Supplementary Data

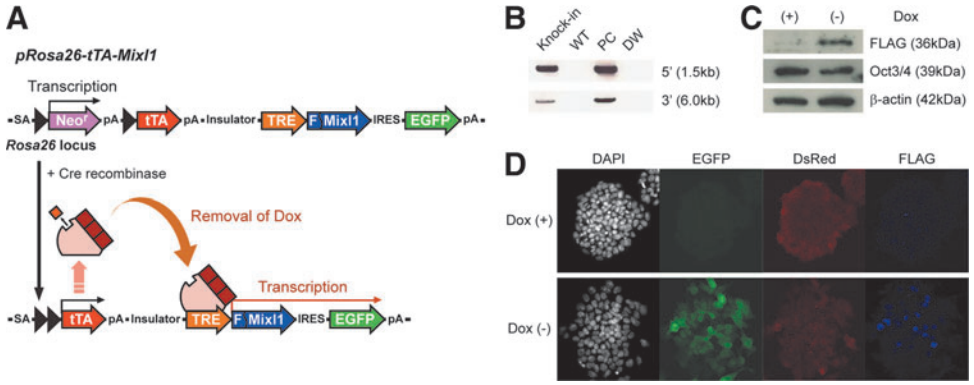
Generation and Characterization of *Mixl1*-Inducible Embryonic Stem Cells Under the Control of *Oct3/4* Promoter or *CAG* Promoter

We first introduced an expression unit composed of a tet-response element (TRE) and *Mixl1* into the *Rosa26* locus of wild-type mouse embryonic stem cells (ESCs). The construct also enabled ubiquitous tdTomato expression under the control of an endogenous *Rosa26* promoter (pRosa26-TIN-TRE-Mixl1; Supplementary Figs S3A and S4A). For OTiV-ESCs, we next introduced into ESCs a bacterial artificial chromosome containing *tTA* with *Venus*, a gene encoding a variant of yellow fluorescent protein, under the control of an *Oct3/4* promoter (pOct3/4-BAC-tTA-Venus; Supplementary Fig. S4A). OTiV1-ESCs should express tTA and Venus only in undifferentiated ESCs, as their expression is under the control of the undifferentiated ESC-specific promoter *Oct3/4* promoter. Visual analysis of gene expression showed that Venus levels were high in undifferentiated ESCs but dramatically decreased when these cells were cultured under differentiation conditions that included removal of 2 inhibitors (2i), leukemia inhibitory factor (LIF) from the medium and addition of retinoic acid to the medium (Supplementary Fig. S4C). In the absence of Dox, Mixl1 is expressed by the *Oct3/4* promoter at levels corresponding to those in the undifferentiated state (Supplementary Fig. S4B). Furthermore, when OTiV1-ESCs were injected into tetraploid embryos that were then transferred into uteri of foster mothers drinking water that contained Dox, we found Venus expression in epiblasts at E5.5 and E6.5, that is, on days 3 to 4 after ET (Supplementary Fig. S4D). These data clearly indicate success in establishing an ESC line expressing Mixl1 under the control of an *Oct3/4* promoter.

