

—Original—

Generation of bicistronic reporter knockin mice for visualizing germ layers

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Abstract: Knockout mouse models are commonly used in developmental biology to investigate the functions of specific genes, and the knowledge obtained in such models has yielded insights into the molecular mechanisms underlying developmental processes. Gastrulation is the most dynamic process in embryogenesis during which differentiation into three germ layers occurs. However, the functions of genes involved in gastrulation are not completely understood. One major reason for this is the technical difficulty of embryo analysis to understand germ layer location. We have generated three reporter mouse strains in which the germ layers are distinguished by different fluorescent reporters. Using CRISPR/Cas9 genome editing in mouse zygotes, the fluorescent reporter genes, *EGFP*, *tdTomato*, and *TagBFP* including 2A peptide sequences were knocked into the appropriate sites before the stop codon of the *Sox17* (endoderm marker), *Otx2* (ectoderm marker), and *T* (mesoderm marker) genes, respectively. Founder mice were successfully generated in the *Sox17-2A-EGFP*, *Otx2-2A-tdTomato*, and *T-2A-TagBFP* knockin reporter strains. Further, homozygous knockin mice of all strains appeared morphologically normal and were fertile. On stereomicroscopic analysis, fluorescent signals were detected in a germ layer-specific manner from heterozygous embryos at embryonic day (E) 6.5–8.5 in all strains, and were immunohistochemically demonstrated to match their respective germ layer-specific marker protein at E7.5. Taken together, these observations suggest that the *Sox17-2A-EGFP*, *Otx2-2A-tdTomato*, and *T-2A-TagBFP* knockin reporter mice may be useful for comprehensive analysis of gene function in germ layer formation.

Key words: bicistronic reporter mice, gastrulation, germ layers

Introduction

A recent International Mouse Phenotyping Consortium (IMPC) study using knockout (KO) mouse models re-


ported that 5.7% of mouse gene knockouts (107/1,861 strains) resulted in embryonic lethality before embryonic day (E) 9.5 [6]. The study also suggested that there are more than 1,000 essential genes for early embry-

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onic development in mice, but the majority of these genes have yet to be identified. Therefore, advances in studies using KO mouse models will continue to be critical for better understanding the developmental mechanism. Although the IMPC carried out systematic characterization of embryonic phenotypes using high-throughput pipelines, detailed analyses of mouse embryos before E9.5 have not been reported. Gastrulation, which occurs from E6.5 to E8.5, is one of the most important stages before E9.5 in mouse embryogenesis. In gastrulation, dynamic proliferation and migration of cells occur to form three germ layers, i.e., the endoderm, ectoderm, and mesoderm [26]. However, the molecular mechanism underlying germ layer formation is not yet completely understood. Therefore, visualization of each germ layer in various gene KO mice would be an effective means of investigating their functions.

Genetically modified mice carrying reporter genes can be generated by numerous methods. One simple method involves microinjection of DNA fragments, which are derived from plasmids or bacterial artificial chromosomes (BACs) carrying the reporter gene fused to the regulatory region of a target gene, into zygotes to generate transgenic mice [10]. However, this is associated with the risk of ectopic expression or disruption of endogenous genes due to random integration of the transgene into the chromosomal DNA. With traditional knockin strategies, the reporter gene is expected to be expressed according to the endogenous expression pattern of the target gene, but with disruption of the target gene [23]. Occasionally, reporter genes are integrated immediately upstream or downstream of the target gene to generate a fusion protein [4]. However, it is not certain whether the fusion proteins will function in the same way as the target protein. The use of bicistronic knockin mice can overcome the problems. Recently, we produced bicistronic *Ins1*-Cre knockin mice by CRISPR/Cas9-mediated genome editing in mouse zygotes without disruption of the endogenous *Ins1* gene [11]. In this driver mouse strain, the function of insulin was maintained and the *Cre* gene was expressed under the transcriptional regulation of *Ins1* because the *Cre* gene including 2A sequences derived from porcine teschovirus-1 (P2A) was integrated at a site before the *Ins1* gene stop codon. Thus, the bicistronic knockin system is generally helpful for production of a reporter mouse strain that maintains the intact function of the respective target gene.

To generate fluorescent proteins in each germ layer of

the mouse gastrula, we used three genes, orthodenticle homeobox 2 (*Otx2*), *T* encoded Brachyury, and SRY (sex determining region Y)-box 17 (*Sox17*). The ectoderm is the first germ layer to emerge during gastrulation. Early ectodermal lineage cells can be identified by the expression of markers, such as the transcription factor, OTX2 [3, 19, 27]. Next, early mesodermal lineage specification is characterized by the expression of specific markers, such as the transcription factor, Brachyury [14, 18, 28]. The *T* gene is expressed first from the primitive streak during gastrulation [14, 18]. Lastly, establishment of the definitive endoderm is dependent on the activities of transcription factors, including members of the Sox and forkhead domain families. Especially, SOX17 plays an important role in endoderm development [15]. Therefore, in the present study, we used CRISPR/Cas9-mediated genome editing in mouse zygotes to generate *Sox17-2A-EGFP*, *Otx2-2A-tdTomato*, and *T-2A-TagBFP* bicistronic reporter knockin mouse strains, to allow visual identification of the endodermal, ectodermal, and mesodermal tissues, respectively, during gastrulation.

