A comprehensive, non-invasive visualization of primordial germ cell development in mice by the *Prdm1*-mVenus and *Dppa3*-ECFP double transgenic reporter

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Abstract

The ability to monitor the development of a given cell lineage in a non-invasive manner by fluorescent markers both *in vivo* and *in vitro* provides a great advantage for the analysis of the lineage of interest. To date, a number of transgenic or knock-in mouse strains, in which developing germ cells are marked with fluorescent reporters, have been generated. We here describe a novel double transgenic reporter mouse strain that expresses membrane-targeted Venus (mVenus), a brighter variant of yellow fluorescent protein (YFP), under the control of *Prdm1* (*Blimp1*) regulatory elements and enhanced cyan fluorescent protein (ECFP) under the control of *Dppa3* (*Stella/Pgc7*).

The double transgenic strain unambiguously marked *Prdm1* expression in the lineage-restricted precursors of primordial germ cells (PGCs) in the proximal epiblast at embryonic day (E) 6.25 and specifically illuminated *Prdm1*- and *Dppa3*-positive migrating PGCs after E8.5. The double transgenic reporter also precisely recapitulated dynamic embryonic expression of *Prdm1* outside the germ cell lineage. Moreover, we derived ES cells that bore both transgenes. These cells made a robust contribution both to the germ and somatic cell lineages in chimeras with accurate *Prdm1*-mVenus and *Dppa3*-ECFP expression. The transgenic strain and the ES cells will serve as valuable experimental materials not only for analyzing the origin and properties of the germ cell lineage *in vivo*, but also for establishing a culture system to efficiently induce proper germ cells with temporally coordinated *Prdm1* and *Dppa3* expression *in vitro*.

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Introduction

The germ cell lineage is the only source for the creation of new organisms in most multicellular animals, ensuring the perpetuation of the genetic and epigenetic information across generations. In mice, and presumably all mammals, germ cell fate is a property induced during development, rather than a trait inherited from the egg, as seen in some model organisms (Ohinata *et al.* 2006, Seydoux & Braun 2006, Hayashi *et al.* 2007). Accordingly, specification of germ cell fate has been an active area of research, with great relevance to stem cell biology and epigenetics.

Recent studies have shown that germ cell fate in mice is induced in proximal epiblast cells by bone morphogenetic protein (BMP) signals from extraembryonic tissues (Lawson *et al.* 1999, Ying *et al.* 2000, Chang & Matzuk 2001, Tremblay *et al.* 2001, Ying & Zhao 2001,

Hayashi *et al.* 2002, Chu *et al.* 2004) at around embryonic day (E) 6.25 as those expressing PRDM1 (previously known as BLIMP1; Ohinata *et al.* 2005, Vincent *et al.* 2005), a potent transcriptional regulator involved in cell fate specification in diverse developmental contexts (Turner *et al.* 1994, de Souza *et al.* 1999, Shapiro-Shelef *et al.* 2003, Baxendale *et al.* 2004, Roy & Ng 2004, Hernandez-Lagunas *et al.* 2005, Wilm & Solnica-Krezel 2005, Horsley *et al.* 2006, Kallies *et al.* 2006, Martins *et al.* 2006, Magnusdottir *et al.* 2007, Robertson *et al.* 2007). These *Prdm1*-expressing cells increase in number and, at around E7.25, exclusively go on to form primordial germ cells (PGCs), the primary source of both oocytes and spermatozoa, with characteristic alkaline phosphatase activity (Chiquoine 1954, Ginsburg *et al.* 1990) and *Dppa3* (previously known as *Stella* or *Pgc7*) expression (Saitou *et al.* 2002, Sato *et al.* 2002). The *Prdm1*- and *Dppa3*-positive PGCs repress *Hox* gene

expression (Saitou *et al.* 2002, Yabuta *et al.* 2006), regain potential pluripotency (Matsui *et al.* 1992, Yabuta *et al.* 2006), and initiate migration, with concomitant genome-wide epigenetic reprogramming (Seki *et al.* 2005, 2007), toward embryonic gonads where they eventually differentiate into functional gametes. *PRDM1* expression continues in PGCs until around E13.5 in both sexes (Chang *et al.* 2002), whereas *DPPA3* is maintained until around E15.5 in males and around E13.5 in females (Sato *et al.* 2002). *DPPA3* expression resumes specifically in the primordial follicle stage oocytes in new born females and continues to be expressed throughout oocyte development (Sato *et al.* 2002, Payer *et al.* 2003). *DPPA3* is also expressed zygotically from the two-cell stage until around E4.5 (Sato *et al.* 2002, Payer *et al.* 2003) and then is exclusively retained in *Prdm1*-expressing cells at around E7.25.

