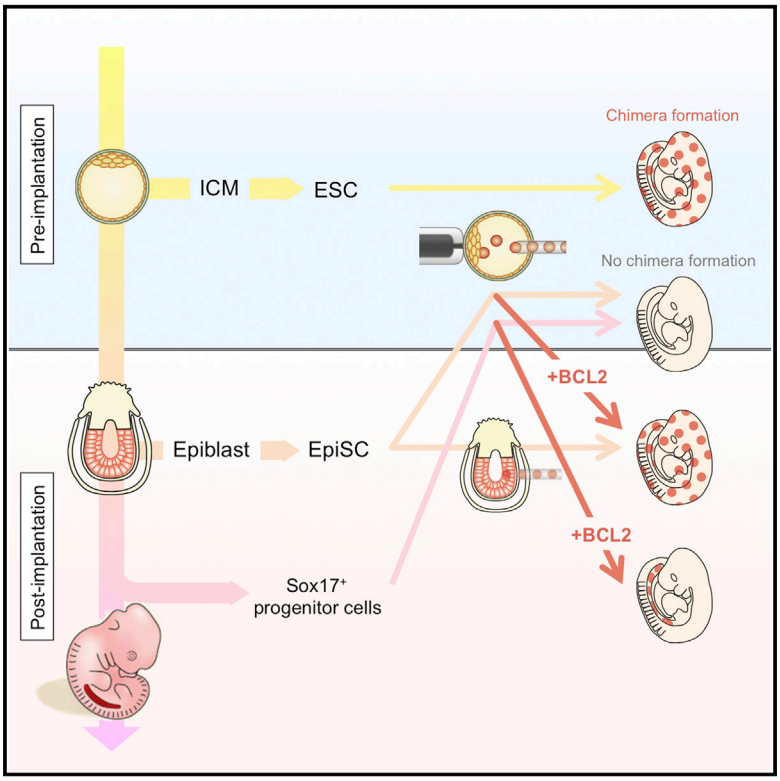
Brief Report

Cell Stem Cell

Inhibition of Apoptosis Overcomes Stage-Related Compatibility Barriers to Chimera Formation in Mouse Embryos

Graphical Abstract



Highlights

- EpiSCs injected into blastocysts rapidly disappear due to apoptosis
- Induced *BCL2* expression enables injected EpiSCs to survive and form chimeras
- *BCL2*-expressing Sox17^{+} endoderm progenitors can also form region-specific chimeras
- *BCL2*-expressing rat EpiSCs form interspecies chimeras that survive to adulthood

Authors

Hideki Masaki, Megumi Kato-Itoh, Yusuke Takahashi, ..., Sean M. Wu, Irving L. Weissman, Hiromitsu Nakauchi

Correspondence

nakauchi@stanford.edu

In Brief

Masaki et al. show that expression of the anti-apoptotic gene *BCL2* allows developmentally incompatible cells such as epiblast stem cells and endoderm progenitors to engraft into mouse blastocysts. This approach can overcome stage-related barriers to cellular integration, allowing effective formation of chimeras within and between species.

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Brief Report

Inhibition of Apoptosis Overcomes Stage-Related Compatibility Barriers to Chimera Formation in Mouse Embryos

Hideki Masaki,[1] Megumi Kato-Itoh,[1] Yusuke Takahashi,[2] Ayumi Umino,[1] Hideyuki Sato,[1] Keiichi Ito,[1,7] Ayaka Yanagida,[1,8] Toshinobu Nishimura,[2] Tomoyuki Yamaguchi,[1] Masumi Hirabayashi,[3] Takumi Era,[4] Kyle M. Loh,[5] Sean M. Wu,[6] Irving L. Weissman,[5] and Hiromitsu Nakauchi[1,2,9,*]

