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In Vivo Differentiation Potential of Epiblast Stem Cells Revealed by Chimeric Embryo Formation

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SUMMARY

Chimera formation after blastocyst injection or morula aggregation is the principal functional assay of the developmental potential of mouse embryonic stem cells (ESCs). This property, which demonstrates functional equivalence between ESCs and the preimplantation epiblast, is not shared by epiblast stem cell (EpiSC) lines. Here, we show that EpiSCs derived either from postimplantation embryos or from ESCs in vitro readily generate chimeras when grafted to postimplantation embryos in whole embryo culture. EpiSC derivatives integrate and differentiate to derivatives of all three embryonic germ layers and primordial germ cells. In contrast, grafted ESCs seldom proliferate in postimplantation embryos, and fail to acquire the identity of their host-derived neighbors. EpiSCs do not incorporate efficiently into embryonic day 8.5 embryos, a stage by which pluripotency has been lost. Thus, chimera formation by EpiSCs requires a permissive environment, the postimplantation epiblast, and demonstrates functional equivalence between this cell type and EpiSCs.

INTRODUCTION

The preimplantation epiblast generates all embryonic cell types (reviewed in Gardner, 1998). When injected into blastocysts, embryonic stem cells (ESCs) derived from preimplantation epiblast cells (Brook and Gardner, 1997) integrate into the preimplantation embryo, generating chimeras with high efficiency. ESCs can contribute to all cell types in the body, including germ cells (Bradley et al., 1984), demonstrating their ability to undergo normal in vivo cell differentiation.

Clonal analysis shows that during gastrulation, single postimplantation epiblast cells can contribute to all three germ layers up to early gastrulation (Lawson et al., 1991; Tzouanacou et al., 2009). Together with the ability of pre- and postimplantation epiblast cells to form teratocarcinomas (tumors containing derivatives of all three germ layers and embryonal carcinoma cells) this shows that the epiblast before and after implantation can be considered pluripotent (Stevens, 1970). Epiblast stem cells (EpiSCs) are cell lines derived from the postimplantation epiblast (Brons et al., 2007; Tesar et al., 2007) or in vitro from ESCs (Guo et al., 2009). Like ESCs, EpiSCs express the core pluripotency genes *Oct4*, *Nanog*, and *Sox2*, and are able to form teratocarcinomas and differentiate into multiple lineages in vitro.

Despite these functional similarities, ESCs and EpiSCs differ in several distinct respects (for reviews, see (Chenoweth et al., 2010; Nichols and Smith, 2009, 2011). EpiSCs and ESCs have distinct signaling requirements for self-renewal. Female EpiSCs exhibit X chromosome inactivation, whereas ESCs do not. The gene expression profiles and colony morphologies of the two cell types are distinct. Crucially, unless EpiSCs are selected for rare subpopulations that resemble very early postimplantation epiblast (Han et al., 2010) or are genetically engineered to express a high level of E-cadherin (Ohtsuka et al., 2012), they are unable to contribute to chimeras upon blastocyst injection or morula aggregation. Mouse EpiSCs are of particular interest because human ESCs share many of their distinctive properties (reviewed in Chenoweth et al., 2010). However, the lack of an in vivo assay for the potency of EpiSCs leaves the issue of whether these cells represent a true in vitro counterpart of an in vivo cell type in doubt. Alternatively, the preimplantation embryo environment may be incompatible with EpiSC survival and integration. The latter possibility implies that EpiSCs should integrate into the postimplantation embryo.

To address this issue, we grafted EpiSCs to postimplantation embryos that were then cultured in vitro. Mouse embryos undergo normal development in culture for periods of 24–48 hr (Copp et al., 1990), permitting analysis of cell integration in the epiblast. We find that EpiSCs, but not ESCs, efficiently form chimeras in gastrulation-stage embryos, colonizing somatic and germ cell lineages. The inability of EpiSCs to form blastocyst-injection chimeras is therefore explained by their incompatibility with the environment of the preimplantation epiblast, not an inability to differentiate in the context of the embryo.

RESULTS AND DISCUSSION

EpiSCs Incorporate Efficiently into Postimplantation Embryos

Initial experiments to test the assimilation of EpiSCs into gastrulation-stage embryos using ultrasound-guided injection