To date, transgenic reporter strains expressing enhanced green fluorescent protein (EGFP) under the control of *Prdm1* or *Dppa3* upstream elements (*Blimp1*-mEGFP or stella-EGFP) have been generated (Ohinata *et al.* 2005, Payer *et al.* 2006). These strains have been useful to monitor the origin and development of the germ cell lineage *in vivo* and are considered to surpass reporter strains based on other genes (MacGregor *et al.* 1995, Yeom *et al.* 1996, Yoshimizu *et al.* 1999, Anderson *et al.* 2000, Toyooka *et al.* 2003, Tanaka *et al.* 2004) in terms of their early onset of expression and germ line specificity (Payer *et al.* 2006). Moreover, the ES cells derived from these strains are expected to be excellent tools to monitor the efficient generation of PGCs and their subsequent development *in vitro* (Payer *et al.* 2006), which will provide abundant experimental materials and may serve as a critical basis for future regenerative medicine applications. However, both strains have some disadvantages. The *Blimp1*-mEGFP strain precisely recapitulates *Prdm1* expression *in vivo* and shows expression in the germ line as early as E6.25 (Ohinata *et al.* 2005). However, endogenous *Prdm1* expression is not restricted to the germ line but is more widespread, especially after E7.5 (Chang *et al.* 2002, Vincent *et al.* 2005, Robertson *et al.* 2007). The stella-EGFP strain exhibits specific expression of EGFP in PGCs but only after E7.5, and is therefore not useful for detecting PGC precursors or monitoring the process of PGC specification (Payer *et al.* 2003, 2006).

To circumvent these drawbacks, we generated a transgenic reporter strain that expresses membrane-targeted Venus (mVenus), a brighter variant of YFP (Nagai *et al.* 2002), under the control elements of *Prdm1* and another strain that express ECFP under the control of *Dppa3*. (We also generated strains that express Venus under the control of *Dppa3*.) By crossing these strains, we obtained a double transgenic reporter strain that homozygously bears both *Prdm1*-mVenus and *Dppa3*-ECFP (*Blimp1*-mVenus and stella-ECFP, BVSC). We report here that the BVSC strain faithfully recapitulates endogenous *Prdm1* and *Dppa3* expression, thereby

enabling the specific real-time monitoring of the development of the germ cell lineage from its incipience. Moreover, we established an ES cell line bearing the BVSC transgenes, which will serve as a useful experimental tool for establishing an *in vitro* culture system reliably generating germ cells from ES cells.

Results

Generation of *Prdm1*-mVenus, *Dppa3*-ECFP, and *Dppa3*-Venus transgenic mice

To generate a transgenic strain that simultaneously marks the expression of *Prdm1* and *Dppa3* with two different colors, we decided to use Venus and ECFP for monitoring *Prdm1* and *Dppa3* respectively. The *Prdm1*-mVenus bacterial artificial chromosome (BAC) construct, spanning a total of ~230 kb (~140 kb upstream of the *Prdm1* transcription start site), bears Venus targeted to and anchored in the plasma membrane by the Ig κ leader and platelet-derived growth factor receptor (PDGFR) transmembrane sequences respectively, after the initial in-frame ATG of the exon 3 of the *Prdm1* gene (Fig. 1A). This construct is thus identical to the *Blimp1*-mEGFP transgene reported previously (Ohinata *et al.* 2005), except that EGFP is replaced by Venus. The *Dppa3*-ECFP construct includes ~16 kb upstream of the *Dppa3* gene and bears ECFP followed by an SV40 polyadenylation sequence recombined in the exon 2 of *Dppa3* in a frame-matched manner, as well as the downstream ~1.3 kb sequence up to exon 4 (Fig. 1A). We also generated a *Dppa3*-Venus construct. These transgenic constructs for *Dppa3* do not bear any neighboring genes, such as *Gdf3*, *Apobec1*, and *Nanog*, which could influence the germ cell development if overexpressed from the transgenes.

We generated 8, 11, and 3 independent strains for *Prdm1*-mVenus, *Dppa3*-ECFP, and *Dppa3*-Venus strains respectively, by pronuclear injection of the transgenes. Three of the *Prdm1*-mVenus lines showed apparently correct *Prdm1* expression patterns, and we decided to use the strain that exhibited the brightest expression for subsequent analysis. The *Dppa3*-ECFP and *Dppa3*-Venus strains (4 out of 11 and 3 out of 3 respectively) also showed specific expression of ECFP and Venus respectively, and we decided to use the ones that exhibited earlier and brighter expression in the developing PGCs for further analysis. We determined the chromosomal localizations of the transgenes by FISH analysis, which revealed that the selected *Prdm1*-mVenus strain harbors the transgene on the A1–A2 region of the chromosome 2, and the *Dppa3*-ECFP and *Dppa3*-Venus strains bear the transgenes on the M5 region of the chromosome 1 and the C1 region of the chromosome 19 respectively (Fig. 1B, data not shown).