Materials and Methods

Animals

C57BL/6J mice were purchased from Charles River Laboratories (Yokohama, Japan). Mice were maintained in plastic cages under pathogen-free conditions in a room maintained at $23.5 \pm 2.5^\circ\text{C}$ and $52.5 \pm 12.5\%$ relative humidity under a 14-h light:10-h dark cycle. Mice had free access to commercial chow (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and filtered water. Animal experiments were carried out in a humane manner with approval from the Institutional Animal Experiment Committee of the University of Tsukuba in accordance with the Regulations for Animal Experiments of the University of Tsukuba and Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Cell culture and transfection

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, in an atmosphere of 5% CO₂ at 37°C. Briefly, 3.5×10^4 HEK293T cells were transfected with *pX330* and *pCX-EGxxFP* vectors of the

target genes by Lipofectamine LTX (Thermo Fisher Scientific, Wilmington, DE, USA) or 6×10^5 HEK293T cells were transfected with *pCX-V5-Sox17-P2A-EGFP-HA*, *pCX-V5-Otx2-P2A-tdTomato-HA*, and *pCX-V5-T-P2A-TagBFP-HA* by polyethylenimine (Sigma-Aldrich, St. Louis, MO, USA). After 24 h, the HEK293T cells were observed by fluorescence microscopy (BZ-X710; Keyence, Osaka, Japan).

Vector construction

Sox17, *Otx2*, and *T* CRISPR oligo DNAs (Supplementary Table 1) were annealed, purified, and inserted into the *pX330* vector (Addgene plasmid 42230, a gift from Dr. Feng Zhang [5]) to yield constructs designated as *pX330-Sox17*, *pX330-Otx2*, and *pX330-T*, respectively [22]. To construct the vector for the EGxxFP system, parts of the final exon of each of the *Sox17*, *Otx2*, and *T* genes containing the CRISPR target were amplified with the primers listed in Supplementary Table 2 and inserted into the *pCX-EGxxFP* vector to produce *pCX-EGxxFP-Sox17*, *pCX-EGxxFP-Otx2*, and *pCX-EGxxFP-T*, respectively, as described previously [22]. The 5' arm upstream and the 3' arm downstream of the stop codon of *Sox17*, *Otx2*, and *T* were amplified (Supplementary Table 3) and the PCR products were cloned into the reporter platform plasmids *pBS-GSG-P2A-EGFP-rGpA*, *pBS-GSG-P2A-tdTomato-rGpA*, and *pBS-GSG-P2A-TagBFP-rGpA*, respectively, to construct the knockin plasmids *pBS-Sox17-GSG-P2A-EGFP-rGpA* (*Sox17* donor plasmid), *pBS-Otx2-GSG-P2A-tdTomato-rGpA* (*Otx2* donor plasmid), and *pBS-T-GSG-P2A-TagBFP-rGpA* (*T* donor plasmid), respectively.

Total RNAs from 12–15 knockin embryos at E7.5 were extracted using Isogen (Nippon Gene, Toyama, Japan) and cDNA was synthesized using oligo-dT primers (Thermo Fisher Scientific) and SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). The cDNAs from mouse *Sox17-2A-EGFP*, *Otx2-2A-tdTomato*, and *T-2A-TagBFP* were then amplified with PrimeSTAR® GXL DNA Polymerase (Takara, Otsu, Japan) (Supplementary Table 4) and cloned into pCX-V5-HA platform plasmids. The resultant plasmids were designated as *pCX-V5-Sox17-P2A-EGFP-HA*, *pCX-V5-Otx2-P2A-tdTomato-HA*, and *pCX-V5-T-P2A-TagBFP-HA*, respectively. To construct the vector, the inserts were amplified by PCR with PrimeSTAR® GXL DNA Polymerase (Takara) and cloned into plasmids using an In-Fusion HD Cloning Kit (Takara).

Microinjection

After superovulation with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG), female C57BL/6J mice were mated with male mice. According to the standard protocol [9], the pronuclei of collected oocytes were injected with 5 ng/ μ l circular *pX330* vector and 10 ng/ μ l donor plasmid of each target gene. The injected embryos were then transferred into pseudopregnant ICR mice.

Genomic PCR and sequence analysis

Genomic DNA obtained from the tails of 3-week-old mice was extracted and subjected to genotyping and sequence analysis. PCR was performed using PrimeSTAR® GXL DNA Polymerase (Takara) and the primers listed in Supplementary Tables 5 and 6. The PCR products were purified with a FastGene Gel/PCR Extraction Kit (Nippon Genetic) and used as the template for PCR with a BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). The sequences were then analyzed using a 3500 Series Genetic Analyzer (Thermo Fisher Scientific).

Fluorescence reporter analysis

The pregnant mice were sacrificed by cervical dislocation and embryos were then dissected from the decidua in 10% fetal bovine serum (FBS)/PBS. Mouse embryos were staged according to Downs and Davies (1993) [7] or described as the number of days of development. Dissected embryos were transferred into PBS and observed by fluorescence microscopy (Leica M165 FC; Leica Microsystems, Wetzlar, Germany) with an appropriate filter set (GFP, #10447408; DSR, #10447412; BFP, #10450329).