[1]Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-8639, Japan
[2]Institute for Stem Cell Biology and Regenerative Medicine, Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305, USA
[3]Center for Genetic Analysis of Behavior, National Institute for Physiological Sciences, Okazaki-shi, Aichi-ken 444-0864, Japan
[4]Department of Cell Modulation, Institute of Molecular Embryology and Genetics, Kumamoto University, Chuo-ku, Kumamoto 860-8555, Japan
[5]Institute for Stem Cell Biology and Regenerative Medicine and Ludwig Center for Cancer Stem Cell Biology and Medicine, Departments of Pathology and Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305, USA
[6]Stanford Cardiovascular Institute, Division of Cardiovascular Medicine, Department of Medicine, Institute for Stem Cell Biology and Regenerative Medicine and Child Health Research Institute, Stanford University School of Medicine, CA 94305, USA
[7]Present address: Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA
[8]Present address: Wellcome Trust-Medical Research Council Cambridge Stem Cell Institute, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QR, UK
[9]Lead Contact: nakauchi@stanford.edu
*Correspondence: nakauchi@stanford.edu
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SUMMARY

Cell types more advanced in development than embryonic stem cells, such as EpiSCs, fail to contribute to chimeras when injected into pre-implantation-stage blastocysts, apparently because the injected cells undergo apoptosis. Here we show that transient promotion of cell survival through expression of the anti-apoptotic gene *BCL2* enables EpiSCs and Sox17$^+$ endoderm progenitors to integrate into blastocysts and contribute to chimeric embryos. Upon injection into blastocyst, *BCL2*-expressing EpiSCs contributed to all bodily tissues in chimeric animals while Sox17$^+$ endoderm progenitors specifically contributed in a region-specific fashion to endodermal tissues. In addition, *BCL2* expression enabled rat EpiSCs to contribute to mouse embryonic chimeras, thereby forming interspecies chimeras that could survive to adulthood. Our system therefore provides a method to overcome cellular compatibility issues that typically restrict chimera formation. Application of this type of approach could broaden the use of embryonic chimeras, including region-specific chimeras, for basic developmental biology research and regenerative medicine.

Naive and primed pluripotent stem cells, originating from mouse early pre-implantation blastocysts (~E3.5–E4.5 of mouse development) and later post-implantation egg cylinders (~E5.5–E7.5), respectively, have long been thought to harbor distinct developmental potentials based on the outcome of chimera assays (Silva and Smith, 2008; Nichols and Smith, 2009). Naive mouse embryonic stem cells (mESCs) can integrate into early blastocysts and subsequently contribute to all bodily tissues in chimeric adult animals. By contrast, primed pluripotent stem cells, i.e. post-implantation mouse epiblast cells (Gardner et al., 1985) or cultured epiblast stem cells (EpiSCs) (Brons et al., 2007; Tesar et al., 2007), generally fail to engraft into early blastocysts, although they can sparsely integrate into post-implantation egg cylinders and differentiate into small numbers (dozens) of cells (Huang et al., 2012; Kojima et al., 2014; Wu et al., 2015) and *E-cadherin* expression can enhance blastocyst integration to some extent (Ohtsuka et al., 2012). Based on these findings, the developmental potential of primed mouse EpiSCs has been questioned (Silva and Smith, 2008), and there has been a general recognition that injected cells optimally contribute to chimeras only if introduced back into the developmental stage from which they were originally derived (Mascetti and Pedersen, 2016). However, why the developmental fates of naive and primed mouse pluripotent cells separated by only 24 hr of development (from E4.5 versus E5.5) differ so significantly after blastocyst injection remains unclear (Loh et al., 2015).

Previously we have shown that, after injection into pre-implantation embryos, most EpiSCs rapidly disappeared within 24 hr (Masaki et al., 2015). Based on that observation, we reasoned that EpiSCs might be prone to apoptosis upon introduction into a heterologous (mismatched) developmental environment. Here we show that this is the case, and that prevention of apoptosis enables primed mouse and rat EpiSCs, and even lineage-committed Sox17$^+$ endoderm progenitors,

to form chimeras upon injection into pre-implantation stage embryos.