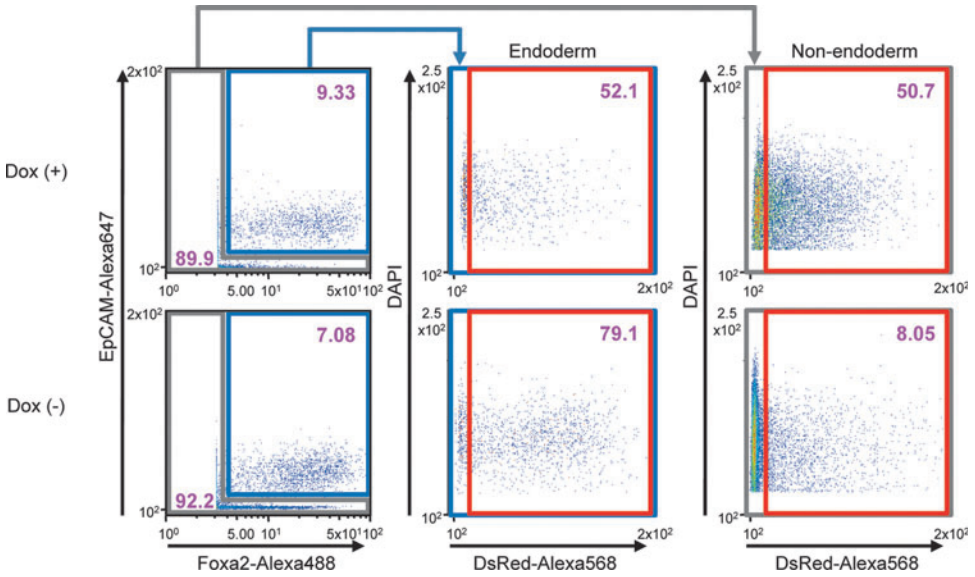
For CHT5-ESCs, we introduced pCAG-tTA-IP, instead of Oct3/4-tTA (Supplementary Fig. S3A). In the resultant CHT5-ESCs, we could regulate the expression of Mixl1 just as we did with the RT5 cells (Supplementary Fig. S3B). As expected, in contrast to Dox(+) settings where generalized chimerism was observed, a preferential contribution of CHT5-ESC-derived cells to the endoderm in settings lacking Dox was confirmed after an injection of CHT5-ESCs into blastocysts (Fig. 2B and Supplementary Fig. S3C).

Supplementary Reference

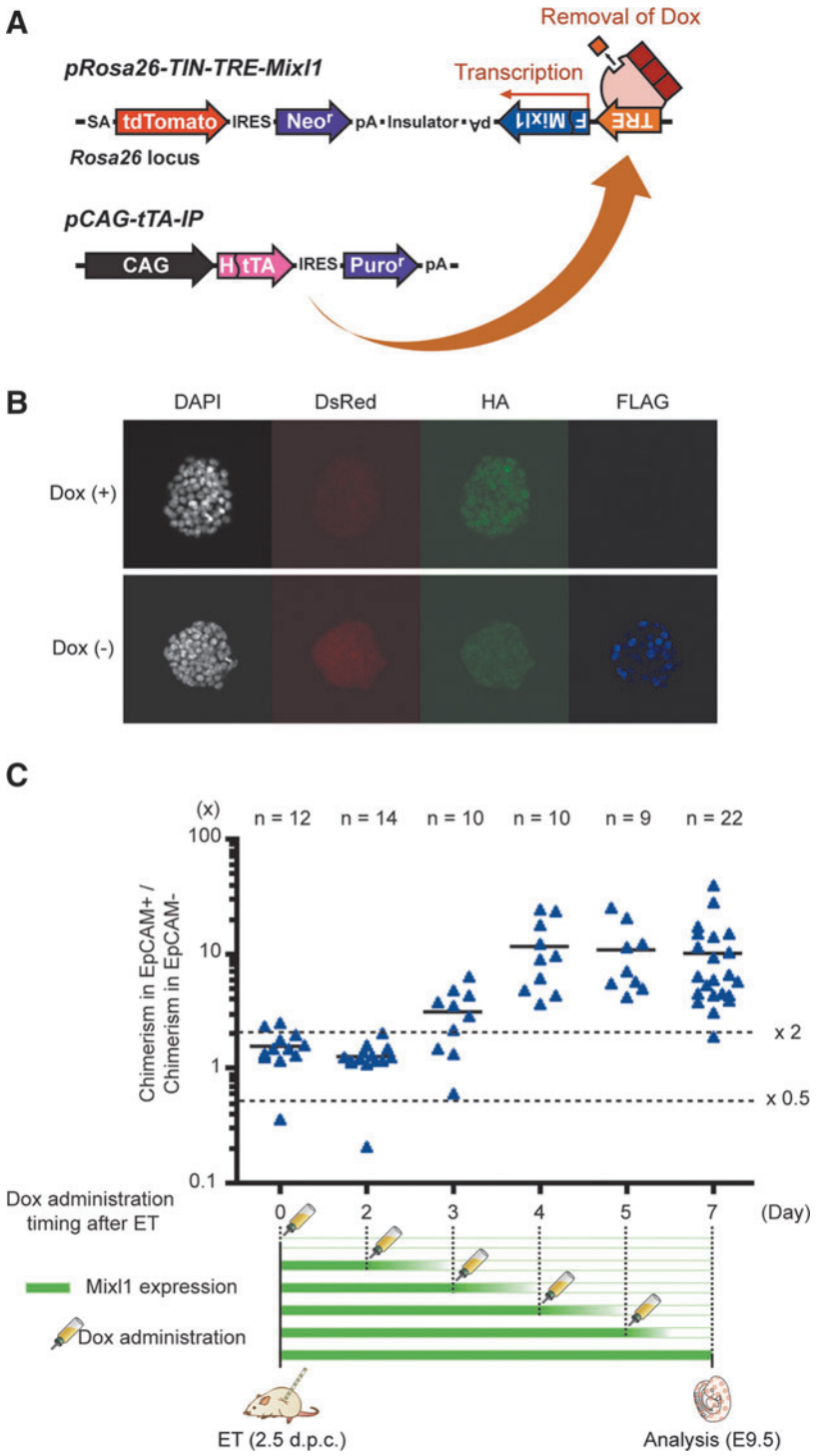
1. Matsumoto K, T Isagawa, T Nishimura, T Ogaeri, K Eto, S Miyazaki, J Miyazaki, H Aburatani, H Nakauchi and H Ema. (2009). Stepwise development of hematopoietic stem cells from embryonic stem cells. PLoS One 4:e4820.