We performed several generations of matings between the *Prdm1*-mVenus and the *Dppa3*-ECFP strains to obtain the double homozygous strain (BVSC). We

Figure 1 *Prdm1*-mVenus, *Dppa3*-ECFP, and *Dppa3*-Venus transgenes. (A) Schematic of the *Prdm1*-mVenus, *Dppa3*-ECFP, and *Dppa3*-Venus transgenes. The genomic loci and exon-intron structures of the *Prdm1* and *Dppa3* genes are shown. The positions at which sequences of the fluorescent reporters are recombined are also shown. The membrane-targeted Venus construct includes an Igk leader sequence, an epitope of hemagglutinin antigen, a Venus coding sequence, an epitope of c-myc, and the transmembrane sequence of the PDGF receptor of the pDisplay vector (Invitrogen). (B) Chromosomal localization of the (a) *Prdm1*-mVenus and (b) *Dppa3*-ECFP transgenes. The *Prdm1*-mVenus transgenes are on the A1-A2 region of chromosome 2 and the *Dppa3*-ECFP transgenes are on the M5 region of chromosome 1 (yellow arrows). FISH analyses were performed using the chromosome spreads of splenocytes from a (a) *Prdm1*-mVenus heterozygous mouse or (b) *Dppa3*-ECFP homozygous mouse.

crossed the double homozygous candidates with non-transgenic mice and judged the candidates as double homozygous when all the embryos or offspring from at least two litters showed expression of both transgenes. Thereafter, the double homozygous mice were maintained by intermating.

BVSC expression in pre-implantation and early post-implantation embryos

We first determined the expression of the BVSC transgenes in pre-implantation (Fig. 2A) and early post-implantation embryos (Fig. 2B) from matings between the BVSC males and non-transgenic females. As expected from the previous finding that *Dppa3* expression from the paternal allele is initiated as early as the two-cell stage concomitant with the onset of bulk zygotic transcription (Payer *et al.* 2003, 2006), we detected strong expression of the *Dppa3*-ECFP transgene

in the morula stage embryos (Fig. 2A). In the early blastocyst stage, the embryos at E3.5, the *Dppa3*-ECFP expression was very strong in the inner cell mass (ICM) cells as well as in the trophoblast (Fig. 2A). *Prdm1*-mVenus expression was barely detectable in these pre-implantation embryos. In peri-implantation embryos at E4.5, we observed relatively weak expression of *Dppa3*-ECFP in all the embryonic and extraembryonic cells, whereas, notably, we detected specific expression of *Prdm1*-mVenus in the incipient primitive endoderm (Fig. 2A). At E5.5, we still observed weak *Dppa3*-ECFP expression throughout the embryos, which was probably attributable to residual ECFP activity from earlier embryos. By contrast, *Prdm1*-mVenus expression was specifically detected in the visceral endoderm (VE) cells (Fig. 2B). At E6.5, there seemed almost no expression of *Dppa3*-ECFP, while we detected *Prdm1*-mVenus expression in the embryonic part of the VE and, most importantly, in a number of the most proximal epiblast

Figure 2 Expression of the BVSC transgenes in pre- and early post-implantation embryos. (A) BVSC expression in pre-implantation embryos. BVSC expression in a morula (top row), a blastocyst (middle row), and an E4.5 embryo (bottom row) are shown with bright field images in the left column, *Prdm1*-mVenus expression in the middle column, and *Dppa3*-ECFP expression in the right column. An arrow indicates *Prdm1*-mVenus expression in the incipient primitive endoderm. Bars, 25 μ m. (B) BVSC expression in post-implantation embryos. BVSC expression in an E5.5 embryo (top row) and an E6.5 embryo (bottom row) are shown with bright field images in the left column, *Prdm1*-mVenus expression in the middle column, and *Dppa3*-ECFP expression in the right column. Arrows indicate *Prdm1*-mVenus expression in the visceral endoderm and an arrowhead indicates *Prdm1*-mVenus in most proximal epiblast cells, most likely, the lineage-restricted PGC precursors. The bar at the top of the figure indicates 50 μ m, and that at the bottom indicates 100 μ m.

cells, which most likely correspond to the emerging precursors of PGCs (Fig. 2B, see below) (Ohinata *et al.* 2005). The *Dppa3*-Venus and *Dppa3*-ECFP transgenes showed essentially identical expression in early embryos (data not shown). These observations demonstrate that the BVSC transgenes recapitulate endogenous expression of *Prdm1* and *Dppa3* faithfully, although both fluorescent markers, especially *Dppa3*-ECFP, seemed perhaps more stable than the endogenous proteins (see Discussion). It has also become evident that *Prdm1* and *Dppa3*, two early markers of the PGCs (Saitou *et al.* 2002, Sato *et al.* 2002, Ohinata *et al.* 2005, Vincent *et al.* 2005), exhibited highly different expression patterns prior to PGC specification, indicating that the expressions of these two genes are under essentially distinct regulations.

Embryonic expression of *Prdm1*-mVenus outside the germ cell lineage

We next went on to define the expression of *Prdm1*-mVenus outside the germ cell lineage after E7.5 at the whole-mount resolution (Fig. 3A). *Prdm1* has been shown to be critical in the specification of diverse cell lineages both in developing embryos and adults (Turner *et al.* 1994, de Souza *et al.* 1999, Shapiro-Shelef *et al.* 2003, Baxendale *et al.* 2004, Roy & Ng 2004, Hernandez-Lagunas *et al.* 2005, Wilm & Solnica-Krezel 2005, Horsley *et al.* 2006, Kallies *et al.* 2006, Martins