To initiate this investigation, we verified our previous findings by injecting EpiSCs constitutively expressing *tdTomato* into pre-implantation mouse morulae. We found that after 1 day in culture almost all of the engrafted cells were apoptotic, as indicated by Annexin V staining (Figures 1A–1C and 1G). We then tested whether forced expression of the anti-apoptotic gene *BCL2* (Hockenbery et al., 1990) might enable injected EpiSCs to survive and form chimeras. To transiently augment EpiSC survival, we engineered an EpiSC line carrying a doxycycline (dox)-inducible *BCL2* expression cassette (Yamaguchi et al., 2012). When treated with dox starting from 24 hr before embryo injection, 42.1% ± 21.2% of engrafted *BCL2*-overexpressing EpiSCs (*BCL2*-EpiSCs) were viable (e.g., negative for Annexin V) within recipient blastocysts (Figures 1D–1F and 1G). Therefore *BCL2*-EpiSCs resisted apoptosis upon injection into pre-implantation embryos. Likewise, upon dox treatment *BCL2*-EpiSCs also exhibited higher single-cell cloning efficiencies in vitro compared to parental EpiSCs (Figure 1H), consistent with findings in *BCL2*-expressing human ESCs (Ardehali et al., 2011). Collectively, these data indicate that forced expression of *BCL2* prevents apoptosis and promotes the survival of EpiSCs both in vivo and in vitro.

After injection into pre-implantation embryos, *BCL2*-expressing EpiSCs contributed to all fetal tissues in the resulting chimeras, as would be expected for pluripotent cells. To look at longer-term integration, we transferred injected embryos into surrogate mothers continuously maintained on dox-infused water to enhance survival of the implanted cells. *BCL2*-EpiSCs from three distinct genetic backgrounds all contributed to chimeric mice whereas neither cells from the parental EpiSC lines nor EpiSCs in which a mock vector had been introduced ever formed chimeras (Figures 1I, 1J, and 1L; Table S1). *BCL2* expression therefore broadly affords EpiSCs with the ability to integrate into blastocysts. The resulting EpiSC-derived chimeras developed to adulthood (Figure 1K). The progeny of *BCL2*-EpiSCs were distributed across the entire embryo (Figure 1J) and included cells in all three germ layers (Figures S1D–S1F). Quantitatively, *BCL2*-EpiSCs contributed to fibroblasts, hematopoietic cells, and hepatoblasts in E13.5 chimeras to an extent that was comparable to, or indistinguishable from, injected control ESCs (Table S1, Figure S1J). Collectively, these results show that upon introduction into pre-implantation embryos, *BCL2*-EpiSCs can survive and form extensive chimeras comparable to those formed by ESCs. Overexpression of other anti-apoptotic genes (*Bcl-xl* or *CrmA*) also enabled EpiSCs to form pre-implantation chimeras, similar to *BCL2* (Figures S1A–S1C). In summary, therefore, enhancement of cell survival enabled primed EpiSCs to engraft into blastocysts and form chimeras, providing formal evidence of the full pluripotency and developmental competence of primed EpiSCs.

To investigate whether *BCL2* expression might also enable more developmentally advanced tissue progenitors to engraft blastocysts and form chimeras, we focused on endoderm progenitors. Endoderm progenitors are broadly classified into primitive endoderm (which principally gives rise to the yolk sac) (Gardner and Rossant, 1979) and definitive endoderm (which is the precursor to various internal organs, including the lungs, pancreas, liver, and intestines) (Švajger et al., 1986). Primitive and definitive endoderm are both characterized by expression of *Sox17* (Kanai-Azuma et al., 2002) and *Foxa2* (Sasaki and Hogan, 1993).

To conduct these experiments, we differentiated cells from an mESC line carrying human CD25 knocked into the *Sox17* locus (Yasunaga et al., 2005) into Sox17$^+$ endoderm progenitors. With this reporter line, differentiated mouse endoderm progenitors can be purified by virtue of human CD25 protein expression. We found that Sox17-CD25$^+$ cells expressed archetypical endodermal markers (*Gata4*, *Gata6*, and *Foxa2*) but lacked markers of pluripotency, either naive or primed, or of mesoderm, indicating that they were committed endoderm progenitors (Figure 2A).

