

Chicken Interspecies Chimerism Unveils Human Pluripotency

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SUMMARY

Human pluripotent stem cells (hPSCs) are commonly kept in a primed state but also able to acquire a more immature naive state under specific conditions *in vitro*. Acquisition of naive state changes several properties of hPSCs and might affect their contribution to embryonic development *in vivo*. However, the lack of an appropriate animal test system has made it difficult to assess potential differences for chimera formation between naive and primed hPSCs. Here, we report that the developing chicken embryo is a permissive host for hPSCs, allowing analysis of the pluripotency potential of hPSCs. Transplantation of naive-like and primed hPSCs at matched developmental stages resulted in robust chimerism. Importantly, the ability of naive-like but not of primed hPSCs to form chimera was substantially reduced when injected at non-matched developmental stages. We propose that contribution to chick embryogenesis is an informative and versatile test to identify different pluripotent states of hPSCs.

INTRODUCTION

Pluripotent stem cells (PSCs) are classically defined by their capacity to generate the entire set of somatic and germline cell types existing in the human body (Hassani et al., 2019). The use of PSCs has revolutionized research in developmental biology and disease modeling and paved the way for new advances in regenerative medicine (Abbasalizadeh and Baharvand, 2013; Martyn et al., 2018). It is important to validate the capability of human PSCs (hPSCs), including embryonic stem cells (hESCs) and induced PSCs (hiPSCs), to differentiate into the three embryonic germ layers. The gold standard for testing pluripotency of mouse ESCs (mESCs) is the creation of allogeneic chimeras, which is not possible in humans, requiring the need to develop hPSCs in a xenogeneic environment for generation of interspecies chimeras (Mascetti and Pedersen, 2016b).

Thus far, most studies relied on mice as hosts for evaluating the competency of hPSCs to form chimeras. However, only a few of these experiments have been successful (Theunissen et al., 2016; Wu et al., 2017). Numerous efforts were made for improving the efficiency of hPSCs to form interspecies chimeras with mice or other mammals (Wu et al., 2016). A crucial factor for success is matching of the developmental stages of hPSCs with the host embryo. hPSCs are in a phase of pluripotency primed to differentiation, preventing functional integration into mouse blastocysts, which is not a problem for inner cell mass-like naive

PSCs, such as rodent ESCs and iPSCs (Chen et al., 2015; James et al., 2006; Masaki et al., 2015). Similar to mouse post-implantation epiblast stem cells (EpiSCs), integration of hPSCs into mouse cultured early gastrula-stage embryos seems to indicate that stage matching (SM) is a crucial determinant to accomplish robust xenogeneic chimerism (Hassani et al., 2019). To further prove the hypothesis that SM between donor hPSCs and host embryos is crucial for generation of interspecies chimeras (Cohen et al., 2018; Mascetti and Pedersen, 2016a; Wu et al., 2015), hPSCs were developed that resemble rodent naive PSCs (Wu et al., 2017). However, derivation of authentic naive hPSCs has been challenging, requiring the need to overcome several technical problems, such as single-cell cloning efficiency, scalable cell expansion, and enhanced directed differentiation (Taei et al., 2020). Despite that, some studies showed that naive-like hPSCs can develop in early mammalian embryos to form interspecies chimera, although with relatively low efficiency (Gafni et al., 2013a; Wu et al., 2017; Yang et al., 2017a). On the other hand, some studies indicated that interspecies chimerism can be achieved with non-stage-matching (NSM) rat EpiSCs or primed hPSCs and mouse pre-implantation embryos when the survival of primed cells was enhanced by overexpression of anti-apoptotic genes. These findings questioned the necessity of an SM approach (Huang et al., 2018; Masaki et al., 2016; Wang et al., 2018).

The evolutionary distance between the host and donor cells is another important factor for efficient interspecies





chimerism that needs to be considered (Masaki et al., 2015; Wu et al., 2016). Pre-implantation embryos from ungulates, which are evolutionary closer to humans, might be more efficient for generation of interspecies chimera when using naive and intermediate (a pluripotent state between naive and primed states) hPSCs (Wu et al., 2017). Ungulates might also offer additional advantages, such as production of human organs through blastocyst complementation, when distinct cell lineages of the host are compromised (Wu et al., 2017). However, these approaches are neither widely accessible, nor particularly versatile, to assess the pluripotency potential of each hPSC line.

Here, we investigate whether similarities between different species in gastrulation, the most critical stage for lineage differentiation, might enable efficient engraftment and lineage contribution of hPSCs of different pluripotent states. We chose chicken embryos as hosts assuming that the high degree of similarity between the gastrulating chicken embryo and early human embryo in the primitive streak (PS), notochord, and neural tube stages might provide an appropriate xenogeneic environment (Boulland et al., 2010; Goldstein, 2010; Goldstein et al., 2002). We observed efficient interspecies chimerism of primed and naive-like hPSCs in chicken embryos. Our study introduces the chicken embryo as a powerful and welcoming host for analysis of different states of human pluripotency.

RESULTS

Optimization of hPSC Injections at Different Stages of Chicken Embryogenesis

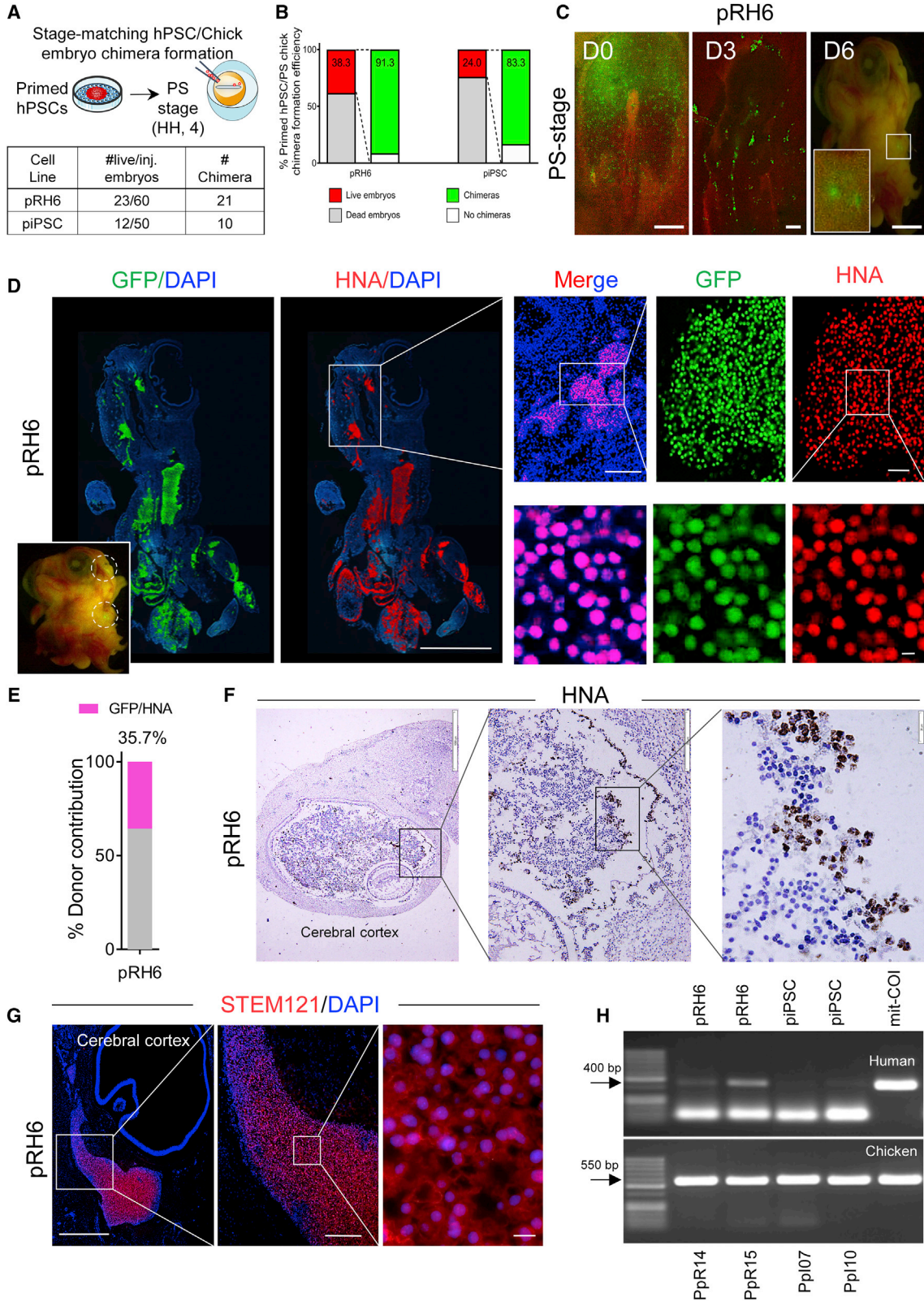
At the time of laying, fertilized chicken eggs are in the blastodisc (BLD) stage with the capability of continuous growth under *in ovo* conditions when incubated at 37.5°C (Hamburger and Hamilton, 1951). After 19–24 h of incubation, the chick embryo progresses to the PS stage. Based on early embryonic development in other animals, we assumed that the time between onset of development until the peri-gastrulation process represents a window of opportunity to introduce PSCs of different states of pluripotency. Following an SM approach for chimera formation (Cohen et al., 2018), we injected primed hPSCs into PS and naive-like hPSCs into BLD stages of chicken embryos. Naive-like hPSCs were generated using NHSM culture conditions, which were reported as naive-like cells that could contribute in mouse embryo (Gafni et al., 2013a) and alternatively by our recently developed protocol for the conversion of primed hPSCs toward naive-like hPSCs. The latter approach relies on the use of agonists for the nuclear receptors LRH-1 and RAR γ , combined with 2i and LIF (2a2iL) (Taei et al., 2020).