et al. 2006, Magnusdottir *et al.* 2007, Robertson *et al.* 2007), and a transgenic reporter strain faithfully recapitulating its entire expression should be of general use. At E7.5, as in E6.5 embryos, we detected *Prdm1*-mVenus expression in VE and PGCs (see also below). In addition, *Prdm1*-mVenus was observed in the anterior axial mesendoderm region, which would include the anterior definitive endoderm and prechordal plate (Vincent *et al.* 2005). At E8.5, *Prdm1*-mVenus was lost from the midline, and we observed a prominent expression of *Prdm1*-mVenus in the anterolateral definitive endoderm, most strongly in the region from which the branchial arch will emerge (Vincent *et al.* 2005). At E9.5, we detected *Prdm1*-mVenus in many parts of the embryo: it was observed in a region of the ventral forebrain, in the pharyngeal endoderm of the first branchial arch, more broadly in the more caudal region from which the second and third arches will emerge, in the otic vesicles, very strongly throughout the mesenchyme of the incipient forelimb and hind limb buds, throughout the gut endoderm, and in the myotome of the most rostral somites, which is the site where myogenesis initiates (reviewed by Tajbakhsh & Buckingham (2000)). At E10.5, *Prdm1*-mVenus expression in the branchial arch region seemed substantially down-regulated, whereas it was strongly expressed in the myotome compartment of the somites. We also detected *Prdm1*-mVenus in the intersegmentary arteries between the somites and in the endothelial cells of the superficial

Figure 3 Dynamic and specific expression of *Prdm1*-mVenus and *Dppa3*-Venus respectively, in gastrulating and mid-gestation embryos. (A) Expression of *Prdm1*-mVenus (bottom rows) in embryos from (a–j) E7.5 to E16.5 with the corresponding bright field images (top rows). The arrowhead in (a) indicates PGCs and the arrow indicates the endoderm. ame, anterior mesoendoderm; ade, anterior definitive endoderm; ot, otic vesicle; ba, branchial arch; mt, myotome; hg, hindgut; mfl, mesenchyme of incipient forelimb; mhl, mesenchyme of incipient hind limb; fl, forelimb; hl, hind limb; sv, sensory vibrissae; hf, hair follicle. Bars in (a and b), 100 μ m; in (c and d), 250 μ m; in (e–h), 500 μ m; in (i and j) 1 mm. (B) Expression of *Dppa3*-Venus (right columns, arrowheads) at E9.5 (a) and E14.5 (b) with corresponding bright field images (left columns). Bar in (a), 250 μ m; in (b), 500 μ m.

capillaries (data not shown, Fig. 4B). The expression of *Prdm1*-mVenus in the limb bud seemed to become restricted to the posterior region encompassing the zone of polarizing activity (ZPA) and to the apical ectodermal ridges (AER). At E11.5, we detected *Prdm1*-mVenus more strongly in the myotome of more caudal somites and in the ZPA and AER of both limb buds. At E12.5, *Prdm1*-mVenus began to be expressed in the mesenchyme of the emerging sensory vibrissae, and at E14.5, it began to be expressed in the mesenchyme forming the dermal papillae of the hair follicles of the coat region. All these expression domains of *Prdm1*-mVenus are essentially in good agreement with those of *Prdm1* mRNA and protein expression previously demonstrated by *in situ*

hybridization and immunohistochemistry (Chang *et al.* 2002, Vincent *et al.* 2005, Robertson *et al.* 2007). Thus, these observations demonstrate that *Prdm1*-mVenus monitors the dynamic embryonic expression of endogenous *Prdm1* with remarkable precision.

BVSC expression in the germ cell lineage

We next determined the expression of BVSC in the germ cell lineage. At E6.5 just after the onset of gastrulation, we detected the expression of *Prdm1*-mVenus in a subset of POU5F1-positive most proximal epiblast cells that are considered to be the lineage-restricted precursors of PGCs (Ohinata *et al.* 2005) (Fig. 4Aa). At E7.5,

Figure 4 (continued)

Figure 4 Expression of the BVSC transgenes in the germ cell lineage. (A) Expression of *Prdm1*-mVenus (left column) and POU5F1 (middle column) in (a) E6.5 and (b) E7.5 embryos, and expression of *Prdm1*-mVenus (left column) and DPPA3 (middle column) in (c) E7.75 embryos. Merged images are shown on the right. Arrowheads in (a) indicate *Prdm1*-positive presumable lineage-restricted PGC precursors and an arrow indicates the visceral endoderm. Cells expressing *Prdm1*-mVenus at a weaker level and negative for DPPA3 in (c) are visceral endoderm cells. Bar in (a and c), 50 μ m; in (b), 20 μ m. (B) Expression of the BVSC transgenes in the region where PGCs reside at (a) E8.5, (b) E9.5, (c) E10.5 and (d) E11.5 with bright field images in the left column, *Prdm1*-mVenus expression in the middle column, and *Dppa3*-ECFP expression in the right column. Arrowheads in (c and d) indicate the nascent genital ridges. hg, hindgut; isc, intersegmentary capillary; mt, myotome. Bars, 50 μ m. (C) Co-expression of BVSC in the mesentery at E9.5 (a) and genital ridge at E11.5 (b), and co-expression of *Dppa3*-ECFP and *Dppa3*-Venus in the genital ridge at E11.5 (c), with *Prdm1*-mVenus (a, b) or *Dppa3*-Venus (c) expression in the left column, and *Dppa3*-ECFP expression in the second-left column. Merged images are shown on the right. BVSC-positive cells at E11.5 were positive for mouse vasa homologue (MVH) (b, second-right, magenta). hg, hindgut. Bar in (a), 100 μ m; in (b), 25 μ m; in (c), 25 μ m. (D) Expression of the BVSC transgenes in the developing ovaries (from E12.5 to P (postnatal day) 0, and P10 and 10 wk (10 weeks old)) with bright field images in the top row, *Prdm1*-mVenus expression in the middle row (except the 10-week column), and *Dppa3*-ECFP expression at the bottom row. *Dppa3*-ECFP shows specific expression in oocytes of various developmental stages in an ovary of 10-week-old female. Md, Müllerian duct. Bars in the E12.5-P0 columns, 150 μ m; in the P10 column, 100 μ m; in the 10 wk column, 200 μ m. (E) Expression of the BVSC transgenes in the developing testes (from E12.5 to P0 and P10) with bright field images in the top row, *Prdm1*-mVenus expression in the middle row, and *Dppa3*-ECFP expression in the bottom row. Md, Müllerian duct. Bars, 150 μ m.

