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Article

Dazl Promotes Germ Cell Differentiation from Embryonic Stem Cells

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It has been demonstrated that through the formation of embryoid bodies (EBs) germ cells can be derived from embryonic stem (ES) cells. Here, we describe a transgene expression approach to derive germ cells directly from ES cells *in vitro* without EB formation. Through the ectopic expression of Deleted in Azoospermia-Like (Dazl), a germ cell-specific RNA-binding protein, both motile tailed-sperm and oocytes were induced from mouse ES (mES) cells in culture. Furthermore, transient overexpression of Dazl led to suppression of Nanog but induced germ cell nuclear antigen in mES cells. Dazl knockdown resulted in reduction in the expression of germ cell markers including Stella, MVH and Prdm1. Our study indicates that Dazl is a master gene controlling germ cell differentiation and that ectopic expression of Dazl promotes the dynamic differentiation of mouse ES cells into gametes *in vitro*.

Keywords: embryonic stem cells, Dazl, sperm, oocyte

Introduction

Embryonic stem (ES) cells have been proposed as a useful model for study of germ cell development (Daley, 2007). Several studies demonstrated that germ cells can be derived from ES cells *in vitro* (Hübner et al., 2003; Toyooka et al., 2003; Clark et al., 2004; Geijsen et al., 2004; Nayernia et al., 2006). In these studies, the derivation of germ cells relied on the formation of embryoid bodies (EBs) from ES cells. Although retinoic acid (RA) enhances the germ cell differentiation in EBs, the number of germ cells from RA-treated EBs was quite low (Geijsen et al., 2004). Therefore, the mechanisms underlying germ cell commitment of ES cells *in vitro* have not been well documented due to the limitation associated with spontaneous differentiation of germ cells from EBs.

Deleted in Azoospermia-Like (Dazl) (Reijo et al., 1995), is a germ cell-specific gene and plays critical roles in germ cell development and differentiation. In the Dazl knockout mice, germ cell-specific genes including Oct4, Dppa3/Stella, germ cell nuclear antigen (GCNA) and MVH are reduced in the embryos (Lin and Page, 2005). In postnatal mice, loss of Dazl results in multiple defects including impairment in progression from $A_{aligned}$ to A_1 spermatogonia and meiotic arrest in the male, and lack of germ cells and follicles in the adult ovary (Ruggiu et al., 1997; Schrans-Stassen et al., 2001). Furthermore, Dazl also is involved in the translational regulation of MVH and SCP3 in meiotic cells (Reynolds et al., 2005, 2007). A very recent report demonstrated that Dazl is a key intrinsic factor in the initiation of meiosis in response to extrinsic signaling (Lin et al., 2008). Thus, it has been indicated that Dazl is a controlling factor in the regulation of germ cell differentiation. On the basis of this fact, we chose Dazl to explore the possibility of establishing a dynamic approach to differentiate ES cells into germ cells *in vitro*. Our study indicates that ectopic expression of Dazl induces germ cell differentiation from mES cells giving rise to both motile tailed-sperm and oocytes in culture. This study should provide a useful *in vitro* system for further dissecting the molecular basis of germ cell development.

Results

Motile and tailed-sperm generated from monolayer-cultured mES cells induced by ectopic expression of Dazl

To promote mES cells to differentiate into germ cells *in vitro*, we carried out ectopic expression of Dazl in mES cells. The mES cells were cultured under standard conditions. The mES cells were characterized by examining the expression of Oct4, Nanog and alkaline phosphatase prior to transfection (data not shown). Dazl cDNA was cloned into a pMSCVneo plasmid in which the expression of Dazl is under the control of the MSCV promoter, which is optimized for introducing and expressing target genes

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in pluripotent cell lines (Cherry et al., 2000; McKinney-Freeman and Daley, 2007). The pMSCVneo-Dazl-transfected mES cells were cultured under standard culture conditions for 2 days, then were trypsinized and plated in gelatin-coated 6-well plates followed by G418 selection 24 h later. The mES cell colonies were obtained after 2 weeks of selection. The overexpression of Dazl in the colonies was examined by Western blotting (Supplemental Figure S1A). To induce spermatogenesis, we cultured Dazl-overexpressing colonies in DMEM/F-12 medium containing 15% fetal bovine serum (FBS) but without leukemia inhibitory factor (LIF). These Dazl-transfected mES colonies grew as a monolayer attaching to the bottom of the plate. Three to 5 days later, subcolonies of small cells appeared in the middle of large colonies (Figure 1A and Supplemental Figure S1B). These small cells formed subcolonies at high density and some of them later changed their morphology into tadpole like shapes. These tadpole-like cells were released from attaching. In most of the cultures, sperm-like floating cells with a 'tail' were observed (Figure 1B). Elongating spermatids were also observed (Figure 1C). These spermatid-like cells further developed into sperm-like cells with a long tail and a head, and occasionally, sperm with two heads connected by one tail were also observed (Figure 1E). Giemsa staining indicated that the sperm contained compact chromatin in the head (Supplemental Figure S2). The tail structure was positive for acetyl-α-tubulin (Marh et al., 2003) as examined by immunofluorescent staining (Figure 1D and F). Some of the spermatid- and sperm-like cells were also positive for FE-J1, a specific marker of acrosome of spermatids and sperm (Fenderson et al., 1984) as shown by immunofluorescent staining (Figure 1G). Interestingly, motile sperm were also

