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Continuing primordial germ cell differentiation in the mouse embryo is a cell-intrinsic program sensitive to DNA methylation

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Abstract

The initial cohort of mammalian gametes is established by the proliferation of primordial germ cells in the early embryo. Primordial germ cells first appear in extraembyronic tissues and subsequently migrate to the developing gonad. Soon after they arrive in the gonad, the germ cells cease dividing and undertake sexually dimorphic patterns of development. Male germ cells arrest mitotically, while female germ cells directly enter meiotic prophase I. These sex-specific differentiation events are imposed upon a group of sex-common differentiation events that are shared by XX and XY germ cells. We have studied the appearance of GCNA1, a postmigratory sex-common germ cell marker, in cultures of premigratory germ cells to investigate how this differentiation program is regulated. Cultures in which proliferation was either inhibited or stimulated displayed a similar extent of differentiation as controls, suggesting that some differentiation events are the result of a cell-intrinsic program and are independent of cell proliferation. We also found that GCNA1 expression was accelerated by agents which promote DNA demethylation or histone acetylation. These results suggest that genomic demethylation of proliferative phase primordial germ cells is a mechanism by which germ cell maturation is coordinated.

Keywords: Primordial germ cells; Gonocytes; GCNA1

Introduction

In mammalian embryos germ cell development is dependent on a complex array of cell-extrinsic and cell-intrinsic signals. In the mouse embryo, initial primordial germ cell (PGC) differentiation occurs at about 7.2 days post coitus (dpc) in the extraembryonic mesoderm (Ginsburg et al., 1990) and requires several members of the BMP ligand family (Fujiwara et al., 2001; Lawson et al., 1999; Ying et al., 2000; Ying and Zhao, 2001). By 8.5 dpc, the PGCs have entered the embryo proper and soon begin migrating along the hindgut endoderm toward the genital ridge. Most PGCs arrive at the genital ridge on the 10th day of embryonic development. During this period the PGCs are proliferating rapidly such that the initial founder population of about 40 PGCs gives rise to about 25,000 germ cells when proliferation ceases at 13.5 dpc (Tam and Snow, 1981). By 13.5 dpc, the germ cells are also undertaking sexually dimorphic patterns of development. Male germ cells enter a mitotic arrest that will be maintained until after birth. In contrast, female germ cells directly enter meiotic prophase. Progression through meiotic prophase is protracted, but most germ cells will have reached diplotene near the time of birth (McLaren, 1984).

The process of sexual differentiation of germ cells coincides with a constellation of differentiation events that occur in both XX and XY PGCs. These sex-common differentiation events include alterations in cell adhesion properties (De Felici et al., 1992; Donovan et al., 1986; ffrench Constant et al., 1991; Garcia-Castro et al., 1997), induction of cell death programs (Coucouvanis et al., 1993; Rucker et al., 2000), similarly timed cessation of proliferation (McLaren, 1984), and loss of ability to form pluripotent stem cells (Labosky et al., 1994; Matsui et al., 1992). These differentiation events are reflected in decreases in the level of expression of several marker gene products including

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stage-specific embryonic antigen 1 (SSEA1) (Cooke et al., 1993), tissue nonspecific alkaline phosphatase (TNAP) (Cooke et al., 1993; Hahnel et al., 1990), c-kit (Coucouvanis and Jones, 1993; Manova and Bachvarova, 1991), pem (Pitman et al., 1998), and Blimp-1 (Chang and Calame, 2002). Other markers, such as c-myc (Coucouvanis and Jones, 1993), E-cadherin (Bendel-Stenzel et al., 2000), c-mos (Coucouvanis et al., 1993), SCP3 (Di Carlo et al., 2000), mvh (Fujiwara et al., 1994), mili (Kuramochi-Miyagawa et al., 2001), and mage-b4 (Osterlund et al., 2000), show increased gene expression in both XX and XY germ cells between 10.5 and 14.5 dpc.