Prdm1-mVenus was detected in a cluster of cells at the border between the embryonic and extraembryonic mesoderm, contiguous from the most proximal epiblast (Fig. 4Ab). These cells are considered to include the founding PGC population (Ohinata *et al.* 2005). At E7.75, *Prdm1*-mVenus strong-positive cells that migrated into the endoderm showed robust endogenous DPPA3 expression (Fig. 4Ac; Saitou *et al.* 2002, Yabuta *et al.* 2006, Seki *et al.* 2007), indicating that *Prdm1*-mVenus expression indeed marks the emerging PGC population. We did not detect *Dppa3*-ECFP expression at this stage. At E8.5, *Prdm1*-mVenus was detected in the cells apparently migrating in the hindgut endoderm, which also began to show *Dppa3*-ECFP expression (Fig. 4Ba). At E9.5, we again detected robust BVSC co-expression in the cells in the hindgut, many of which seemed to have already left the endoderm and to be moving through the mesentery toward the developing genital ridges (Fig. 4Bb and Ca). At both E10.5 and E11.5, we found strong BVSC

expression in the cells in the incipient genital ridges (Fig. 4Bc and d). Immunofluorescence analysis showed that the BVSC-expressing cells in the E11.5 genital ridges were exclusively positive for MVH, one of the well-recognized germ cell markers (Fig. 4Cb) (Tanaka *et al.* 2000, Toyooka *et al.* 2000), demonstrating that BVSC-positive cells were indeed PGCs. From E12.5 onward, the genital ridges had a sexually dimorphic appearance (reviewed by Capel 2000), and we could easily distinguish the sex of the embryos. In the female genital ridges, *Prdm1*-mVenus showed strong expression until E13.5 and was down-regulated acutely at E14.5 in an anterior-to-posterior order (Fig. 4D). We did not detect *Prdm1*-mVenus in female germ cells, thereafter. We also detected strong *Dppa3*-ECFP expression until E13.5, after which it became somewhat weaker from the entire female genital ridges. However, we consistently observed some level of *Dppa3*-ECFP fluorescence, which was probably attributable to the higher stability

of ECFP (see Discussion). The neonatal ovaries that contain oocytes of the primordial follicle stage (Pepling 2006), re-initiated strong *Dppa3*-ECFP expression, which persisted thereafter in the developing oocytes (Fig. 4D). In the male genital ridges, we also detected strong *Prdm1*-mVenus expression until E13.5. Similar to the expression in the female germ cells, *Prdm1*-mVenus became down-regulated after E14.5 in an anterior-to-posterior order, and we did not detect *Prdm1*-mVenus in the male germ cells after E16.5. The *Dppa3*-ECFP transgene continued to robustly mark male germ cells until E16.5, after which it gradually became weaker. We did not detect *Dppa3*-ECFP in the adult testis (data not shown). The *Dppa3*-Venus transgenic strains also recapitulated *Dppa3* expression specifically in the germ cell lineage after E8.0 and exhibited precise co-expression with *Dppa3*-ECFP in the genital ridge at E11.5 (Figs 3B and 4Cc, data not shown). Interestingly, we also noted that *Prdm1*-mVenus was expressed in the Müllerian duct but not in the Wolffian duct at E13.5 both in the females and males, and it continued to be expressed in the Müllerian duct up until at least postnatal day 0 in the female embryos, but disappeared after E14.5

in the males, in which the Müllerian duct itself degenerates. Thus, collectively, these observations demonstrate that the BVSC reporter enables the efficient monitoring of the specification, migration, and further differentiation of PGCs *in vivo*.