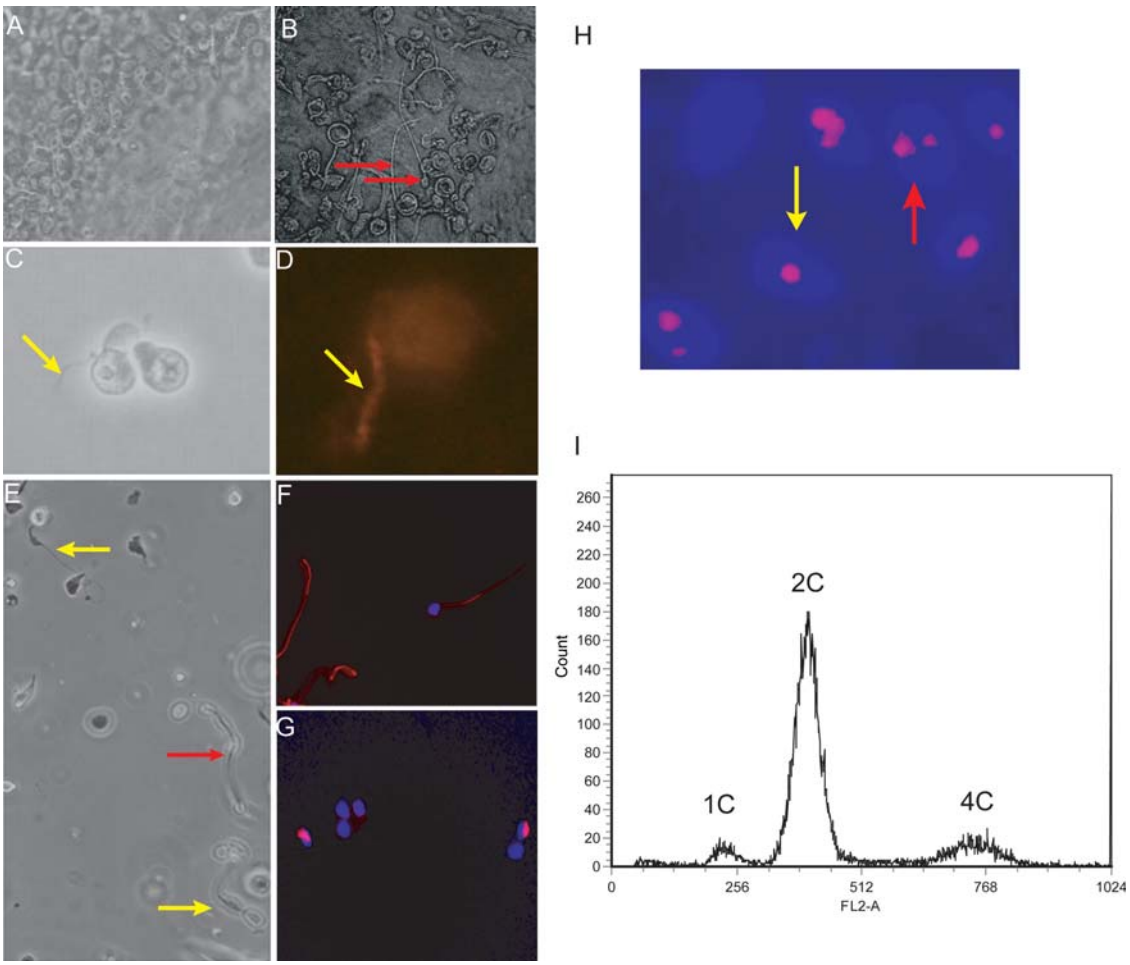

Figure 1 Characterization of spermatids and sperm induced by Dazl from mES cells. (**A**) Phase contrast microscopy analysis shows a colony of small cells derived from Dazl-transfected mES cells. (**B**) Floating cells with a tail (red arrows) were produced from Dazl-transfected mES cells (phase contrast microscopy image). (**C**) Elongated spermtids with a tail (yellow arrow) produced from Dazl-transfected mES cells (phase contrast microscopy image). (**E**) Phase contrast microscopy analysis shows that sperm were produced from Dazl-transfected mES cells, sperm with a long tail and a head (yellow arrows) and two sperm heads connected by one tail (red arrow). (**D, F**) Immunofluorescence analysis shows acetyl-α-tubulin staining of the tails of sperm produced from Dazl-transfected mES cells. (**G**) Immunofluorescent staining of FE-J1 shows acrosome of spermatids and sperm produced from Dazl-transfected mES cells. (**H**) Fluorescence *in situ* hybridization analysis of chromosome 8 demonstrates diploid (red arrow) and haploid (yellow arrow). (**I**) Flow cytometry analysis of DNA content shows haploid cells as 1C peak followed by diploid cells (2C) and tetraploid cells (4C).

observed (Supplemental Vedio) in culture. Therefore, ectopic Dazl expression might promote mES cells to undergo spermatogenesis in culture. To further identify the product of haploid cells, fluorescence *in-situ* hybridization analysis of chromosome 8 was carried out. The haploid cells were found in the culture of the Dazl-transfected mES cells (Figure 1H). The product of the haploid cell population in culture was also examined by flow cytometric analysis (FCA). The FCA data clearly showed a typical 1C peak followed by a 2C peak and a 4C peak indicating that ~9% of haploid cells were induced in early culture (Figure 1I). A higher 1C peak was observed in long-term culture but these cells were accompanied by a large population of dead cells (data not shown). This would be expected if the haploid cells underwent degeneration during extended culture. Given all these data, we concluded that ectopic expression of Dazl can induce spermatogenesis from mES cells.

Oocytes derived from Dazl-transfected XY mES cells

Since it was reported that oocytes could be derived from XY mES cells *in vitro* (Hübner et al., 2003), we were interested to test whether Dazl can induce oogenesis from XY mES cells. To achieve this, we cultured the Dazl-transfected mES cells in DMEM/F-12 medium containing 15% FBS but without LIF to form large colonies. The large colonies were manually collected and cultured in non-coated Petri dishes for 3 days, and then were transferred into gelatin-coated 6-well plates. After 2 days of culture, large round cells growing at the edges of colonies (Figure 2A) were observed. With these large cells, we carried out immunflourescent staining with an antibody against growth differentiating factor-9 (GDF-9), an oocyte-specific marker (Dong et al., 1996). We found that many of these large cells were

