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A possible role of *Reproductive homeobox 6* in primordial germ cell differentiation

CHANG LIU, PAICHI TSAI, ANA-MARIE GARCÍA, BRANDON LOGEMAN and TETSUYA S. TANAKA*

Department of Animal Sciences, Institute for Genomic Biology, University of Illinois at Urbana-Champaign, USA

ABSTRACT ***Rhox6*** **is one of the *R*eproductive *Ho*meobox genes on the *X* chromosome (*Rhox*) that is expressed in the placenta and the post-migratory primordial germ cells (PGCs) in the nascent gonad. Despite its novel expression pattern, the significance of *Rhox6* expression in the differentiation of these cell types remains unknown. To investigate the role that *Rhox6* plays in PGCs, cDNA encoding *Rhox6* and short-hairpin (sh) RNA directed against *Rhox6* transcripts were introduced by unique expression vectors into a genetically engineered mouse embryonic stem cell (ESC) line. This ESC line expresses enhanced green fluorescent protein (EGFP) under the *Oct3/4* promoter, thereby allowing us to monitor the presence of undifferentiated ESCs and PGCs in culture in real time. This ESC line was used to isolate clones that stably expressed *Rhox6* cDNA, shRNA against *Rhox6* transcripts, or controls. Quantitative RT-PCR results validated that overexpression had been achieved, as well as knockdown of *Rhox6* transcripts in these ESC clones. However, these clones exhibited a normal appearance of undifferentiated ESCs and expressed EGFP. Next, these ESC clones were induced to differentiate into PGCs by generating embryoid bodies (EBs) in culture medium without leukemia inhibitory factor. Detection of EGFP expression by fluorescence microscopy and germ cell markers by RT-PCR validated the differentiation of PGCs in EBs. The *Rhox6* transgene had little, if any, effect on EGFP expression in EBs, whereas *Rhox6* knockdown significantly decreased EGFP expression in EBs. Thus, it is suggested with these results that *Rhox6* is necessary for determination of the germ cell lineage.**

KEY WORDS: *mouse embryonic stem cell, primordial germ cell, Rhox, short-hairpin RNA*

Introduction

A cluster of 12 homeobox genes was originally identified on the X chromosome of the mouse that exhibit unique colinearity in their spatial and temporal expression in reproductive organs, such as the ovary, testis and placenta (MacLean *et al.*, 2005). These homeobox genes are known as the *R*eproductive *ho*meobox genes on the *X* chromosome (*Rhox*) and are numbered according to their physical proximity to the centromere (MacLean *et al.*, 2005). The *Rhox* family now includes more than 30 genes (MacLean and Wilkinson, 2005).

A member of the *Rhox* family, *Rhox5*, was originally referred to as transcripts expressed in the *p*lacenta and *em*bryos, *Pem*, and is expressed in post-migratory primordial germ cells (PGCs) (Daggag *et al.*, 2008) and extraembryonic cells (Wilkinson *et al.*, 1990; MacLean *et al.*, 2005). In mature male mice, *Rhox5* is expressed in Sertoli cells during spermatogenesis (Pitman *et al.*, 1998). Mice with a targeted mutation in *Rhox5* exhibited normal fecundity, with no obvious alteration in testicular development or function (Pitman *et al.*, 1998). However, more detailed analysis revealed that the mutant male mice exhibited increased germ cell apoptosis and reduced sperm production, sperm motility, and fertility (MacLean *et al.*, 2005). Thus, it is suggested with these data that the *Rhox* family plays important roles in the development of the reproductive organs.

Another member of the *Rhox* family, *Rhox9*, was originally identified as a homeobox gene expressed in PGCs in nascent bipotential gonads and the placenta (also known as *G*erm cell and

Abbreviations used in this paper: EB, embryoid body; ESC, embryonic stem cell; PGC, primordial germ cell; Rhox, reproductive homeobox gene on the X chromosome; sh, short hairpin.

***Address correspondence to:** Tetsuya S. Tanaka. 330 ASL, MC-630, 1207 West Gregory Drive, Urbana IL 61801, USA. Tel: +1-217-244-2522. Fax: +1-217-333-8286. e-mail: ttanaka@illinois.edu

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*p*lacenta-specific homeo*box*, *Gpbox*, or *P*lacenta-*s*pecific homeobox 2, *Psx2*; Han *et al.*, 2000; Takasaki *et al.*, 2000). Comparable levels of *Rhox9* transcripts were initially detected in the urogenital ridge at the onset of gonadal dimorphism, whereas later in embryonic development, *Rhox9* transcripts accumulated in female germ cells more abundantly (Takasaki *et al.*, 2000). However, targeted mutagenesis of *Rhox9* has demonstrated that *Rhox9*-null mice are fertile and exhibit no obvious gonadal dysfunction (Takasaki *et al.*, 2001). Importantly, 91% of the protein-coding nucleotide sequence from *Rhox9* is identical to that of *Rhox6*, which was originally referred to as *P*lacenta *s*pecific homeobox 1 (*Psx1*) and was identified by expression-based screening of homeobox genes involved in mouse embryonic development (Han *et al.*, 1998; Chun *et al.*, 1999). Similar to *Rhox9*, the expression of *Rhox6* transcripts is localized to the placenta (Chun *et al.*, 1999; Tanaka *et al.*, 2002) and PGCs in nascent bipotential gonads (Takasaki *et al.*, 2000; Daggag *et al.*, 2008). Therefore, due to the high sequence homology and the similar expression pattern, the function of *Rhox9* may be complemented by that of *Rhox6*. To the best of our knowledge, no *Rhox6*-deficient mouse has yet been studied.

Interestingly, mouse embryonic stem cells (ESCs) maintained in an undifferentiated state exhibit fluctuating expression of *Rhox6* and/or *Rhox9* (Carter *et al.*, 2008). In addition, mouse ESCs can give rise to germ cells *in vitro* by manipulating the chemical composition of the culture media and by maintaining ESCs as cellular aggregates, specifically as embryoid bodies (EBs), which mimics the gastrulation process in developing embryos (Geijsen *et al.*, 2004; Young *et al.*, 2010). Therefore, mouse ESCs provide a simple assay system to evaluate the significance of the unique *Rhox6* expression pattern in ESCs and PGCs. In particular, because the POU-domain transcription factor *Oct3/4* (*Pou5f1*) is expressed in undifferentiated ESCs and PGCs (Yeom *et al.*, 1996; Yoshimizu *et al.*, 1999; Niwa *et al.*, 2000; Tanaka *et al.*, 2002), the use of a mouse ESC line that expresses enhanced green fluorescent protein (EGFP) under the *Oct3/4* promoter (Walker *et al.*, 2007; Chowdhury *et al.*, 2010; Li *et al.*, 2011; Tedesco *et al.*, 2011) allows us to evaluate the pluripotency of ESCs and the differentiation of PGCs in real time. In this study, the cDNA encoding *Rhox6* and short-hairpin (sh) RNA directed against *Rhox6* were introduced into ESCs, although they were not found to induce the immediate differentiation of ESCs. However, *Rhox6* knockdown significantly decreased the efficiency of the maintenance of Oct3/4-positive cells under the culture conditions that induce the differentiation of PGCs. Therefore, it is suggested with these results that *Rhox6* may play an important role in the determination of the germ cell lineage.