We have been interested in the regulation of germ cell differentiation as PGCs colonize the gonad. Cessation of germ cell proliferation has been suggested to result from a cell-intrinsic program (Ohkubo et al., 1996). To study germ cell differentiation, we have used the anonymous germ cell antigen GCNA1. GCNA1 is first detectable in germ cells of either sex as they enter the gonad at 11.5 dpc (Enders and May, 1994). We previously found that premigratory 8.5-dpc PGCs explanted into culture initiate expression of GCNA1 with timing similar to that observed in vivo (Richards et al., 1999b). We now present evidence that germ cell differentiation is independent of proliferative status, and thus unlikely to be controlled by the number of preceding mitotic events. This result further suggests that germ cell development is not dependent on dilution of a differentiation inhibitor present in premigratory cells. Instead, our data support the interpretation that the ability to undergo differentiation is an intrinsic property of premigratory cells.

PGCs play a critical role in genomic reprogramming and the phenomenon of genetic imprinting. By 11.5 dpc, some imprinted genes are biallelically expressed in fetal germ cells, indicating that some inherited imprints have been erased in the germline by this time (Szabo and Mann, 1995). More recently, investigators using PGC nuclear transplantation, as well as direct analysis of PGC gene expression, have found evidence that some imprints may be active in 8.5-dpc PGCs, but are erased between 10.5 and 12.5 dpc as germ cells colonize the gonad (Lee et al., 2002; Szabo et al., 2002). Furthermore, the germ cell genome is hypomethylated at 12.5 dpc, consistent with the notion that methylcytosine is essential for marking imprinted genes (Davis et al., 2000; Hajkova et al., 2002; Kafri et al., 1992; Lee et al., 2002; Monk et al., 1987; Ueda et al., 2000).

We have also explored the role of DNA methylation in germ cell development. We present evidence that DNA demethylation is a rate limiting step in the expression of GCNA1. These results suggest that demethylation of the germ cell genome serves not only to erase imprints during genetic reprogramming, but also plays an important role in regulation of germ cell differentiation.

Materials and methods

Germ cell isolation and culture

All experiments were performed on timed matings of B6C3F1 mice obtained from Jackson Laboratories (Bar Harbor, ME). Noon of the day on which a mating plug was first visible was taken to be 0.5 dpc. Primordial germ cells were recovered and cultured in 96-cell wells on irradiated Sl/Sl4 m220 feeder layers (Toksoz et al., 1992) in QBSF-58 (Quality Biological, Gaithersburg, MD) medium supplemented with 100 U penicillin, 50 μg streptomycin, 2 μmole glutamine, and 1000 U LIF (ESGRO, Chemicon, Temecula, CA) per ml as previously described (Richards et al., 1999a). In some experiments, fetal bovine serum (Hyclone, Logan, UT) was added to 0.5% to stabilize feeder layers. PGCs from 8.5-dpc embryos were plated at 0.7–0.9 embryo equivalents per well. PGCs at 10.5 and 11.5 dpc were plated at 0.2–0.4 equivalents per well.

Primordial germ cells were immunomagnetically purified by using the TG-1 antibody as described (Pesce and De Felici, 1995). Purified preparations were regularly greater than 80% PGCs as judged by alkaline phosphatase staining.

Phorbol 12-myristate 13-acetate (TPA), all-trans-retinoic acid (retinoic acid, RA), 5-azacytidine, and trichostatin A (TSA) were purchased from Sigma (St. Louis, MO).

Immunological and cytochemical methods

The number of cells expressing alkaline phosphatase and GCNA1 was sequentially determined as previously described (Richards et al., 1999a). Each data point represents the mean and standard deviation of at least five wells. Each experiment has been repeated to confirm reproducibility, and a representative result is shown. We frequently observed that regardless of culture conditions purified PGCs exhibited a number of small GCNA1-positive nuclear fragments which may be representative of apoptotic cells. Only full, intact nuclei were scored.

5-Bromo-2′-deoxyuridine (BrdU) labeling was performed by using a 1:100 dilution of labeling reagent, and detected after fixation in 70% ethanol with a BrdU detection kit as suggested by the supplier (Zymed, San Francisco, CA).

Results

Differentiation of PGCs toward gonocytes is a cell-intrinsic program independent of the rate of cell division

We have previously shown that premigratory PGCs obtained from 8.5-dpc embryos will differentiate in culture to express the postmigratory germ cell antigen GCNA1 (Richards et al., 1999b). In cultures of 8.5-dpc PGCs, the rate of appearance of GCNA1-positive cells increased after 2–3

days of culture, suggesting that differentiation in vitro occurs on schedule similar to that observed in vivo. These observations, plus the observation that germ cells express GCNA1 in Ftz-1 null embryos which are deficient in genital ridge development, suggest that gonocyte differentiation is a cell-intrinsic program (Wang et al., 1997).