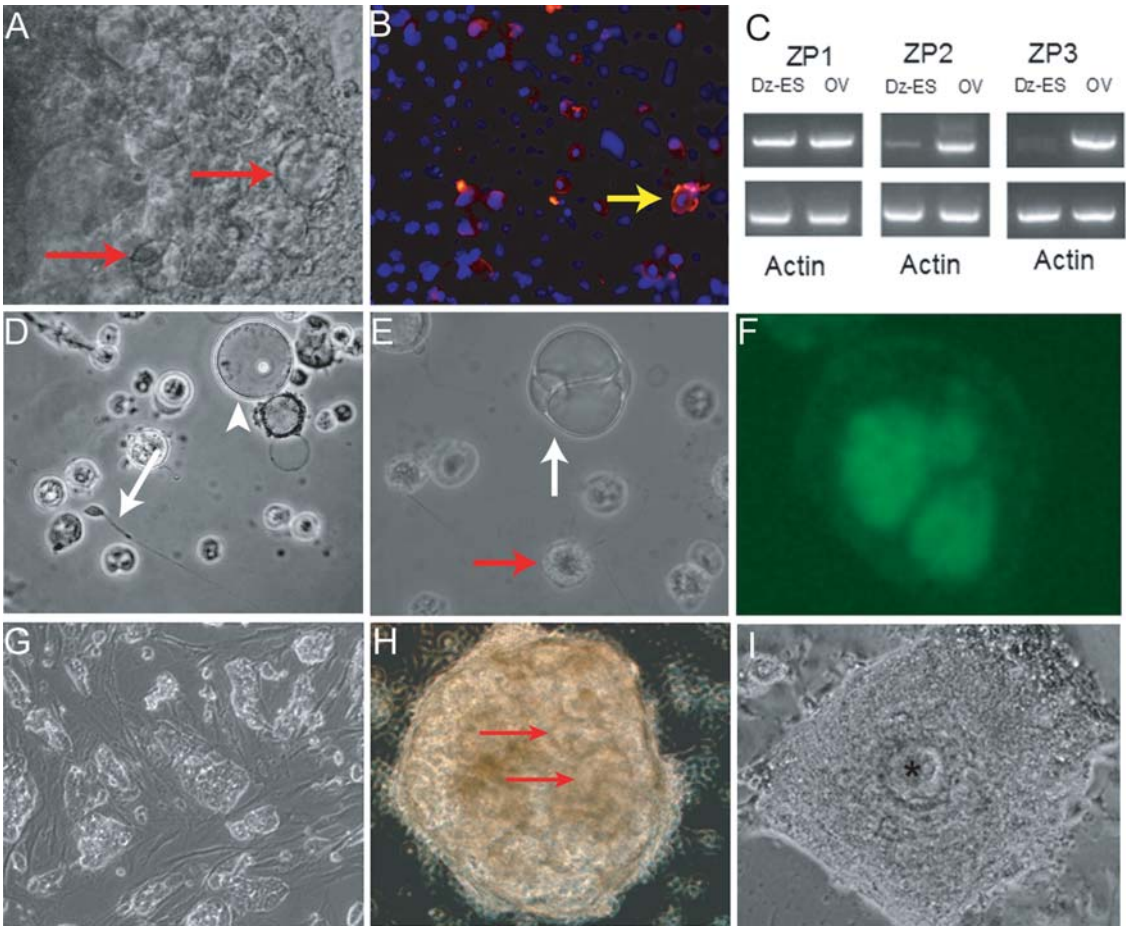

Figure 2 Characterization of oocytes induced by Dazl from mES cells. (**A**) Phase contrast microscopy analysis shows the large cells (arrows) produced from the culture of Dazl-transfected mES cells. (**B**) Immunofluorescence analysis demonstrates that the large cells from Dazl-transfected mES cells were positive for CDF-9 (arrows), which is a marker for oocytes. (**C**) RT–PCR analyses show the expression of ZP genes (Dz-ES, Dazl-transfected mES cells; OV, ovarian tissue). (**D**) Phase contrast microscopy analysis shows a sperm with a long tail (arrow) and oocytes (arrow head) produced from the same culture of Dazl-transfected mES cells. (**E**) Phase contrast microscopy analysis shows a 2-cell embryo (white arrow) and clusters of sperm (red arrow). (**F**) Immunofluorescent staining of Oct4 shows a 3-cell embryo. (**G**) Phase contrast microscopy analysis shows the mES cells cultured under standard conditions. (**H**) Phase contrast microscopy analysis shows seminiferous tubule-like structures (arrows) formed from the culture of Dazl-transfected mES cells mixed with untransfected mES cells. (**I**) Phase contrast microscopy analysis shows a follicle-like structure formed from the culture of Dazl-transfected mES cells mixed with untransfected mES cells, one oocyte (*) located in the middle of a follicle surrounded by layers of supportive cells.

positive for GDF-9 (Figure 2B). These cells became larger and were released into the culture medium. We also carried out RT–PCR assays for the expression of zona pellucida (ZP) genes (Epifano et al., 1995) in the large cells, and found that ZP1 was strongly expressed in these cells but ZP2 and ZP3 were expressed at low levels (Figure 2C). Interestingly, during the induction of oocytes from Dazl-transfected mES cells, some cells still underwent spermatogenesis instead of oogenesis, therefore, there were both tailed-sperm and oocytes in the same culture (Figure 2D, sperm with a long tail). Some of the oocytes derived from Dazl-transfected mES cells appeared to undergo parthenogenesis activation giving rise to embryos of early developmental stages (Figure 2E). These embryos were further characterized as Oct4 positive (Figure 2F).

Embryo development from Dazl-induced sperm via intracytoplasmic sperm injection

We also observed sperm surrounding and attaching on oocyte in the culture of Dazl-transfected mES cells (Figure 3A). However, there did not seem to be any fertilization from the Dazl-induced oocytes and sperm in culture. To further address whether the