Whole-mount immunofluorescence analysis

After dissection, embryos were fixed in 4% paraformaldehyde (PFA) for 30 min at 4°C and washed twice in PBS containing 0.1% Tween 20 (PBST). Embryos were then permeabilized in PBS with 0.5% Triton-X (TPBS) for 30 min at room temperature (RT) and blocked in blocking solution consisting of 10% serum, 0.1% bovine serum albumin (BSA), and PBST for 1 h at 4°C. The primary antibody reaction was performed in blocking solution overnight at 4°C with anti-SOX17 polyclonal goat IgG (1:300, AF1924; R&D Systems, Minneapolis, MN, USA), anti-Otx2 polyclonal rabbit IgG (1:200; ab21990; Abcam, Cambridge, UK), or anti-

Brachyury polyclonal goat IgG (1:200; AF2085, R&D Systems). After washing three times in 0.5% TPBS at RT, embryos were incubated with secondary antibodies for 3 h at RT: rabbit anti-goat IgG (H+L) superclonal secondary antibody, Alexa Fluor 555 (1:500, A27017; Thermo Fisher Scientific), or goat anti-rabbit IgG (H+L) superclonal secondary antibody, Alexa Fluor 488 (1:500, A27034; Thermo Fisher Scientific). Embryos were washed twice in PBST and treated with DAPI (1:1000, D9542; Sigma-Aldrich) or Nuclear Green DCS1 (1:1,000, ab138905; Abcam) and observed by confocal microscopy (Leica SP5; Leica Microsystems). Fluorophores were excited with a 405 nm diode laser (TagBFP, DAPI), a 488 nm argon laser (EGFP, Alexa Fluor 488, Nuclear Green DCS1), and a 543 nm HeNe laser (tdTomato, Alexa Fluor 555). Whole embryo images were captured by 20×/0.70 dry objective lens. High magnification images were captured by 63×/1.4 Oil objective lens. Raw data were processed using microscope software LAS X (Leica Microsystems).

Western blotting

Twenty-four h after transfection, HEK293T cells were washed and protein was extracted using T-PER™ Tissue Protein Extraction Reagent (Thermo Scientific). The cell lysate was then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After blocking in 5% skim milk, the membranes were probed overnight at 4°C with anti-V5-tag monoclonal antibody (1:500, M167-3; MBL International, Woburn, MA, USA) or anti-HA-tag polyclonal antibody (1:500, 561; MBL International). After washing three times in 0.05% Tween in TBS, the membranes were incubated for 1 h at RT with secondary antibody: anti-mouse IgG HRP-linked whole antibody (1:5,000, NA931; GE Healthcare Life Sciences, Isquah, WA, USA) or anti-rabbit IgG HRP-linked whole antibody (1:5,000, NA934; GE Healthcare Life Sciences) and detected using an iBright™ CL1000 Imaging System (Invitrogen, Carlsbad, CA, USA).

Results

Generation of specific reporter mice for gastrula germ layers

Germ layer formation is a key time point during gastrulation, which is critical for body plan and early development [26]. To monitor the morphology of mouse

embryos during gastrulation, we first generated specific reporters for each germ layer using the CRISPR/Cas9 system. In this study, the *Sox17*, *Otx2*, and *T* genes were fused with *EGFP*, *tdTomato*, and *TagBFP* and used as reporters for the endoderm, ectoderm, and mesoderm, respectively. Moreover, to express the reporter gene under the control of the endogenous promoter and without any disruption of the marker gene, P2A peptide [16] was inserted between the target gene and reporter gene (Figs. 1A–C top). The CRISPR target sites for three knockin models were designed, and the efficiencies of CRISPR vectors were confirmed using the EGxxFP system [20] (Supplementary Fig. 1). Finally, three reporter strains, C57BL/6J-*Sox17^{em1}(EGFP)Utr*, C57BL/6J-*Otx2^{em1}(tdTomato)Utr*, and C57BL/6J-*T^{em1}(TagBFP)Utr* (hereafter, referred as *Sox17-2A-EGFP*, *Otx2-2A-tdTomato*, and *T-2A-TagBFP*, respectively) were generated by co-microinjection of *pX330* and donor plasmids into the pronuclei of C57BL/6J mouse zygotes (Table 1).

The founder mice were genotyped by: (1) detection of knockin fluorescent reporter gene; (2) screening to exclude random integration of *pX330* and knockin donor plasmids by *Cas9* and *Amp* detection; and (3) confirmation of sufficient P2A-reporter knockin downstream of the target marker gene (Figs. 1A–C bottom, Supplementary Fig. 2). Four founders of *Sox17-2A-EGFP* (#7, #8, #41, #45), two founders of *Otx2-2A-tdTomato* (#30, #50), and nine founders of *T-2A-TagBFP* (#6, #19, #20, #35, #40, #45, #51, #54, #62) were obtained. After excluding founders that were positive for *Amp* and *Cas9*, we selected *Sox17-2A-EGFP* #45, *Otx2-2A-tdTomato* #30, and *T-2A-TagBFP* #40 as endoderm, ectoderm, and mesoderm reporter mouse strains, respectively.

Normal development of homozygous reporter mice

To confirm reproduction and development in knockin models, heterozygous mice were intercrossed to obtain homozygous progeny. First, heterozygous mice were propagated by *in vitro* fertilization using sperm from each founder strain and C57BL/6J wild-type oocytes. Heterozygous mice were obtained at an efficiency of around 50% (data not shown) in all strains. Homozygous mice were also obtained by intercrossing heterozygotes in all strains (Table 2, Fig. 2). There was a tendency that the frequency of each genotype in all reporter mouse strains followed Mendelian pattern of inheritance (Table 2, Supplementary Table 7). Moreover, the fertility and physical appearance were normal in both heterozygous