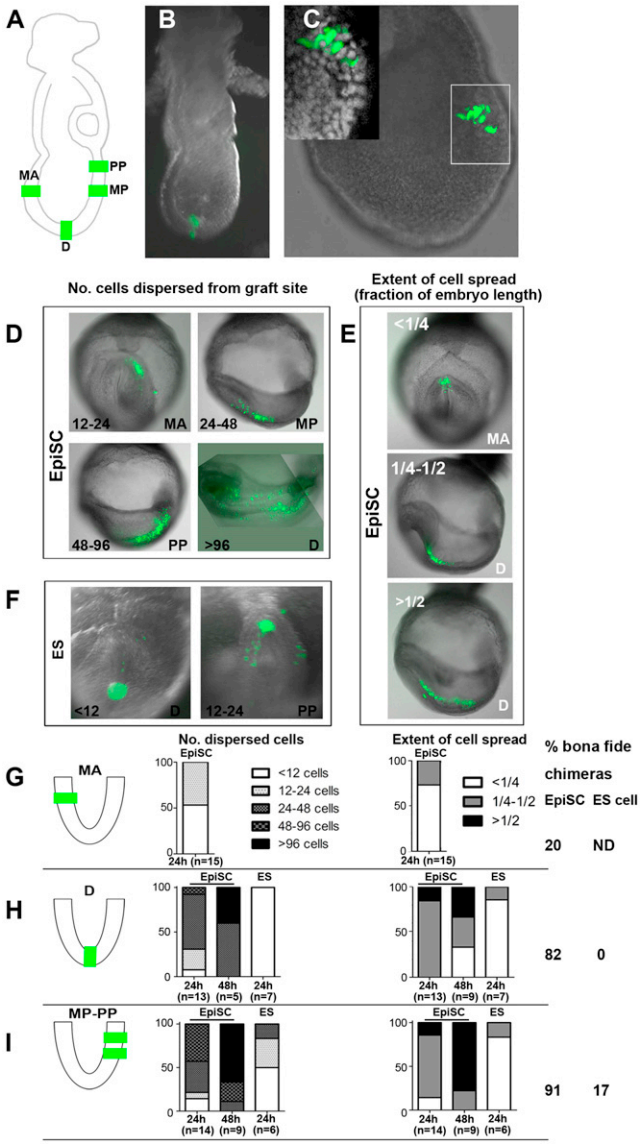

Figure 1. Grafting Procedure and Analysis of Cell Contribution to Cultured Embryos

(A) Diagram of late-streak embryo, showing graft sites (MA, D, MP, and PP) in the egg cylinder.

(B and C) GFP-labeled cells (green) overlaid on a brightfield or DAPI-counterstained image (C, inset).

(B) Embryo-derived EpiSC (r04-GFP) grafted to the D region of a late-streak-stage embryo.

(C) Confocal z-stack showing a mid-streak-stage embryo with C2 in-vitro-derived EpiSC in the MP region. Inset: confocal z-slice showing individual grafted cells.

(D–F) Examples of cell contribution to embryos grafted at indicated sites (lower right of each panel) after culture. Culture period: 24 hr, except for lower right in (D), when it was 48 hr.

(D) Embryos with different numbers of dispersed embryo-derived EpiSCs.

(E) Embryos with different extents of embryo-derived EpiSC cell spread along the anteroposterior axis.

(F) Embryos grafted with ESCs (AGFP7).

(G–I) Histograms showing the percentage of embryos for each category of graft containing different numbers of dispersed donor cells and extents of cell spread. The percentage of bona fide chimeras is shown at the right. Note: cells grafted to the D site of four embryos cultured for 48 hr were located internally and consequently only the extent of spread, not the number of dispersed cells, was scored.

See also Figure S1.

of EpiSC cell suspensions to the amniotic cavity in utero did not result in any chimeras at birth (data not shown). Similar injections of single-cell suspensions in ex-vivo-cultured embryos formed free-floating unincorporated clumps in the amniotic cavity (data not shown). In contrast, EpiSC clusters that were manually dislodged from the culture dish and grafted in the epiblast (Figures 1A–1C) survived, proliferated, and dispersed from the original graft site (Figures 1D, 1E, and S1A–S1D). This implies that EpiSC integration in embryos depends upon cell-cell contacts, and that single-cell suspensions are unable to form such contacts to integrate into the host.

To assess EpiSC incorporation in defined regions of the postimplantation epiblast, we grafted EpiSCs constitutively expressing green fluorescent protein (GFP) in four sites of gastrulation-stage (early-streak to late-streak) embryos: mid-anterior (MA), distal (D), mid-posterior (MP), and proximal-posterior (PP; Figures 1A–1C). Embryos were cultured in vitro and analyzed after 24–48 hr (Figures 1D and 1E). The distribution of donor cells was assessed by fluorescence microscopy (Figures 1D–1F).

Two EpiSC lines that ubiquitously express GFP (r04-GFP, derived from E6.5 epiblast, and C2, derived in vitro from ESCs) were injected. Two ESC lines (AGFP7, which ubiquitously expresses GFP, and TßC44cre6, which expresses GFP under *Nanog* regulatory control) were injected for comparison. Both ESC lines efficiently form chimeras following blastocyst injection (Chambers et al., 2007; Gilchrist et al., 2003). To determine the number of cells that initially grafted into the embryos, the embryos were fixed immediately after grafting. An examination of confocal z-stacks (Figure 1C) showed that 12 ± 2.83 cells/embryo were grafted (range: 10–16 cells; $n_{embryos} = 4$).

