBS was added at 3 DIV to raise the final FBS concentration to 20%. After attachment at 4 DIV, medium was replaced with CMRL medium supplemented with 10% FBS and 30% rat IC (immediately centrifuged) serum [Charles River Laboratories Japan, Yokohama]. CMRL medium supplemented with 50% rat IC serum was used from 5 DIV and was exchanged daily. All media were placed in an incubator for 1-2 hours before feeding to adjust temperature and pH. Rat IC serum was supplemented with 2 mg/ml glucose. Stirred culture supported normal development beyond egg-cylinder stage.

Preparation of rodent ESC / iPSC lines

A *DsRed*-expressing mouse ESC line (EB3DR), a *tdTomato*-expressing rat ESC line (BLK-RT2) (Kobayashi et al., 2012), and a rat iPSC line (T1-3) (Hamanaka et al., 2011) were assayed. T1-3 cells were labeled with tdTomato as were hiPSCs. These rodent PSC lines were cultured in mouse ESC medium (DMEM / F12: Neurobasal medium supplemented with 1% volume of fraction V bovine serum albumin, 2 mM Glutamax, 1% NEAA, 0.5% N2 supplement, and 1% B27 supplement [all Life Technologies], and 1,000 U/ml of mouse LIF [Millipore, MA], 1 μM of CHIR99021, and 1 μM of PD0325901. Rat ESC and iPSC lines were maintained on mitomycin-C-treated MEF-coated dishes. Mouse ESCs were maintained on 0.1% gelatin-coated dishes.

Preparation of rat EpiSC lines

The rat EpiSC line was derived from a BLK-RT2-injected interspecies chimera developed *in vivo*, and was established from a BLK-RT2-injected interspecies chimeric E6.5 embryo. Rat EpiSCs that expressed tdTomato were purified by cell sorting. Rat EpiSCs were maintained in hESC medium on mitomycin-C-treated MEF feeder cells.

Preparation of nonhuman primate ESC lines

We used an established CMK6 cynomolgus monkey ESC line (Suemori et al., 2001). Cell culture and labeling with *tdTomato* were performed as with hiPSCs.

Immunohistochemistry for primordial germ cells

Frozen sections were made from genital ridges obtained from E13.5 EpiSC-sub cells derived chimeric embryos. The sections were incubated with anti-Mvh antibody (ab13840; 1:200 dilution; Abcam, Cambridge, UK), were washed with PBS, and then were stained with Alexa Fluor 647 conjugated anti-rabbit IgG antibody (Life Technologies) to mark primordial germ cells. Nuclei were stained with DAPI (Wako Pure Chemicals). Fluorescence microscopy was performed using a BZ-9000 microscope (Keyence, Osaka, Japan).

Flow-cytometry analysis

For flow-cytometry analysis, cells were dissociated into single cells using Accutase (Innovative Cell Technologies, San Diego, CA), and then stained with APC-conjugated anti-mouse CD31 antibody (390; BioLegend, San Diego, CA), isotype control (R35-95; BD Biosciences, San Jose, CA), or anti-SSEA1 antibody (MC-480; eBioscience, San Diego, CA) antibody. The stained cells were analyzed using a FACSCanto flow cytometer (BD Biosciences).