Germ line-competent ES cells bearing the BVSC transgenes

We derived ES cell lines from the blastocysts from BDF1 females mated with the BVSC males. The undifferentiated BVSC ES cells cultured in the ES cell maintenance medium (see Materials and Methods) showed neither *Prdm1*-mVenus nor *Dppa3*-ECFP expression (Fig. 5A). To investigate the ability of the BVSC ES cells to contribute to the germ line, we generated diploid chimeras and observed their contribution to the developing germ cell lineage. At E9.5, we detected a good contribution of the ES cells throughout the entire embryo and to the migrating PGCs in the hindgut endoderm: a majority of the migrating PGCs expressed BVSC transgenes (Fig. 5B). At E13.5, the ES cells also contributed substantially to the embryos, showing *Prdm1*-mVenus expression in the mesenchyme

Figure 5 The BVSC ES cells made a robust contribution both to the germ and somatic lineages in diploid chimeras. (A) The BVSC ES cells with bright field images are shown on the left, *Prdm1*-mVenus expression is shown in the middle, and *Dppa3*-ECFP expression is shown on the right. Bar, 100 μ m. (B) A BVSC ES cell E9.5 chimera showing high contribution of the BVSC ES cells to the developing gut endoderm and migrating PGCs. (a) bright field image, (b) *Prdm1*-mVenus expression, (c) *Dppa3*-ECFP expression, and (d) merged BVSC image. Bar, 50 μ m. (C) A BVSC ES cell E13.5 chimera. (a) Bright field image, (b) *Prdm1*-mVenus expression, (c) *Dppa3*-ECFP expression, (d) bright field image of the urogenital region of the same chimera, (e) *Prdm1*-mVenus expression in (d), (f) *Dppa3*-ECFP expression in (d), and (g) merged BVSC image in (d). gn, gonad; mn, mesonephros. Bar in (a), 500 μ m; in (d), 150 μ m.

of the incipient sensory vibrissae and the mesenchyme encompassing the ZPA, recapitulating the endogenous *Prdm1* expression. In the genital ridges, robust expression of BVSC was observed in PGCs (Fig. 5C). Thus, we demonstrated that the BVSC ES cells have the ability to efficiently form germ cells *in vivo*.

Discussion

We described here the generation of BVSC transgenic mice that simultaneously monitor endogenous expression of *Prdm1* and *Dppa3*, two key markers for PGCs (Saitou *et al.* 2002, Sato *et al.* 2002, Ohinata *et al.* 2005, Vincent *et al.* 2005). The BVSC transgenic strain has critical advantages as a germ line reporter over other transgenic lines: it detects the germ cell lineage from its very beginning by *Prdm1*-mVenus expression and compensates the subsequent relatively widespread expression of *Prdm1* by exclusive germ line expression of *Dppa3*-ECFP after E8.5. Thus, the BVSC strain is considered to be the first reporter that guarantees efficient and reliable visualization of *in vivo* germ cells from their incipience to at least their colonization and proliferation in the embryonic gonads, the period in which germ cells exhibit one of the most complicated behaviors in their development (Sasaki & Matsui 2008). The BVSC transgenic strain provided explicit evidence that *Prdm1* and *Dppa3* expression are regulated very differently (Figs 2–5), which is quite relevant in terms of the mechanism of germ cell specification. We detected *Dppa3*-ECFP expression in PGCs after E8.5, which is ~1 day after the onset of *Dppa3* mRNA expression and slightly later than the stella-EGFP or stella-EGFP BAC transgene expression reported previously (Payer *et al.* 2006). Nonetheless, very bright fluorescence of ECFP assures easy detection of PGCs after E8.5.

We note that, in some cases, both fluorescent markers, especially *Dppa3*-ECFP, appeared to show greater stability over the endogenous proteins: for example, endogenous DPPA3 protein expression becomes undetectable after E4.5 (Sato *et al.* 2002), but we observed persistent fluorescence of *Dppa3*-ECFP until E5.5 (Fig. 2A and B). Similarly, endogenous DPPA3 is down-regulated after E12.5 in the female genital ridges and becomes undetectable at E16.5 (Sato *et al.* 2002). However, we detected *Dppa3*-ECFP fluorescence throughout the development of the embryonic ovary, although it did indeed become weaker and more restricted after E13.5 (Fig. 4D). The half-life of GFP and its variants *in vivo* is ~24–48 h (Chalfie & Kain 2005), and this would depend on when and where they are expressed. Despite these points, the exclusive expression of *Dppa3*-ECFP in the germ cell lineage ensures its reliability as a germ cell reporter.

We established BVSC ES cells that made an efficient contribution to both the germ and somatic lineages in diploid chimeras. It has been shown that the germ cell