Results

Validation of PGC differentiation in embryoid bodies

We followed an established protocol to induce the differentiation of PGCs with mouse ESCs (Geijsen *et al.*, 2004) (Fig. 1A) that has been utilized reproducibly by other groups (Payer *et al.*, 2006; Nicholas *et al.*, 2009). Briefly, three days after mouse ESCs were maintained in hanging drop culture using the EB medium (see Materials and Methods), formed EBs were transferred to non-coated culture dishes in the same medium with continuous motion, which enriched the differentiation of PGCs in EBs (Geijsen *et al.*, 2004).

A male mouse ESC line, OGR1, which expresses enhanced green fluorescent protein (EGFP) under the *Oct3/4* promoter (GOF-18/EGFP; Walker *et al.*, 2007; Chowdhury *et al.*, 2010; Li *et al.*, 2011) was used throughout this study because *Oct3/4* is expressed in both undifferentiated ESCs and germline cells, but not in other differentiated somatic cells (Yeom *et al.*, 1996; Yoshimizu *et al.*, 1999). Immunofluorescence microscopy results using an antibody that reacts with Oct3/4 proteins validated that EGFP expression indicated the presence of Oct3/4 proteins (Fig. 1B).

Differentiation of PGCs was induced in OGR1 ESCs (Fig. 1A and C). The EBs generated in three days of hanging drop culture maintained green fluorescence ("Day 3" in Fig. 1C). It takes three to five days for the transcriptional activity of *Oct3/4* to exhibit a

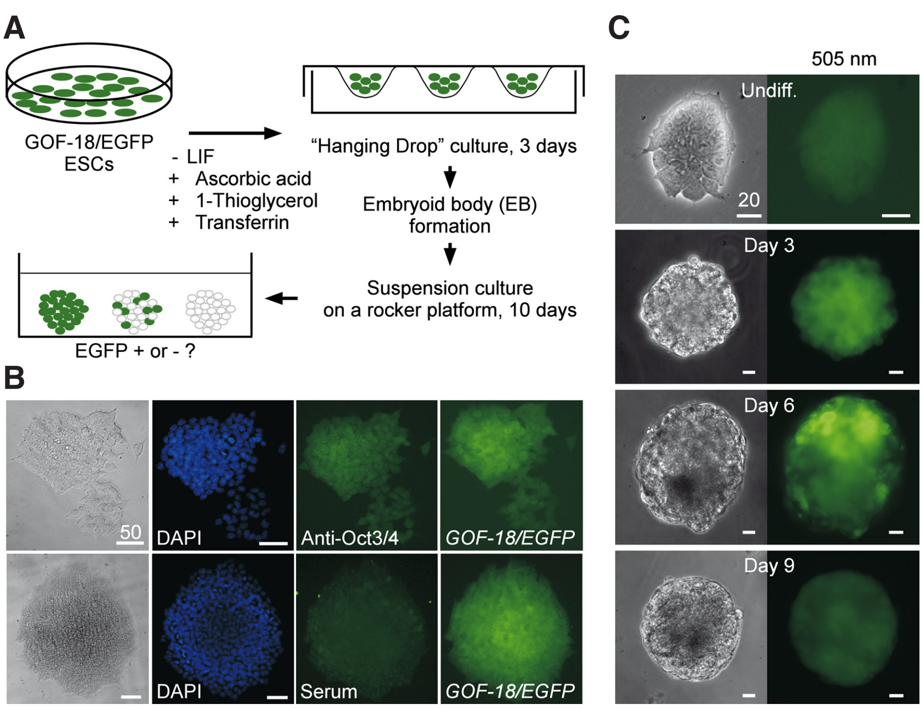

Fig. 1. A mouse embryonic stem cell (ESC) line harboring EGFP driven by the *Oct3/4* promoter allows the monitoring of the differentiation of primordial germ cells (PGCs) in real time. (A) *The culture method used to induce the differentiation of PGCs from mouse ESCs is schematically represented. See Materials and Methods for details.* **(B)** *Phase contrast (far left) and DAPI-stained (2nd from left) images of ESC colonies are shown. These OGR1 ESCs express EGFP driven by the* Oct3/4 *promoter (*GOF-18/EGFP*). The colony on top was immunostained with an anti-Oct3/4 antibody (Anti-Oct3/4). Its fluorescence overlaps with that of EGFP. The colony on the bottom was immunostained with normal mouse serum (Serum). Only non-specific background fluorescence was observed. Bars, 50 μm.* **(C)** *The OGR1 ESCs (Undiff.) were used to generate embryoid bodies (EBs) to initiate the induction of PGC differentiation, as shown in A. Phase contrast and fluorescence (505 nm) images of EBs three, 6 and 9 days after the formation of EBs (Days 3, 6, and 9, respectively) are shown. Due to the 3D structure of the EBs, the fluorescence in the EBs appears to be much brighter than in undifferentiated ESCs. This is also because all images were processed in the same manner. Bars, 20 μm.*

50% reduction in the absence of LIF (Tanaka *et al.*, 2002; Walker *et al.*, 2007). Fluorescence in the EBs became more heterogeneous 6 days after EB formation, such that within each EB, some regions lost EGFP expression, whereas other regions exhibited even higher EGFP expression ("Day 6" in Fig. 1C). On day 9 of EB culture ("Day 9" in Fig. 1C), the overall expression level of EGFP in the EBs was downregulated compared with that in day 3 EBs. However, the day 9 EBs did continue to exhibit fluorescence indicative of the presence of Oct3/4-positive cells such as PGCs.