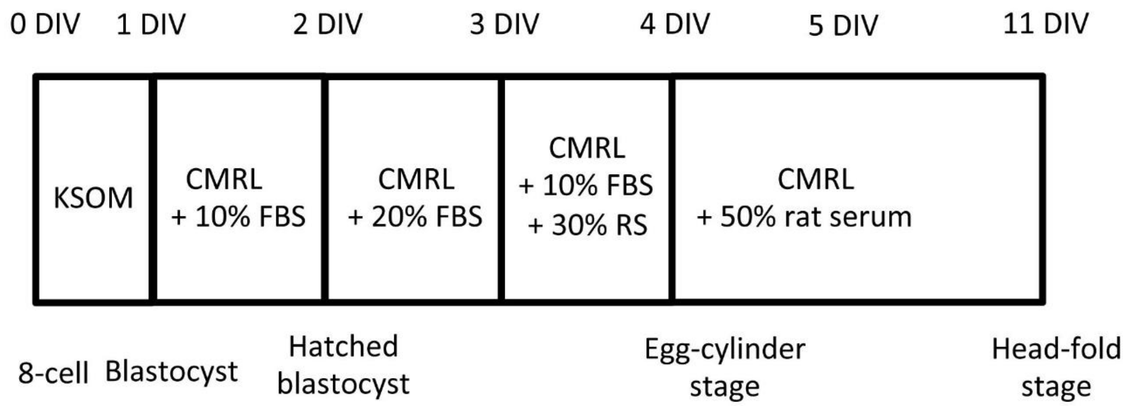

Fig. S1. Stepwise culture conditions for *in vitro* development of mouse pre-implantation embryos

The figure illustrates culture media and expected developmental stages at each culture day.