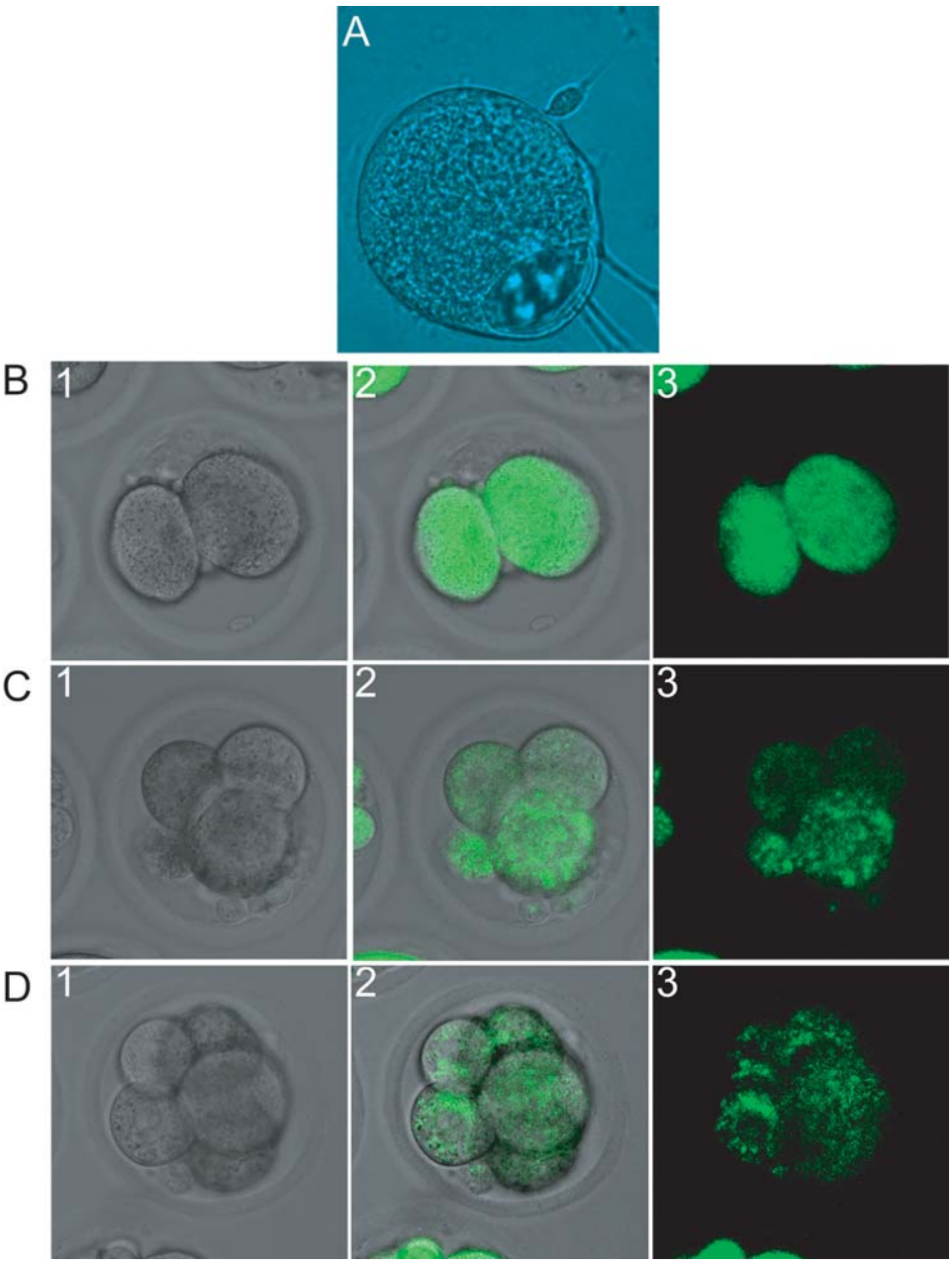

Figure 3 Fertilization and embryo development of Dazl-induced sperm from mES cells. (**A**) Phase contrast microscopy analysis showing sperm attaching on oocyte in the culture of Dazl-transfected mES cells. (**B**) Embryos were developed by ICSI of GFP-labeled Dazl-induced sperm from mES cells: a 2-cell embryo, phase contrast microscopy image (1), and fluorescence microscopy images (2, 3). (**C**) A 4-cell embryo, phase contrast microscopy image (1), and fluorescence microscopy images (2, 3). (**D**) An 8-cell embryo, phase contrast microscopy image (1), and fluorescence microscopy images (2, 3).

Dazl-induced sperm are capable of fertilizing eggs, we carried out Intracytoplasmic Sperm Injection (ICSI) using Dazl-induced sperm and eggs collected from mice. We labeled Dazl-transfected mES cells with a plasmid expressing EGFP under the control of the EF-1α promoter and obtained GFP-labeled sperm from subcultured colonies. After injection of GFP-labeled sperm into eggs, we derived GFP-expressing embryos (Figure 3B–D). The full-term development of embryos from Dazl-induced sperm has not yet been achieved; nevertheless, the ICSI experiment indicated that the Dazl-induced sperm are able to fertilize eggs *in vitro*.

Overexpression of Dazl induced the formation of ovarian follicle- and seminiferous tubule-like structures from mES cells

Although we continually observed oocytes and sperm derived from Dazl-transfected mES cells in culture, we could not find any ovarian follicle- or seminiferous tubule-like structures. We considered that missing formation of these structures might be due to lack of supportive cells since in the culture there were only Dazl-overexpressing mES cell colonies. To solve this problem, we performed transient transfection of mES cells using plasmid plenti6Ubc-Dazl-V5, through which Dazl is expressed as a fusion protein with the V5 tag. The expression of Dazl-V5 fusion protein was examined by Western blotting with V5 antibody (data not shown). Following the culture of the mixture of Dazl-transfected and untransfected cells in DMEM/F-12 medium containing 15% FBS without LIF, both follicle- and seminiferous tubule-like structures (Figure 2H and I) were observed. Furthermore, we continued the culture of the mixed cells and obtained larger tissues for sections. HE staining of these sections indicated that primary ovarian follicle-like structures were formed (Figure 4A); 3,3'-diaminobenzidine (DAB) staining of V5 and GCNA, which is a specific marker of both male and female germ cells, demonstrated that both V5 and GCNA positive cells were located along the inside of seminiferous tubule-like structures

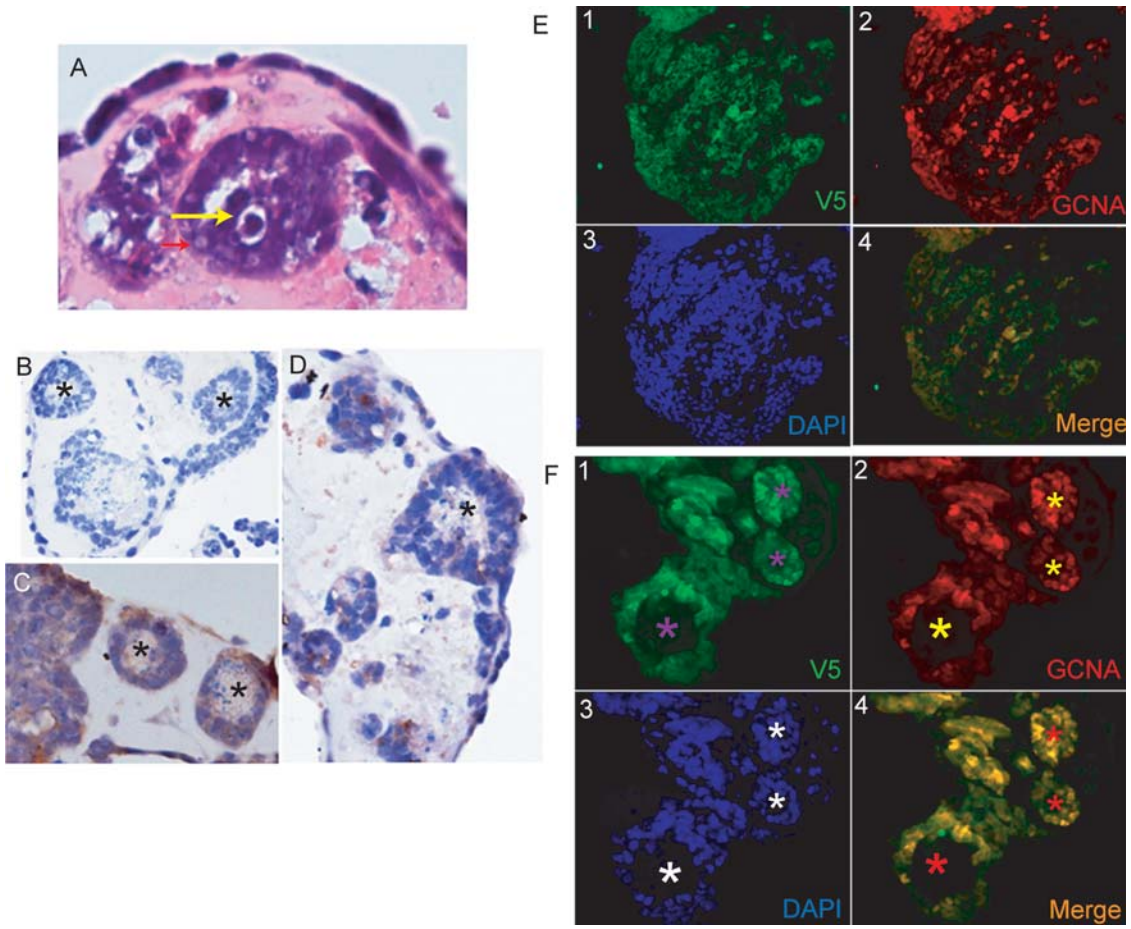

Figure 4 Formation of ovarian follicle- and seminiferous tubule-like structures from mES cells. mES cells transiently transfected with Dazl were cultured in DMEM/F12 containing 15% FBS for 1 week to obtain tissues for sections. (**A**) HE staining of sections shows a structure like the primary ovarian follicle containing one oocyte (yellow arrow) surrounded by small supportive cells (red arrow) produced from Dazl-transfected mES cells co-cultured with untransfected mES cells. (**B**–**D**) DAB staining of V5 (C) and GCNA (D) shows the seminiferous tubule-like structures (*) containing germ cells derived from Dazl-transfected mES cells co-cultured with untransfected mES cells, B is negative control for DAB staining. (**E**) Dual-immunofluorescence staining of V5 and GCNA shows that mES cell-derived V5 positive cells express germ cell marker GCNA, indicating that Dazl can efficiently promote mES cells to differentiate into germ cells *in vitro*. (**F**) Dual-immunofluorescence staining of V5 and GCNA shows multiple tubules (*) containing germ cells (GCNA positive cells) surrounded by supportive cells.

resembling testis cords at the early developmental stages (Figure 4B–D). We also performed dual-immunofluorescent staining of V5 and GCNA, and found that almost all V5-expressing cells were positive for GCNA (Figure 4E) and these GCNA positive cells formed seminiferous tubule-like structures (Figure 4F) in many sections.