A total of 91 embryos received a graft of either EpiSCs or ESCs. Most of these (n = 81) developed normally and contained labeled cells after culture, verifying that the grafting technique is efficient. The remainder either did not contain labeled cells after culture (n = 4) or developed abnormally (n = 6). These embryos were excluded from the analysis. Seventy-three embryos received grafts of cells carrying ubiquitously expressed GFP; 55 of these embryos were analyzed after a 24 hr culture, and the remainder were analyzed after 48 hr (Figure S1E). To describe the distribution of donor cells in the host after culture, we scored wholemount embryos for the approximate number of donor cells that had dispersed from the graft site, and the spread of donor cells within the host (Figures 1D–1I and S1E). We compared EpiSC and ESC grafts, using the number of dispersed cells and their spread as a semiquantitative assessment of donor cell contribution. Following culture, an embryo was considered to be a bona fide chimera if the following criteria were met: (1) at least 12 donor cells had clearly dispersed from the graft site, indicating that dispersed cells had survived and/or proliferated in the host; and (2) dispersed donor cells were not restricted to one area and had spread over >1/4 of the

embryo's anteroposterior length, indicating robust intermixing with the host cells.

The profiles of the contributions made by in-vitro- and embryo-derived EpiSCs to embryos were similar (Figure S1E), and therefore we combined those data (Figures 1G–1I). Both EpiSC lines incorporated efficiently into the D, MP, and PP regions (Figures 1H and 1I). In the D region, 17 of 18 embryos contained ≥12 dispersed donor cells. Donor cells spread over >1/4 of the embryo's length in 19 of 22 embryos (Figure 1H). Similarly, in the MP and PP regions, 21 of 23 embryos contained ≥12 dispersed donor cells, which spread over >1/4 of the embryo's length in all 21 embryos (Figure 1I). Hence, the overwhelming majority (82%–91%) of the embryos grafted with EpiSCs in D, MP, and PP regions formed bona fide chimeras (Figures 1H and 1I). Moreover, after 24 hr of culture, 20 chimeras (74% of those injected in D, MP, and PP regions) contained 24–96 dispersed cells, indicating cell proliferation in the host (Figures 1H, 1I, and S1E). After 48 hr of culture, more than half of the embryos injected into the D, MP, and PP regions contained >96 dispersed donor cells, distributed throughout >1/2 of the embryo's length (Figures S1A–S1D, showing two embryos containing ~300 graft-derived cells), indicating further proliferation and dispersal between 24 and 48 hr. Therefore, EpiSC readily proliferate and disperse from regions that are in the primitive streak (MP and PP regions) or are recruited there during culture (D region).

EpiSCs that were grafted in the MA region showed less cell dispersal. Just under half (47%) of the embryos contained ≥12 dispersed donor cells after culture. Donor cells extending for >1/4 of the embryo's length were seen in only 20% of the embryos (Figure 1G). Fate maps indicate that few, if any, cells in the MA region will be recruited to the primitive streak by the mid-streak to late-streak stage (Lawson et al., 1991). The primitive streak is a site of extensive cell rearrangement and may provide grafted cells in these regions with a greater opportunity for cell intercalation with the host than cells grafted at the MA site. Alternatively, the high levels of Nodal and fibroblast growth factor (FGF) signaling in distal and posterior regions of the embryo may favor EpiSC growth because these pathways are important for EpiSC maintenance.

To determine whether the dispersed donor cells differentiate to acquire the identity of their immediate host-derived neighbors, we sectioned cultured embryos and assessed them for cell integration. Double nuclear and actin filament staining revealed that the morphology of the EpiSC-derived donor cells was similar to that of their neighbors (Figure 2B). Moreover, immunohistochemistry showed that in all assays of somatic lineages (n = 27; Table 1), both in-vitro- and embryo-derived EpiSC descendants expressed markers characteristic of their host location, i.e., T (Brachyury) in the primitive streak and emergent mesoderm (Wilkinson et al., 1990; Figure 2C), Tbx6 in the paraxial presomitic mesoderm (Chapman et al., 1996; Figure 2D), AP-2α (Tcfap2a) in the surface ectoderm (Arkell and Beddington, 1997; Figure 2E), Sox2 in the neurectoderm (Avilion et al., 2003; Figure 2E), Foxa2 in the floor plate and endoderm (Sasaki and Hogan, 1993; Figures 2F and 2G), and Cdx2 in the allantois and posterior mesoderm (Beck et al., 1995; Figure 2H). Therefore, EpiSCs integrate efficiently into all three germ layers within 24–48 hr. Notably, T (Wilson et al., 1995), Foxa2 (Dufort et al., 1998), and Cdx2 (Chawengsaksophak et al., 2004) are required for correct differentiation at this stage, and thus failure to express these markers would preclude functional differentiation.