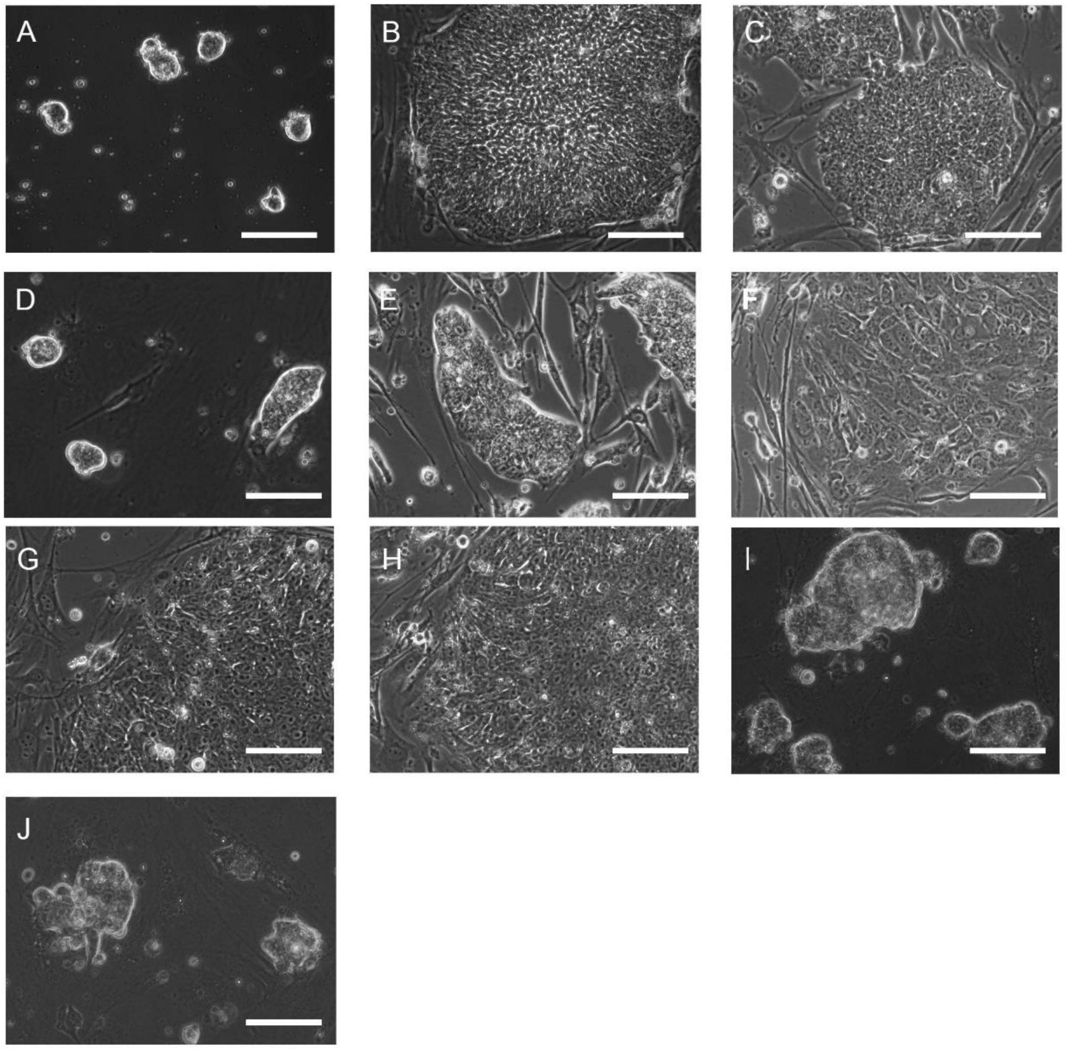

Fig. S2. Appearances of engrafted cell lines

Brightfield images of the lines used in the assay. (A) EB3DR mouse ESC. (B) Parental mouse EpiSC of EpiSC-sub line. (C) EpiSC-sub line. (D) BLK-RT2 rat ESC. (E) BLK-RT2 EpiSC. (F) CMK6 monkey ESC. (G) Parental human iPSC of DR-hiPSC. (H) DR-hiPSC. (I-J) Human naïve-like PSCs cultured with 2iL and Dox (I), or with 2iL and PKCi 2 weeks after withdrawal of Dox (J). Note that EpiSC-sub cells and DR-hiPSCs are morphologically similar to their respective parental cells. Scale bar indicates 100 μm.

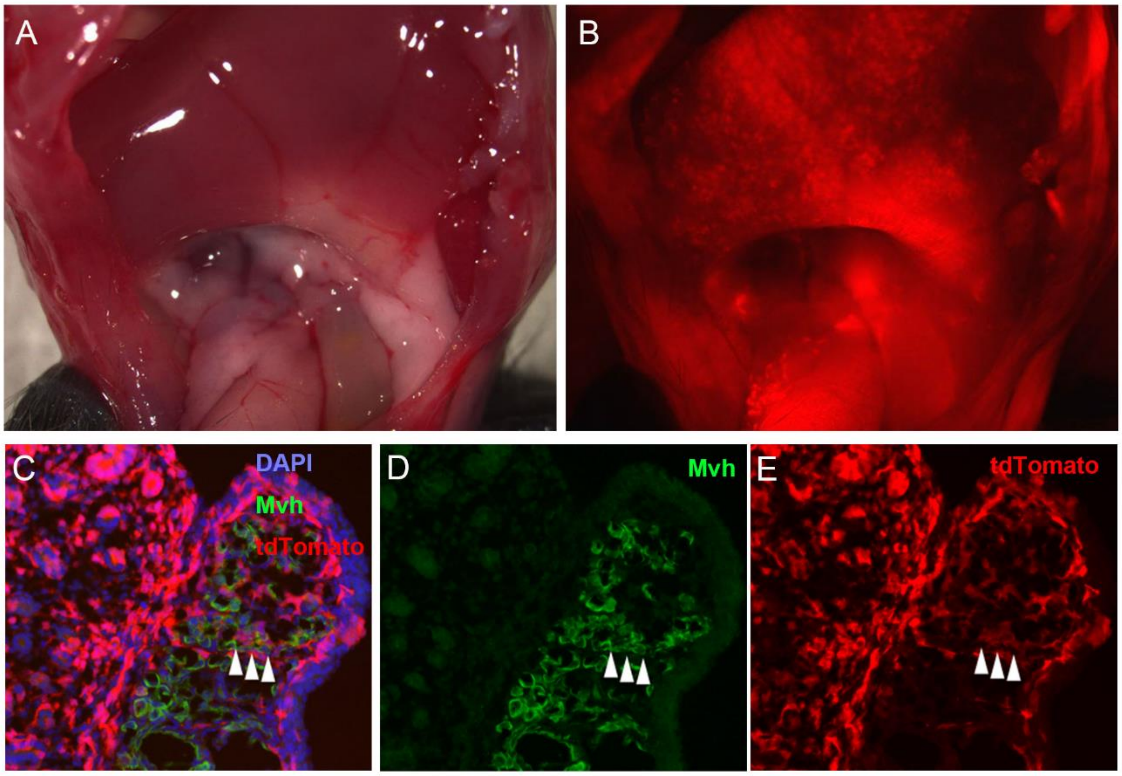

Fig. S3. Distribution of EpiSC-sub cells-derived progeny

(A, B) Chimeric offspring were sacrificed at postnatal day 14 and analyzed for the distribution of tdTomato-labeled descendants of EpiSC-sub cells. Fluorescence images indicate that EpiSC-sub cells contributed to bone, skeletal muscle, liver, pancreas, and most observed organs (B). (C-E) To clarify germline contribution, frozen sections of genital ridges obtained from E13.5 chimeric embryos were stained using antibody against Mvh, a PGC marker. (C) Overlaid image of DAPI staining, Mvh antibody staining (D), and tdTomato expression (E). Arrowheads indicate co-localization of tdTomato expression and Mvh staining.