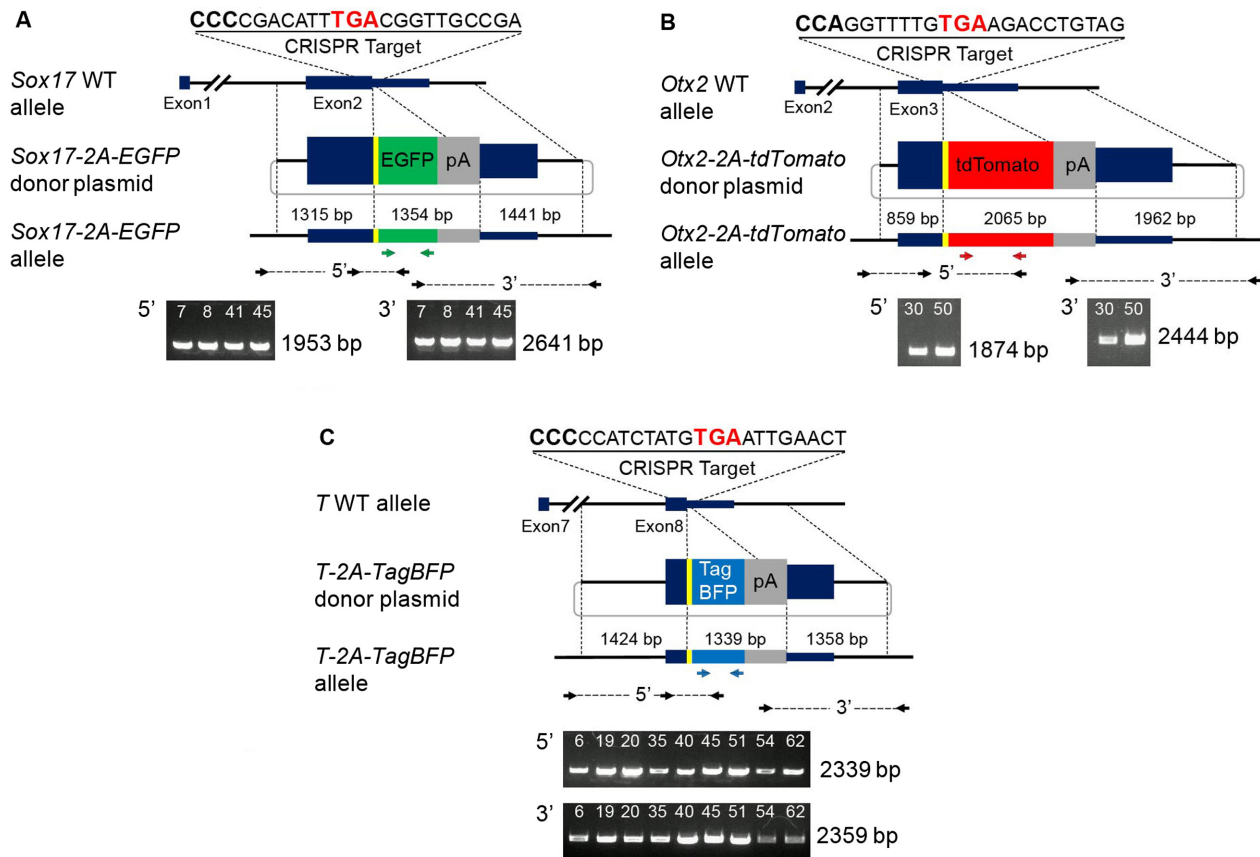


Fig. 1. CRISPR design for three knockin mouse models. The CRISPR target site (underlined) was designed including the protospacer adjacent motif NGG (PAM) (black bold) and stop codon (red bold). Generally, the P2A sequence (yellow box) was fused with the reporter gene *EGFP*, *tdTomato*, or *TagBFP* and polyadenylation signal (pA) was inserted immediately before the stop codon by homology-directed repair (HDR). Primers used for PCR genotyping were reporter detection (green, red and blue arrows), 5' arm detection and 3' arm detection (black arrows). (A) Knockin strategy for the *Sox17-2A-EGFP* allele. Primers used for EGFP detection: EGFP F, EGFP R; 5' detection: Sox17-G5 F, EGFP Tm68 R and Sox17-G5 seq F; 3' detection: EGFP Tm68 F & Sox17-G3 R. (B) Knockin strategy for *Otx2-2A-tdTomato* allele. Primers used for tdTomato detection: tdTomato detect F, tdTomato detect R; 5' detection: Otx2-G5 F, tdTomato Tm68 R and Otx2-G5 seq F; 3' detection: rGpA Tm68 F & Otx2-G3 R. (C) Knockin strategy for *T-2A-TagBFP* allele. Primers used for TagBFP detection: TagBFP detect F, TagBFP detect R; 5' detection: T-G5 F, TagBFP detect R and T-G5 seq F; 3' detection: rGpA Tm68 F & T-G3 R.

Table 1. Generation of bicistronic reporter knockin mice

Strains	Number of			
	Injected zygotes	Neonates	Founders (%) [*]	Founders negative for <i>Amp</i> and <i>Cas9</i>
<i>Sox17-2A-EGFP</i>	301	65	4 (6.2%)	1
<i>Otx2-2A-tdTomato</i>	243	67	2 (3.0%)	1
<i>T-2A-TagBFP</i>	242	66	9 (13.6%)	8

^{*}Numbers in parentheses mean No. of founders/No. of neonates.

and homozygous adult mice. These data strongly suggested that all reporter mouse models were normal and healthy.