We then labeled purified Sox17-CD25$^+$ endoderm cells with *tdTomato*, engineered them to express *BCL2*, and injected them into blastocysts. Strikingly, 6 days after injection, these *BCL2*-overexpressing endoderm cells significantly contributed to mid-gestation mouse embryos. However, their descendants were exclusively distributed in the yolk sac or gut (Figures 2B–2E), contrasting with undifferentiated cells from the same ESC line, which contributed extensively to many tissues (Figures 2F and 2G). Focusing on the embryo proper, the contribution of tdTomato$^+$ Sox17$^+$ cells was restricted to Foxa2$^+$ gut endoderm (Figures 2H–2K).

Because *Sox17* is expressed in both primitive and definitive endoderm (Gouon-Evans et al., 2006; Sherwood et al., 2007; Yasunaga et al., 2005), the injected Sox17$^+$ endoderm population may have included cells of both lineages. Chimerism was noted more frequently in the yolk sac (n = 7/8 independent experiments) than in the embryo proper (n = 4/8 independent experiments; Table S2), implying that the original Sox17$^+$ population included more primitive endoderm than definitive endoderm. Moreover, as some primitive endoderm cells naturally contribute to gut endoderm (Kwon et al., 2008), it is also possible that the purified Sox17$^+$ endoderm population contained primitive endoderm that then contributed to gut tissues.

BCL2 overexpression was a significant factor in the ability of injected Sox17$^+$ endoderm cells to engraft into pre-implantation embryos and form chimeras, as untransduced control Sox17$^+$ cells lacking the *BCL2* overexpression cassette rarely contributed to the yolk sac (Figure 2L, Table S2). Expression of the alternative anti-apoptotic genes *Bcl-xl* or *CrmA* also instilled Sox17$^+$ endoderm progenitors with the ability to form region-specific chimeras (Figure 2L, Table S2), phenocopying the effects of *BCL2* expression.

We then examined the early integration patterns of injected cells in chimeric embryos. EpiSCs and Sox17$^+$ endoderm were both randomly distributed across injected blastocysts after 1 day of in vitro culture (Figures S2A and S2D). However, at the egg-cylinder stage, Sox17$^+$ cells were found only in visceral endoderm (Figures S2E and S2F), whereas EpiSCs were localized to the epiblast (Figures S2B and S2C), as expected. These findings suggest that prevention of apoptosis supports survival of grafted progenitors in pre-implantation embryos, and that once surviving cells synchronize with the developmental stage of the host, they take part in morphogenesis and follow their appropriate developmental fate. Our results show that prevention of apoptosis by *BCL2* overexpression enables chimera formation even by non-pluripotent progenitor cells.

Finally, we looked at whether prevention of apoptosis could enable cells from other species, namely rat EpiSCs, to form