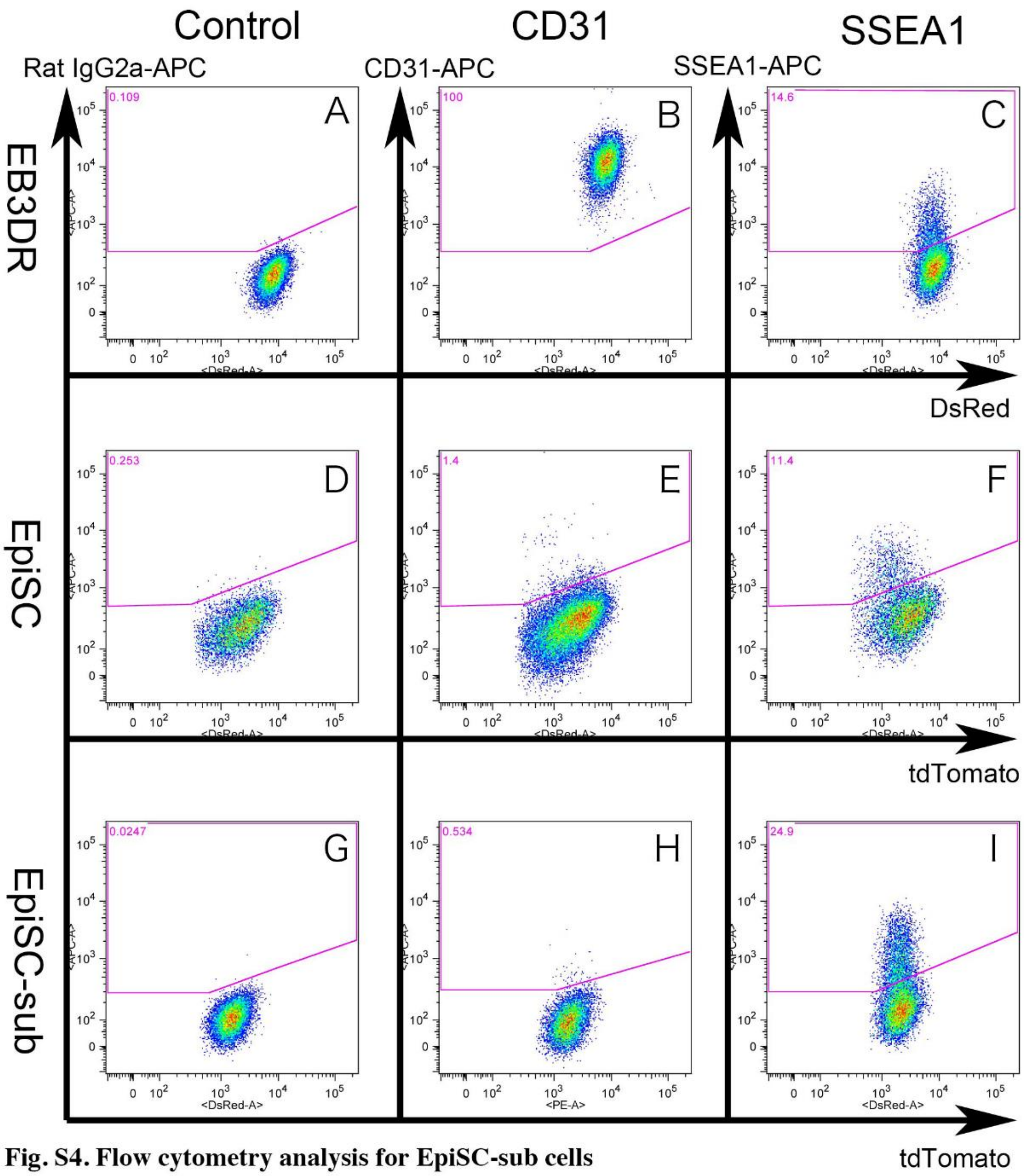

Fig. S4. Flow cytometry analysis for EpiSC-sub cells

EB3DR mouse ESCs (A-C), parental EpiSCs (D-E), and EpiSC-sub cells were stained with APC-conjugated anti-mouse CD31 antibody (B, E, H) or anti-SSEA1 