To confirm sufficient cleavage of the 2A sequence, RNA from E7.5 heterozygous embryos was extracted

and cDNA was synthesized. The cDNAs derived from *Sox17-2A-EGFP*, *Otx2-2A-tdTomato*, and *T-2A-TagBFP* gastrula mRNA were inserted into the V5-HA tag platform plasmid. These constructs were transfected into HEK293T cells for analysis of the cleavage efficiency

Table 2. Genotypes of progeny obtained by intercrossing heterozygous mice

Strains	Numbers of		
	Wild-type mice	Heterozygous mice	Homozygous mice
<i>Sox17-2A-EGFP</i>	3 (23.1%)	6 (46.1%)	4 (30.8%)
<i>Otx2-2A-tdTomato</i>	9 (42.9%)	8 (38.1%)	4 (19.0%)
<i>T-2A-TagBFP</i>	10 (33.3%)	14 (46.7%)	6 (20.0%)

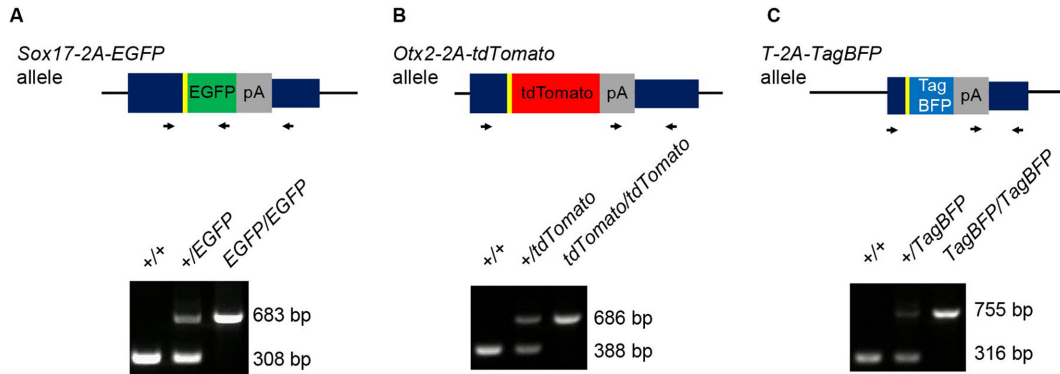


Fig. 2. Genotyping of homozygous knockin mice. Strategies for genotyping knockin strains *Sox17-2A-EGFP* (A), *Otx2-2A-tdTomato* (B), and *T-2A-TagBFP* (C). The arrows indicate primers used for PCR analysis and electrophoresis data show the detection of heterozygous and homozygous mice compare with wild-type. Primers used for genotyping *Sox17-2A-EGFP*: Sox17 to stop F, Sox17 to stop R, and EGFP to out; for *Otx2-2A-tdTomato*: Otx2 to stop F, Otx2 to stop R, and rGpA to Outside F2; *T-2A-TagBFP*: T to stop F, T to stop R, and rGpA to Outside F2. Yellow box: P2A sequence.

of 2A by Western blotting (WB). The results of WB clearly demonstrated successful cleavage of V5-tagged markers and HA-tagged reporters by P2A in the case of SOX17 and OTX2 (Figs. 3A and B). Few bands were detected in HA-tagged tdTomato, which were probably smaller degradation products of the tdTomato protein. A similar phenomenon was reported previously in the eyes of adult transgenic mice [2]. WB of V5-tagged Brachyury showed both cleaved and uncleaved forms of T-2A-TagBFP protein (Fig. 3C). The fainter upper band (red arrow) represented residual uncleaved protein due to incomplete P2A activity [16], but its level was very low compared to that of cleaved TagBFP (black arrow). Taken together, these results indicated that the fluorescent reporter genes were inserted into *Sox17*, *Otx2*, and *T* loci without disrupting or disturbing endogenous genes.

Fluorescence reporter expression in gastrulas

To confirm the expression of reporter proteins under the control of the endogenous promoters of *Sox17*, *Otx2*, and *T* during gastrulation, heterozygous E6.5–E8.5 embryos from each mouse strain were dissected and exam-

ined for fluorescent signals. In the *Sox17-2A-EGFP* strain, EGFP fluorescence was detected in the extraembryonic visceral endoderm at E6.5 as expected (Fig. 4A). At E7.5, fluorescence was expressed simultaneously in the extraembryonic visceral endoderm, embryonic visceral endoderm, and definitive endoderm (Fig. 4B). EGFP was localized along the gut endoderm from the foregut to the hindgut at E8.5 (Fig. 4C). In *Otx2-2A-tdTomato* embryos, tdTomato fluorescence was detected in the whole epiblast with the exception of the extraembryonic region at E6.5 (Fig. 4D). Strong tdTomato expression was observed in the anterior region of the epiblast at E7.5 (Fig. 4E) and was localized to the forebrain and midbrain, but not the hindbrain at E8.5 (Fig. 4F). In the *T-2A-TagBFP* strain, TagBFP fluorescence was detected in the nascent primitive streak (PS) between the extraembryonic and embryonic region at E6.5 (Fig. 4G). Consistent with the normal T expression at E7.5, TagBFP fluorescence was detected in the extraembryonic mesoderm, elongated primitive streak, and notochord (Fig. 4H). At E8.5, fluorescence was detected in the notochord and paraxial mesoderm at E8.5 (Fig. 4I). Generally, there were no morphological abnormalities in het-