To further validate the induction of PGC differentiation in EBs and to correlate the PGC differentiation with EGFP expression, total RNA was isolated from undifferentiated ESCs as well as from EBs at days 3, 6 and 9 and subjected to RT-PCR (Fig. 2). Primer pairs for genes expressed in PGCs (*Dazl*, *Esg1*, *Fgls*, *c-Kit*, *Mvh*, *Nanog*, *Nanos3*, *Oct3/4*, *Piwil2*, *Rex1*, *Rhox6* and *Rhox9*; see Supplementary Table 1) were used. These genes, including *Rhox6* and *Rhox9*, were also expressed in undifferentiated OGR1 ESCs (Fig. 2A), although *Rhox9* was much less abundant than *Rhox6* (Fig. 2; see also "Scrambled" in Fig. 4B). Consistent with previous studies (Geijsen *et al.*, 2004), semi-quantitative (sq) RT-PCR analysis demonstrated that expression levels of these genes were downregulated three days after EB formation (Fig. 2A). Interestingly, the expression levels of *Dazl*, *Esg1*, *c-Kit*, *Mvh*, *Nanos3*, *Oct3/4* and *Piwil2* were upregulated 9 days after EB formation, whereas the levels of *Fgls*, *Nanog*, *Rex1* and *Rhox6* became upregulated 6 days after EB formation (Fig. 2A). In contrast, the expression level of *Rhox9* gradually decreased during EB formation. When relative expression levels of *Dazl*, *Esg1*, *Mvh*, *Oct3/4*, *Piwil2*, *Rhox6* and *Rhox9* during formation of EBs were analyzed by quantitative (q) RT-PCR, most of the markers exhibited similar expression patterns to those obtained by sqRT-PCR (Fig. 2B). However, qRT-PCR results revealed that *Rhox6* reached its maximal level at day 6 after EB formation and that *Mvh* showed steady downregulation between day 6 and 9 (Fig. 2B). Expression of these PGC markers was also confirmed in EBs made from another ESC line (W4; Fig. 2C), which validates the reproducibility of this method. Collectively, these results demonstrated that Oct3/4-positive cells were present in EBs formed in the absence of LIF for 17 days, which was indicated by EGFP expression in real time. Although PGC markers examined were also expressed in ESCs, these markers were not constitutively expressed during EB formation (Fig. 2). Therefore, it is highly likely that the differentiation of PGCs took place in EBs. Oct3/4-positive cells in EBs are referred to as PGC-like cells hereafter. Importantly, we found that the expression of *Rhox6* was temporally regulated during the PGC differentiation process. We did not observe significant differences in the expression of these markers between day 9 and day 17 EBs, except that downregulation of *Rex1* and upregulation of *Rhox9* were observed in day 17 EBs (Fig. 2C). Therefore, for the subsequent experiments, we chose to culture EBs for 10 days after the initiation of hanging drop culture (day 13), which provides sufficient time to induce the differentiation of PGC-like cells in EBs (Geijsen *et al.*, 2004).

Constitutive expression of Rhox6 *during PGC differentiation in EBs*

To investigate the effect of constitutive *Rhox6* expression on mouse ESCs and on the differentiation of PGCs from the ESCs, we first built an expression vector (Fig. 3A; see Supplementary data). This expression vector, pCAGT2AP, allows us to isolate ESC lines that stably express cDNAs of interest and a puromycin-resistant gene product monocistronically via the self-cleaving peptide T2A (Szymczak *et al.*, 2004) (Fig. 3A). cDNAs encoding *Rhox6* and non-toxic *DsRedT3* were separately cloned into pCAGT2AP. The resulting vectors were designated pCAG_Rhox6T2AP and pCAG_DsRedT2AP. pCAG_DsRedT2AP served as a negative control.

Next, OGR1 ESCs were electroporated with

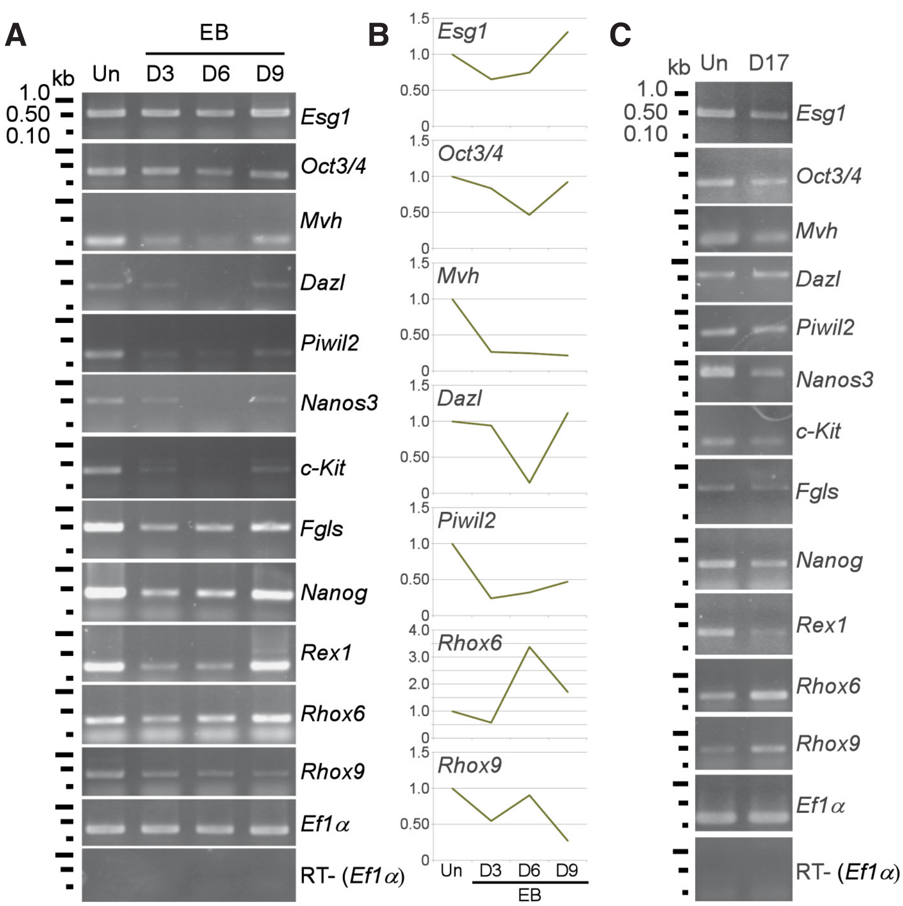

Fig. 2. Differentiation of PGCs is validated by the expression of markers in EBs. (A) *Expression of marker genes for PGCs, as indicated on the right, was examined by semi-quantitative (sq) RT-PCR in undifferentiated OGR1 ESCs (Un) and EBs at days 3 (D3), 6 (D6) and 9 (D9), which were cultured as shown in Fig. 1A.* Ef1α *is a control for PCR. RT- is a negative control. DNA size markers are shown on the left.* **(B)** *Relative expression levels of seven PGC markers, as indicated on the top-left corners, were examined by quantitative (q) RT-PCR using the same set of samples described above and shown as line graphs. Samples are indicated on the bottom. A value for undifferentiated OGR1 (Un) is normalized to 1.* Ef1α *was used as a reference.* **(C)** *Similarly, the expression of PGC markers was examined in W4 ESCs (Un) and EBs at day 17 (D17).*