To further demonstrate that EpiSCs form cell types that are functional at early stages, we injected them into the primitive streak of embryos in the early-streak stage, when cardiac progenitors are present in the primitive streak. After 48 hr in culture, at which point the heart is beating and required for normal development, we observed donor-derived cells in the heart of two chimeras (Figure 2I). These cells expressed the cardiomyocyte marker Nkx2.5 (Lints et al., 1993; Figure 2J; Table 1) at levels comparable to those observed in their neighbors, indicating differentiation of EpiSC toward cell types with an early functional role.

We also observed graft-derived alkaline phosphatase (TNAP)-positive cells with primordial germ cell (PGC) morphology at the junction between the allantois and posterior primitive streak (Figure 2K), conventionally designated as PGCs (Ginsburg et al., 1990). Furthermore, graft-derived cells in this location express the PGC-specific marker Stella (Figure 2L; Saitou et al., 2002). Specific antibody immunofluorescence of Stella was verified by the detection of Stella$^+$, Nanog:GFP$^+$ ESCs (Figure S2H) and Stella$^+$, Nanog:GFP$^+$ cells at the base of the allantois in embryonic day 8.5 (E8.5) transgenic embryos (Figure S2I). Thus, EpiSCs grafted in early- to mid-streak-stage embryos are not restricted to somatic lineages, consistent with earlier reports that EpiSC can undergo PGC differentiation in vitro (Hayashi et al., 2011). Nevertheless, not all graft-derived cells at the base of the allantois expressed PGC-specific markers (Figure S2J; Table 1), and only two of the seven embryos tested contained any putative PGCs, consistent with a short time window of PGC specification that is complete by early to mid-streak stage, and the low abundance of PGCs in the embryo (Lawson and Hage, 1994; Tam and Zhou, 1996).

Sox2, which marks not only neural progenitors but also EpiSCs (Figures S2A and S2B), was downregulated in the vast majority of EpiSC-derived nonneural cells in the embryo (Figures 2E and S2B; five of five assays, comprising three or more sections per assay; Table 1). This indicates that dispersed donor cells lose EpiSC characteristics as they incorporate into the host. Subpopulations of EpiSCs in vitro also express T and Foxa2 (Figures S2C and S2D), but we did not observe ectopic expression of these markers in chimeras (12 of 12 assays; Figure 2C; Table 1). A small minority of EpiSCs in vitro (<1%) expressed Cdx2, AP-2α, and Nkx2.5 (Figures S2E–S2G), yet after injection into the embryo, most EpiSC correctly expressed these markers (11 of 11 assays; Figures 2E, 2H, and 2J; Table 1). AP levels in EpiSCs are uniformly low (Brons et al., 2007; Greber et al., 2010) and we did not detect Stella-positive EpiSCs in vitro (data not shown). In each case, EpiSC-derived cells expressed these markers in appropriate locations in host embryos (Figures 2K and 2L; Table 1). Therefore, given that only 10–16 cells were injected into the embryo, and most grafts produced cells that expressed markers appropriate for the site of integration, it is unlikely that grafted EpiSCs expressing these differentiation markers would have selectively integrated and/or expanded in the host embryos.