antibody (C, F, I). Indicated controls (A, D, G) were stained with APC-conjugated isotype control antibody for anti-mouse CD31 antibody.

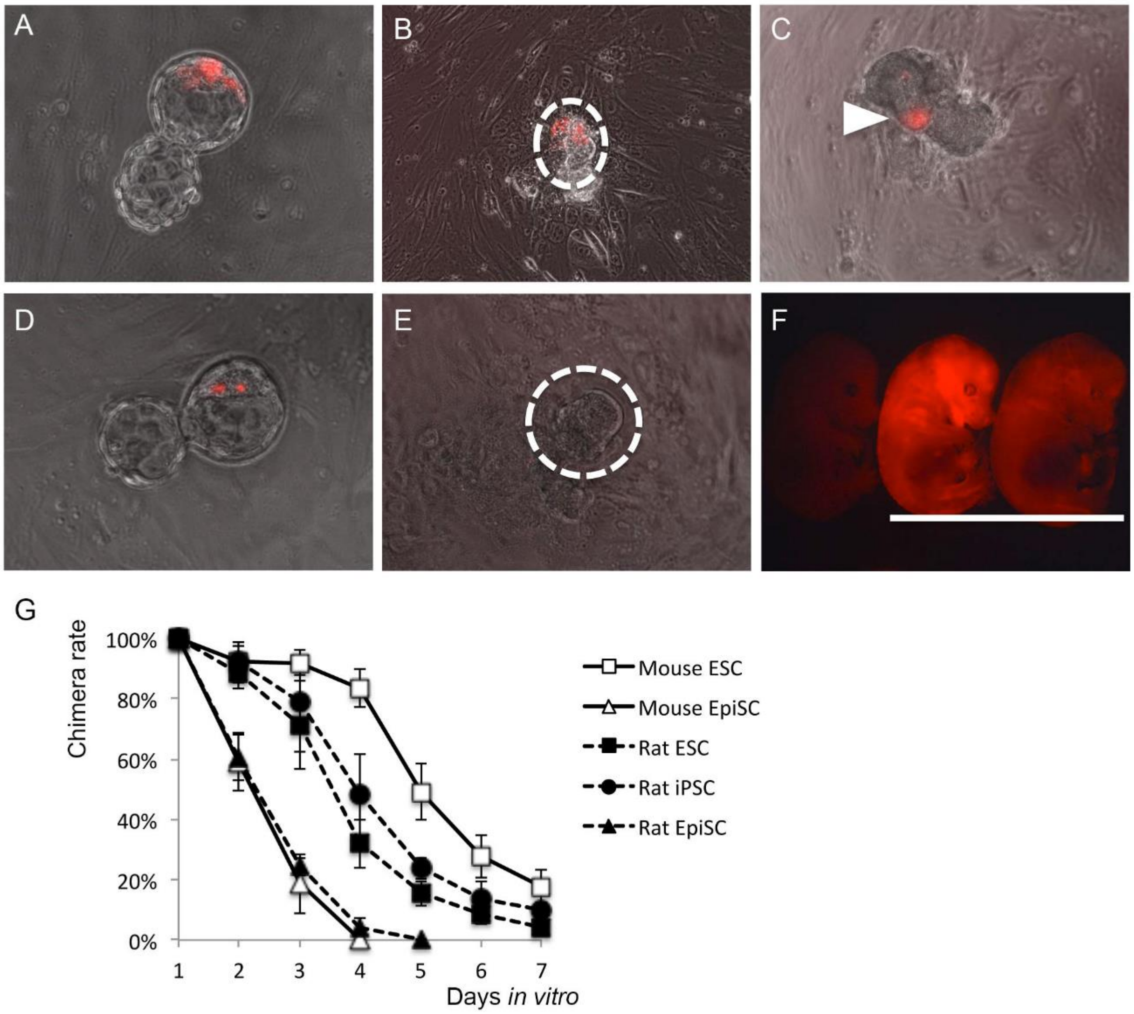

Fig. S5. Interspecies in vitro chimera assay: Rat PSCs.