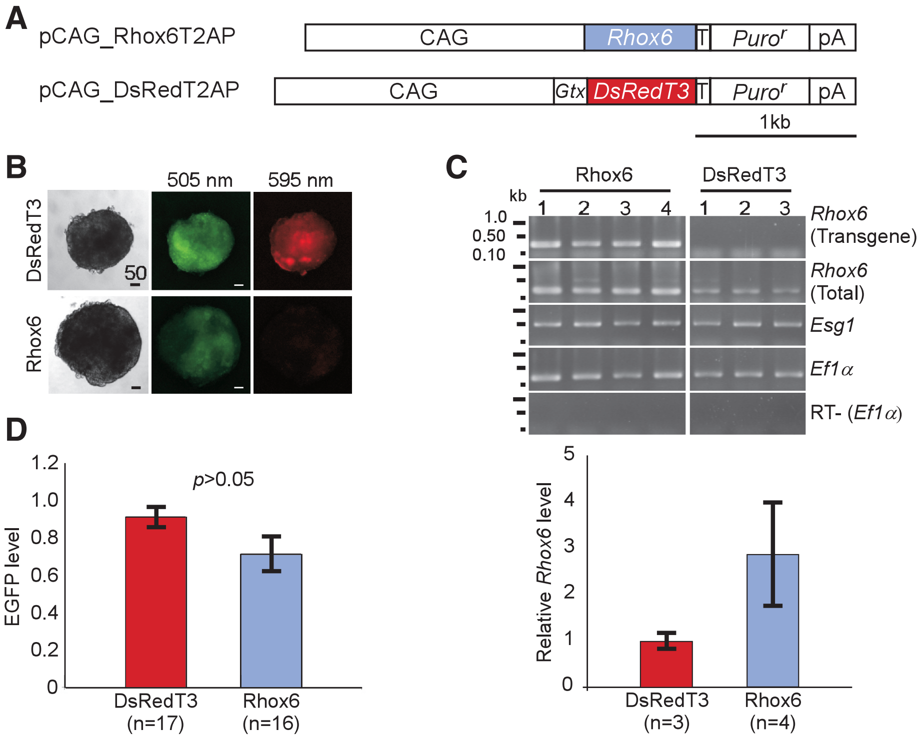

Fig. 3. Overexpression of *Rhox6* has little, if any, effect on PGC differentiation. (A) *The cDNA expression vector pCAGT2AP was built in this study and used to stably overexpress either* Rhox6 *(pCAG_Rhox6T2AP) or* DsRedT3 *(pCAG_DsRedT2AP) in ESCs. CAG, the CAG promoter; Gtx, a translational enhancer (Tanaka* et al., *2008); T, T2A (Szymczak* et al., *2004);* Puro^r*, the puromycin-resistant gene; pA, a bovine growth hormone polyadenylation signal. Bar, 1 kb.* **(B)** *ESC clones that stably express either* DsRedT3 *(top) or* Rhox6 *(bottom) were cultured as EBs (Fig. 1A). At day 13, the expression of EGFP (505 nm) and DsRedT3 (595 nm) was examined. Bars, 50 μm.* **(C, top)** *Expression levels of the* Rhox6 *transgene and total* Rhox6 *transcripts were examined by sqRT-PCR in day 13 EBs generated independently from four or three clones expressing* Rhox6 *or* DsRedT3 *transgenes, respectively, as indicated above. Labels are explained in Fig. 2.* **(C, bottom)** *Relative expression levels of total* Rhox6 *transcripts were examined by qRT-PCR using the same set of samples described above. An averaged value for EBs expressing* DsRedT3 *is normalized to 1.* Esg1 *was used as a reference. Standard errors of the means are indicated by bars.* **(D)** *The "EGFP levels" (see Materials and Methods) were compared between the day 13 EBs generated from 17 or 16 independent clones expressing* DsRedT3 *or* Rhox6 *transgenes, respectively. No statistically significant differences were observed (*$p>0.05$*). Standard errors of the means are indicated by bars.*

each vector, followed by selection with puromycin for 7 days. A total of 16 and 17 puromycin-resistant OGR1 clones were collected from two independent sets of electroporation with pCAG_Rhox6T2AP and pCAG_DsRedT2AP, respectively (see Supplemental Table 2). Isolating multiple independent clones rules out the possibility that any phenotypic differences observed are due to an effect of a specific insertion site of transgenes. The obtained clones were individually plated into wells of 24-well plates. Introduction of these transgenes did not induce immediate differentiation of OGR1 ESCs. Each drug-resistant clone exhibited the appearance of undifferentiated ESCs and expressed EGFP.

The ESC clones were cultured as hanging drops to promote the differentiation of PGCs (Fig. 1A). Twelve days after the induction of PGC differentiation, the number of green fluorescence-expressing EBs per clone ("505 nm" in Fig. 3B) was counted. In addition, the expression of DsRedT3 in each clone was examined ("595 nm" in Fig. 3B). Overexpression of *Rhox6* was first validated by sqRT-PCR with primer sets that amplified products of transcripts derived from the *Rhox6* transgene or products of total *Rhox6* transcripts (Fig. 3C). Based on image analysis, the expression level of *Rhox6* in clones harboring pCAG_Rhox6T2AP became twice the amount of that in clones harboring pCAG_DsRedT2AP (Fig. 3C, top). Furthermore, qRT-PCR analysis demonstrated that the expression level of *Rhox6* in clones harboring pCAG_Rhox6T2AP showed about three-fold increase compared to that in clones harboring pCAG_DsRedT2AP (Fig. 3C, bottom). These data confirmed that the *Rhox6* and *DsRedT3* transgenes were stably integrated into the genome and transcribed, even though the induction of PGC-like cell differentiation was carried out in the absence of any antibiotics for 12 days.