To better understand the relationship between PGC proliferation and differentiation, we investigated PGC differentiation in cultures in which proliferation was either inhibited or stimulated. While several agents are known to promote PGC proliferation, few proliferation inhibitors have been identified. In an initial screen of several putative agents, we found that the phorbol ester TPA strongly reduced the accumulation of PGCs in culture. Fig. 1 shows that incorporation of BrdU into cultured PGCs is reduced in the presence of TPA, indicating that the observed reduction in PGC numbers in the presence of TPA is at least partly due to an inhibition of PGC cell division.

Next, we investigated the extent of continuing germ cell differentiation in cultures containing agents that alter PGC proliferation in order to better understand the regulation of this program. In these experiments, premigratory PGCs from 8.5-dpc embryos were allowed to adhere to feeder layers overnight, cultured an additional 3 days in either control media or media containing agents which alter PGC proliferation, and the extent of GCNA1-expressing cells determined. We chose 4 days of culture as an end point for these experiments. By the 5th day of culture, total numbers of PGCs reliably start to decline, a process that could complicate an analysis of the extent of differentiation.

In the first of these experiments, PGCs were cultured in the presence of TPA. In control cultures, the number of PGCs increased over fourfold between 1 and 4 days, while the increase was less than twofold in cultures containing TPA. The number of GCNA1-expressing cells at day 4 in control cultures was about one-third of the total, similar to our previous findings (Richards et al., 1999b). In TPA-treated cultures, the number of GCNA1-expressing cells was also reduced, so that the ratio of GCNA1-expressing cells was also about one-third of the total (Table 1).

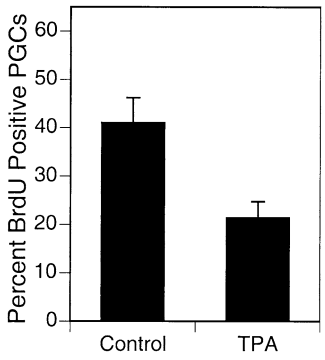

Fig. 1. TPA inhibits PGC proliferation. PGCs from 8.5-dpc embryos were seeded onto feeder layers in control media, or media containing 100 ng TPA per ml. Forty-eight hours later, the cultures were refed control media supplemented with BrdU labeling mixture for 1 h. The percentage and s.d. of PGCs which incorporated BrdU are shown. The values were significantly different by Student's t test at $P < 0.01$.

Table 1
Gonocyte differentiation in culture

Sample	Alkaline phosphatase	GCNA1	Ratio	P value
Control day 1	56.4 ± 5.7	6.8 ± 4.5	0.10 ± 0.07	
Control day 4	243 ± 16.3	74.4 ± 22.0	0.31 ± 0.10	0.60
TPA day 4	93.4 ± 20.5	32.2 ± 9.3	0.34 ± 0.17	

Note. PGCs from 8.5-dpc embryos were seeded onto feeder layers. The following morning cultures were refed either control media or media containing 100 ng TPA per ml. The mean and s.d. of alkaline phosphatase, GCNA1, and ratio of GCNA1-positive to alkaline phosphatase-expressing cells present in five-cell wells were determined after 1 or 4 days of culture.

To further explore the relationship between PGC proliferation and differentiation, we investigated the appearance of GCNA1-expressing cells in cultures containing the potent PGC mitogen retinoic acid (Koshimizu et al., 1995). Fig. 2 shows that retinoic acid treatment led to an expected increased proliferation of PGCs obtained from 8.5-dpc embryos. The rate of appearance of GCNA1-expressing cells also increased, so that the rate of appearance of positive cells remained proportional to the total germ cell population. As previously observed, the rate of appearance of GCNA1-expressing cells increased after a 2- to 3-day delay (Richards et al., 1999b). This delay was preserved in the presence of retinoic acid, suggesting that the mechanism controlling differentiation operates independently of proliferation.