fate in mice is induced by BMP signals (Lawson *et al.* 1999, Ying *et al.* 2000, Chang & Matzuk 2001, Tremblay *et al.* 2001, Ying & Zhao 2001, Hayashi *et al.* 2002, Chu *et al.* 2004) and the specified PGCs repress a somatic program represented by *Hox* gene expression and regain potential pluripotency as indicated by their *Sox2* re-expression (Saitou *et al.* 2002, Yabuta *et al.* 2006). It is also notable that, almost immediately after their specification and concomitant with their migration, PGCs have been shown to embark on an ordered reprogramming of their epigenome, which includes the erasure of significant levels of genome-wide DNA methylation and histone H3-lysine (K) 9 dimethylation, followed by the up-regulation of H3K27 trimethylation (Seki *et al.* 2005, 2007). Quantitative single-cell expression profiling showed that the transitions from *Prdm1*-positive PGC precursors to *Dppa3*-positive PGCs and to more advanced migrating PGCs involve a highly dynamic, stage-dependent transcriptional orchestration (Yabuta *et al.* 2006). Furthermore, a number of gene mutations have been shown to affect PGC development (Mintz & Russell 1957, McCoshen & McCallion 1975, Buehr *et al.* 1993, Beck *et al.* 1998, Tsuda *et al.* 2003, Youngren *et al.* 2005, Covello *et al.* 2006). Despite these recent critical advances on the genetics of early germ cell development, the underlying biochemical mechanisms remain almost entirely unexplored, mainly because PGCs *in vivo*, especially at earlier stages, are too small in number for such an analysis. Thus, a precise recapitulation of the PGC specification process and subsequent development using ES cells in culture, which would provide abundant experimental materials, will be an important goal in the relevant fields.

We assume that one of the reasons why it has been difficult to induce functional PGC-like cells from the ES cells may be because most such attempts have used single transgene reporters with germ line expression (reviewed by Daley 2007), despite the fact that the ES cells can potentially up-regulate such single reporters inadequately due to their unique chromatin state (Bernstein *et al.* 2006, Lee *et al.* 2006). The use of BVSC ES cells as a starting material and monitoring of the temporally coordinated expression of *Prdm1*-mVenus and *Dppa3*-ECFP will circumvent this problem and may serve as a paradigm for this line of exploration.

Materials and Methods

Generation of *Prdm1*-mVenus, *Dppa3*-ECFP, and *Dppa3*-Venus transgenic mice

BACs bearing the *Prdm1* or *Dppa3* genomic locus of the C57BL/6 background were purchased from BACPAC Resources Center (Children's Hospital Oakland Research Institute, Oakland, CA, USA). For preparing Venus (a kind gift from A Miyawaki) targeted to the plasma membrane, the coding sequence of Venus was subcloned after the Igk leader sequence of the pDisplay vector

(Invitrogen). The resultant sequence from the Igk leader to the bovine growth hormone polyadenylation signal was recombined after the initial in-frame ATG of the exon 3 of the *Prdm1* gene by Red/ET recombineering (Gene Bridges, Heidelberg, Germany), according to the protocol provided by the manufacturer. The entire genomic sequence (~230 kb) was excised by NotI digestion and separated from the vector by gel filtration using CL-4B sepharose (Yang *et al.* 1997). For construction of the *Dppa3*-ECFP and *Dppa3*-Venus transgenes, ECFP (Takara, Tokyo, Japan) or Venus coding sequences including an SV40 polyadenylation sequence were recombined after the initial in-frame ATG of the exon 2 of the *Dppa3* gene by Red/ET recombineering. To remove the *Gdf3* gene from the transgene, the recombined BAC was digested with SacII and SmaI and an ~16 kb element encompassing the upstream region, exon 1, and part of intron 1 of the *Dppa3* gene was isolated and subcloned into the SacII and SmaI sites of the pKO919 vector (Lexicon Genetics Incorporated, Woodlands, TX, USA). The sequence from the intron 1 to part of exon 4 including the recombined ECFP or Venus sequence (~4.6 kb) was then amplified by Pyrobest DNA polymerase (Takara) using 5'-GGTGAAGCCTGTAATCACTGC-3' and 5'-AAAAGCGGCCCATCTGAATGGCTCACTGTCC-3' primer pairs and subcloned into the SmaI and NotI sites of the pKO919 vector bearing the upstream element. The sequence of the amplified portion was confirmed by DNA sequencing. The entire resultant insert was excised by AscI and NotI digestion and purified by QIAEX II (Qiagen) using the manufacturer's protocol.

The *Prdm1*-mVenus, *Dppa3*-ECFP, and *Dppa3*-Venus constructs were then injected into pronuclei of B6DBA F2 zygotes to generate transgenic mice, which were genotyped by PCR using the primer pair DisplayF, 5'-ACTCATCTCAGAAGAGGATCTG-3'; DisplayR, 5'-CACAGTCGAGGCTGATCTCG-3' for *Prdm1*-mVenus and the primer pair SV40F, 5'-CGACTCTAGATCATAATCAGCC-3'; SV40R, 5'-TAAGATACATTGATGAGTTTGGAC-3' for *Dppa3*-ECFP and *Dppa3*-Venus. The selected transgenic lines (see Results) were backcrossed onto the C57BL/6 background at least five times.

Isolation of transgenic embryos, immunofluorescence staining, and imaging

All the animals were treated with appropriate care according to the RIKEN ethics guidelines. Noon of the day when the vaginal plugs of mated females were identified was scored as E0.5. Female BDF1 mice were mated with male BVSC transgenic mice and were killed at the designated stages to recover embryos. Pre-implantation embryos were collected by flushing the oviduct or uteri with M2 medium, and post-implantation embryos were isolated in DMEM with 10% fetal bovine serum (FBS) (Stem Cell Science, Melbourne, Australia) and 1 mM HEPES. They were imaged immediately thereafter either with an Olympus IX71 upright or SZX16 dissection fluorescent microscope equipped with a DP70 cold CCD camera (Olympus, Tokyo, Japan).