The number of green fluorescent EBs was divided by the total number of EBs examined per clone (see Materials and Methods). The resulting values were used to evaluate the "EGFP level" in the EBs (Fig. 3D). Each clone was assigned an "EGFP level" value between 0 and 1, such that a value of "1" indicated that all of the EBs generated from an individual clone exhibited green fluorescence, whereas a value of "0" indicated that none of the EBs generated from a clone exhibited green fluorescence. Although the constitutive expression of *Rhox6* transcripts appeared to decrease the "EGFP level" in the EBs (0.72 ± s.e.m. 0.095, Fig. 3D; see Series I in Supplementary Table 2), no statistically significant difference was found when compared with the "EGFP level" of the negative control (i.e., DsRedT3, 0.91 ± s.e.m. 0.064, Fig. 3D; see Series I in Supplementary Table 2). In addition, the expression of *Esg1* (Tanaka *et al.*, 2002) was also consistent between these two groups (Fig. 3C). Similarly, qRT-PCR analysis demonstrated that the expression of *Esg1*, *Mvh* and *Oct3/4* was consistent between these two groups (data not shown). Therefore, we conclude that constitutive expression of *Rhox6* does not have a significant impact on the differentiation of PGCs. Additionally, we did not observe any significant impact on PGC differentiation when the cDNA encoding a close homologue of *Rhox6*, *Rhox9*, was introduced into OGR1 cells (data not shown).

Constitutive knockdown of Rhox6 *during PGC differentiation in EBs*

Next, forced downregulation of *Rhox6* was carried out by expression of short-hairpin (sh) RNA directed against *Rhox6* in the OGR1 ESCs. First, we built the shRNA-expression vectors phH1CCP and phH1CRB (Fig. 4A; see Supplementary Data). Both vectors contain the human H1 promoter to drive expression of the shRNA. Additionally, phH1CCP and phH1CRB include another cassette that independently expresses either a puromycin (*Puro*^{r})- or blasticidin (*Bsd*^{r})-resistant gene product fused either to enhanced cyan fluorescent protein (ECFP) directly or to DsRedT3 via the self-cleaving T2A peptide, respectively. Thus, with these vectors, it is possible to isolate stable mouse ESC lines that constitutively express shRNA and to validate its stable integration into the host genome by ECFP or DsRedT3 fluorescence. An initial set of data was obtained using phH1CCP (see Supplementary Table 2). Because the use of DsRedT3 was preferred under standard settings used in typical fluorescence microscopy, phH1CCP was modified to build phH1CRB.

Eight different shRNA constructs were designed: 1) three against *Rhox6*, 2) three against *Rhox9,* 3) one against both *Rhox6* and *Rhox9* (referred to as shRhox6&9 hereafter), and 4) one containing a scrambled oligonucleotide sequence that does not target any specific sequence in the mouse genome (control hereafter). After the transfection of each shRNA expression vector, followed by the isolation of drug-resistant ESCs, total RNA was extracted from each pool of drug-resistant ESC clones. The expression levels of *Rhox6*, *Rhox9*, *Oct3/4* and *Esg1* were examined by sqRT-PCR. The most specific shRNA constructs for *Rhox6* and *Rhox9* were chosen and used for subsequent analyses (referred to as shRhox6 and shRhox9 hereafter). As shown in Fig. 4B left, controls were not observed to exhibit any changes in gene expression. In contrast, shRhox6, shRhox9 and shRhox6&9 downregulated the expression level of their targets (by least 50% compared to the *Rhox6* or *Rhox9* levels in controls, according to image analysis; results obtained by qRT-PCR using samples from individually isolated drug resistant clones will be described below). On the other hand, these shRNA constructs did not affect the expression levels of *Ef1α*, *Esg1* and *Oct3/4* (Fig. 4B, left). Consistent with these results, introduction of these expression vectors did not induce immediate differentiation of OGR1 ESCs. Therefore, although mouse ESCs exhibit fluctuating expression of *Rhox6* and/or *Rhox9* (Carter *et al.*, 2008), these results indicate that *Rhox6* and *Rhox9* do not play critical roles in maintaining the self-renewal of mouse ESCs. Because *Rhox9*-deficient mice present no specific phenotype (Takasaki *et al.*, 2001), we initially focused on the knockdown of *Rhox6* (see Supplementary Table 2). However, because of this, the effect of shRhox9 was also examined in subsequent datasets.

After the expression vectors for shRhox6, shRhox9, shRhox6&9 or the control were delivered into the OGR1 ESCs (see Materials and Methods), drug-resistant colonies were individually isolated and subjected to induction of PGC-like cell differentiation (Fig. 1). As described in the previous section, isolation of multiple independent clones rules out any possible effects due to transgene insertion sites. Each drug-resistant clone exhibited the appearance of undifferentiated ESCs and expressed EGFP. The OGR1 clones harboring the expression vectors for shRhox6, shRhox9, shRhox6&9 or the control are hereafter referred to as shRhox6-, shRhox9-, shRhox6&9- or Control-OGR1, respectively.

Quantitative RT-PCR analysis revealed that shRhox6 achieved significant downregulation of *Rhox6* in shRhox6-OGR1 (about 90