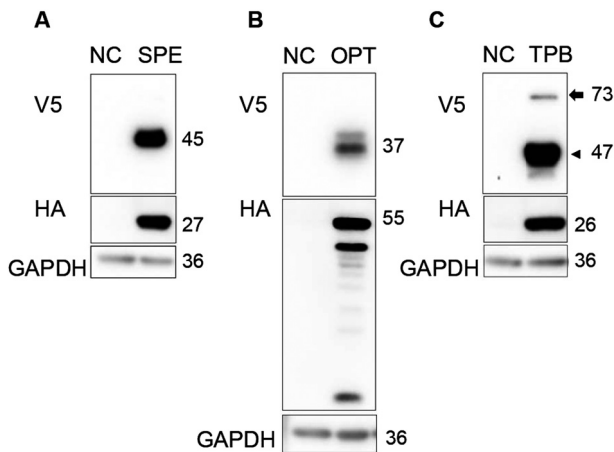


Fig. 3. P2A cleavage efficiency in HEK293T cells. The V5-HA platform plasmids containing *Sox17-2A-EGFP* (SPE), *Otx2-2A-tdTomato* (OPT), and *T-2A-TagBFP* (TPB) cDNAs, respectively, were transfected into HEK293T cells. The cell lysates were analyzed by SDS-PAGE with V5 and HA antibodies to detect cleavage of the target marker protein and reporter protein. (A) Western blotting of V5-tagged SOX17 and HA-tagged EGFP showed complete cleavage of P2A. (B) Western blotting of V5-tagged OTX2 and HA-tagged tdTomato showed some ladder bands by HA antibody due to degradation of tdTomato proteins under reducing conditions. (C) Western blotting of V5-tagged Brachyury and HA-tagged TagBFP showed less efficiency of P2A with the faint upper band of uncleaved protein (arrow) and the denser band of cleaved TagBFP (arrowhead). The number indicated molecular weight (kDa) of protein.

erozygous embryos from E6.5 to E8.5, suggesting that all knockin mouse models sufficiently expressed the fluorescent reporter during gastrulation.

Comparative expression level and fluorescent reporter pattern at E7.5

To determine whether it was possible to visualize each germ layer with the same expression pattern as the endogenous target marker protein in the transgenic reporter strains, fluorescence reporter signals were examined simultaneously with endogenous SOX17, OTX2, and T expression using whole-mount immunofluorescence in heterozygous embryos at E7.5. In the *Sox17-2A-EGFP* gastrulas at E7.5, both EGFP and SOX17 signals were detected in definitive endoderm expanding from posterior to anterior, with the exception of the anterior notochordal mesoderm (Fig. 5A). In the *Otx2-2A-tdTomato* gastrulas, TdTomato and OTX2 showed similar expression patterns in the anterior neuroectoderm and head-fold (Fig. 5B). In the *T-2A-TagBFP* gastrulas,

TagBFP and Brachyury were expressed in the same region along the primitive streak at the posterior of the embryos (Fig. 5C). Taken together, the results confirmed fluorescence reporter expression in mouse models with the same expression pattern as the target endogenous marker protein, thus enabling visualization of each germ layer during gastrulation.

Discussion

Here, we generated three novel bicistronic reporter knockin mouse strains, i.e., the *Sox17-2A-EGFP* mouse strain to visualize the endoderm, the *Otx2-2A-tdTomato* mouse strain to visualize the ectoderm, and the *T-2A-TagBFP* mouse strain to visualize the mesoderm containing the primitive streak and notochord. In all of these strains, both heterozygous and homozygous knockin reporter adults showed normal physical appearance and fertility. Further, during gastrulation, the knockin reporter embryos showed expression patterns of fluorescent reporter proteins specific for each germ layer. Moreover, in the gastrulas of all strains, the fluorescent protein expression domains overlapped with those of the target marker protein at E7.5.

The CRISPR/Cas9-based genome editing has superseded traditional gene targeting methods for knockout and knockin mouse production, and a number of useful methods have been developed over a very short period. We used the previous standard strategy [11] to obtain precise knockin individuals with CRISPR/Cas9-mediated gene modification, in which plasmids including the transgene donor with targeted homology arm are injected into mouse zygotes with the all-in-one pX330 vector. Successful production was confirmed from all of the bicistronic reporter knockin mouse strains. The efficiency rates of knockin mouse generation for the *Sox17-2A-EGFP*, *Otx2-2A-tdTomato*, and *T-2A-TagBFP* mouse strains were 6.2%, 3.0%, and 13.6%, respectively (Table 1). The differences in efficiencies between the three stains may have been due to differences in stability or in cleavage activity of sgRNA [24]. The generation efficiencies in this study were comparable to those in our previous study [11]. New strategies, such as Tild-CRISPR (targeted integration with linearized dsDNA-CRISPR), should be investigated to improve the efficiency of knockin reporter mouse production [29].

Many transcription factors expressed in the gastrula, including SOX17, OTX2, and Brachyury, play critical