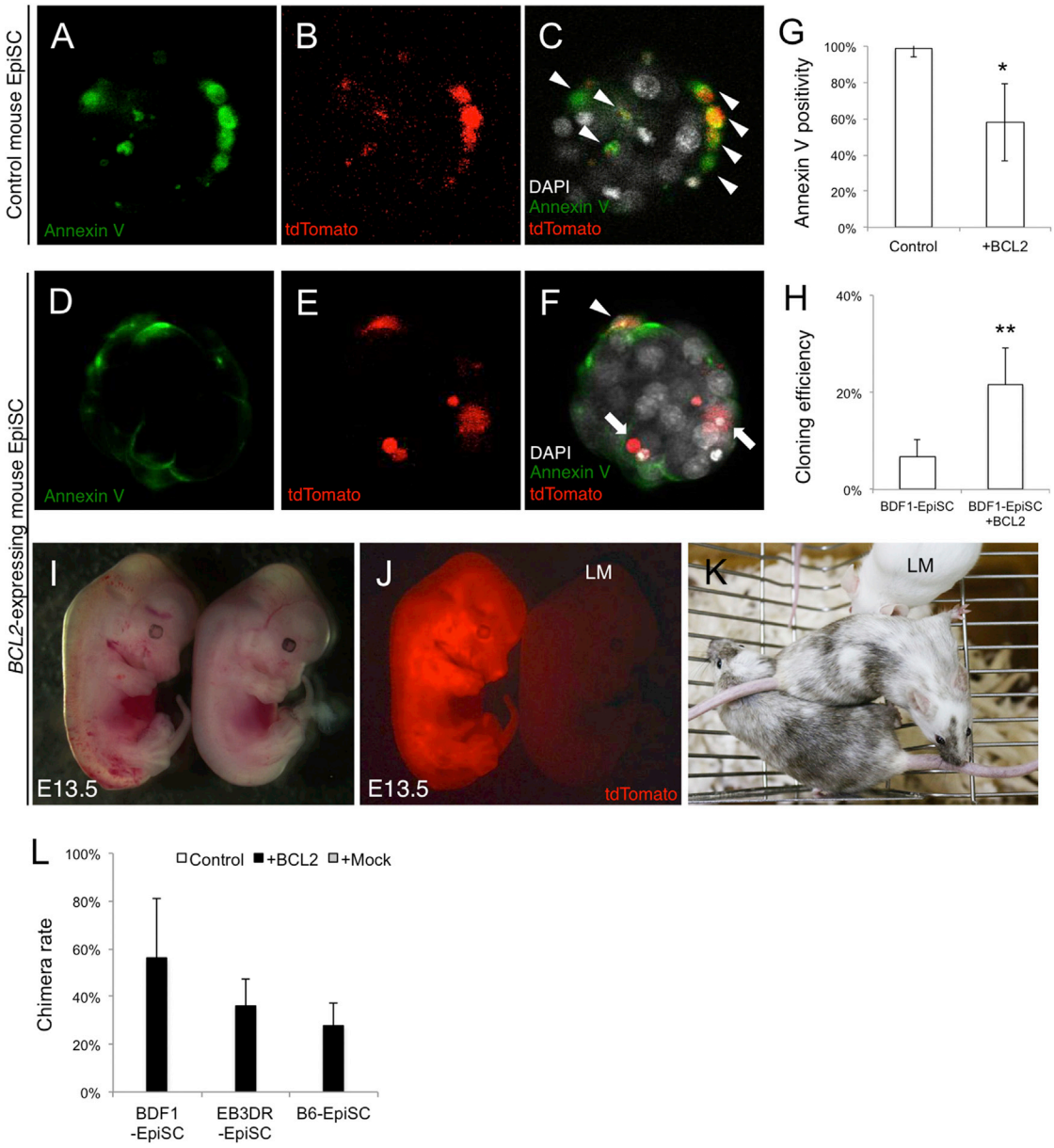

Figure 1. BCL2 Overexpression Enables EpiSCs to Form Chimeras with Pre-implantation Embryos

(A–F) Annexin V staining of embryos injected with tdTomato$^+$ cells, 1 day after injection. Annexin V staining (A and D), anti-DsRed antibody staining for tdTomato$^+$ cells (B and E), and overlaid images (C and F) of embryos injected with either control EpiSCs (A–C) or *BCL2*-overexpressing EpiSCs (*BCL2*-EpiSCs) (D–F) are shown. Arrowheads indicate injected cells stained with Annexin V, and arrows indicate cells not stained.

(G) Histogram of the percentage of engrafted tdTomato$^+$ cells that were Annexin V$^+$ in injected blastocysts (mean ± SD of 20 samples each, *$p < 0.0001$). *BCL2* overexpression supported survival of engrafted EpiSCs. We enumerated the percentage of Annexin V$^+$ cells by dividing the number of Annexin V$^+$ cells over the total number of tdTomato$^+$ cells.