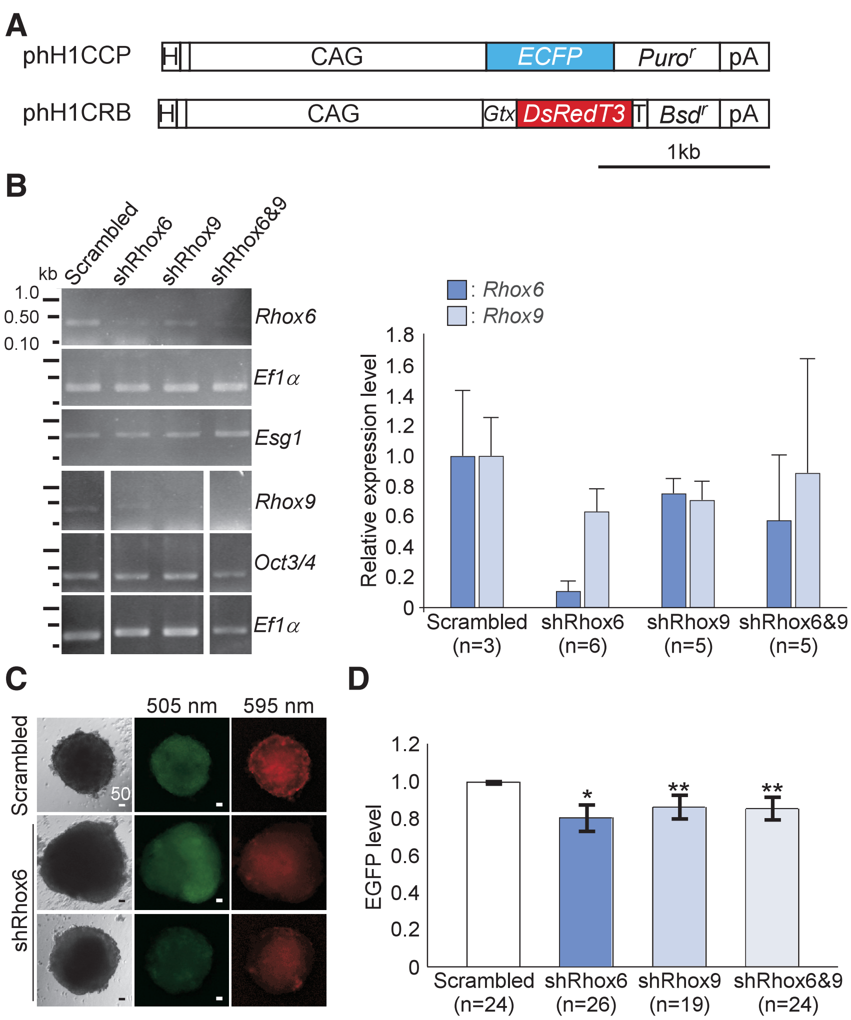

Fig. 4. Continuous knockdown of *Rhox6* expression significantly impairs PGC differentiation in EBs. (A) *The short-hairpin (sh) RNA expression vectors phH1CCP and phH1CRB were built in this study and used to stably express shRNA against* Rhox6, Rhox9 *or both* Rhox6 *and* Rhox9 *(Rhox6&9). The abbreviations used are described in Fig. 3A, except for: H, human H1 promoter; ECFP, enhanced cyan fluorescent protein;* Bsd^r*, the blasticidin resistant gene. Bar, 1 kb.* **(B, left)** *The efficiency of shRNA knockdown was evaluated by sqRT-PCR in pools of drug-resistant ESCs transfected with the shRNA expression vectors, as indicated above. "Scrambled" indicates a negative control for shRNA expression. phH1CCP was used to drive shRNA expression in this experiment. Expression levels of genes indicated on the right were examined. Labels are explained in Fig. 2.* **(B, right)** *Relative expression levels of* Rhox6 *and* Rhox9 *transcripts were examined by qRT-PCR in individual drug-resistant ESC clones transfected with the shRNA expression vectors indicated on the x-axis. An averaged value for ESCs that expressed control shRNA (Scrambled) is normalized to 1.* Esg1 *was used as a reference. Standard errors of the means are indicated by bars. Parentheses indicate the number of biological replicates (i.e., independent ESC clones transfected with the shRNA expression vectors).* **(C)** *ESC clones that stably expressed either control shRNA (Scrambled, top) or shRNA against* Rhox6 *(middle and bottom) were cultured as EBs (Fig. 1A). phH1CRB was used. At day 13, expression of EGFP (505 nm) and DsRedT3 (595 nm) was examined. EGFP was not detected in the EB in the bottom panel, whereas DsRedT3 was detected in all EBs. Bars, 50 μm.* **(D)** *The "EGFP levels" (see Materials and Methods) were compared among day 13 EBs generated from 24, 26, 19 or 24 independent clones expressing control shRNA, or shRNA against* Rhox6, Rhox9 *or both (Rhox6&9), respectively. Pair-wise comparisons with the control were performed in statistical tests. *:* $p<0.01$*; **:* $0.01<p<0.025$*.*

% reduction; Fig. 4B right). On the other hand, both shRhox9 and shRhox6&9 moderately downregulated their targets in shRhox9- and shRhox6&9-OGR1 (about 30 % reduction in *Rhox9* by shRhox9, and 40 % reduction in *Rhox6* and 10 % reduction in *Rhox9* by shRhox6&9; Fig. 4B right). In addition, both shRhox6 and shRhox9 exhibited downregulation of *Rhox9* and *Rhox6* in shRhox6- and shRhox9-OGR1, respectively (about 40% reduction in *Rhox9* by shRhox6, and 25% reduction in *Rhox6* by shRhox9; Fig. 4B right). Perhaps this is due to the high sequence similarity of *Rhox6* to *Rhox9*.

In the initial dataset, OGR1 clones were individually collected into 96-well plates (16~32 clones per shRNA construct). Induction of PGC-like cell differentiation was carried out in a 96-well format. Each clone was used to generate three or four EBs, which were pooled into individual wells of new 96-well plates. Experiments were independently repeated three times (see Series II in Supplementary Table 2). These preliminary data showed that *Rhox6* knockdown significantly decreased EGFP expression in the EBs (e.g., see "505 nm" in Fig. 4C). However, only 33% of the control-OGR1 clones exhibited EGFP expression in their EBs (see Series II in Supplementary Table 2). To rule out the possibility of off-target effects of shRNA and sub-optimal culture conditions due to the 96-well culture format, similar experiments were conducted by reducing the amount of expression vector delivered (1 or 7 μg per vector) as well as the size of the multi-well format used (24-well plates; Fig. 4D and Series III in Supplementary Table 2).

The data presented in Fig. 4D were collected from two independent experiments, which generated 24, 26, 19 and 24 individual clones for control-, shRhox6-, shRhox9-, and shRhox6&9-OGR1, respectively (see Series III in Supplementary Table 2). When phH1CRB was used to obtain OGR1 clones, all collected clones exhibited red fluorescence ("595 nm" in Fig. 4C). As discussed in the previous section, the "EGFP levels" were measured for each clone. Comparison of the "EGFP levels" between the control- and shRhox6-OGR1 clones (0.996 ± s.e.m. 0.0042 vs. 0.805 ± s.e.m. 0.069) revealed that the shRhox6-OGR1 clones exhibited significantly downregulated EGFP expression in their EBs ($p<0.01$). The EBs derived from the shRhox6&9-OGR1 clones consistently exhibited a reduced "EGFP level" (0.856 ± s.e.m. 0.061, $0.01<p<0.025$ compared with controls), which is associated with the modest downregulation of *Rhox6* by shRhox6&9 (Fig. 4B right). In addition, the EBs derived from the shRhox9-OGR1 clones exhibited a decreased "EGFP level" (0.864 ± s.e.m. 0.062, $0.01<p<0.025$ compared with controls). This result is consistent with the fact that shRhox9 induced downregulation of *Rhox6*, which was revealed by qRT-PCR (Fig.4B right). Taken together, it is suggested with these results that the downregulation of *Rhox6* impairs the differentiation of PGC-like cells in EBs.