To clarify whether our in vitro chimera assay results held for interspecies chimeras, rat ESCs, iPSCs, and EpiSCs were tested for chimera formation ability in vitro. (A-E) Overlaid images show the distribution of engrafted rat ESC- (A-C) or EpiSC (D, E) -derived cells in red at 2 DIV (A, D), 4 DIV (B, E), and 7 DIV (F). (F) Fluorescence-microscopy image of rat ESC-derived interspecies chimera developed in vivo. (G) Plots indicate proportion of rat ESCs-, iPSCs-, or EpiSCs-injected chimeric embryos compared with mouse ESC- or EpiSC-injected embryos at each culture day.

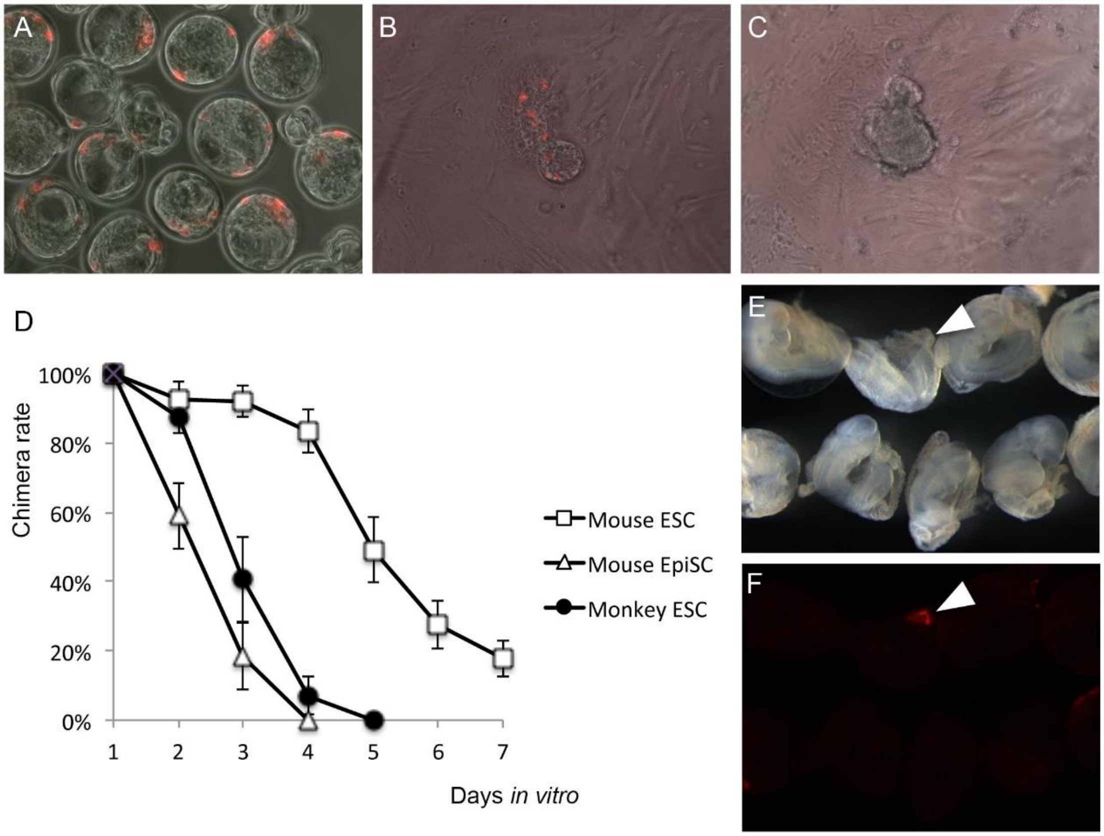

Fig. S6. Interspecies in vitro chimera assay: Monkey PSCs.