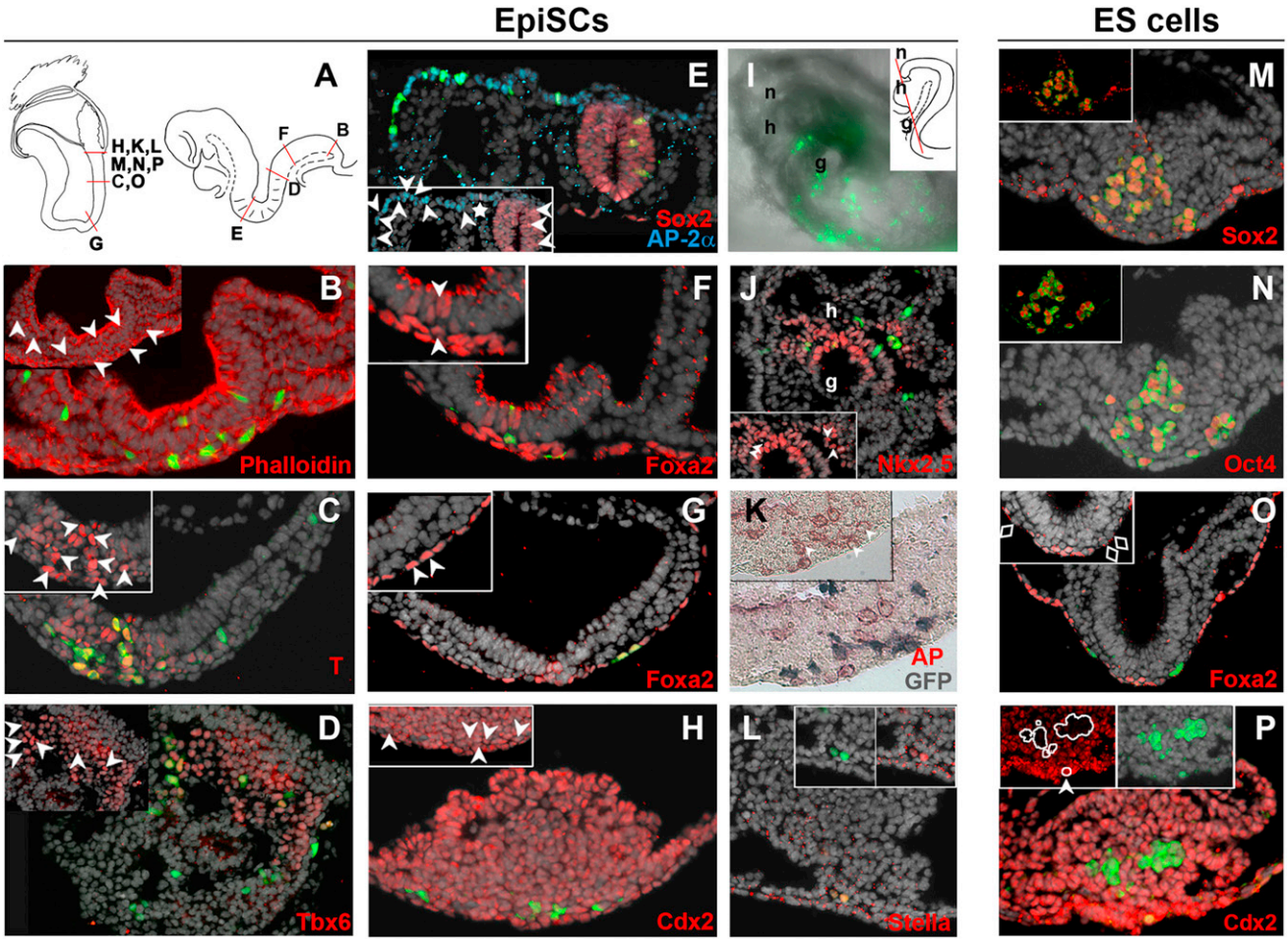

Figure 2. Analysis of Donor Cell Differentiation in Chimeras

(A) Schematic diagrams showing approximate sectioning planes in embryos cultured for 24 hr (left) or 48 hr (right).

(B–J and L–P) Specific stains (red/cyan). Insets: position of graft-derived cells with channels removed to allow comparison of graft-derived (green) and host (gray, DAPI counterstain) cells. Arrowheads: donor cells showing correct marker expression.

(B–L) EpiSC grafts.

(B) Phalloidin, showing donor cells in the neural groove and mesoderm.

(C) T (brachyury), showing donor cells in the primitive streak and paraxial mesoderm.

(D) Tbx6, showing donor cells in the paraxial presomitic mesoderm.

(E) Sox2 (red) and AP-2a (cyan) of embryo in (D), showing donor cells in the neural tube and surface ectoderm. Asterisk: single cell ectopically expressing Sox2.

(F and G) Foxa2, showing donor cells in the floor plate (F) and endoderm (G).

(H) Cdx2 staining on the embryo in (D), showing donor cells in allantois.

(I) Frontal view of a 48-hr-cultured, early-streak-stage embryo grafted in the PP region with in-vitro-derived EpiSC. Inset: position of section J in this embryo. n, neurectoderm; h, heart; g, gut.

(J) Nkx2.5 staining of embryo shown in (I), showing donor cells in the embryonic heart.

(K) Alkaline phosphatase (AP) (red) and GFP immunohistochemistry (gray), showing PGC differentiation. Inset: TNAP staining prior to immunohistochemistry. Arrowheads: $TNAP^+$, GFP^+ cells with PGC morphology.

(L) Stella staining, showing PGC differentiation. Insets: removal of either the green (GFP) or red (Stella) channel.

(M–P) ESC grafts.

(M and N) Donor cells ectopically express Sox2 (M) and (N) Oct4. Inset: nuclear stain removed.

(O) Foxa2, showing expression only in host-derived cells. Diamonds: GFP-labeled cells.

(P) Cdx2 staining of embryo in M and N, showing donor cells in allantois. A single cell (arrowhead) correctly expresses Cdx2. Left inset: Cdx2 immunofluorescence. White lines outline the position of donor cells. Right inset: donor cells.

See also Figure S2.

Because morphological integration correlated with functional integration (as verified by marker expression) in all embryos analyzed, we used morphological integration as a criterion to score the remaining sectioned embryos (Table 1). In all cases, the cellular distribution of dispersed EpiSCs in their host is consistent with the fate map of the epiblast (Table 1; Figure S3).