(H) Histogram of cloning efficiency after single-cell sorting. *BCL2*-EpiSCs showed higher cloning efficiency than untreated control cells (mean ± SD of four technical replicates, **$p < 0.05$).

(I and J) Chimeric E13.5 embryos derived from *BCL2*-EpiSCs; bright field (I) and fluorescence (J) images are displayed.

(K) Black coat color indicates EpiSC-derived chimeric mice developed to adulthood. LM, non-chimeric littermate (negative control).

(L) Histogram of chimera-forming rate of *BCL2*-EpiSC lines and their parental EpiSCs as controls (mean ± SD). The AiLV-mCherry BDF1-EpiSC line was also assessed as a mock control.

See also Figure S1.

chimeras. When untreated primed rat EpiSCs were injected into mouse blastocysts, interspecies chimeras never formed (Figures S2G, S2H, and S2L). However, *BCL2*-overexpressing rat EpiSCs successfully formed interspecies chimeras (Figures S2I–S2K). These interspecies chimeras developed to adulthood (Figure S2K) and survived as long as chimeras derived from rat ESCs or iPSCs.

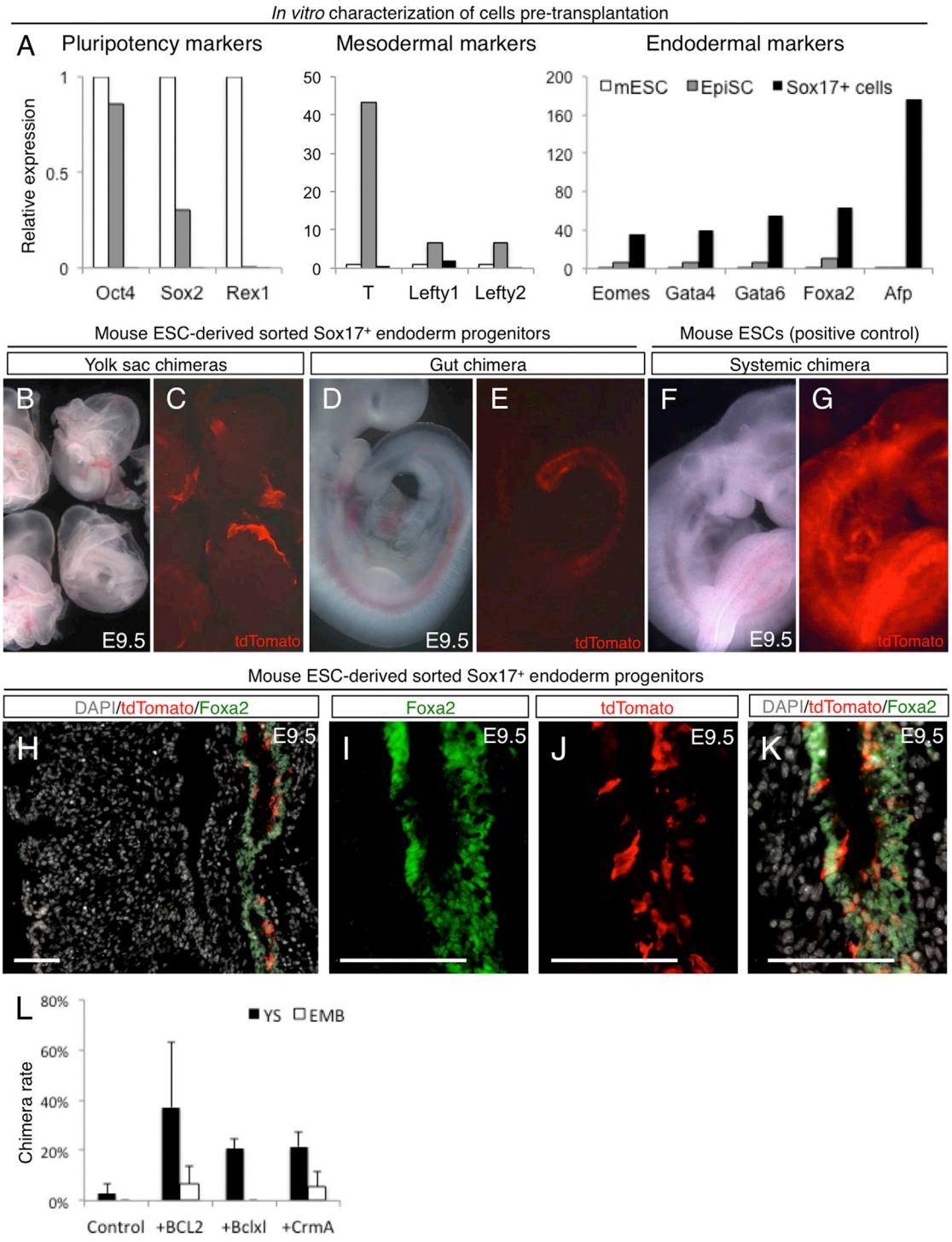

Figure 2. Region-Specific Chimera Formation with Sox17⁺ Progenitor Cells

(A) Gene-expression profiles of mouse ESCs, EpiSCs, and Sox17⁺ cells derived from the K17-5 mouse ESC line, assayed by quantitative PCR. Vertical axis indicates relative gene-expression levels normalized to those in ESCs.

(B–G) Bright field (B, D, and F) and fluorescence (C, E, and G) images of E9.5 chimeric embryos. K17-5 cells carrying tet-ON *BCL2* and *CAG-tdTomato* were differentiated for 6 days. Isolated Sox17⁺ cells were then injected into mouse blastocysts (B–E). The progeny of injected Sox17⁺ cells indicate that chimerism was observed only in yolk sac (B and C) or gut endoderm (D and E). In contrast, chimeras obtained from undifferentiated ESCs from the same experiment show whole-body chimerism (F and G).

(legend continued on next page)