A similar experiment was performed on PGCs obtained from 11.5-dpc embryos, a time at which many germ cells have colonized the gonad but are still motile (Donovan et al., 1986; Molyneaux et al., 2001). At this time, the fraction of GCNA1-expressing PGCs was initially higher, and there was no delay in the appearance of GCNA1-expressing cells (Fig. 3). As observed with premigratory cells, the ratio of GCNA1-expressing cells remained proportional between control and retinoic acid-treated cultures. Together, these experiments indicate that differentiation to express GCNA1 is a cell-intrinsic program rather than inductive, and is independent of the rate of cell proliferation.

We have found similar results with a number of agents that affect PGC proliferation including forskolin, cholera toxin, transforming growth factor β, and urogenital ridge-conditioned media. Each of these agents altered the accumulation of germ cells in culture, but in no case did we observe a consistent change in the ratio of GCNA1-expressing cells to the total (data not shown).

PGC differentiation is sensitive to DNA methylation status

To better understand the nature of the putative cell-intrinsic program controlling PGC differentiation, we cul-

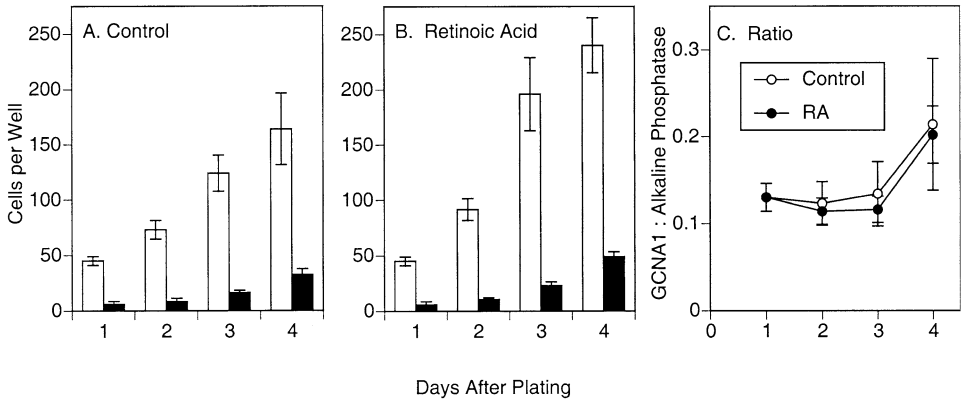

Fig. 2. Differentiation of 8.5-dpc PGCs in the presence of retinoic acid. PGCs from 8.5-dpc embryos were seeded onto feeder layers and the following morning refed either control media (A) or media containing 1 μM retinoic acid (RA) (B). Open bars: Mean and s.d of alkaline phosphatase-positive cells per well. Closed bars: Mean and s.d. of GCNA1-positive cells per well. (C) The mean ratio and s.d of the number of GCNA1 and alkaline phosphatase-positive cells in each cell well.

tured premigratory PGC in several agents that might be anticipated to alter cellular programs of development. Some agents such as aphidocolin and cytochalasin were highly toxic to PGCs (data not shown). However, we did find that 5-azacytidine, which promotes DNA demethylation (Juttermann et al., 1994), could increase both the rate and extent of GCNA1 expression in cultures of both 8.5-dpc premigratory (Fig. 4) and 10.5-dpc postmigratory PGCs (Fig. 5).

In 5-azacytidine-treated cultures, we frequently observed that the number of GCNA1-expressing cells exceeded the number of alkaline phosphatase-expressing germ cells. Several explanations for this observation seemed feasible, including the possibility that 5-azacytidine treatment caused somatic cells present in the assay to express GCNA1. GCNA1 is not detectable in control wells of feeder cells treated with 5-azacytidine, indicating that the feeder layer does not contribute to the GCNA1-positive population (data not shown). We investigated the possibility that embryonic somatic cells express GCNA1 by plating immunopurified 11.5-dpc PGCs into 5-azacytidine-containing medium. Fig. 6 shows that in these cultures of highly purified germ cells treated with 5-azacytidine, the number of GCNA1-positive cells again exceeded the number of alkaline phosphatase-expressing cells. Although this experiment does not exclude the possibility that somatic cells express GCNA1 in the presence of 5-azacytidine, it does demonstrate that under these conditions PGCs increase expression of the GCNA1 marker.