Table 1. Distribution of Donor Cells in Host Embryos after Culture

Donor Cells	Stage of Recipients	Graft Site	Number of Sectioned Embryos	Number of Embryos with Incorporated GFP+ Cells	Number of Sectioned Embryos Containing Incorporated Cells in Tissue (Number of Embryos Confirmed/Number Tested by Immunohistochemistry)					
					Surface Ectoderm (AP-2a)	Neural Ectoderm (Sox2, Foxa2)	Mesoderm (T, Cdx2, Nkx2.5, Tbx6)	Endoderm (Foxa2)	PS (T)	Allantois (Cdx2, Stella, TNAP)
Embryo-derived EpiSC (r04-GFP)	M-LS	MA	4[a]	3		3	1			
		D	9[a]	9	1(1/1)	8(4/4 Sox2, 2/2 Foxa2)	8(2/2 T, 2/2 Cdx2)		3	1(0/1 Stella)
		MP	4	4	1(1/1)	1(1/1 Sox2)	1(1/1 Tbx6)		2(2/2)	3(2/2 Cdx2, 0/1 Stella)
		PP	3	3			3	1	3(2/2)	3(3/3 Cdx2)
	E-MS	PP	4	4		2	4	4	3	2(1/2 Stella)
In vitro EpiSC (C2)	M-LS	D	2[b]	2		2	2	2		
		MP	4[a]	4			4(1/1 Tbx6)	2(2/2)	4(1/1)	4(1/2 TNAP, 0/1 Stella)
	ES	PP	4	4		1(1/1 Foxa2)	2(1/1 Cdx2, 2/2 Nkx2.5)	4	2	
ESCs (AGFP7)	M-LS	D	5[a]	2			2[c]	1(0/1)		
		PP	3	3			1+1[c]		2(0/1[d])	2(0/1 Cdx2[d])

Brackets: number of sectioned embryos containing GFP cells that expressed the indicated marker/number of embryos tested. For each embryo, three or more sections were tested for immunoreactivity to any one antibody. Staging: ES, early streak; E-MS, early to mid streak; M-LS, mid to late streak. See also Figure S3.

[a]One sectioned embryo contained a small unincorporated clump of cells (three or fewer sections).

[b]Both embryos also contained small unincorporated clumps of cells.

[c]GFP+ cells present in three or fewer sections.

[d]GFP+ cells showed ectopic Sox2 and Oct4 expression, and only one cell correctly expressed Cdx2.

Greater endoderm contribution is observed in embryos that received an EpiSC graft at the early-streak stage, consistent with the early segregation of endoderm (Tzouanacou et al., 2009). Thus, EpiSC chimera formation is efficient and follows the normal fates of cells at the graft site. Moreover, the similar chimera-forming capacity of embryo- and in-vitro-derived EpiSCs highlights that cell lines produced by these distinct routes share common functional properties. Because mouse EpiSCs also share features with human ESCs (Tesar et al., 2007), these data suggest that human ESCs also represent a true in vitro counterpart of an in vivo cell type.

Occasionally, clumps of cells were observed in sections of the embryo at the graft site (Table 1). Six out of 34 sectioned embryos contained such clumps, two of which were very small, extending over one to two sections. These clumps may represent a subpopulation of EpiSCs that are incapable of integrating into the host embryonic environment, or EpiSCs may require contact with neighboring host cells to ensure their appropriate differentiation. In support of the latter possibility, when we performed additional grafts containing larger numbers of EpiSCs (>12 cells per embryo, n = 4), this did not result in more extensive chimeras after 24 hr (in all four embryos, 24–48 dispersed cells extended between 1/4 and 1/2 of the embryo length). Instead, all embryos additionally contained partially integrated clumps. Importantly, Sox2 was ectopically expressed at low levels in the center of the clump but was absent in nearby dispersed cells (Figure S4A). This strongly suggests that the primary influence on EpiSC differentiation is dispersal from the grafted cell cluster, rather than variations in the abilities of subpopulations to integrate and differentiate. Additionally, unincorporated clumps were previously observed in orthotopic grafts of E7.5 epiblast (Beddington, 1981), indicating that even tissue from an origin equivalent to the host site can fail to incorporate, presumably because there are too many cells to be assimilated by the surrounding host cells.

ESCs Do Not Incorporate into E7.5 Embryos

The efficient integration of EpiSCs into embryos prompted us to compare them with AGFP7 ESCs in terms of their ability to form chimeras. The vast majority of ESC-derived cells typically remained as nonintegrated clumps either in the amniotic cavity (data not shown) or at the graft site (Figures 1F, left, and S4B). Only one of 13 embryos grafted with AGFP7 ESCs contained >12 dispersed donor cells extending >1/4 of the embryo length (Figures 1F, right, and 1I), suggesting that was a bona fide chimera. However, immunohistochemistry on sections of this embryo showed that nearly all ES-derived cells ectopically expressed the ESC markers Sox2 and Oct4 (Figures 2M and 2N). Moreover, differentiation markers were not upregulated appropriately: T and Foxa2 were not expressed, and only one cell expressing Cdx2 was detected (Figures 2O and 2P; Table 1). To further test whether ES-cell-specific gene expression persists in ESCs after grafting, we grafted TßC44cre6 ESCs, which contain a GFP transgene integrated at the Nanog locus (Nanog:GFP) in the MA, D, and MP regions of eight E7.5 embryos. If these donor cells incorporate into the host after