To determine to what extent a 1-day chick embryo tolerates manipulations, we initially injected 8–10 μ L of cell-free primed culture medium around the Henson's node of 20 embryos at different stages of elongating PS, which includes the initial, intermediate, and definitive stages, corresponding to Hamburger and Hamilton (HH) 2 to 4 (6–19 h post-incubation). Injected embryos were incubated for further development for 7 days using a surrogate egg shell platform (Farzaneh et al., 2017). Chicken embryos at early stages of PS proved to be rather sensitive to injections. At HH2 and HH3, we only observed a survival of 5% and 15%, respectively. However, embryos at the fully elongated PS stage (HH4) showed a relatively high survival rate (45%) after the injection (Table S1). We next determined the survival rate of chicken embryos after injection of primed hPSCs into different stages of the developing PS ($n = 50$ embryos). hPSCs ($3\text{--}5 \times 10^3$) (hESCs, primed RH6; pRH6) suspended in 8–5 μ L culture medium were engrafted into HH2, HH3, and HH4 PS stages (Table S1). Since anti-apoptotic factors improve interspecies chimera formation of primed pluripotent cells (Huang et al., 2018; Masaki et al., 2016; Wang et al., 2018), the cell suspension was supplemented with 10 μ M Y27632 (ROCK inhibitor). Similar to the results obtained for injection of medium, 40% of chicken embryos injected at HH4 survived, while survival after injection of HH2 and HH3 was poor (6% and 14% survival, respectively) (Table S1). These findings suggested that HH4 chicken embryos at the fully elongated PS stage are suitable hosts for cell injections.

Similar experiments were performed with BLD-stage chicken embryos (stage X; nearly 20 h uterine age or freshly laid eggs based on the Eyal-Giladi and Kochav morphological staging system). We found that 46.7% of chicken embryos ($n = 30$) injected with 3–5 μ L cell-free naive culture media survived, suggesting that BLD-stage chicken embryos show a higher tolerance for injections compared with PS-stage embryos (Table S2). Accordingly, we observed that 41.7% of chicken embryos injected at the BLD stage (stage X) with $0.5\text{--}1 \times 10^3$ naive 2a2iL-induced RH6 (2a2iL-RH6) survived for 6–7 days. The same experiment using NHSM-induced RH6 (NHSM-RH6) cells yielded a survival rate of 35% ($N = 60$, each) (Table S2). The results indicate that stage-matched (BLD and PS) chicken embryos accept naive and primed hPSCs, survive, and continue to develop.

Primed hPSCs Contribute Robustly to Formation of Chicken Chimera when Injected at the PS Stage

To track injected hPSCs in the developing chick embryo, we labeled pRH6 cells with enhanced green fluorescent protein (eGFP). Pluripotency of GFP-expressing pRH6 cells was confirmed by morphological criteria and by NANOG expression after serial passaging (Figure S1A). GFP-labeled



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pRH6 cells ($3\text{--}5 \times 10^3$) supplemented with Y26732 were injected into HH4 chicken embryos ($n = 60$). Assessment at 6–8 days of development revealed that 60% of the embryos exhibited growth-retarded morphology (Figures 1A and S1B). A total of 38.3% of the embryos showed beating hearts, demonstrating that they were still alive (Figures 1B and S1C). Notably, surviving chimeric embryos exhibited different malformations after extended incubation at day 6 (D6), including changes in head size, lack of the prominence of the telencephalon area, reduced eye size, changes in the oral area, lack of chicken tip formation, malformation of the anterior limbs, and lack of tail formation (Figure S1C). The type and degree of malformations varied strongly among individual chimeric embryos.

The transparency of the chick embryos during the first days of incubation allowed tracing of GFP-expressing pRH6 cells during D0, D3, and D6 of development by time-lapse imaging (Figure 1C). After 6 days of incubation, it became difficult to detect the GFP signal, since most fluorescence signals were absorbed by the growing chicken embryo (Figure 1C). However, histological studies demonstrated that 91.3% of live embryos contain extensive numbers of GFP-expressing pRH6 cells at D6 (Figure 1B), which were found throughout the embryos (Figures 1D and S1D). The contribution of pRH6 to developing chicken chimera was confirmed by immunohistochemistry (IHC) or immunohistochemistry (IHC) staining for two human-specific proteins, human nuclear antigen (HNA), and STEM121, which are widely used to detect engraftment of human cells in host animals (Figures 1D, 1F, S1D, and 1G). The HNA signal co-localized with GFP fluorescence and was readily detectable in whole-mount preparations of pRH6-derived chimeras (Figure 1D). Quantification of

GFP/HNA-expressing pRH6-derived cells in sagittal and coronal sections of three independent chimeras revealed a contribution of 35.7% (Figure 1E).

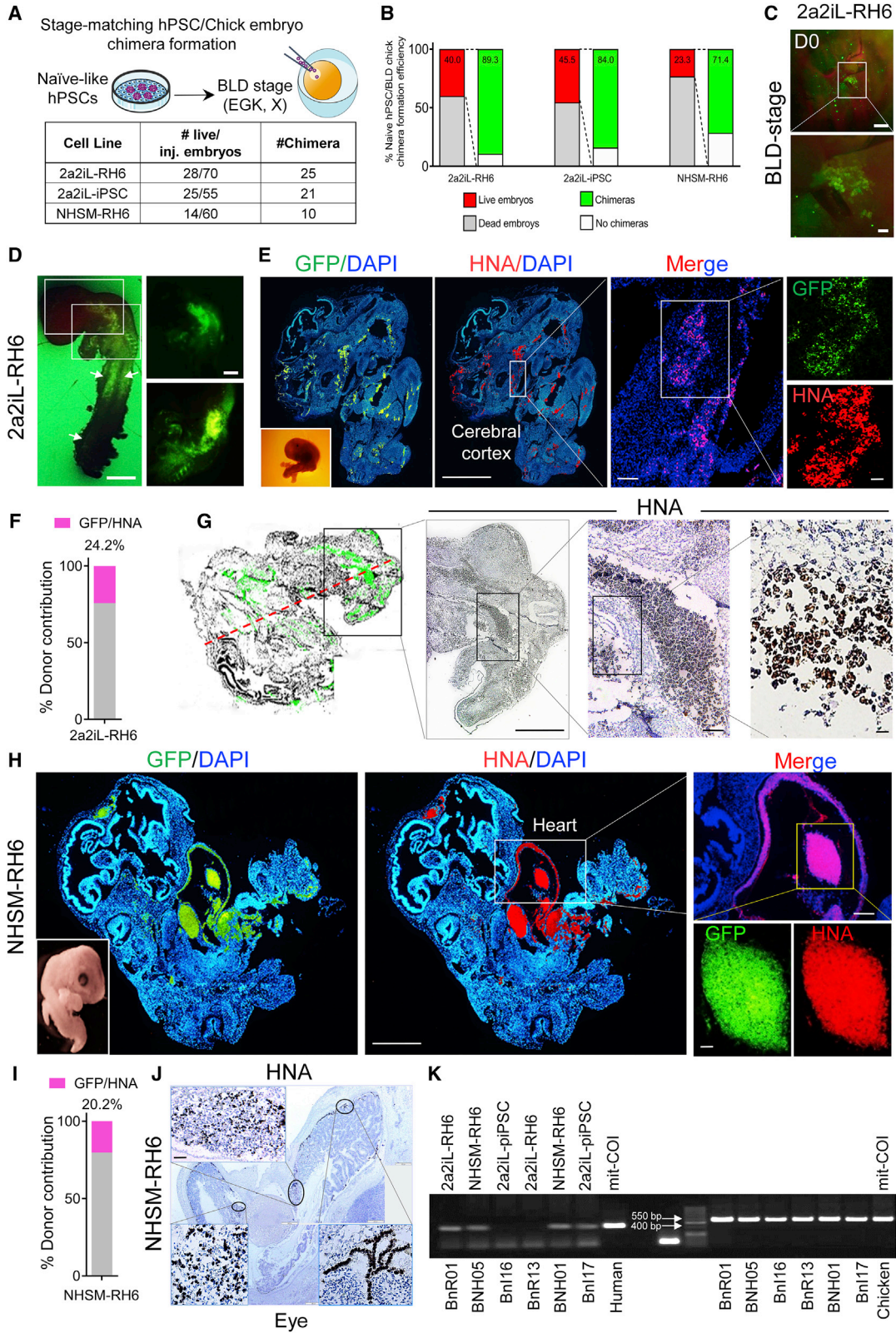
We also investigated interspecies chimera formation using a human primed iPSC line (piPSC; CAG-GFP-chr13-hetero, IP-001-ZIV8-A-1V), which was confirmed to maintain pluripotency (Figure S1E). Twelve out of 50 (24%) embryos injected with piPSCs survived, of which 83.3% displayed chimera formation (Figures 1A and 1B). Quantification of GFP/HNA-expressing cells in whole-body sections from three independent chimeras revealed a 16.3% contribution of piPSC-derived cells (Figures S1F and S1G). Furthermore, we performed species-specific PCR experiments to exclude any potential artifacts associated with fluorescence analysis. We clearly detected the presence of human mitochondrial cytochrome *c* oxidase subunit I (mit-COI) genes, thereby validating the presence of human-derived cells in the chimeras (Figure 1H). Taken together, we demonstrated that primed hPSCs contribute to chimera formation in chicken host embryos at the PS stage, when the developmental stage between donor cells and host embryos were matched. Our results indicate that the PS stage of the chick embryo is able to form interspecies chimeras with different primed hPSCs, confirming the robustness of this model.