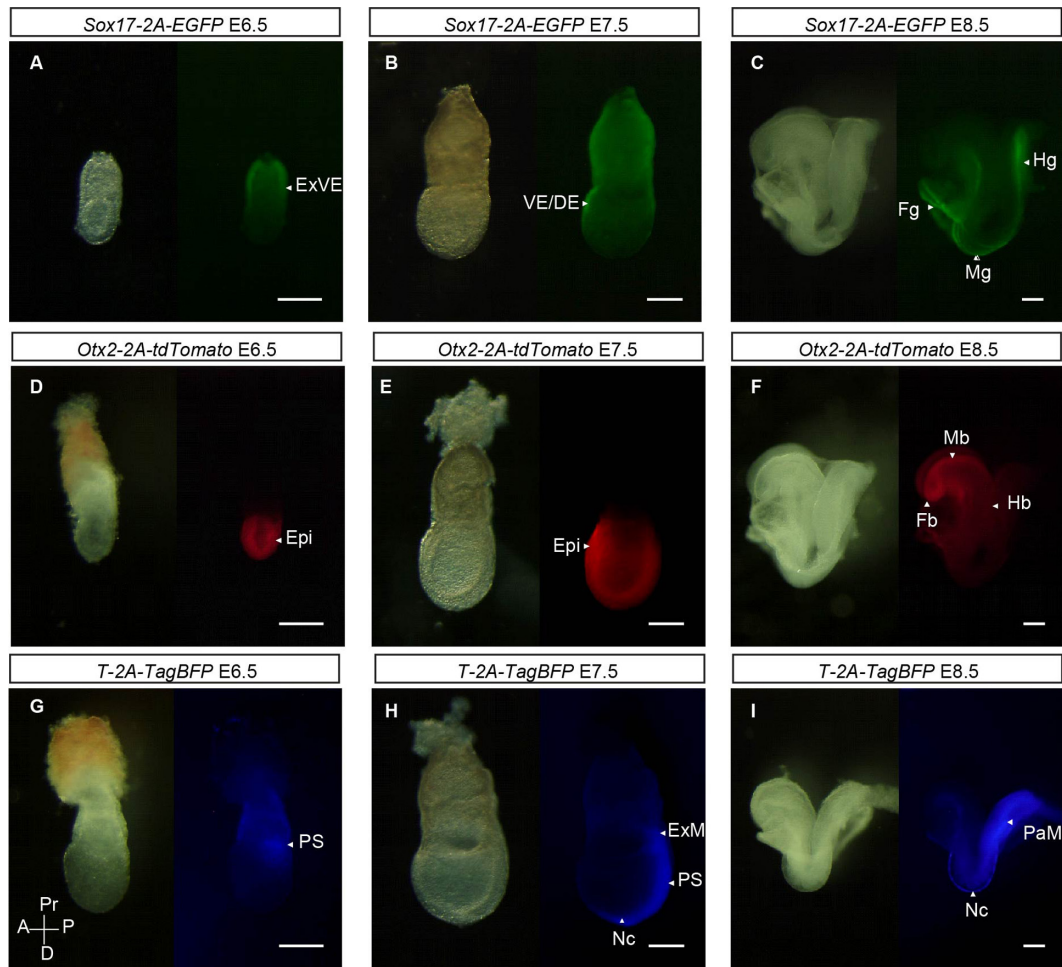


Fig. 4. Fluorescence reporter expression in gastrulas. Heterozygous *Sox17-2A-EGFP* (A–C), *Otx2-2A-tdTomato* (D–F), and *T-2A-TagBFP* (G–I) embryos were collected from E6.5–E8.5 and the reporter signals were examined by fluorescence microscopy. ExVE: extraembryonic visceral endoderm; VE: visceral endoderm; DE: definitive endoderm; Fg: foregut; Mg: midgut; Hg: hindgut; Epi: epiblast; Fb: forebrain; Mb: midbrain; Hb: hindbrain; PS: primitive streak; ExM: extraembryonic mesoderm; Nc: notochord; PaM: paraxial mesoderm; A: anterior; P: posterior; Pr: proximal; D: distal. Scale bar: 100 μ m.

roles in embryonic development. Although *Sox17* heterozygous mice show no phenotypic abnormalities and were viable, *Sox17* homozygous mutant mice showed embryonic lethality around E10.5 due to abnormal endoderm formation [15]. In the *Otx2* gene, approximately 30% of newborns are lethal in heterozygous null mutants and homozygous *Otx2*-null mutants show complete embryonic lethality around E9.5 because of defects of the forebrain or midbrain [1, 21]. There are many mutant alleles of the *T* gene in mice and several mutants have been studied [13]. Heterozygous for *T^{vis}* allele lacking normal gene ability causes no tail phenotype. Loss of function of the *T* gene leads to embryonic lethality around E10.5 due to mesoderm defect [12]. On the other hand,

there were no abnormal development and viable in heterozygous *Sox17-2A-EGFP*, *Otx2-2A-tdTomato*, and *T-2A-TagBFP* mice generated in this study. Interestingly, homozygous knockin mice also showed viable, normal appearance, and fertility. Therefore, these results suggest that functions of the target genes are intact by the bicistronic knockin strategy which integrates fluorescence reporter gene including P2A sequences into the site just before the stop codon sequences. Actually, our Western blotting analysis revealed that the gastrulas with the *Sox17-2A-EGFP*, *Otx2-2A-tdTomato*, and *T-2A-TagBFP* constructs expressed Sox17, Otx2, and Brachyury proteins with their original molecular weights (Fig. 3).