For standard whole-mount immunofluorescence analysis, isolated embryos were fixed in 4% paraformaldehyde (PFA) in PBS for 4 h at 4°C, washed three times with PBS-0.2% Triton (PBS-T), and blocked with PBS-T with 2% normal goat serum (Vector Laboratories, Burlingame, CA) overnight. The embryos were then incubated with primary antibodies (anti-POU5F1

(1:500, rabbit polyclonal, a kind gift of H Hamada; Shimazaki *et al.* 1993), anti-DPPA3 (1:1000, rabbit polyclonal; Seki *et al.* 2007), and anti-GFP (1:500, rat monoclonal, Nakarai, Kyoto, Japan) in blocking solution for 96 h at 4°C, washed eight times with PBS-T, incubated with secondary antibodies (1:500, Alexa Fluor 488 goat anti-rat IgG and Alexa Fluor 568 anti-rabbit IgG (Invitrogen)), and DAPI for 48 h at 4°C in blocking solution, washed eight times with PBS-T, and mounted in Vectashield (Vector Laboratories) for observation by confocal microscopy (LSM 510 META (Zeiss, Jena, Germany)).

For immunostaining of BVSC genital ridges with anti-MVH (DDX4) antibody (rabbit polyclonal; Abcam, Cambridge, UK), isolated genital ridges at E11.5 were fixed in 4% PFA in PBS for 1 h at room temperature, washed three times with PBS-T, blocked with PBS-T with 0.5% normal goat serum for 30 min, incubated with the primary antibody (1:250) in blocking solution for 1 h, washed three times with PBS-T, incubated with a secondary antibody (1:1000, Alexa Fluor 633 goat anti-rabbit IgG (Invitrogen)) for 30 min, washed three times with PBS-T, and mounted in Vectashield (Vector Laboratories) for observation by confocal microscopy (LSM 510 META (Zeiss)).

Fluorescence in situ hybridization to determine the chromosomal localization of the transgenes

To determine the chromosomal localization of the transgenes, the splenocytes were collected from the spleen of the transgenic mice in RPMI 1640 (Invitrogen) supplemented with kanamycin. The isolated splenocytes were cultured in RPMI 1640 supplemented with fetal calf serum (15%), concanavalin A (3 µg/ml), lipopolysaccharide (10 µg/ml), and β-mercaptoethanol (5×10^{-5} M) for a few days, then for 3.5 h in the presence of 30 µg/ml bromodeoxyuridine, and an additional 30 min with 0.02 µg/ml colcemid. The cells were then collected, treated with a hypotonic buffer (0.075 M KCl), and fixed in methanol with acetic acid (methanol:acetic acid 3:1), and the chromosomal spreads were prepared on a glass slide. The spreads were air dried for a few days, stained with Hoechst 33258, and irradiated with u.v., and a Hoechst G-band staining pattern was prepared.

The BAC clone bearing the *Prdm1* gene or the plasmid bearing *Dppa3* cDNA were labeled with digoxigenin-11-dUTP using a nick translation kit (Invitrogen). The chromosome spreads were denatured in 70% formamide in $2 \times$ SSC at 70°C for 2 min, washed with 70% and 100% ethanol, air dried, and hybridized with the denatured probe cDNAs at 37°C overnight. The hybridized chromosomal spreads were washed stringently and the hybridized signals were detected using anti-digoxigenin antibody conjugated with Cy3. The images were obtained by Leica DMRA2 fluorescent microscopy (Leica, Wetzlar, Germany) and analyzed using Leica CW4000 FISH software.

Derivation of transgenic ES cells and generation of chimeras

The derivation of ES cells from blastocysts was performed essentially as described previously (Wakayama *et al.* 2001, 2005). Female BDF1 mice were mated with male BVSC transgenic mice and the morulae were flushed out from the

oviduct or uteri at E2.5 and cultured overnight in KSOM medium. The developed blastocysts were treated with acid Tyrode's solution to remove the zona pellucida, and each blastocyst was seeded onto mouse embryonic feeder cells in ES cell derivation medium (Knockout DMEM (Invitrogen) with 2 mM L-glutamine (Invitrogen), 1 × MEM non-essential amino acids (0.1 mM; Invitrogen), 1 × nucleosides (0.03 mM each; Chemicon, Temecula, CA, USA), 1 × β-mercaptoethanol (0.1 mM; Chemicon), 2 × 10³ units/ml leukemia inhibitory factor (LIF; ESGRO; Chemicon), and 20% KSR (Invitrogen)) in a 96-well plate and cultured at 37 °C under 5% CO₂ until the ICM cells had grown sufficiently (~10 days). The developed cells were passaged in ES cell maintenance medium (knockout DMEM with 2 mM L-glutamine, 1 × MEM non-essential amino acids, 1 × nucleosides, 1 × β-mercaptoethanol, 1 × 10³ units/ml LIF, and 20% FBS (Stem Cell Science)) under a feeder-free condition, and undifferentiated ES cells were eventually established. Generation of diploid chimeras was performed with a standard protocol using C57BL/6 blastocysts as recipients.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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