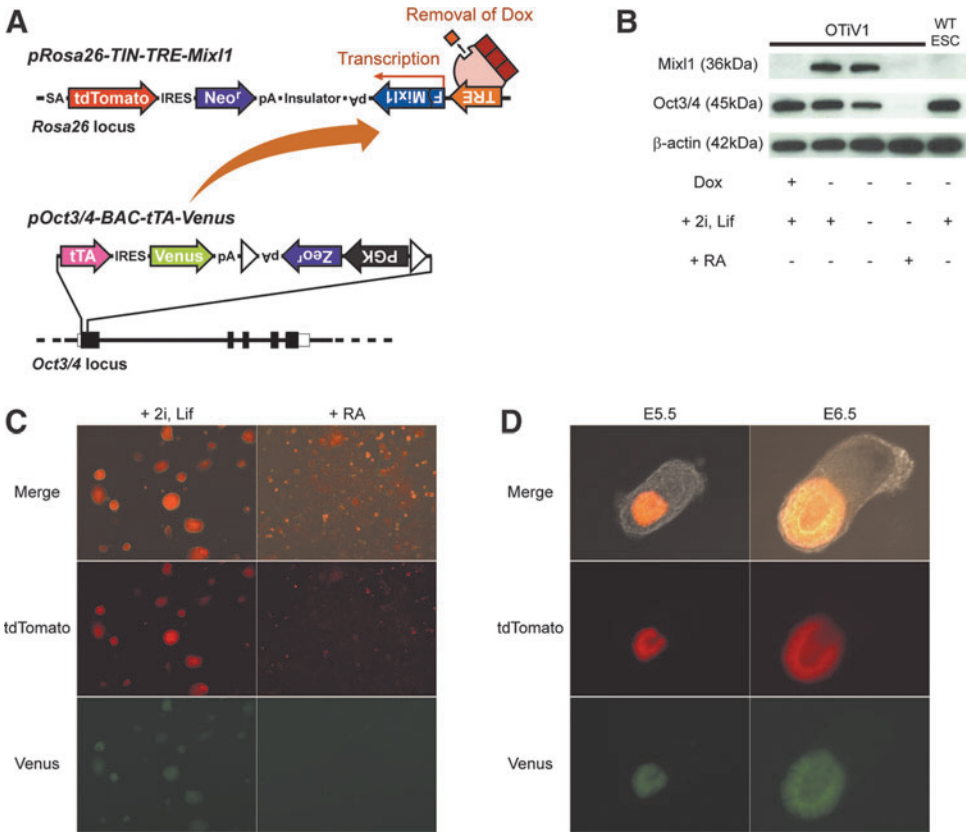
SUPPLEMENTARY FIG. S1. Generation of Mixl1-inducible ESCs using a single cassette Tet-Off regulatory system, related to Fig. 1. **(A)** Schema of system for inducing *Mixl1* expression in *Rosa26* locus. After insertion of the *Mixl1*-containing cassette into the *Rosa26* locus of mouse ESCs, a neomycin resistance gene (*Neo*) flanked by 2 *loxP* sequences was activated by transient expression of *Cre* recombinase. After removal of doxycycline (Dox) from culture medium, the tetracycline (tet) trans-activator (tTA) can bind to a tet-response element (TRE) and activate the expression of *FLAG*-tagged *Mixl1* with EGFP. **(B)** Confirmation of gene targeting in *Rosa26* locus by genomic PCR for 5′ and 3′ arms. Positive control (PC), genomic DNA from similarly modified cells (Matsumoto et al., 2009); negative controls, genomic DNA from wild-type ESCs (WT) and distilled water (DW). **(C)** Expression of exogenous *FLAG*-tagged *Mixl1* and endogenous *Oct3/4* in Mixl1-inducible ESCs (RT5-ESCs) in presence of Dox [Dox(+)] or absence of Dox [Dox(−)]. Internal control, β-actin, western blots. **(D)** RT5-ESCs [Dox(+), Dox(−)] immunostained for FLAG-tagged Mixl1 with EGFP. Cells were immunostained for FLAG (*blue*) and EGFP (*green*); nuclear counterstaining, 4′,6-diamidino-2-phenylindole (DAPI; *white*). ESCs, embryonic stem cells; PCR, polymerase chain reaction.