To clarify whether interspecies-in vitro chimera assay results held for primates, monkey ESC-line CMK6 cellslabeled with tdTomato were subjected to interspecies-in vitro chimera assay. (A-C) Overlaid images show the distribution of monkey ESC-derived cells in red at 2 DIV (A), 4 DIV (B), and 6 DIV (C). (D) Plots indicate proportion of CMK6-derived chimeric embryos among started embryos at each culture day. (E, F) In vivo chimera assay was also performed for CMK6 cells. E8.5 embryos were observed by brightfield (E) and fluorescence (F) microscopy. Arrowhead indicates autofluorescent placental tissue.

Table S1. Results of in vitro chimera assay with mouse embryos

Mouse ESC

	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate
1DIV	50	100%	21	100%	20	100%	30	100%
2DIV	49	98%	20	95%	18	90%	26	87%
3DIV	48	96%	20	95%	18	90%	26	87%
4DIV	45	90%	18	86%	15	75%	25	83%
5DIV	31	62%	10	48%	8	40%	14	47%
6DIV	18	36%	5	24%	6	30%	6	20%
7DIV	11	22%	4	19%	4	20%	3	10%

Mouse EpiSC

	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate
1DIV	40	100%	30	100%	31	100%	28	100%	33	100%
2DIV	23	58%	15	50%	17	55%	21	75%	19	58%
3DIV	5	13%	4	13%	5	16%	10	36%	5	15%
4DIV	0	0%	0	0%	0	0%	0	0%	0	0%
5DIV	0	0%	0	0%	0	0%	0	0%	0	0%
6DIV	0	0%	0	0%	0	0%	0	0%	0	0%
7DIV	0	0%	0	0%	0	0%	0	0%	0	0%

EpiSC-sub

	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate
1DIV	30	100%	30	100%	33	100%
2DIV	25	83%	27	90%	24	73%
3DIV	20	67%	21	70%	24	73%
4DIV	15	50%	13	43%	21	64%
5DIV	8	27%	7	23%	12	36%
6DIV	3	10%	4	13%	5	15%
7DIV	2	7%	3	10%	4	12%

Rat ESC

	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate
1DIV	36	100%	30	100%	27	100%	40	100%	24	100%
2DIV	31	92%	28	93%	24	89%	36	90%	19	79%
3DIV	27	75%	25	83%	21	78%	30	75%	11	46%
4DIV	8	22%	11	37%	11	41%	14	35%	6	25%
5DIV	5	8%	5	17%	5	19%	7	18%	4	17%
6DIV	2	6%	3	10%	3	11%	5	13%	1	4%
7DIV	2	6%	1	3%	2	7%	2	5%	0	0%

Rat iPSC

	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate
1DIV	50	100%	50	100%	24	100%	27	100%
2DIV	47	94%	41	82%	23	96%	26	96%
3DIV	37	74%	29	58%	23	96%	24	89%
4DIV	21	42%	16	32%	15	63%	15	56%
5DIV	11	22%	11	22%	7	29%	6	22%
6DIV	4	8%	8	16%	5	21%	3	11%
7DIV	2	4%	6	12%	4	17%	2	7%

Rat EpiSC

	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate
1DIV	23	100%	27	100%	24	100%
2DIV	12	52%	18	67%	15	63%
3DIV	6	26%	7	26%	5	21%
4DIV	0	0%	2	7%	1	4%
5DIV	0	0%	0	0%	0	0%
6DIV	0	0%	0	0%	0	0%
7DIV	0	0%	0	0%	0	0%

Monkey ESC

	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate
1DIV	36	100%	40	100%	40	100%	40	100%
2DIV	32	89%	33	83%	34	85%	31	78%
3DIV	11	31%	13	33%	19	48%	12	30%
4DIV	2	6%	0	0%	4	10%	2	5%
5DIV	0	0%	0	0%	0	0%	0	0%
6DIV	0	0%	0	0%	0	0%	0	0%
7DIV	0	0%	0	0%	0	0%	0	0%