Naive-like hPSCs Contribute to Interspecies Chimera Formation when Injected into Blastula Stage Chicken Embryos

Next, we determined whether naive-like hPSC contribute to chimera formation when injected in an SM manner into chicken embryos at the BLD stage. Chimeras showed similar morphological abnormalities as after injection of primed hPSCs at the PS stage. Twenty-eight out of 70

Figure 1. SM Injection of Naive hPSCs into BLD of Chicken Embryos

- (A) Schematic outline of the strategy to generate SM interspecies chimera with primed hPSCs injected into PS-stage embryos with a list of the used hPSC lines and their efficiencies in chimera formation.
- (B) Bar graphs showing the percentage of live (red) and dead embryos (gray) until D6 of development, and the ratio of chimeric (green) and non-chimeric (white) embryos in the surviving cohort.
- (C) Tracing of GFP-labeled pRH6 cells during D0, D3, and D6 of incubation after injection at the PS stage. Scale bars, 2 mm (left and right images) and 1 mm (middle image).
- (D) Representative images of a whole-mount collage from sagittal sections of a GFP-expressing pRH6 chicken chimera (left panel). The morphology is shown in the bright-field image in the lower left corner. All sagittal sections are stained with HNA (middle panel; red). The sequential higher magnifications of a piece of the collage are placed within boxes (right panels). The insets (right lower panel) are zoomed-in images of the areas in the white boxes. Scale bars, 500 μm (left and middle panels), 200 μm (upper right panel: right image), 100 μm (upper right panel: middle and left images), and 50 μm (lower right panel).
- (E) Percentage of donor cell contributions to the chimeras. Cell numbers of three whole-mount collages in three chimeras were counted ($n = 9$).
- (F) IHC analysis of the HNA marker in the chimera. The insets are zoomed-in images of the black boxes. Scale bars, 1,000 μm (left image), 200 μm (middle image), and 20 μm (right image).
- (G) Sections stained with STEM121 antibody (red) show the contribution of pRH6 cells to the chimeras. The insets are zoomed-in images of the white boxes. Scale bars, 500 μm (left panels), 200 μm (middle panel), and 100 μm (right panel).
- (H) Gel electrophoresis image of PCR analyses for species-specific (human or chicken) detection of mit-COI gene in chicken chimeras at D6. PCR analyses were performed for two randomly selected chimeras. All nuclei are stained with DAPI (blue).



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(40%) chick embryos were viable 6 days after receiving naive-like cells, among which 25 (89.3%) were chimeras (Figures 2A and 2B). *In ovo* time-lapse imaging revealed the distribution of 2a2iL-RH6-derived cells in chimeras at D0 and D3 post-incubation (Figures 2C and 2D). We found that GFP-RH6 cells, which were converted from a primed to a naive-like state by the 2a2iL protocol (Figure S2A), contributed massively to the extraembryonic vascular network that surrounds the embryos (Figure S2B). In addition, we detected a broad contribution of 2a2iL-RH6-derived cells to different parts of the embryo in sagittal sections at D6 of development (Figures 2E and S2C), which was further confirmed by co-staining against HNA (Figures 2E and 2G).

The average contribution of GFP/HNA-expressing cells in whole-body sagittal sections was 24.2% (Figure 2F). In particular, we observed a strong presence of 2a2iL-RH6-derived cells in the mesonephros and liver (Figure 2G). Injection of hiPSC cells converted from a primed into a naive-like state by the 2a2iL protocol (2a2iL-iPSCs) (Figure S2D) yielded similar results. A total of 45.5% of injected embryos survived (25 out of 55 embryos), of which 84% (21 embryos) generated interspecies chimeras (Figures 2A and 2B). The contribution of 2a2iL-iPSCs in different regions of chimeras amounted to 22.4%, according to GFP fluorescence and HNA immunostaining (Figures S2E and S2F).

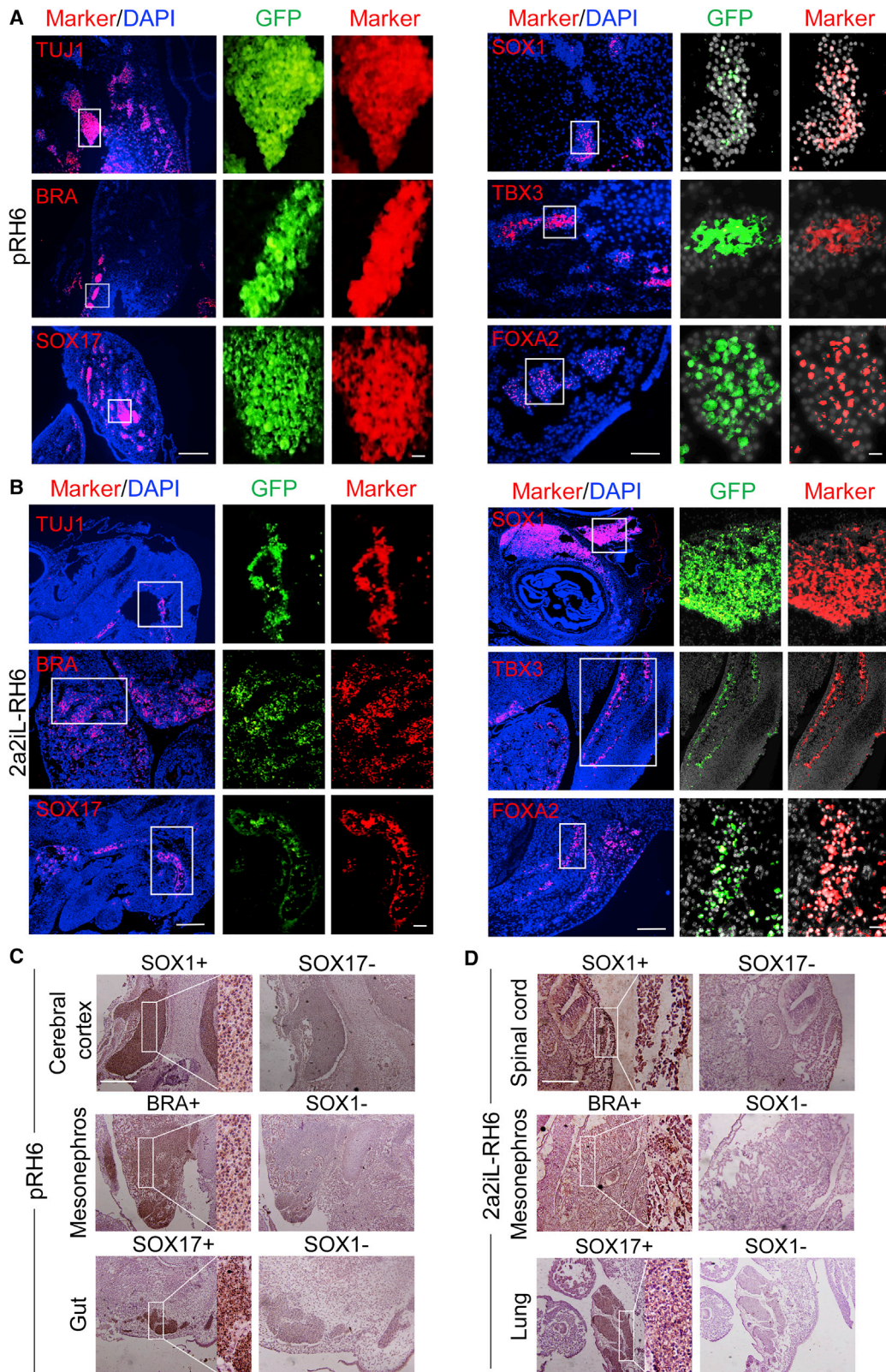
In contrast to hPSCs that were converted to a naive-like state by the 2a2iL protocol, RH6-GFP cells converted by the NHSM protocol contribute as small clusters in some regions of chimeric embryos particularly in heart and eye (Figures 2H and S2H). In total, the contribution reached 20.2% (Figure 2I), which represents a 1.5-fold decline in comparison with 2a2iL-RH6 cells. The presence of NHSM-RH6-derived cells was also confirmed by immunostaining against HNA (Figures 2H and 2J) and by PCR analysis using human- and chick-specific mit-COI sequences (Figure 2K). On average, 14 out of 60 (23.3%) host embryos survived, with 71.4% (10 embryos) forming chimeras (Figures 2A and 2B).

hPSC-Derived Cells Differentiate into Different Cell Lineages in Chicken Chimeras

To identify the fate of injected hPSCs, we stained sections of 6-day-old chimeras with human-specific antibodies against TUJ1 and SOX1, which identify neural derivatives, Brachyury (BRA) and TBX3, which identify mesodermal lineages, and SOX17 and FOXA2 to recognize endodermal cells. Staining of non-chimeric D6 chicken embryos demonstrated that the antibodies do not react with chicken proteins (Figure S3A). IHF analysis of pRH6-based chimeras revealed the presence of immune-reactive human cells in different regions of chicken chimeras