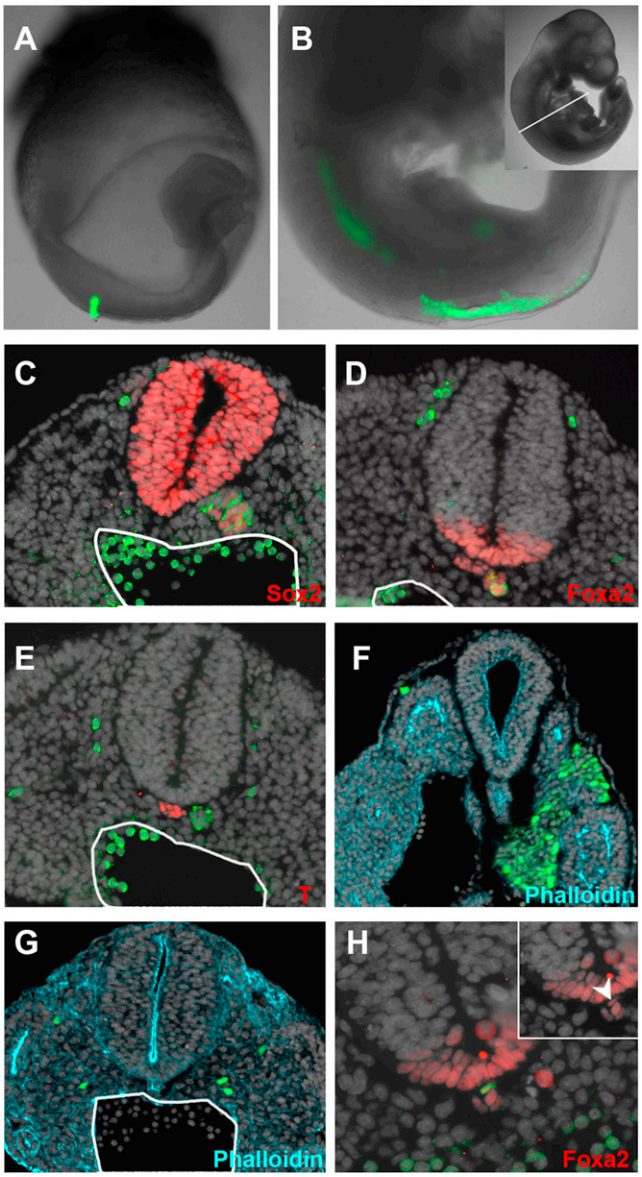

Figure 3. EpiSC Grafted in E8.5 Embryos

(A and B) Grayscale, brightfield images. (A) Representative E8.5 embryo receiving a graft of embryo-derived EpiSC in the NSB. (B) Aggregated donor cells in the recipient embryo after 48 hr. Inset: whole embryo. White line: plane of transverse sections (C–H).

(C–H) DAPI-counterstained images. Immunofluorescence (C–E) and phalloidin staining (F) show unincorporated cell clumps. Distinct clumps beneath the neural tube express Foxa2 (D) but not T (E). (G) Phalloidin staining shows dispersed donor cells in the mesoderm. The boundary of the dorsal aorta (containing highly autofluorescent circulatory blood cells) is indicated by a hand-drawn white line. (H) One Foxa2-expressing donor cell (arrowhead) is present in the notochord. Inset: green (GFP) channel removed.

See also Figure S4.

culture, they should downregulate GFP in somatic tissue, since Nanog expression is restricted to the PGCs by around E8.0 (Hart et al., 2004; Yamaguchi et al., 2005). However, like the AGFP ESC grafts, Nanog:GFP ESCs generally formed small self-adherent clumps (in seven of eight embryos) after culture. One embryo contained apparently dispersed cells at the base of the allantois; because PGCs, like ESCs, express Nanog:GFP (Osorno et al., 2012), this embryo was uninformative. In two of eight embryos, dispersed GFP^+ cells were also detected distant from the PGC location at the base of the allantois, indicating their failure to downregulate pluripotency markers (Figures S4C and S4D). Thus, unlike EpiSCs, donor ESCs incorporate very inefficiently into the host, and even when they do, they do not differentiate correctly.