As for mouse *BCL2*-overexpressing EpiSCs, the progeny of *BCL2*-overexpressing rat EpiSCs were distributed across the entire body of chimeras (Figure S2J) and differentiated into cells of all three germ layers (Figures S1G–S1I). Prevention of apoptosis can therefore also enable primed rat EpiSCs to form interspecies chimeras with mouse blastocysts, overriding barriers related to both developmental stage and species.

Generation of embryonic chimeras is of both practical and conceptual importance as it provides a method to assess the developmental competence of injected cells. It could therefore be used to test the properties of differentiated or reprogrammed cells in an in vivo setting. Chimera formation also holds potential as a source of organs for regenerative medicine. We have pursued the in vivo generation of human organs in chimeric livestock through blastocyst complementation of organ-deficient animals (Kobayashi et al., 2010; Matsunari et al., 2013; Rashid et al., 2014), but it has been very challenging. A prerequisite to this potentially useful approach for obtaining donor organs is the successful generation of interspecies chimeras between human and livestock. Current difficulties in obtaining interspecies chimeras might reflect genuine xenogeneic barriers and/or might stem from transplantation of cells into heterologous developmental environments (Rashid et al., 2014; Mascetti and Pedersen, 2016). Our present findings suggest that both barriers might be at least partially overcome by transiently inhibiting apoptosis in transplanted cells. Hence our findings might also offer a technological platform by which targeted organs might one day be made in vivo by the mixture of developmentally advanced tissue progenitors (i.e., endoderm progenitors or their derivatives) with pre-implantation embryos to form tissue-specific chimeras in a way that avoids contribution to host gametes or neural tissues (Kobayashi et al., 2015). If properly realized, this approach could mitigate the ethical concerns that exist and thus could constitute an important step toward targeted in vivo organ generation.