Figure 2. Highly Efficient Interspecies Chimera Formation with Naive-like hPSCs Injected into BLD of Chicken Embryos

- (A) Schematic outline of the strategy to generate SM interspecies chimera using naive-like hPSCs injected into the BLD-stage embryos with a list of hPSC lines and their efficiencies in chimera formation.
- (B) Bar graph showing the percentage of live (red) and dead embryos (gray) until D6 of development, as well as the ratio of chimeric (green) and non-chimeric (white) embryos in the surviving cohort.
- (C and D) Live images of D0 and D6 chicken chimeras derived from injections of 2a2iL-RH6 cells into BLD-stage embryos. Higher magnifications of the white boxes are shown in the right panels. Scale bars, 1 mm (C, upper image; D, right panel), 2 mm (D, left image), and 0.5 mm (C, lower image).
- (E) Representative images of a whole-mount collage from sagittal sections of GFP-expressing 2a2iL-RH6-derived chick chimera (left panel). The morphology is shown in the bright-field image in the lower left corner. All sagittal sections were stained with HNA (middle panel; red). Sequential higher magnifications of a piece of the collage are shown in boxes (right panels). The insets (right lower panel) are the zoomed-in pictures of the areas of the white boxes. Scale bars, 500 μ m (left and middle panels), 200 μ m (right panel: larger image), and 100 μ m (right panel: smaller images).
- (F) Percentage of contribution of donor cells in chimera embryos. Cell numbers of three whole-mount collages in three chimeras were counted ($n = 9$).
- (G) IHF staining for HNA in chimera embryos. The insets are zoomed-in images of the white boxes.
- (H) Representative images of a whole-mount collage from sagittal sections of GFP-expressing NHSM-RH6-derived chimera embryos (left panel). The morphology is shown in the bright-field image in the lower left corner. All sagittal sections were stained for HNA (middle panel; red) showing a presumptive heart. Sequential higher magnifications of a piece of the collage are boxed (right panels). The insets (right lower panel) are the zoomed-in images of the areas of white boxes. Scale bars, 500 μ m (left and middle panels), 200 μ m (upper right panel), and 100 μ m (lower right panel).
- (I) Percentage of contribution by donor cells in NHSM-RH6-derived chimera embryos. Cell numbers of three whole-mount collages in three chimeras were counted ($n = 9$).
- (J) IHF analysis of the HNA marker in embryo chimera showing the contribution of hPSC in eye structure. The insets are the zoomed-in images of the black boxes.
- (K) Gel electrophoresis image of PCR analyses for species-specific detection of mit-COI gene in chicken embryo chimeras at D6. PCR analyses were done for two randomly selected chimers derived from 2a2iL-RH6, 2a2iL-iPSC, or NHSM-RH6. All nuclei were stained with DAPI (blue).



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(Bellairs and Osmond, 2014). We distinguished different tissues and their presumptive organs according our hematoxylin and eosin (H&E) staining of normal chicken embryo with Bellairs Atlas of Chick Development (Figure S3B). TUJ1- and SOX1-positive cells were found in cerebral cortex, BRA in the thoracic, TBX3 in pre-muscles mass of body wall and heart, SOX17 in the liver, and FOXA2 in the cloacal membrane (Figures 3A and S3D). By IHC, we also showed contribution of pRH6 in appropriate regions of chicken chimeras as well as elimination of possible auto-fluorescence signals in IHF results (Figure 3C). According to H&E (Figure S3C) and GFP/HNA (Figures 1D and 2E) staining, no duplicated and independent organs or teratomas were observed. We also detected TUJ1 in the wall of the optic lobe, BRA in the heart, and SOX17 in the esophagus of embryos injected with piPSCs (Figure S3E). We concluded that hPSC derivatives participate in the development of different cell lineages of chicken embryo.

Similar experiments using different naive-like hPSCs essentially showed the same results. TUJ1 expression was detected in the optic lobe, SOX1 in cerebral cortex, and BRA in the mesonephros area. TBX3, SOX17, and FOXA2 signals were found in the pre-muscle cell of wing bud, esophagus, mesentery, and liver of embryos injected with 2a2iL-RH6 cells (Figures 3B and S5A). Contribution of 2a2iL cells in appropriate locations of chick chimeras were verified by IHC (Figure 3D). Regarding H&E and GFP/HNA staining of sections, no duplicated and independent organs or teratomas were detected (Figure S5B). In addition, IHF staining against STEM121 and BRA showed the expression of STEM and lack of BRA expression in the spinal cord region of the chick chimera while its surrounding pre-muscle cells were stained with BRA, indicating that engrafted hPSCs are harmonized with the area in which they are located and they correctly differentiated in appropriate tissue (Figure S4C). TUJ1 expression was observed in the wall of the optic lobe, BRA in the ductus venosus, and SOX17 in the gut of embryos injected with 2a2iL-iPSC cells (Figure S3F). Furthermore, NHSM-hPSCs showed structurally functional participation in the eye, as an ectodermal organ (Figures 2I and S2H), and the mesodermal tissue heart (Figure 2H). To ascertain that antibody staining is specific, and also that engrafted hPSCs differentiated along the

ectodermal lineage of surrounding tissues, we co-stained an OTX2 antibody, which has a cross-species reactivity for human and chick, with human-specific marker, STEM121, and observed that OTX2 showed extensive expression in cranial tissue and rare expression in the engrafted human PSCs (Figure S4D).

In general, both primed and naive-like hPSCs showed increased propensity to participate to head and mesonephros development. We also examined expressions of several other neural markers (PAX6, NF, and Tubulin III) in different chimeras. However, only PAX6 was detectable in the 2a2iL-RH6 chimeras, which might indicate the requirement of extended incubation to allow further maturation (Figures S4E and S4F). We did not observe NANOG-positive cells in any of the hPSC-derived chimeras, demonstrating that injected cells underwent differentiation and lost their initial pluripotency (Figure S4G). Overall, these findings indicated robust contribution and differentiation of both primed and naive-like hPSCs to multiple tissues in the developing chicken embryo.

Validation of the Developmental Potential of hPSCs in Chicken Chimeras Using an ISL1 Reporter Cell Line

To further trace progenies of injected hPSCs in chimeric chicken embryos, we used our recently established ISL1 reporter hPSC line, which was developed by a CRISPR-Cas9-mediated gene-editing approach. hPSC-ISL1 cells efficiently activate the GFP reporter during directed differentiation into cardiac progenitor cells *in vitro* (Z.B., unpublished data; Figure S5). ISL1 is expressed in various cells and organs during mouse embryo development (Zhuang et al., 2013). After injection of primed hPSC-ISL1 (piSL1) cells into PS-stage chick embryos, we observed that 13 out of 45 embryos (28.9%; Figure 4A) survived, of which 10 embryos formed chimeras (76.9% efficiency; Figure 4B). We also injected piSL1 cells into BLD-stage chicken embryos after conversion into the naive state using the 2a2iL protocol. Under these conditions, 14 out of 45 embryos (31.1%) survived (Figure 4A) and formed chimeras with 78.6% efficiency (Figure 4B).

GFP expression from piSL1- and 2a2iL-ISL1-derived chimeras was seen in different locations within the chimeras, including the spinal cord and brain. However, we were

Figure 3. Contribution of hPSCs to Different Tissues of Chick Chimeras

(A) IHF staining with TUJ1, BRA, SOX17, SOX1, TBX3, and FOXA2 human antibodies (red) of D6 chimeras formed by pRH6 injected into PS-stage embryos. Nuclei were stained with DAPI (blue) and GFP. The insets represent zoomed-in images of areas in the white boxes. Scale bars, 500 μ m (merge images) and 100 μ m (GFP and markers).

(B) IHF of D6 chimera formed by 2a2iL-RH6 injected into the BLD-stage embryos using antibodies as in (A). Scale bars, 500 μ m (merge images) and 100 μ m (GFP and markers).