Dazl plays a critical role in the regulation of the expression of Nanog and germ cell markers

To investigate the mechanisms through which Dazl induces germ cell differentiation from mES cells, we searched and compared the gene expression profiles between mES cells and germ cells in the literature (Imamura et al., 2006), and sorted out a number of key molecules expressed in mES cells but suppressed during germ differentiation. One of them is Nanog, which is required for self-renewal of mES cells but decreased during germ cell differentiation, and absent in Dazl-enriched germ cells (Mitsui et al., 2003; Hart et al., 2004; Hatano et al., 2005; Yamaguchi et al., 2005). To determine whether Nanog expression is affected at the early stage of germ cell induction by Dazl from mES cells, we carried out transient transfection of mES cells with plasmid plenti6Ubc-Dazl-V5 and cultured transfected cells in standard mES cell medium containing LIF. Dual-immunofluorescence analysis of V5 and Nanog indicated that Nanog was reduced in V5 positive cells after 24 h of culture under standard conditions in the presence of LIF (Figure 5A and B). Interestingly, we also found that GCNA was expressed in the Nanog-negative cells (Figure 5C and D) and co-expressed with V5 under the same culture conditions (Figure 5E–H). These data indicate that Nanog suppression might be an early event in Dazl-induced germ cell differentiation from mES cells. Furthermore, we carried out Western blotting using a cell pool from plenti6Ubc-Dazl-V5-infected mES cells (Figure 6A) and confirmed that Nanog expression was suppressed in Dazl-overexpressing mES cells, although GCNA was induced. Moreover, we also used siRNA approach to knock down Dazl (Figure 6B) to determine whether Dazl regulates other key germ cell-specific molecules and found that Stella, MVH and Prdm1 (Tanaka et al., 2000; Saitou et al., 2002; Ohinata et al., 2005; Vincent et al., 2005) were reduced in Dazl knockdown cells (Figure 6C). Apparently, Dazl is required for the expression of Stella, MVH and Prdm1. Additionally, we performed time course analyses of the expression of germ cell markers of different developmental stages, and found that Prdm1, Stra8 and Protamine1 were induced temporally, and the mRNA for haploid germ cell marker Protamine1 was induced by Dazl at day 3 after plenti6Ubc-Dazl-V5 infection (Figure 6D). Finally, we performed transient transfection of mES cells with plenti6Ubc-Dazl-V5 and carried out dual-immunofluorescence analyses of V5, by which Dazl is tagged, along with GCNA, or Stra8, or β-Tubulin. Consistently, GCNA, Stra8 and β-Tubulin, which mark primordial germ cells, premeiotic cells and postmeiotic cells, respectively, were activated temporally in V5 positive cells (Figure 7).

Discussion

ES cells have been shown to be ideal materials for study of the early molecular regulations underlying the derivation of specific tissues as well as for understanding of the genetic and molecular mechanisms of disease development (Lerou and Daley, 2005). Our current study provides a novel approach to differentiating mES cells into sperm and oocytes *in vitro*. Significantly, motile sperm can be produced from mES cells through our approach. Therefore, this might provide an ideal *in vitro* system for studying the molecular mechanisms underlying germ line development.

Although a number of key germ cell-specific genes have been identified, the mechanisms and networks involved in the regulation of germ cell specification and development remain largely unknown. Dazl is an RNA-binding protein and involved in the post-translational regulation of gene expression in germ cells. Previous studies demonstrated that Dazl plays critical roles in germ cell development and differentiation (Ruggiu et al., 1997; Lin and Page, 2005; Lin et al., 2008). Thus, Dazl might function as a master gene during germ cell specification and differentiation. In our study, we used Dazl to induce germ cells from mES cells and have demonstrated that ectopic expression of Dazl induces efficient germ cell differentiation from mES cells. Furthermore, under standard mES cell culture conditions, siRNA knockdown of Dazl in mES cells led to reduction in the expression of Stella, MVH and Prdm1 (Tanaka et al., 2000; Saitou et al., 2002; Ohinata et al., 2005; Vincent et al., 2005). Importantly, our data also indicated that ectopic Dazl expression led to Nanog suppression but GCNA induction in mES cells. Nanog is required for the maintenance of pluripotency of mES cells; however, Nanog is down-regulated during germ cell development as evidenced by the fact that Nanog mRNA was not detected at later stages including neonatal testis, mature ovaries and testes (Yamaguchi et al., 2005). Thus, it is indicated that Nanog is progressively silenced during germ cell differentiation (Mitsui et al., 2003; Hart et al., 2004; Hatano et al., 2005; Yamaguchi et al., 2005). Our data suggest that Nanog is suppressed during the germ cell differentiation induced by Dazl from mES cells. GCNA is a germ cell-specific marker and expressed in germ cells from the early developing gonads to the adult ovary and testis (Enders, 1994). Our data suggest that GCNA might be a downstream mediator of Dazl in the regulation of germ cell development. To probe the dynamics of the Dazl-induced germ cell differentiation from mES cells, we carried out time course analyses of the expression of genes marking different developmental stages of germ cells such as Prdm1 (Ohinata et al., 2005; Vincent et al., 2005), Stra8 (Oulad-Abdelghani et al., 1996; Koubova et al., 2006; Anderson et al., 2008) and Protamine1 (Caldwell and Handel, 1991). We found that these genes were temporally activated in the Dazl-transfected mES cells. This dynamic germ cell differentiation triggered by ectopic expression of Dazl was further examined by dual-immunofluorescence analyses showing the temporal expression of GCNA, Stra8 and Tubulin.