Discussion

In the present study, we showed that the expression of *Rhox6* is temporally regulated during the differentiation of PGC-like cells in EBs derived from male mouse ESCs and that the continuous knockdown of *Rhox6* expression impairs the PGC-like cell differentiation process. Therefore, it is suggested with these data that *Rhox6* is necessary for the determination of the germ cell lineage.

In developing embryos, *Oct3/4* is expressed in preimplantation embryos, in the inner cell mass of blastocysts, in the epiblast and in the germline, which is governed by its regulatory sequence (Yeom *et al.*, 1996). Therefore, both the expression of *Oct3/4* and the use of its regulatory sequence are valuable tools to monitor the undifferentiated state of ESCs (Walker *et al.*, 2007; Chowdhury *et al.*, 2010; Li *et al.*, 2011) as well as the differentiation of germ cells (Yoshimizu *et al.*, 1999; Geijsen *et al.*, 2004; Young *et al.*, 2010). On the other hand, the formation of EBs mimics the gastrulation process in embryos. However, cell differentiation in EBs generally relies on absolute random events. To enrich a cell type of interest, genetically modified ESC lines are often used, in which a reporter, such as EGFP, is expressed under the regulatory sequence of a genetic marker specific to the cell type of interest. Then, based on the expression of EGFP, the differentiated cell type of interest can be sorted from disaggregated EBs (e.g. Nicholas *et al.*, 2009). In the present study, we applied an established protocol to induce the differentiation of PGCs from ESCs (Geijsen *et al.*, 2004). By means of the OGR1 ESC line, we were able to reproduce the PGC differentiation process *in vitro*, as demonstrated by EGFP expression, as well as the expression profiles of PGC markers including *Oct3/4* (Figs. 1 and 2). Although PGC markers examined were also expressed in ESCs, these markers were not constitutively expressed but exhibited downregulation followed by upregulation during EB formation (Fig. 2). In addition, EBs were cultured in the absence of LIF for at least 10 days. Therefore, it is highly likely that the majority of the Oct3/4-positive cells in EBs are not remaining undifferentiated ESCs but differentiated PGCs.

The data obtained in this study demonstrate that *Rhox6* is expressed in undifferentiated ESCs derived from the inner cell mass of blastocysts. Interestingly, we found that the expression of *Rhox6* was temporally regulated during the PGC differentiation process in EBs (Fig. 2A) and reached its maximal level at day 6 after EB formation (Fig. 2B). Based on the examination of the DNA methylation marks on differentially methylated regions at the *Igfr* and *H19* loci, PGC-like cells in EBs completely lose their methylation as early as day 7 (Geijsen *et al.*, 2004). In developing embryos, clearance of parent-of-origin DNA methylation in PGCs occurs at around embryonic day (E) 10.5 and is completed by E13.5 (Yamazaki *et al.*, 2003). Thus, PGC-like cells differentiated in EBs at day 7 are similar to the post-migratory PGCs in developing embryos. *Rhox6* expression is consistently detected in male gonads in E10.5 embryos and reaches its maximal expression level in the postmigratory PGCs of E11.5 embryos (Takasaki *et al.*, 2000).

We showed that constitutive knockdown of *Rhox6* significantly inhibited the differentiation of PGC-like cells in EBs (Fig. 4C). These data indicate that *Rhox6* is necessary for the determination of the germ cell lineage and is expressed in differentiating PGCs in embryos. Each ESC line isolated in this study harbors different levels of *Rhox6* knockdown or overexpression. Therefore, when introduced into host blastocysts in the future study, they will serve as an excellent tool to determine the effect of quantitative *Rhox6* expression on PGC determination and differentiation. However, it remains unknown whether *Rhox6* is expressed in PGCs at E9.5 or earlier, when germline establishment takes place in developing embryos (discussed below in more detail). Detection of *Rhox6* in embryos at E9.5 or earlier stages is rather challenging because it is difficult to obtain a probe or antibody specific to *Rhox6* due to its high sequence similarity to *Rhox9*. Perhaps future studies using embryos that express EGFP driven by the *Oct3/4* promoter will be able to reliably determine the complete expression profile of *Rhox6* in PGCs at different stages by isolating PGCs based on EGFP expression.

According to the data obtained in this study, overexpression of *Rhox6* resulted in little, if any, effect on PGC differentiation (Fig. 3D). In mice, germline determination is initiated by an inducing signal from the extraembryonic ectoderm to the epiblast by E7.2 (Yoshimizu *et al.*, 2001). Specifically, this signal is Bone morphogenetic protein 4 (Bmp4) (Lawson *et al.*, 1999; Ohinata *et al.*, 2009). Therefore, PGC differentiation is a rather passive process in mice, such that *Rhox6* may not play a role in autonomously directing the fate of ESCs to form PGCs. However, because the OGR1 clones examined in our experiments were isolated according to the appearance of undifferentiated ESCs, this may have resulted in an enrichment of clones in which the expression levels of *Rhox6* were not greater than three-fold those of a negative control. Nevertheless, at the level of analysis employed here, we did not observe any significant differences in obtaining colonies with the appearance of differentiated cells after electroporation of ESCs with either *Rhox6* or *DsRedT3* transgenes. In addition, transgenes encoding *Oct3/4*, *Gata6* and *Cdx2* were introduced in ESCs in separate experiments, whose expression was driven by the CAG promoter as described in this manuscript. Upregulation of these genes is known to induce differentiation of either primitive endoderm (Niwa *et al.*, 2000; Fujikura *et al.*, 2002) or trophoblasts (Niwa *et al.*, 2005). As expected, we were able to reproduce induced differentiation of ESCs (data not shown). Although we did not measure levels of transcripts or proteins from the transgenes in these experiments, it is a logical deduction for us to conclude that these transgenes achieved similar levels of upregulation in ESCs to those we found in the *Rhox6* transgene. Although the expression level of the *Rhox6* transgene could be increased by using other systems, *Rhox6* does not appear to play a significant role in directly inducing PGC differentiation.

Materials and Methods

Vector construction

Expression vectors were built by standard molecular cloning techniques. A stepwise description of vector construction is presented in the supplementary data.