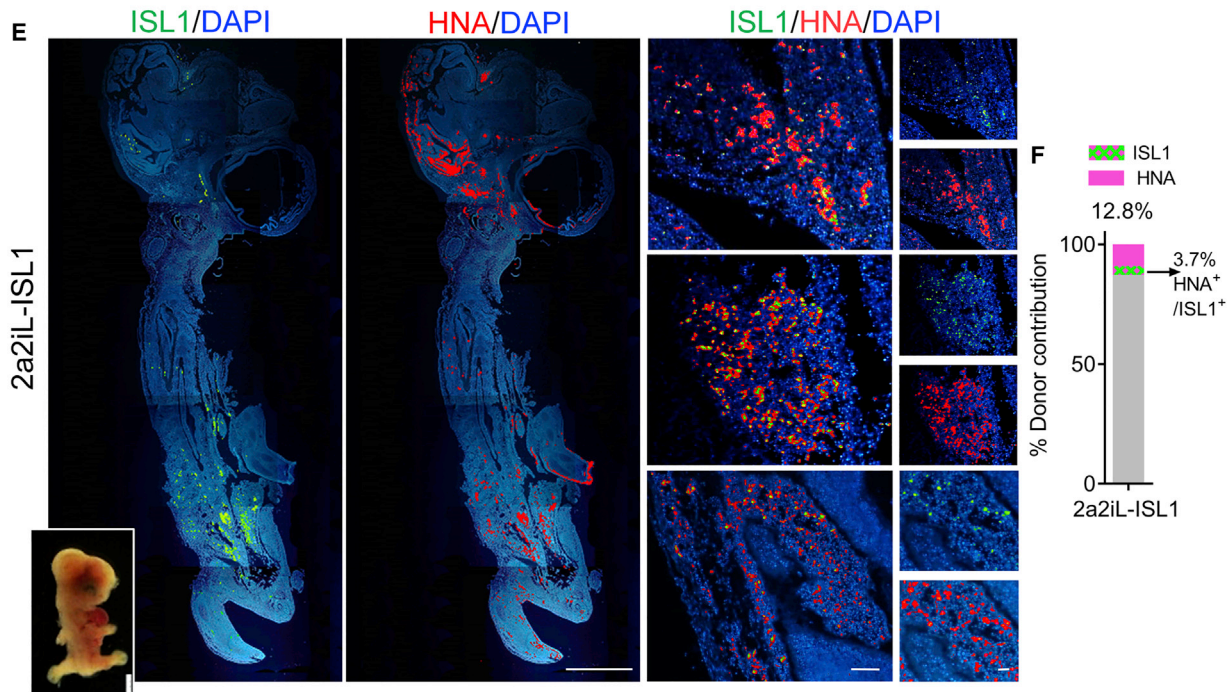
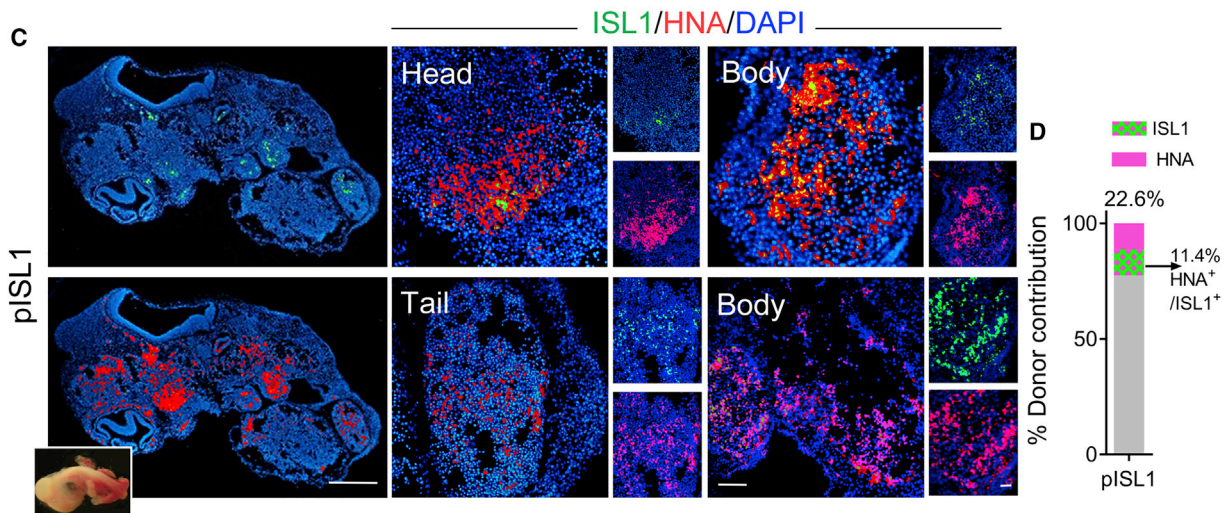
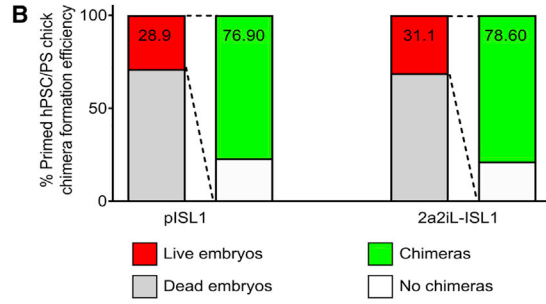
(C) IHC staining of D6 chimeras formed by pRH6 injected into PS-stage embryos with human antibodies against SOX1, BRA, SOX17, and TBX3. Scale bars, 200 μ m.

(D) IHC of D6 chimera formed by 2a2iL-RH6 injected into the BLD-stage embryos using antibodies as in (C). Scale bars, 200 μ m.



A Stage-matching hPSC/Chick embryo chimera formation

| Cell Line | # live/ inj. embryos | # Chimera |
|------------|-------------------------|-----------|
| pISL1 | 13/45 | 10 |
| 2a2iL-ISL1 | 14/45 | 11 |



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unable to determine precisely the nature of GFP-expressing structures due to alterations in embryonic development that correlate with chimerism (Figures 4C and 4E). HNA staining revealed that 22.6% of cells in chimeras were derived from pISL1, of which 11.4% were double-positive for GFP and HNA cells. In 2a2iL-ISL1 chimeras we found 12.8% of HNA-expressing cells, of which 3.7% were double-positive for GFP and HNA cells (Figures 4D and 4F). Although primed and naive-like hPSCs both form chick chimeras (nearly 70%–80% efficiency of chimera formation; Figures 1B, 2B, and 4B) and the degree of chimerism is similar (16%–35% for primed hPSCs and 12%–24% for naive-like hPSCs), we noted clear differences in differentiated progenies in the 6-day-old chick embryo chimeras. At present it is not clear whether there are inherent differences in the potential of naive-like and primed hPSCs to differentiate into the ISL1 cell lineage or whether investigation of different time points will reveal a different picture.

Injection of Primed hPSCs in an NSM Approach Allows Formation of Chicken Chimera

Previous studies indicated that heterochronic chimera formation is feasible when enhancing survival of primed PSCs (Huang et al., 2018; Kang et al., 2018; Masaki et al., 2016; Wang et al., 2018). To test this hypothesis, we injected primed pRH6 and piPSCs into BLD-stage chicken embryos after treatment with Y27632. We observed that 21 out of 60 embryos survived injection of pRH6 cells (35.0% survival rate at D6) and 11 out of 50 injections of piPSC cells (22.0% survival rate at D6). Eleven out of 21 (52.4%) pRH6-injected embryos and 4 out of 11 (36.4%) piPSC-injected embryos formed interspecies chimeras (Figures 5A and 5B). We concluded that the BLD stage supports survival and maintenance of primed hPSCs, although the efficiency is clearly lower when compared with injections of primed hPSCs at the PS stage. IHF analysis by GFP and HNA re-

vealed a broad contribution of both the pRH6 and piPSC progenies to chicken embryogenesis. For example, we observed the presence of pRH6-derived cells in the cranial region and of piPSC-derived cells in the ventricular cortex (Figures 5C and S6A). Quantification revealed a chimerism of 23.7% for pRH6 chimeras and of 9.2% for piPSC chimeras, indicating lower proliferation and incorporation of primed hPSCs after injection at the BLD compared with the PS stage (Figures 5D and S6B).

Further analysis of heterochronic chimera revealed that primed hPSCs participated in the development of different cell lineages, albeit with a lower efficiency. Primed hPSC-derived cells expressed TUJ1 in the cerebral cortex, SOX1 in the cerebral cortex, BRA in the heart, TBX3 in the myotome, SOX17 in the liver, and FOXA2 in the gut (Figure 5E). We also validated the presence of human cells in chimeras using human-specific mit-COI sequences (Figure 5F). The results indicate that primed hPSCs contribute to broad interspecies chimera formation in chicken embryos, even when introduced at non-matched stages, although the contribution is lower compared with matched stages.

Naive-like hPSCs Show Only Limited Chimera Formation when Introduced into PS-Stage Chicken Embryos in an NSM Approach

To complete the study, we investigated whether naive-like hPSCs generate interspecies chimeras when introduced into PS-stage chicken embryos. Two different 2a2iL-induced naive-like hPSCs were injected. We found that 7 out of 50 embryos survived injection of 2a2iL-RH6 cells (14% survival rate at D6) and 6 out of 40 embryos survived injection of 2a2iL-iPSCs (15.0% survival rate at D6) (Figures 6A and 6B). Interestingly, only 2 out of the 7 surviving embryos injected with 2a2iL-RH6 cells and 2 out of 6 embryos with 2a2iL-iPSCs were chimeric (Figures 6A and 6B). GFP fluorescence and IHF staining for HNA confirmed the

Figure 4. The ISL1-Reporter Cell Line Validates the Developmental Potential of hPSCs in Chicken Embryo Chimeras

(A) Summary of results from injections of pISL1 and 2a2iL-ISL1 cell lines into PS and BLD-stage chicken embryos, and the efficiency of chimera formation.