EpiSCs Do Not Incorporate Well into E8.5 Embryos

The epiblast loses pluripotency at the beginning of somitogenesis (Osorno et al., 2012). However, axial progenitors in the node-streak border (NSB) contribute to two germ layer derivatives, neurectoderm and mesoderm, during organogenesis (Tzouanacou et al., 2009). The NSB can change the fates of E8.5 streak regions on heterotopic grafting (Cambray and Wilson, 2007), suggesting a strong inductive influence on grafted cells. To investigate whether EpiSCs can colonize older embryos, we grafted embryo-derived EpiSCs into the NSB of two to five somite-stage (E8.5) embryos (Figure 3A) and analyzed embryos cultured for 24–48 hr. Although the donor cells proliferated extensively in recipients (Figures 3B and S4E), the majority remained as discrete clumps in the medial somite area and ectopically expressed Sox2 (Figures 3C and S4F; n = 5). Despite expression of notochord marker Foxa2, the cells remained distinct from the endogenous notochord and did not express T (Figures 3D and 3E). Few dispersed cells were observed (Figures 3G and 3H). Thus, EpiSC integration is severely compromised in embryos later than E7.5, suggesting that the period of their compatibility with the host embryo corresponds to the period during which the postimplantation epiblast is pluripotent (Osorno et al., 2012)

In summary, both embryo- and in-vitro-derived EpiSCs are able to form chimeras and differentiate appropriately in postimplantation embryos, and thus can be considered functionally equivalent to postimplantation epiblast cells in vivo prior to somitogenesis. Therefore, although future technological advances are required to demonstrate the formation of liveborn chimeras, embryo grafting and culture provide an accessible means to test the differentiation capacity of both wild-type and mutant EpiSC lines in the context of normal early development.

EXPERIMENTAL PROCEDURES

Mouse Husbandry and Staging Embryos

Wild-type, outbred MF1 recipient mice and Nanog:GFP [129-$Nanog^{tm1(GFP-IRES-Puro)}$] females derived from TNG-targeted ESCs (Chambers et al., 2007) were maintained on a 12-hr-light/12-hr-dark cycle. Timed matings were set up overnight. Noon on the day of finding a vaginal plug was designated E0.5. At E7.0–E7.5, early-streak, mid-streak, and late-streak-stage embryos (Downs and Davies, 1993) were selected for grafting. At E8.0–E8.5, two to five somite-stage embryos were used for grafting.

Cell Lines

r04-GFP EpiSCs were derived as previously described (Tesar et al., 2007) from an E6.5 embryo of mixed genetic background. Tps/tb;$HPRT^{TREdsRed2}$-C2 (C2) EpiSCs (which carry a doxycycline-inducible dsRed transgene not used in these experiments) were differentiated as previously described (Guo

et al., 2009) from a feeder-free ESC line. Both EpiSC cell lines carry a random integration of a CAG-driven GFP, conferring ubiquitous GFP expression. Each line was passaged at least five times after genetic manipulation and before injection into the embryo. AGFP7 ESCs (Gilchrist et al., 2003) carry a randomly integrated CAG-GFP transgene and TßC44Cre6 (Chambers et al., 2007), and contain a GFP reporter integrated at the *Nanog* locus. TNG ESCs (Chambers et al., 2007) were used as a positive control for Stella immunocytochemistry.

Grafting Cells into Embryos and Embryo Culture In Vitro

Grafting was performed by hand in a dissecting stereomicroscope using a hand-pulled micropipette. ESCs or EpiSCs were scraped from culture dishes using a 20–200 μl pipette tip. The resulting cell clumps were placed close to the embryos, and a small clump was drawn into the micropipette by gentle suction. The embryo was held loosely in place with forceps while the micropipette was inserted into the region of interest to create an opening. Cells were then gently expelled as the micropipette was drawn out of the embryo, leaving a short string of cells lodged in the epiblast (Figure 1A). Embryos were cultured in 50% rat serum as previously described (Copp et al., 1990) except that E7.5 embryos were cultured in static four-well dishes (Nunclon) in an incubator at 5% CO_2 in air.

Embryo Sectioning, Staining, and Immunofluorescence

After culture, embryos were fixed with 4% paraformaldehyde in PBS at 4°C for 2 hr (E7.5 + 24–48 hr culture embryos) or overnight (E8.5 + 24–48 hr culture embryos), embedded in 7% gelatin blocks, and snap-frozen in liquid nitrogen. The blocks were stored at −80°C and sectioned at 7 μm in a cryostat (Leica). AP staining was performed with a leukocyte AP kit (Sigma-Aldrich), and actin filament staining with phalloidin-Alexa 647 (Invitrogen) was performed according to the manufacturer's instructions. Immunohistochemistry was performed as described in the Extended Experimental Procedures. Immunocytochemistry was performed as described previously (Osorno et al., 2012).

Imaging

Images of whole embryos were captured with the use of Volocity software (Perkin Elmer) on a digital camera (Qimaging) attached to a Zeiss Stemi SV11 or a Nikon NZ100 dissecting microscope. To count grafted GFP-labeled cells, whole embryos were imaged in a Leica DM IRE2 inverted confocal microscope (Leica Microsystems). Images were processed with Volocity software. A compound photomicroscope with fluorescence optics (Olympus BX61) was used to capture images of the sections. Images were processed with Adobe Photoshop software (Adobe Systems).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.10.022.

LICENSING INFORMATION

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