SUPPLEMENTARY FIG. S2. Dot plot analysis of contribution of *Mixl1*-inducible ESCs to the endoderm in settings with or without Dox, related to Fig. 1. Sections immunostained in Fig. 1C were imaged by ArrayScan technology, with collected data analyzed by FloJo software.



SUPPLEMENTARY FIG. S3. Generation and characterization of *Mixl1*-inducible ESCs using CAG-tTA transgene, related to Fig. 2. **(A)** Schema of system for inducing *Mixl1* expression under the control of the *CAG* promoter. ESCs into which TRE-driven *Mixl1* had been inserted were transduced with a transgene containing *HA*-tagged *tTA* under the control of the *CAG* promoter. **(B)** CHT5-ESCs [Dox(+), Dox(−)] immunostained for FLAG-tagged Mixl1 with HA-tagged tTA. Cells were immunostained for FLAG (*blue*) and HA (*green*); nuclear counterstaining, DAPI (*white*). **(C)** Degrees of chimerism in embryonic endodermal tissues in Dox(+) and Dox(−) settings. *Triangles* indicate values for individual chimeric embryos.



SUPPLEMENTARY FIG. S4. Generation and characterization of *Mixl1*-inducible ESCs using Oct3/4-tTA transgene, related to Fig. 2. **(A)** Schema of system for inducing *Mixl1* expression under the control of the *Oct3/4* promoter. TRE-driven *Mixl1* was inserted into the *Rosa26* locus of ESCs with *tdTomato*. In addition, a bacterial artificial chromosome containing tTA with *Venus* under the control of the *Oct3/4* locus was transduced into the ESCs. **(B)** Expression of exogenous *Mixl1* and endogenous *Oct3/4* in OTiV1-ESCs after 3 days' culture under various conditions. Internal control, β-actin, western blots. +2i, LIF, maintaining undifferentiated state of ESCs and +RA, inducing differentiation of ESCs. **(C)** Expression patterns in vitro of *tdTomato* and *Venus*. OTiV-ESCs were cultured in the presence of 2i and Lif (+2i, Lif) for maintenance of the undifferentiated state or in the presence of retinoic acid (+RA) for differentiation. **(D)** Expression patterns in vivo of *tdTomato* and *Venus*. OTiV-ESC-injected blastocysts were transferred into uteri of foster mothers given drinking water with Dox, with embryos analyzed at 3 days (E5.5) and 4 days (E6.5) after embryo transfer. 2i, 2 inhibitors; LIF, leukemia inhibitory factor.