Collectively, our study provides further support that Dazl is a master gene in mediating germ cell development and

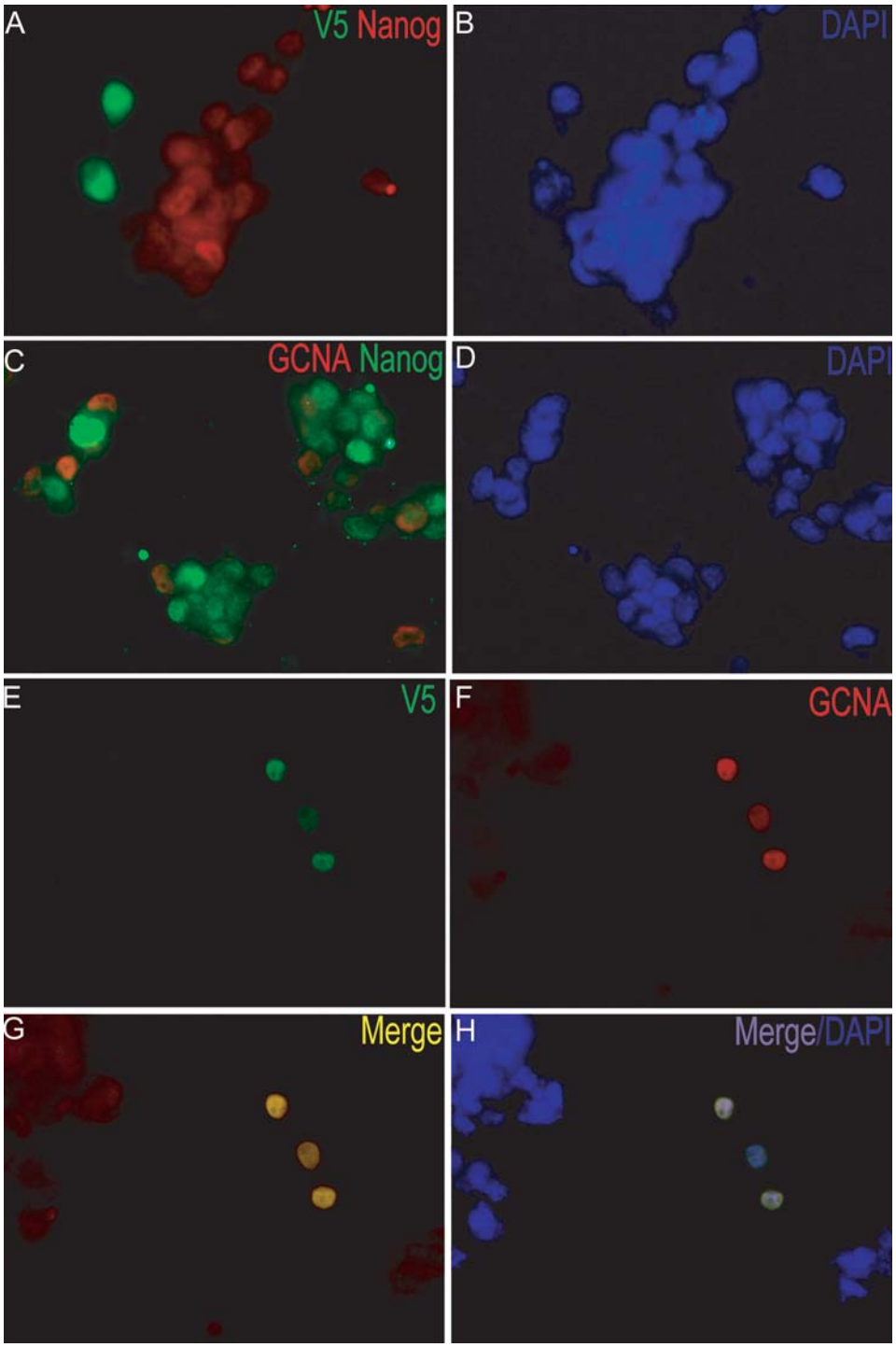

Figure 5 Suppressed Nanog expression and induced GCNA expression in Dazl-transfected mES cells. Mouse ES cells were transiently transfected with plenti6Ubc-Dazl-V5, by which Dazl is expressed as a fusion protein with the V5 tag, and cultured under standard ES medium with LIF for 24 h. (**A**, **B**) Dual-immunofluorescence staining of V5 and Nanog shows that Nanog expression was suppressed in Dazl-overexpressing mES cells. (**C** and **D**) Dual-immunofluorescence staining of GCNA and Nanog. (**E**–**H**) Dual-immunofluorescence staining of V5 and GCNA, G is the overlap of E and F, H is the overlap of V5, GCNA and DAPI; this result indicates that the expression of GCNA was induced by Dazl.

differentiation. Significantly, ectopic expression of Dazl directly drives germ cell differentiation of mES cells independent of EB formation, which was shown to be required for germ cell derivation from ES cells in the previous studies (Hübner et al., 2003; Toyooka et al., 2003; Clark et al., 2004; Geijsen et al., 2004). Thus, our approach should provide a novel system for further dissecting the intrinsic mechanisms underlying germ cell differentiation from ES cells.

Materials and methods

Cultivation and transfection of mES cells and knockdown of Dazl transcript by siRNA

A 129/SV mES cell line was cultured on MEF feeders in standard DMEM medium containing 15% FBS (Invitrogen, Carlsbad, CA 92008, USA) and 1000 units/ml of LIF (Chemicon, Temecula, CA 92590, USA). The Dazl cDNA was amplified and cloned into pMSCVneo (Clontech Laboratories, Inc. Mountain View, CA 94043, USA) in that Dazl expression is driven by a MSCV promoter. Mouse ES cells were transfected with the plasmid pMSCVneo-Dazl using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA 92008, USA). After 2 days of culture, the transfected mES cells were reseeded and selected with 100 μg/ml G418 (Clontech Laboratories, Inc. Mountain View, CA 94043, USA) for 2 weeks in standard mES cell culture medium with LIF. Then, the selected cell colonies were cultured in DMEM/F12 medium (Invitrogen, Carlsbad, CA 92008, USA) containing 15% FBS but in the absence both of LIF and feeder cells for the generation of sperm and oocytes. For generation of oocytes, the colonies of transfected mES cells were first cultured in non-coated Petri dishes for 3 days then were transferred to coated dishes to continue the culture. For *in vitro* cytoplasm sperm injection, Dazl-transfected mES cells were further transfect by a plasmid expressing EGFP under EF-1α promoter. Green colonies were isolated to continually produce spermatids *in vitro* using the same procedure as above. To knock down Dazl, the following siRNAs targets were applied: for siRNA1, 5′-GAGAGCAGAGGAGTTAT GTTA-3′, for siRNA2, 5′-GTGGATGTGCAGAAGATAGTA-3′.

Preparation of lentivirus

Lentiviral medium was prepared using the combination of plenty-UBC-Dazl-V5, PLP1, PLP2 and PLP-VSVG. Briefly, 293FT cells were used as the packaging cells with 50–60% confluent in culture, then refreshed with half of an amount of fresh medium and transfected with 30–40 mg of the combined plasmid DNAs (per transfection of a 10 cm dish) using the standard $CaCl_2$ method 4 h later. The transfected cells were incubated overnight and fresh medium was added the next day. Lentiviral medium was collected every 24 h after change of medium from day 1 to day 2 after transfection (Store at 4°C for up to 72 h until spin). One day before infection, 40 000/ml of mES cells were plated in a 12-well-plate. The same volume of viral supernatant was added into one well of target cell to a final volume of 2 ml as well as 1× polybrane. After 12 h of infection, the media containing virus was replace with 1 ml of fresh medium and continue the culture for planned experiments.