Mouse embryonic stem cell culture

An R1 male mouse embryonic stem cell (ESC) line, which expresses EGFP driven by the *Oct3/4* promoter (GOF-18/EGFP; Walker *et al.*, 2007; Chowdhury *et al.*, 2010; Li *et al.*, 2011), was kindly provided by Dr. William L. Stanford, University of Toronto, Ontario, Canada. After being thawed on mitotically inactivated mouse embryonic fibroblasts, this ESC line, referred to as OGR1, was passaged onto 0.1 % gelatin (Sigma-Aldrich, St. Louis, MO)-coated tissue culture dishes in standard ESC culture medium, essentially as described previously (Li *et al.*, 2011). Expression vectors were linearized with ScaI and delivered by electroporation into feeder-free ESCs (7 or 10 μg DNA/ 1.0 x 10^7 cells/ cuvette, 0.8 kV/cm, 12 pulses of 99 μsec/pulse, BTX ECM200). Alternatively, FuGene HD (Roche Applied Science, Indianapolis, IN) was used to deliver vectors for shRNA expression into OGR1 ESCs (1 μg each per well of a 6-well plate). After selection with 2 μg/ml puromycin (InvivoGen, San Diego, CA) for pCAG_DsRedT2AP, pCAG_Rhox6T2AP and phH1CCP (Supplementary Data, see Figs. 3A and 4A), or 10 μg/ml blasticidin (InvivoGen) for phH1CRB (Supplementary Data, see Fig. 4A) for 7-10 days, the drug-resistant colonies were collected into 24-well or 96-well plates and expanded. When these ESC clones reached 80 to 100 % confluence, one-fifth or one-tenth of trypsinized ESCs from the wells of the 96- or 24-well plates was diluted with 2X embryoid body (EB) medium at 1:1 and used to generate three or four hanging drops (20-30 μl each) on bacterial Petri-dishes, respectively. This was considered to be day 1 of EB culture. The exact number of cells used to generate hanging drops is unknown. The 1X EB medium used was made with Iscove's MEM (Invitrogen, Carlsbad, CA) supplemented with 15 % FBS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM GlutaMax I, 4.5 mM 1-Thioglycerol (Sigma), 20 μg/ml Ascorbic acid (Sigma) and 200 μg/ml Holo-transferin (Sigma) (Geijsen *et al.*, 2004). Three days after the initiation of hanging drop culturing, the EBs generated from each clone were transferred into individual wells of a new 96- or 24-well plate for suspension culturing and placed on a shaker under conditions of 5% CO2 and 37ºC for three to 10 days (days 6-13 of EB culture). Fluorescence microscopy was carried out with an epifluorescent Leica DMI4000B microscope. Fluorescence images were taken using the same exposure time for all images (750 msec for 505 nm, 950 msec for 595 nm) and enhanced in the same way. EB formation was also validated using another male mouse ESC line, namely, W4 (Taconic, Hudson, NY).

Data analysis

Due to human error during the handling of EBs, the number of EBs in each well at day 13 exhibited variability. Therefore, to compare the data obtained under different conditions, the number of larger EBs with or without EGFP expression at day 13 was recorded for each clone and divided by the total number of EBs counted per clone. Thus, each clone had a final value of between 0 and 1, which indicates the "level" of EGFP expression per clone. We considered that the smaller EBs at day 13, which were possibly derived from EBs that were disturbed during transfer, failed to properly represent the PGC differentiation process. Thus, they were excluded from data analysis. Because most of the clones had a value of 0 or 1, the sum of the values per condition typically equaled the number of clones that exhibited EGFP expression in EBs at day 13. Complete datasets are presented as Supplementary Table 2. These values were averaged within each condition and statistically analyzed using Student's t-test. Standard errors of the mean (s.e.m.) were calculated.

Immunofluorescence microscopy

Immunofluorescence microscopy using OGR1 ESCs was carried out essentially as described previously (Chowdhury *et al.*, 2010; Li *et al.*, 2011). After EGFP expression was observed and recorded, ESCs were fixed with Dent's fixative (methanol: DMSO = 4:1) at – 20ºC. An anti-human Oct4 mouse monoclonal antibody (sc-5279, Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:100 with 2 % skimmed milk, 0.1% Tween-20 in PBS (-) (PBSMT) and used to detect Oct4 in the ESCs. As a negative control, normal mouse serum diluted 1:1,000 with PBSMT was used. For use as a secondary antibody, Alexa Fluor 488 goat anti-mouse IgG (H+L; Invitrogen) was diluted 1:400 with PBSMT. After washing with PBSMT, samples were incubated with 0.5 μg/ml DAPI in PBS (-) before being mounted with Glycerol Gelatin (Sigma). Fluorescence images were taken using the same exposure time for all images (750 msec for 505 nm) and enhanced in the same way.

Semi-quantitative and quantitative RT-PCR

Total RNA extraction from ESCs and EBs, first-strand cDNA synthesis and semi-quantitative reverse transcriptase polymerase chain reaction (sqRT-PCR) assays were carried out essentially as described previously (Tanaka *et al.*, 2008; Tanaka *et al.*, 2010; Li *et al.*, 2011). To determine the time course of PGC differentiation in EBs, 100 - 200 EBs were pooled. Undifferentiated ESCs (1.6 x 10^5 cells) were used as a reference. PCR mixtures were prepared using Phusion DNA polymerase (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. The PCR conditions were as follows: initial denaturing at 98ºC for 1 min, followed by 21 - 30 cycles of denaturing at 98ºC for 10 sec, annealing at 60ºC for 30 sec, extension at 72ºC for 30 sec and a final extension at 72ºC for 7.5 min. PCR cycle numbers were optimized for each primer set to ensure that products were linearly amplified, as follows: *Ef1α*, *Esg1*, *Fgls*, *Oct3/4* and *Rex1*, 21-23 cycles; *Dazl*, *c-Kit*, *Mvh*, *Nanog*, *Nanos3*, *Piwil2* and *Rhox6*, 27 cycles; *Rhox9*, 29-30 cycles. Primers used for RT-PCR are listed in Supplementary Table 1. Image analysis was carried out using Image J 1.42q. PCR products for *Esg1* were used to normalize the expression levels of *Rhox6*.

For quantitative RT-PCR, PCR mixtures were prepared using EXPRESS SYBR® GreenER™ qPCR superMixes (Invitrogen) and ran on the Eco™ Real-Time PCR System (Illumina, Inc.,San Diego, CA) according to the manufacturer's instructions, after concentrations of each primer were optimized. Melting curve analysis validated that each primer pair gave rise to single amplicons. Relative expression levels were determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using *Esg1* or *Ef1*α as a reference.

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