Our findings also suggest that the concept of developmental competence is more sophisticated than once thought. Indeed, if performed in heterologous contexts, current in vivo transplantation assays may underestimate, or fail to reveal, the intrinsic developmental competence of a given lineage. Although the maturation of E4.5 naive pluripotent epiblast into E5.5 primed pluripotent epiblast is accompanied by changes in transcription factor expression, it is also accompanied by structural reorganization of post-implantation cells into a pseudostratified epithelium (Bedzhov and Zernicka-Goetz, 2014; Snow, 1977), which Loh et al. (2015) suggested could make primed pluripotent cells structurally incompatible with the pre-implantation blastocyst environment. Our results now show that the inability of EpiSCs to form chimeras with pre-implantation embryos (Brons et al., 2007; Tesar et al., 2007) is not due to an innate defect in developmental competence, and blockade of cell death allows their developmental competence to be fully realized in vivo in pre-implantation chimeras.

The reason why these otherwise competent cells are normally unable to engraft and survive may suggest an underlying physiological mechanism in the embryo in which developmentally unmatched "straggler" or "outlier" cells are normally eliminated. Such deviant cells might be apoptotically purged (Wakamatsu et al., 1998) by deprivation of trophic signals present only at correct developmental stages or alternately by active elimination (Clavería et al., 2013). However, when apoptosis was temporarily prevented by expression of *BCL2*, surviving cells seemed to maintain their developmental fate until their developmental stage was synchronized with that of the host and then resume development in step with host cells.

To what extent can developmental incompatibility be overcome by *BCL2* expression? The EpiSC lines that we used were established from E6.5 embryos (Masaki et al., 2015; Murayama et al., 2015) and these cells were injected into ~E2.5 mouse morulae. We infer that the engrafted EpiSCs "paused" their developmental program for 3–5 days (a long time in mice, where gestation is ~21 days) until synchronization with host development had occurred. We expect that once the engrafted cells enter their appropriate developmental environment, e.g., *BCL2*-EpiSCs in the epiblast of the ~E5.5–E6.5 egg cylinder embryo, the cells should not require *BCL2* expression for survival. If this is the case, engrafted lineage-committed precursor cells would most likely require a longer duration of *BCL2* expression to attain developmental synchrony with recipient embryos. The duration of anti-apoptotic factor expression needed to support chimera formation in heterologous contexts remains an outstanding question. In our experiments, we gave dox-containing water to the surrogate mothers until day 4 or day 7 after transfer of *BCL2*-EpiSC-injected blastocysts (at which point the transferred embryos developed to either E6.5 or E9.5, respectively) and we did not see significant differences in the degree of chimerism (data not shown). As transgene expression persists for several days in vivo in the tet-ON system even after withdrawal of dox (Traykova-Brauch et al., 2008), the precise timing of obligate *BCL2* expression remains unclear. Nevertheless, once this and other questions are resolved, we envisage that *BCL2* expression or other apoptosis blocks could see broad application in the generation of embryonic chimeras for basic research or regenerative medicine.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Supplemental Experimental Procedures, two figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2016.10.013.

AUTHOR CONTRIBUTIONS

H.M. developed the concepts and performed all the experiments and data analysis; M.K. contributed to all the experiments; A.U. and H.S. contributed to Figures 1, S1, and S2; K.I., A.Y., and T.E. contributed the experiments related to Figures 2 and S2; and Y.T. and T.N. contributed to Figure S2.

(H–K) Immunohistochemistry of frozen sections of Sox17$^+$ progenitor-derived chimera stained with anti-Foxa2 antibody to mark gut endoderm. (H) Overlaid image showed that tdTomato$^+$ progeny were only distributed in gut endoderm. (I–K) Higher-magnification images of anti-Foxa2 antibody staining (I), anti-DsRed antibody staining for tdTomato$^+$ cells (J), and overlay (K) are shown. Scale bars represent 100 μm.

(L) Histogram of the percentage of chimeras that demonstrated either yolk sac (YS) or embryo body (EMB) chimerism (mean ± SD). Control cells only carried *CAG-tdTomato*.

See also Figure S2.

K.M.L., S.M.W., and I.L.W. edited the manuscript. T.Y., M.H., I.L.W., and H.N. supervised all the studies.

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