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

Hideki Masaki1,2,§, Megumi Kato-Itoh1,2, Ayumi Umino1,2, Hideyuki Sato1,2, Sanae Hamanaka1,2, Toshihiro Kobayashi1,2,¶, Tomoyuki Yamaguchi1,2, Ken Nishimura3,4,‡, Manami Ohtaka3, Mahito Nakanishi3 and Hiromitsu Nakauchi1,2,4,§

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

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.
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 hiPSCs 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.
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 trophoectoderm (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 naive-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 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.](image-url)
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...
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 mesodermal 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 embryos.

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.
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 andreset cells as non-chimera-forming 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 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 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 and molecules: rodent gastrulae uniquely form cylinders, whereas gastrulae of most other species form disks, and many growth factors

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 hiPSCs.

**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 EB13DR-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 L21+Dox and reset cells cultured with L21+PKCi after withdrawal of Dox for 14 days. Harvested RNA was processed into cRNA and hybridized against a SurePrint G3 Hymann 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. Kyono 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.

Acknowledgements

We thank Dr A. Kinski, Y. Yamazaki, A. Fujita, Y. Ishii, Dr S. Kaneko and Dr M. Watanabe for supporting this work; Dr H. Niwa for providing the EB13DR mouse ESC line; Dr H. Suemori for providing the CMK6 monkey ESC line; and Dr N. Osumi for technical advice.

Competing interests

H.N. is a founder and shareholder of iCELL and ChimERA, 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.

Funding

This work was supported by grants from the Japan Science and Technology Agency (JST), Exploratory Research for Advanced Technology (ERATO), Nakauchi Stem Cell and Organ Regeneration Project, Tokyo, Japan.

Supplementary material

Supplementary material available online at http://dev.biologists.orglookup/suppl/doi:10.1242/dev.124016/-/DC1

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