Immunohistochemistry and immunofluorescence microscopy

Cell aggregates were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at a thickness of 5 μm. For DAB staining, the slides were stained with antibodies against V5 (Invitrogen) and GCNA (Rat IgG, kindly provided by George C. Enders, The University of Kansas) using a standard avidin–biotin complex method. Briefly, slides were deparaffinized with xylene, hydrated through a graded alcohol series, and then rehydrated in distilled water. Endogenous peroxidase activity was blocked by placing the slides in 0.5% hydrogen peroxidase for 10 min followed by

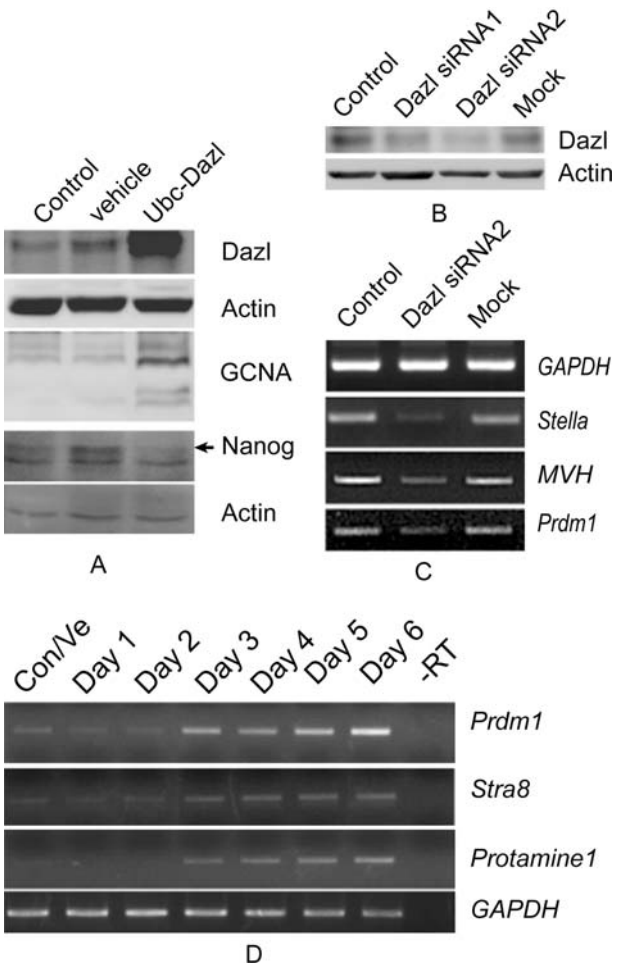

Figure 6 Dazl regulates the expression of germ cell specific genes. (**A**) Mouse ES cells were infected by plenti6Ubc-Dazl-V5 lentivirus medium for the overexpression of Dazl. Western blotting analyses shows that Dazl was overexpressed and that overexpression of Dazl induced the expression of GCNA (top panel, control was of mES cells without transfection), whereas the expression of Nanog was suppressed by Dazl (bottom panel). (**B**) Western blotting analysis shows that Dazl was knocked down by siRNA in mES cells; Dazl siRNA2 was more efficient than Dazl siRNA1, thus, the cells transfected with Dazl siRNA2 were used for further experiments. (**C**) RT–PCR analyses were performed to test the expression of early germ cell markers including Stella, MVH and Prdm1, in the Dazl knockdown mES cells. (**D**) RT–PCR analyses show that germ cell markers including Prdm1 for PGCs, Stra8 for premeiotic germ cells and Protamine1 for postmeiotic cells, were activated temporally in Dazl-transfected mES cells cultured under DMEM/F12 medium containing 15% FBS without LIF. (Con/Ve is for mixed total RNAs collected from non-infected cells and vector-infected cells cultured over the same period of culture time as did for pLenti-UBC-Dazl-V5 infected cells, Day 1 is for 1 day after infection, Day 2 is for 2 days after infection and so on).

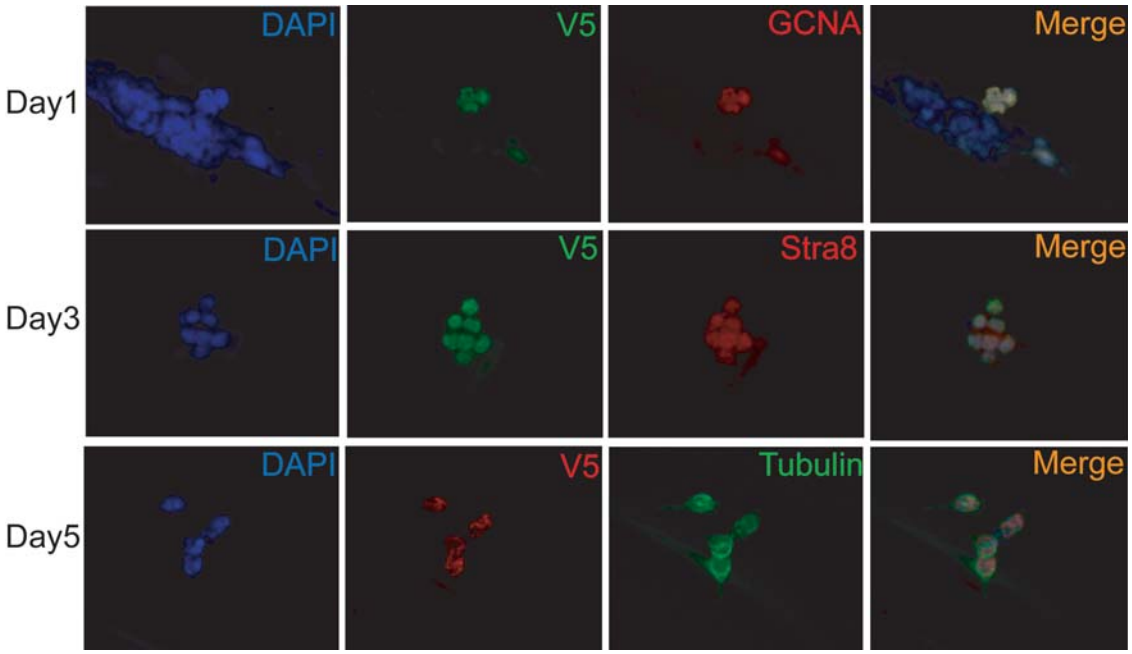

Figure 7 Temporal expression of germ cell markers of different developmental stages in Dazl-transfected mES cells. Mouse ES cells were cultured in standard mES culture medium with LIF and transfected by plenti6Ubc-Dazl-V5. The transfected cells were then cultured in DMEM/F12 medium containing 15% FBS without LIF for 1 day, or 3 days, or 5 days, and cells at different time points were fixed for dual-immunofluorescence analyses of V5, by which Dazl is tagged, along with GCNA, or Stra8, or Tubulin.