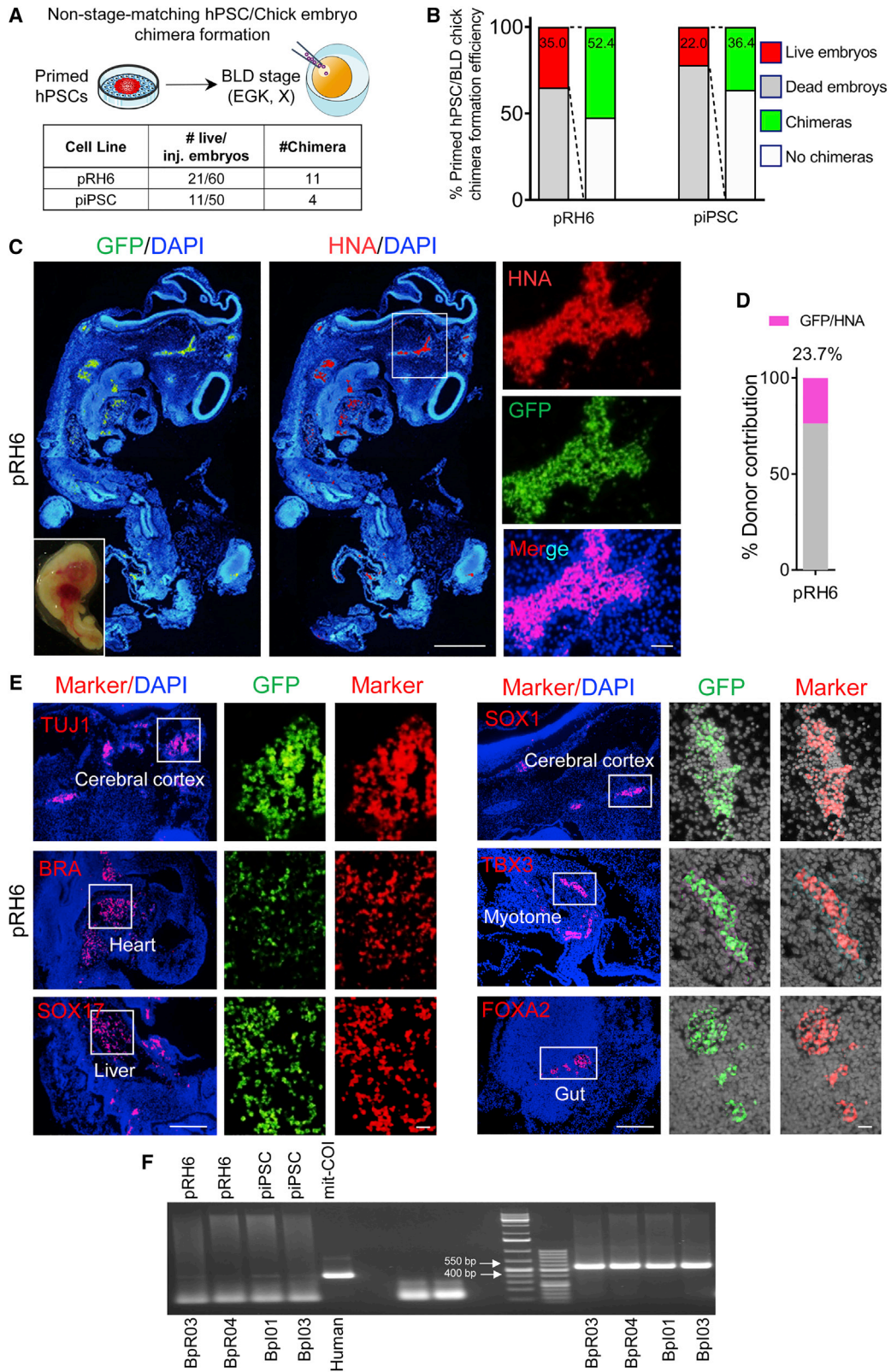
(B) The bar graph shows the percentage of live (red) and dead embryos (gray) until D6 of development, as well as the ratio of chimeric (green) and non-chimeric (white) embryos in the cohort.

(C) Representative images of a whole-mount collage (left panel, up and down) from the sagittal sections of a pISL1-PS chicken chimera at D6. GFP was expressed under the control of ISL1 gene promoter. Tissue sections were stained with HNA antibody (red). The image in the left corner (lower panel), shows the morphology of the chimeras. The higher magnifications of the head, body, and tail regions are shown in the right panels. Scale bars, 500 μ m (left panel), 200 μ m (middle panel), and 100 μ m (right panel).

(D) The contribution percentage of the donor cells in chimeras and the ratio of GFP-ISL1 and HNA double-positive cells. Cell numbers were assessed for three chimeras and three whole-mount collages per chimera ($n = 9$).

(E) Representative sagittal sections of chicken chimeras injected with 2a2iL-ISL1 cells at the BLD stage (left panel). The GFP expression marks activity of the ISL-locus in 2a2iL-ISL1-derived cells. Sections were stained with HNA (middle panel). Sequential higher magnifications (right panels) are shown. The morphology is shown in the bright-field image in the lower left corner. Scale bars, 500 μ m (left panel), 200 μ m (middle panel), and 100 μ m (right panel).

(F) Percentage of contribution of donor cells in 2a2iL-ISL1-derived chimeras and the ratio of GFP-ISL1 and HNA double-positive cells. Cell numbers of three whole-mount collages in three chimeras were counted ($n = 9$). All nuclei are stained with DAPI (blue).



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presence of hPSC-derived cells in different tissues in the mesonephros (2a2iL-RH6-derived chimeras) and the cerebral cortex regions (2a2iL-iPSC-derived chimeras) (Figures 6C and S7A). The degree of chimerism amounted to 10.9% in 2a2iL-RH6 and 9.8% in 2a2iL-iPSCs in injected chimeras, which is substantially lower compared with the stage-matched injections (Figures 6D and S7B). The contribution of naive-like hPSCs to the chimera was confirmed using HNA antibody and the PCR assay (Figures 6E and 6G). Injection of hPSCs converted to the naive state by the NHSM protocol using PS-stage chicken embryo confirmed decreased efficiency of chimera formation (two embryos; 12.5% survival rate) and lower donor cell contribution (11.5%), when an NSM approach was chosen (Figures S7C and S7D).

Next, we examined the contribution of naive-like hPSCs injected into PS-stage chicken embryos for generation of different cell lineages by IHF staining. Some naive-like hPSC-derived cells expressed the ectodermal markers TUJ1 and SOX1 in the optic lobe and otic capsule, the mesodermal markers of BRA and TBX3 in the mesonephros, and the endodermal markers SOX17 and FOXA2 in the liver and gizzard (Figure 6F). Taken together, the results indicate that naive hPSCs are able to form chimeras and give rise to different cell types when injected in non-stage-matched PS-stage chicken embryos. However, the number of chimeric embryos was lower and the contribution was reduced, offering the opportunity to use generation of xenogeneic chimeras as a versatile test system to distinguish different states of pluripotency.

DISCUSSION

The current study introduces a versatile assay for evaluation of the differentiation potential of naive-like and

primed hPSCs. We demonstrate that naive-like and primed hPSCs engraft into chicken embryos and participate in different processes of embryonic development. The ability to form chimeras is considered to be the most rigorous functional assays for PSCs. However, the formation of interspecies human-animal chimeras was not possible for a long time. Recent studies identified a number of limitations, which prevent successful generation of interspecies human-animal chimeras, including the type of host embryo, evolutionary distance, developmental SM between donor cells and host, and the pluripotency state of hPSCs (Cohen et al., 2018; Masaki et al., 2015; Mascetti and Pedersen, 2016a, 2016b; Theunissen et al., 2014; Wu et al., 2016; Wu et al., 2015; Wu et al., 2017; Yang et al., 2017b).

The main drawback for generation of human interspecies chimeras is lack of an acceptable animal. So far, most experiments with primed hPSCs for chimera formation were based on the use of mouse blastocysts, which showed a low contribution of hPSCs to host tissues (Masaki et al., 2015; Wu et al., 2017). Although early mouse embryogenesis is similar to human embryos during the pre-implantation stage, the morphology differs dramatically during post-implantation stages. In mice, a cylinder-shaped embryo forms, while human embryos develop a discoidal-like shape. Since differentiation into the three embryonic lineages starts at the gastrula stage, we have chosen chicken embryos, which develop a morphology that is very similar to post-implantation humans. We used immunostaining and PCR analysis to demonstrate the capacity of chicken embryos to form chimeras with different types of hPSCs that were injected during different developmental stages. Injected hPSCs differentiated and integrated into various regions of host embryos. To the best of our knowledge our study represents the attempt to use early developmental stages of chicken embryos for xenotransplantation experiments. Previous studies using chicken embryos to

Figure 5. NSM hPSC/Chick Chimera Formation with Primed hPSCs Injected into BLD-Stage Chicken Embryos

(A) Schematic outline of the strategy to generate NSM interspecies chimera using primed hPSCs injected into BLD-stage embryos. The table summarizes the results from injections and the efficiencies of chimera formation.

(B) The bar graphs indicate the percentage of live (red) and dead embryos (gray) until D6 of development, as well as the ratio of chimeric (green) and non-chimeric (white) embryos.

(C) Representative images of a whole-mount collage from sagittal sections through chimeras formed by injection of GFP-labeled pRH6 into BLD-stage embryos (left panel). Sections were stained for HNA (middle panel). Sequential higher magnifications are shown in boxes (right panels). The image in the lower left corner shows the morphology of an NSM pRH6-derived chimera. Scale bars, 500 μ m (left and middle panels) and 100 μ m (right panel).

(D) Percentage of contributions by donor cells in chimeras derived from injections of pRH6 cells into BLD-stage embryos. Cell numbers of three whole-mount collages in three chimeras were counted ($n = 9$).

(E) IHF staining of D6 chimeras formed by injection of GFP-labeled pRH6 into BLD-stage embryos using TUJ1, BRA, SOX17, SOX1, TBX3, and FOXA2 human antibodies (red). The right panels represent zoomed-in pictures of the areas of the white boxes. Scale bars, 500 μ m (merged images) and 100 μ m (GFP and markers).