The illumination patterns of reporter gene product in

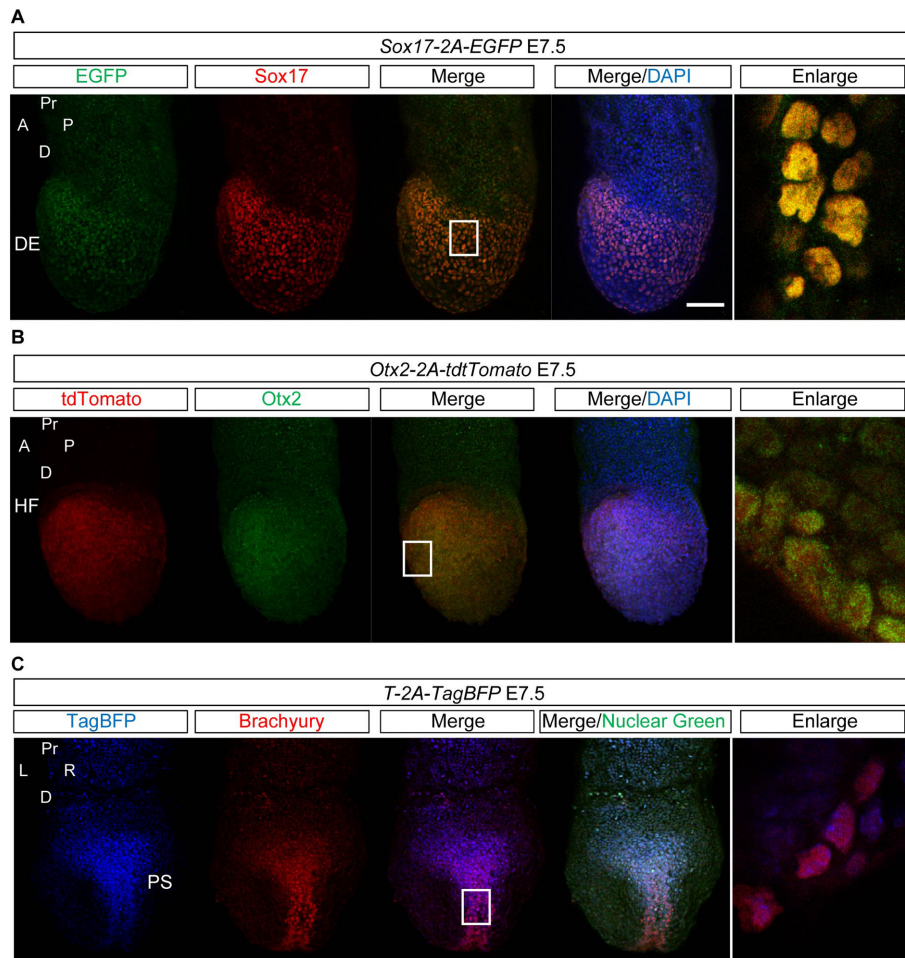


Fig. 5. Merged expression pattern of reporter and target marker expression in gastrulas at E7.5. Whole-mount immunofluorescence analyses of heterozygous *Sox17-2A-EGFP* (A), *Otx2-2A-tdTomato* (B), and *T-2A-TagBFP* (C) E7.5 embryos were performed using SOX17, OTX2, and Brachyury antibodies, respectively. The nucleus was stained with DAPI or Nuclear Green DCS1. The whole embryos in (A–C) were captured by Z-stack of confocal microscope Leica-SP5. The enlarge images showed the section of indicated area (white square) with high magnification. DE: definitive endoderm; HF: head-fold; PS: primitive streak; A: anterior; P: posterior; Pr: proximal; D: distal; L: left; R: right. Scale bar: 100 μm .

the *Sox17-2A-EGFP*, *Otx2-2A-tdTomato*, and *T-2A-TagBFP* gastrulas were largely consistent with gene expression patterns in the *Sox17* [4, 15], *Otx2* [2, 19, 27], and *T* genes [28], respectively. Furthermore, confocal microscopic analysis indicated the colocalization of reporter signals with the endogenous target proteins in gastrulas at E7.5. These observations indicated that our knockin reporter models are reliable with reporter expression patterns faithfully reflecting the localization of the endogenous target proteins during gastrulation. Although *Sox17-GFP* [17] and *Otx2-GFP* [8] knockin reporter mice were previously produced to investigate the function/localization of SOX17 and OTX2, respec-

tively, their homozygotes caused embryonic lethality in both cases due to target gene disruption. Burstscher [4] reported viable knockin reporter mice homozygous for *Sox17-mCherry*, which expressed Sox17-mCherry fusion protein from the knockin allele. Although the fusion strategy is interesting, it is unclear whether it can be applied to other transcription factors. To our knowledge, no knockin reporter mouse strains have been reported for the *T* gene. Under these circumstances, the *Sox17-2A-EGFP*, *Otx2-2A-tdTomato*, and *T-2A-TagBFP* mouse strains described here are the first knockin reporter mice with intact target gene products for the *Sox17*, *Otx2*, and *T* genes, respectively.

Although our knockin reporter mice represent conventional tools for visualizing each germ layer, a few issues remain. First, mRNA from the knockin allele could be strongly stabilized by the inclusion of rabbit β -globin poly(A) sequences [25], so the expression levels of the target protein in the knockin reporter mice may be higher than the endogenous levels in wild-type mice. Although all three knockin reporter models showed no phenotypic abnormalities, it will be necessary to compare the target gene mRNA and protein levels with the respective endogenous levels in wild-type mice. Second, due differences in the half-life between the target and reporter proteins, it is possible that the fluorescence of the reporter protein may be maintained after degradation of the target protein. Further studies are required to determine the expression patterns of the reporters and target proteins in gastrulas not only at E7.5 but also at E8.5.

A recent IMPC study [6] suggested that germ layer formation involves a great many as yet unidentified genes. Therefore, it will be important to develop high-throughput analytical methods to evaluate the roles of genes in germ layer formation in the mouse gastrula. The CRISPR/Cas9-based genome editing using embryos that allow visualization of the three germ layers will be useful in such high-throughput analyses. The three bicistronic reporter knockin mouse strains produced in the present study allow maintenance of the homozygous knockin allele. Further, the three reporter genes produce visually distinguishable fluorescent proteins and are integrated into different chromosomes in the mice. Therefore, simple crossing will give rise to homozygous triple knockin reporter mice carrying *Sox17-2A-EGFP*, *Otx2-2A-tdTomato*, and *T-2A-TagBFP* genes. Such mice would provide valuable spatial information about the three germ layers simultaneously at the gastrulation stage. We expect that such triple knockin reporter mice will become a useful tool for high-throughput analysis to evaluate the functions of genes in germ layer formation.

In conclusion, we have generated three novel bicistronic reporter knockin mouse strains, designated as *Sox17-2A-EGFP*, *Otx2-2A-tdTomato*, and *T-2A-TagBFP*, using CRISPR/Cas9-mediated genome editing in mouse zygotes. These mouse strains will be valuable tools to gain a greater understanding of the functions of genes in differentiation of the three germ layers in developing embryos.

Conflicts of Interests

The authors declare that there are no competing financial interests.

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