a tap water rinse. Background staining was reduced by incubating slides in 1% bovine serum albumin (BSA)/Tris-buffered saline. Antigen was retrieved by placing the slides in a pressure cooker with an antigen unmasking solution (0.01 M citrate buffer, pH 6.0) for 15 min. Slides were subsequently incubated with the primary antibodies (4°C overnight), then with biotinylated secondary antibodies and streptavidin–biotin peroxidase. DAB was used as chromogen and sections were counterstained with hematoxylin. For immunofluorescent staining of V5 and GCNA, the slides were incubated with antibodies against V5 and GCNA. The primary antibodies were revealed with Alexa-488 and Cy3 conjugated secondary antibodies together with hoechst to stain nuclei. The sections were mounted and viewed under a microscopy. For immunofluorescence with cells, the following antibodies were used: FE-J1 antibody (The Developmental Studies Hybridoma Bank, University of Iowa); acetyl-α-tubulin antibody (Sigma-Aldrich Corp, St. Louis, MO, USA); Anti-CDF-9 antibody (R&D Systems Inc., Minneapolis, MN 55413, USA); Oct4 antibody (c-10 monoclonal ab from Santa Cruz Biotech, CA, USA); V5 antibody (Invitrogen); Nanog antibody (R&D Systems); GCNA antibody (Rat IgG, kindly provided by George C. Enders, The University of Kansas); Stra8 antibody (Abcam, Cambridge, MA, USA); β-Tubulin (Sigma, Clone Tub2.1). Cells were fixed with 2% paraformaldehyde in PBS for 20 min followed by permeabilization with 0.3% Triton X-100 in PBS. After blocking in 10% goat serum containing 3% BSA (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA 19390, USA) and 0.05% Triton X-100 for 1 h at room temperature, the primary antibody was diluted in blocking buffer, added to the cells and incubated for 1 h at room temperature. Then, the cells were washed three times with PBS containing 0.1% NP-40 (15 min each time). Cells were incubated with Texas Red- or fluorescein-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA 19390, USA) diluted in blocking buffer for 1 h. Cells were washed three times as above and stained with DAPI in PBS for 15 min. Cells were washed three times as above and one more time with distilled water. Slides were mounted in Vectashield (Vector Laboratories) and examined under a fluorescence microscope.

Western blotting

The proteins were extracted from cells using the lysis buffer [20 mM Tris–HCl (pH 7.5), 500 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate and 1 mM Na_3VO_4] containing protease inhibitors (2 μg/ml leupeptin and 2 mM phenylmethylsulfonyl fluoride). Cell extract samples containing 30 μg of proteins were subjected to 10% SDS-PAGE followed by being electrophoretically transferred to polyvinylidene difluoride membranes. The filter was probed with the Dazl antibody (1:500 dilutions, the Developmental Studies Hybridoma Bank, University of Iowa), V5 antibody (Invitrogen) or Nanog antibody (R&D Systems). Appropriate secondary antibodies were used and the antibody-antigen complexes in the membranes were visualized using an enhanced-chemiluminescent detection kit (Millipore).

Fluorescence *in situ* hybridization

Dazl-induced cells were pretreated with 2.2% sodium citrate solution at 37°C for 5 min. Then they were placed on pre-cleaned glass slides and dried in air overnight. The slides were fixed with 3:1 of methanol: acetic acid, air dried and incubated in

10 mM dithiothreitol for 30 min on ice. The slides were washed with distilled water and then were completely air-dried. The slides were denatured in 2× SSC (pH 7.0) containing 70% formamide for 10 min at 78°C, and then dehydrated in an ice cold ethanol series (70, 85, 90 and 100%) for 2 min each. Slides were again air dried prior to hybridization. Centromeric probe for chromosome 8 was purchased from Vysis (Vysis, Dowers Grove, IL, USA). Hybridizations were performed using the standard procedure. The probe mixture contained 30 μl of mouse Cot-1 DNA (Invitrogen, Carlsbad, CA 92008, USA), 2 μl of herring sperm (Invitrogen, Carlsbad, CA 92008, USA), and 4 μl of centromeric probe for chromosome 8. After ethanol precipitation, the DNA was resuspended in 7 μl of CEP hybridization buffer (Vysis, Dowers Grove, IL, USA) and 3 μl of distilled water. The probe mixture was denatured at 78°C for 10 min and pre-annealed for 30 min at 37°C. The hybridization was carried out at 37°C for 48 h. The slides were then washed three times in 2× SSC (pH 7.0) containing 50% formamide, then once for 5 min at 45°C in 2× SSC and PN buffer (0.1 M NaH_2PO_4/0.1 M Na_2HPO_4, pH 8.0, 0.1% Nonidet P-40), respectively. The slides were washed finally in PN buffer for 5 min at room temperature. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted with 0.1 μg/ml mounting medium (Vector Laboratories, Burlinggame, CA, USA).

RT–PCR

RT–PCR analyses were carried out using the following primers: ZP1, 5′-GAGTGACTGTGTTGCCATAG-3′ and 5′-GCCACACTGGTCTC ACTACG-3′; ZP2, 5′-GCTACACACATGACTCTCAC-3′ and 5′-GGTGAC TCACAGTGGCACTC-3′; ZP3, 5′-TTGAGCAGAAGCAGTCCAGC-3′ and 5′-CGGTTGCCTTGTGGATGGTC-3′; Stella, 5′-GACGCTTTGGATGATAC AGACG-3′ and 5′-GGTCTTTCAGCACCGACAACA-3′; GAPDH, 5′-TCAC TGCCACCCAGAAGA-3′ and 5′-AAGTCGCAGGAGACAACC-3′; Stra8, 5′-CTGAGTGACTGACTCGTCA-3′ and 5′-GTCCTATTCAGTACCTGCCAC -3′; MVH, 5′-ATGATGCGGGATGGAATAACT-3′ and 5′-ACTTGCCCA ACAGCGACAAAC-3′; Prdm1, 5′-GAGGAGTTCCACCACTTCATTGAT GGC-3′ and 5′-TCACTGTGAGCTCTCCAGGATAAGGGT-3′; Prdm14, 5′-AGCAGGCTGTCTGAGTCCTCTGAAGAGT-3′ and 5′-GAGTGCTGTCT GATGTGTGTTCGGAGT-3′; Protamine1, 5′-TTCCACCTGCT CACAGGTTG-3′ and 5′-CGAGATGCTCTTGAAGTCTGG-3′. Reaction conditions: for ZP1, ZP2, ZP3, 94°C for 2 min, then 94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec, 28 cycles, then 72°C for 10 min; for MVH, 94°C for 2 min, then 94°C for 30 sec, 53°C for 30 sec, 72°C for 45 sec, 28 cycles, then 72°C for 10 min; for Prdm1and Prdm14, 94°C for 2 min, then 94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec, 28 cycles, then 72°C for 10 min; for time course analysis, Prdm1, 94°C for 2 min, then 94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec, 25 cycles, then 72°C for 10 min, Stra8, 4°C for 2 min, then 94°C for 30 sec, 56°C for 30 sec, 72°C for 45 sec, 25 cycles, then 72°C for 10 min, protamine1, 4°C for 2 min, then 94°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec, 25 cycles, then 72°C for 10 min.

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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