(F) Gel electrophoresis image of PCR analyses for species-specific detection of mit-COI gene in chicken chimeras generated by injection of primed hPSCs (RH6 and iPSC) into the BLD stage. PCR analysis was performed for two randomly selected chimers. All nuclei are stained with DAPI (blue).

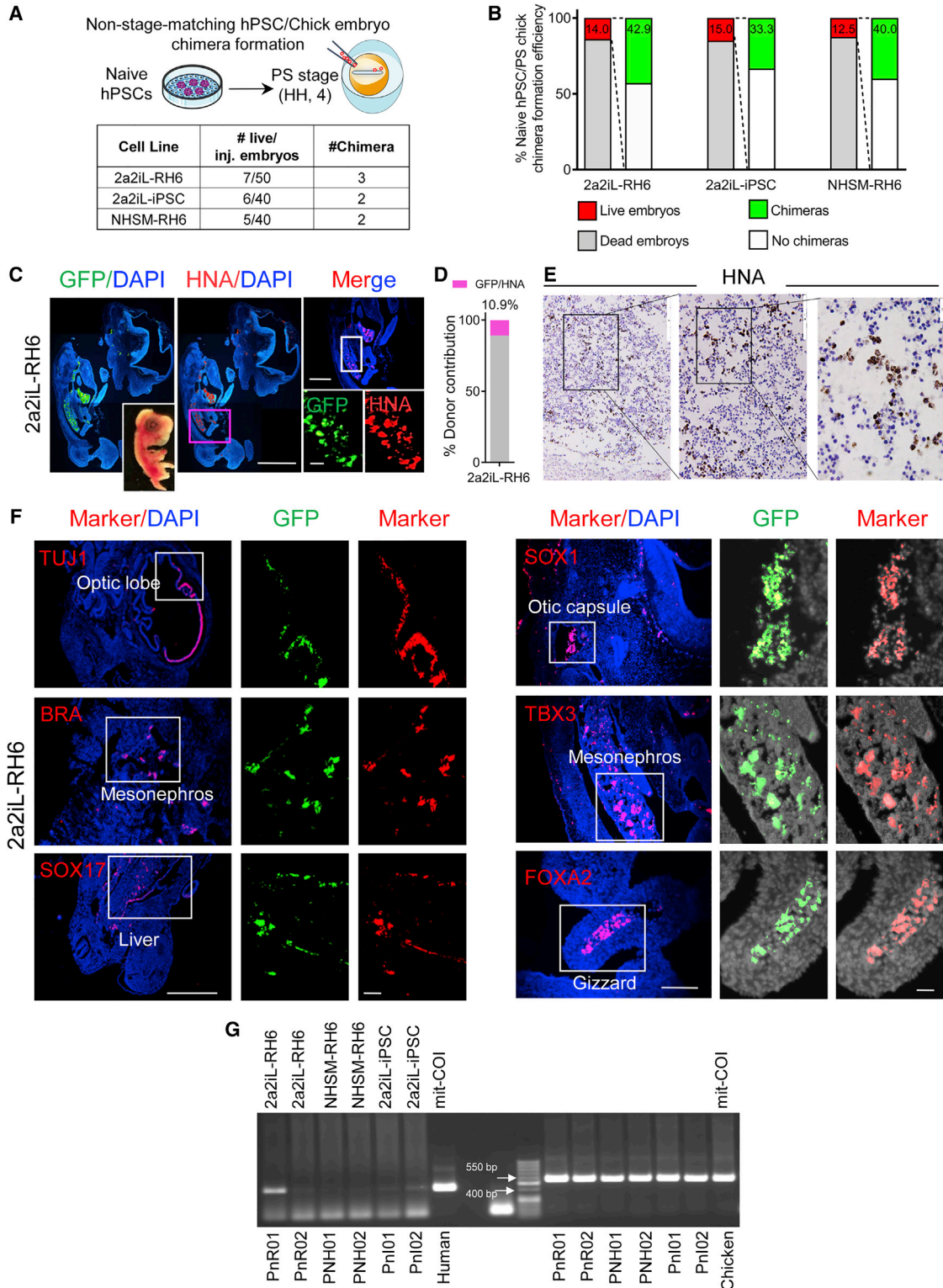


Figure 6. NSM hPSC/Chick Embryo Chimera Formation with Naive-like hPSCs Injected into PS-Stage Chick Embryos

(A) Schematic outline of the strategy to generate NSM interspecies chimera using naive-like hPSCs injected into PS-stage chicken embryos. The table summarizes the results from injections and their efficiencies in chimera formation.

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assay the functional potential of PSCs (Goldstein et al., 2002; Haraguchi et al., 2016; Lee et al., 2006) and their progenies (Martyn et al., 2018) relied on organogenesis stages of chick embryos, which is a completely different scenario.

It was proposed recently that reduction of the evolutionary distances between hosts and donor hPSCs might enable human organ production in chimeras and assessment of pluripotency due to higher similarities in peri-implantation epiblast stages (Wu et al., 2017). Large animals, such as ungulates, have been considered as hosts, but high maintenance cost, low numbers of embryos, difficulties with *in vivo* imaging and lineage tracing, as well as ethical concerns, have limited such approaches (Harding et al., 2013). Chicken embryos and *in ovo* transplantations overcome such limitations and offer a system that provides convenient manipulation, decreased costs, extensive accessibility, and rapid evaluation. While the evolutionary distance between chickens and humans is more than 300 million years (Nanda et al., 1999), our results insinuate that morphological similarities at the gastrula stage are important for successful chimera formation. Furthermore, the chicken embryo lacks a fully developed immune system (Jankovic et al., 1975), allowing implantation of various otherwise immunogenic cell types. In addition, the uncomplicated accessibility of chicken embryos during embryogenesis allows implantation of cells at different development stages (e.g., BLD and PS stages) or choosing between SM and NSM approaches. In fact, our results indicated that the developmental stage of the recipient embryos has a strong effect on chimerism when naive-like hPSCs are used. Our study suggests that the morphological similarity between the donor and host species is a relevant factor for successful interspecies chimerism.

Primed hPSCs are developmentally similar to the epiblast of post-implantation embryos. Therefore, we injected primed hPSCs into epiblasts of PS chick embryos. This approach allowed us to successfully generate interspecies

chimeras with a high contribution of primed hPSCs. The results are in line with previous studies demonstrating that injection of primed PSCs into the gastrula stage of mouse embryos but not in blastocysts leads to chimera formation (Mascetti and Pedersen, 2016b; Wu et al., 2015). Surprisingly, we also observed formation of chimeras when we injected primed hPSCs into BLD-stage chicks, although the efficiency of chimera formation was lower and the contribution of donor cells to embryonic tissues was reduced (Figure 7). Apparently, naive-like cells are not fully compatible with the epiblasts of the gastrula stage in the chicken embryo. In contrast, primed hPSCs efficiently formed chicken chimera when transplanted at the BLD stage in an NSM approach. This interesting feature creates the opportunity to use non-stage-matched injection of hPSCs into chicken embryos as a test system for distinguishing different pluripotent states of hPSCs. Of note, primed hPSCs integrated and differentiated into three embryonic germ layers with no trace of teratoma formation, while injection of hPSCs into 8.5-day-old mouse embryos led to teratoma formation (Cohen et al., 2018). Moreover, hPSCs injected into mouse embryos in a non-stage-matched approach are not able to functionally contribute to mouse tissue (Cohen et al., 2018; Theunissen et al., 2014). We conclude that developmentally matched timing of injection is indeed important but seems to matter less in chicken embryos, which seem to coordinate much better with incoming PSCs of different stages. This property of chicken embryos might be related to the innate ability of chicken BLD for rapid differentiation and exit from pluripotency preventing long-term self-renewal of isolated chicken ES-like cells (Farzaneh et al., 2017).

Another important lesson from our study concerns the use of different media and protocols for generation of naive-like hPSCs. hPSCs cultivated in 2a2iL contribute broadly to chimera formation while hPSCs cultured in NHSM gave rise to small colony-like clusters of cells. The efficiency of chimera formation was much lower with

(B) The bar graphs indicate the percentage of live (red) and dead embryos (gray) until D6 of development and the ratio of chimeric (green) and non-chimeric (white) embryos in the surviving cohort.

(C) Representative images of a whole-mount collage from sagittal sections through chimeras formed by injection of 2a2iL-RH6 cells into PS-stage chicken embryos (left panel). Sections were stained for HNA (middle panel) and sequential higher magnifications are shown in boxes (right panels). The morphology is shown in the bright-field image in the lower right corner. Scale bars, 500 μ m (left and middle panels), 200 μ m (upper right panel), and 50 μ m (lower right panel).

(D) Percentage of contribution by donor cells in chimeras. Cell numbers of three whole-mount collages in three chimeras were counted ($n = 9$).

(E) IHF analysis of HNA expression in embryo chimera. The insets are the zoomed-in pictures of the black boxes. Scale bars, 200 μ m (left image), 100 μ m (middle image), and 20 μ m (right image).

(F) IHF staining of D6 chimeras formed by injection of 2a2iL-RH6 cells into BLD-stage embryos using TUJ1, BRA, SOX17, SOX1, TBX3, and FOXA2 human antibodies (red). The middle and right panels in each column are the zoomed-in images of areas in the white boxes. Scale bars, 500 μ m (left panels in each column) and 100 μ m (middle and right panels in each column).