Human iPSC

	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate
1DIV	40	100%	34	100%	50	100%	36	100%	54	100%
2DIV	33	83%	29	85%	43	86%	32	89%	44	81%
3DIV	11	28%	16	47%	12	24%	13	36%	13	24%
4DIV	5	13%	7	21%	3	6%	5	14%	3	6%
5DIV	0	0%	0	0%	0	0%	0	0%	0	0%
6DIV	0	0%	0	0%	0	0%	0	0%	0	0%
7DIV	0	0%	0	0%	0	0%	0	0%	0	0%
	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate
1DIV	35	100%	30	100%	26	100%	20	100%	20	100%
2DIV	31	89%	19	63%	19	73%	13	65%	14	70%
3DIV	23	66%	7	23%	9	35%	3	15%	6	30%
4DIV	3	9%	0	0%	4	15%	0	0%	0	0%
5DIV	0	0%	0	0%	0	0%	0	0%	0	0%
6DIV	0	0%	0	0%	0	0%	0	0%	0	0%
7DIV	0	0%	0	0%	0	0%	0	0%	0	0%

DR-hiPSC

	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate
1DIV	50	100%	28	100%	30	100%	26	100%	26	100%
2DIV	40	80%	21	75%	25	83%	19	73%	19	73%
3DIV	18	36%	11	39%	13	43%	15	58%	9	35%
4DIV	6	12%	4	14%	2	7%	11	42%	4	15%
5DIV	3	6%	1	4%	0	0%	6	23%	0	0%
6DIV	0	0%	0	0%	0	0%	0	0%	0	0%
7DIV	0	0%	0	0%	0	0%	0	0%	0	0%

Reset cell

	No. of chimera	Chimera rate	No. of chimera	Chimera rate	No. of chimera	Chimera rate
1DIV	40	100%	40	100%	41	100%
2DIV	33	83%	31	78%	31	76%
3DIV	13	33%	16	40%	18	44%
4DIV	4	10%	5	13%	2	5%
5DIV	0	0%	0	0%	0	0%
6DIV	0	0%	0	0%	0	0%
7DIV	0	0%	0	0%	0	0%

Table S2. Results of *in vivo* chimera assay with mouse embryos

Graft cell type	Grafted cell line	Host embryos	Recipients	Analyzed stage	Pups (Fetus)	Chimera	Chimera rate
mouse ESC	EB3DR-P25	60	3	Neonate	13	10	77%
	EB3DR-P27	20	1	E9.5	11	8	73%
	EB3DR-P27	20	1	E13.5	9	6	67%
mouse iPSC	GT3.2-P18	40	2	E13.5	21	16	76%
	GT3.2-P19	80	4	Neonate	22	12	55%
	GT3.2-P19	80	4	Neonate	26	14	54%
	GT3.2-P21	80	4	Neonate	23	16	70%
	GT3.2-P21	80	4	Neonate	21	13	62%
mouse iPSC	EB3DR-EpiSC-P8+3	40	2	E9.5	26	0	0%
	BDF1-EpiSC-tdT-P5+2	20	1	E9.5	11	0	0%
	BDF1-EpiSC-tdT-P5+4	20	1	E7.5	15	0	0%
	BDF1-EpiSC-tdT-P5+4	20	1	E6.5	10	0	0%
	BDF1-EpiSC-tdT-P5+7	20	1	E9.5	12	0	0%
	EpiSC-tdT-P35+15	40	2	E9.5	21	0	0%
mouse EpiSC subclone	EpiSC-sub-P35+7	20	1	E9.5	17	4	24%
	EpiSC-sub-P35+8	20	1	E9.5	20	7	35%
	EpiSC-sub-P35+8	20	1	Neonate	8	3	38%
	EpiSC-sub-P35+15	40	2	E9.5	21	8	38%
rat ESC	BLK-RT2-P24	80	4	Neonate	15	7	47%
	BLK-RT2-P25	20	1	E9.5	12	4	33%
rat iPSC	riPSC-WI-T1-3-P24	80	4	Neonate	17	7	41%
rat EpiSC	BLK-RT2-EpiSC-P6	40	2	E9.5	25	0	0%
	BLK-RT2-EpiSC-P7	17	1	E9.5	12	0	0%
	BLK-RT2-EpiSC-P8	32	2	E9.5	22	0	0%