(G) Gel electrophoresis image of PCR analyses for human/chicken detection of the mit-COI gene in chicken chimeras generated by injection of naive-like hPSCs (2a2iL-RH6, 2a2iL-iPSC, and NHSM-RH6) cells into PS-stage embryos. All nuclei are stained with DAPI (blue).

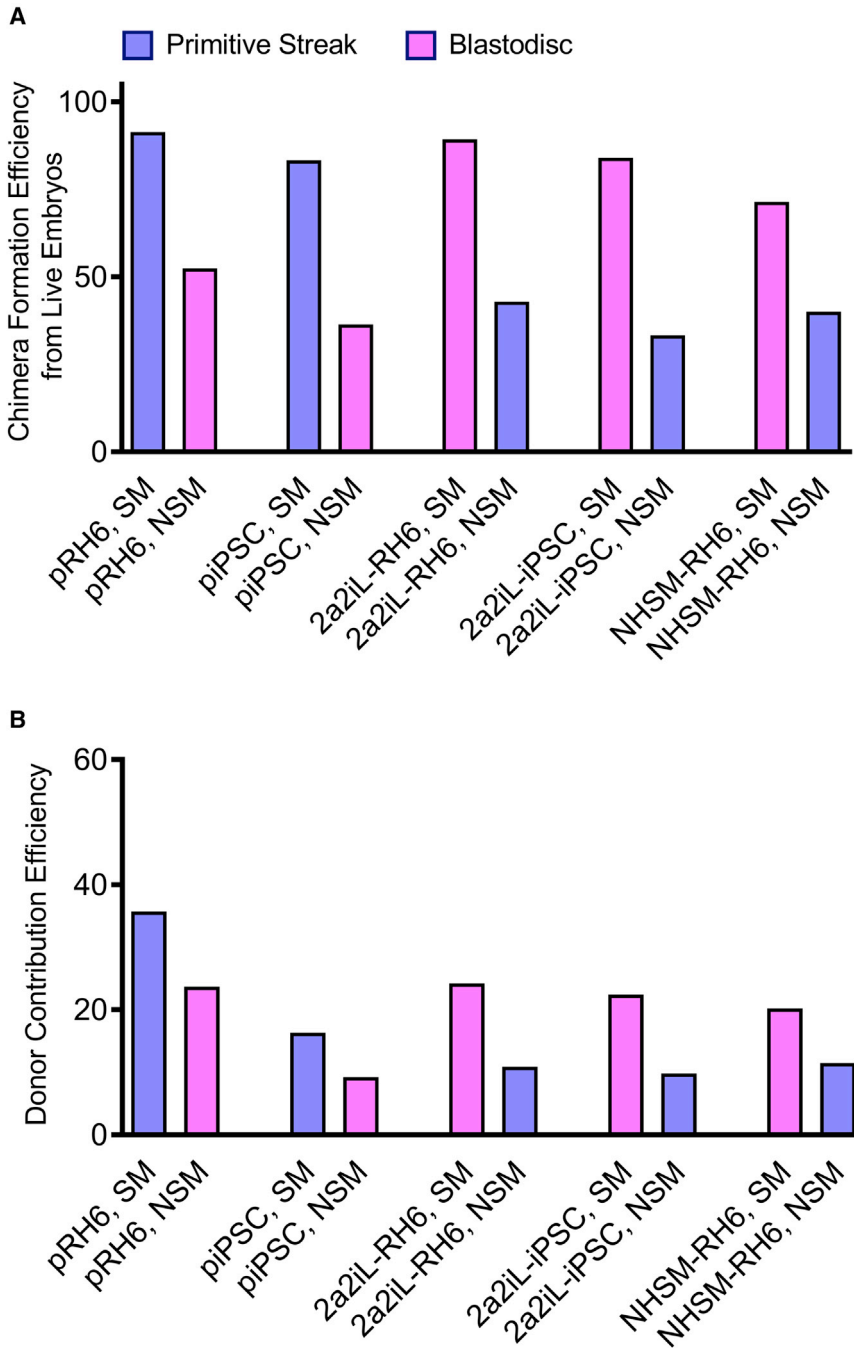


Figure 7. Efficiency of Xenogeneic Chimera Formation after Injection of Different hPSC Lines into Chicken Embryos in Various States of Pluripotency (A) Barplots summarizing the efficiency of chimera formation in surviving embryos using SM and NSM approaches. (B) Barplots summarizing the contribution of donor cells in chimeras generated by SM and NSM approaches.

NHSM-hPSCs compared with 2a2iL-hPSCs. The importance of culture conditions is also evident from previous studies, which reported that a single human expanded PS cell derived in LCDM medium that included human LIF, CHIR99021, dimethindene maleate, and minocycline hydrochloride is able to generate an interspecies chimera when injected into an eight-cell mouse embryo but not into the blastocyst (Yang et al., 2017b). Belmonte and col-

leagues recently suggested that ungulates, which are evolutionary closer to humans than rodents, might serve as hosts for evaluation of pluripotency of naive hPSCs (Wu et al., 2017). However, it was not possible to form efficient post-implantation chimeras with pig embryos when naive hPSCs cultured in non-optimized medium were used. It seems worthwhile to investigate whether the 2a2iL protocol overcomes this limitation or whether species-specific



differences make the chicken embryo a superior host for testing the functional potential of human PSCs and for identifying the state of pluripotency. Our study represents a major step forward to develop robust *in vivo* models for the assessment of hPSC pluripotency. However, further studies are necessary to determine whether hPSCs are also able to contribute to all cell types of chicken embryos.

EXPERIMENTAL PROCEDURES

Cell Culture

We used GFP-labeled RH6, CAG-GFP hiPSC, and ISL1-GFP reporter cell lines in this study. Primed hPSCs were maintained on Matrigel (Sigma-Aldrich). Primed hPSCs were cultured in serum-free medium (SFM)-DMEM/F12 supplemented with 20% KOSR (Invitrogen), 2 mM L-glutamine (Invitrogen), 1% non-essential amino acids (Invitrogen), 1 × ITS-G (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), penicillin-streptomycin (Invitrogen), and 100 ng/mL basic fibroblast growth factor (bFGF) (Royan Biotech). To induce naive-like 2a2iL cells, 2×10^5 hPSCs were seeded on mitomycin C-inactivated MEF in SFM supplemented with 10 μM Y27632 (Sigma-Aldrich) and bFGF. Next day, the medium was switched to 2a2iL including SFM supplemented with 10 μg/mL RJW101 (kindly gifted by Dr. Richard Withby), and 0.1 μg/mL CD437 (Invitrogen) (named as 2a) plus 1 μM PD0325901 (Sigma-Aldrich), 3 μM CHIR99021 (Stemgent), and 10 ng/mL human leukemia inhibitory factor (hLIF; Royan Biotech) (known as 2iL). NHSM naive-like cells were also generated in NHSM medium including knockout DMEM (Invitrogen), 10% AlbuMAXI (Irvine Scientific), N2 supplement (Invitrogen), 1 mM glutamine, 1% NEAA, 1% ITS, 1% penicillin-streptomycin, 0.1 mM β-mercaptoethanol, 10 μg hLIF, 8 ng/mL bFGF, and 1 ng/mL recombinant transforming growth factor β (Fitzgerald), supplemented with the following small molecules: 1 mM PD0325901, 3 μM CHIR99021, 10 μM SP600125 (Tocris), 5 μM SB203580 (Cayman), 5 μM Go6983 (Tocris), and 5 μM Y27632 (Gafni et al., 2013b).

Xenogenic Chicken Chimera Formation

We selected two developmental stages of chick embryos, BLD (stage X) and PS (stage HH4), for injection of naive-like and primed GFP-labeled hPSCs, respectively. For the BLD-stage chick embryo, freshly laid eggs and for the PS stage, 18–24 h incubated fertile eggs were used. Approximately 3–5 μL of a suspension that contained 500–1,000 single hPSCs was injected into the sub-germinal cavity in the BLD or under the marginal zone between the area opaca and area pellucida at PS stage (24 and 27 h after incubation). Subsequently, the injected embryos were returned to the incubator for 6–7 days. Expression of GFP cells was examined under a fluorescence microscope (SZX 16, Olympus) and the main markers of the germinal layers were visualized by histological analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.stemcr.2020.11.014>.

AUTHORS CONTRIBUTIONS

A.A. designed the study and performed most of the experiments. A.T. developed Royan H6 tagged with GFP. S.A.G. contributed to biostatistical analysis and preparation of the diagrams. Z.B. developed Royan H6, targeted it with ISL1-GFP, and characterized this cell line. S.Y. contributed to the chimera formation process. S.M., K.K., and N.H. contributed to the cell culture. A.S. performed PCR and analyzed the results. T.K. contributed to the immunohistological experiments. S.-N.H., A.A., A.T., and Z.G. discussed the results and wrote the manuscript. T.B. contributed to data interpretation and edited the manuscript. H.B. and S.N.H. provided financial and administrative support, designed and analyzed the experiments, interpreted and discussed the results, and approved the manuscript. All authors reviewed and confirmed the manuscript before submission.

CONFLICTS OF INTERESTS

The authors declare that they have no conflicts of interests. A patent is associated with this study and the provisional number is IP NA99021.

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