DNA methylation is often associated with histone deacetylation and subsequent transcriptional repression (Jones et al., 1998; Nan et al., 1998). To investigate the role of chromatin structure in germ cell differentiation, PGCs were cultured in the presence of trichostatin A, an inhibitor of histone deacetylases (Yoshida et al., 1990). Fig. 7 shows that trichostatin A significantly increased the appearance of GCNA1-expressing cells. Together, these experiments indicate that DNA demethylation and chromatin remodeling are rate limiting for continuing germ cell differentiation.

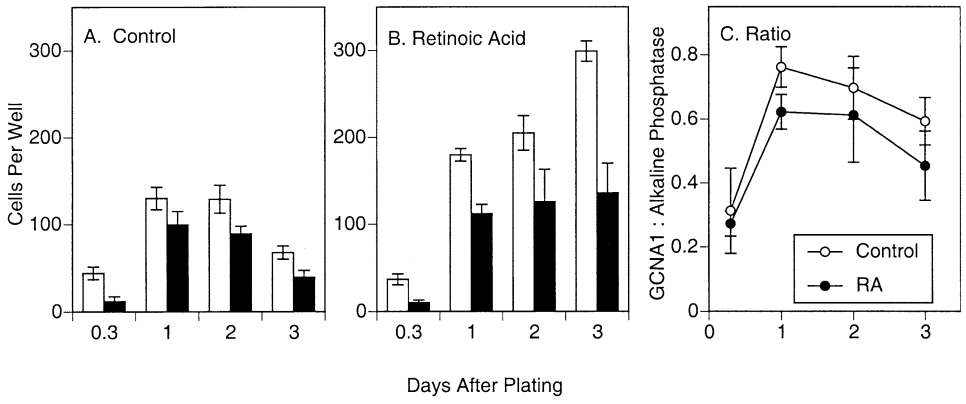

Fig. 3. Differentiation of 11.5-dpc PGCs in the presence of retinoic acid. PGCs from 11.5-dpc embryos were seeded onto feeder layers in either control media (A) or media containing 1 μM retinoic acid (RA) (B). Open bars: Mean and s.d of alkaline phosphatase-positive cells per well. Closed bars: Mean and s.d. of GCNA1-positive cells per well. (C) The mean ratio and s.d of the number of GCNA1 and alkaline phosphatase-positive cells in each cell well.

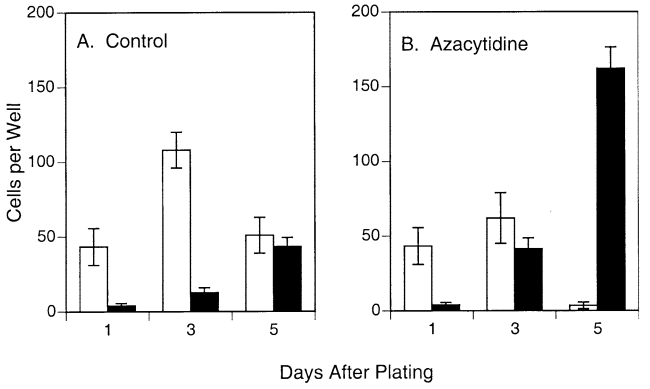

Fig. 4. Differentiation of 8.5-dpc PGCs in the presence of 5-azacytidine. PGCs from 8.5-dpc embryos were seeded onto feeder layers and the following morning refed either control media (A) or media containing 10 μM 5-azacytidine (B). Open bars: Mean and s.d of alkaline phosphatase-positive cells per well. Closed bars: Mean and s.d. of GCNA1-positive cells per well.

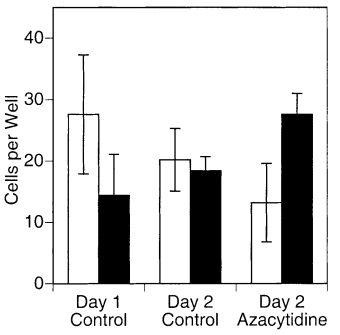

Fig. 6. Differentiation of purified PGCs in the presence of 5-azacytidine. PGCs from 11.5-dpc embryos were immunomagnetically purified and seeded onto feeder layers in either control media or media containing 10 μM 5-azacytidine. Open bars: Mean and s.d of alkaline phosphatase-positive cells per well. Closed bars: Mean and s.d. of GCNA1-positive cells per well.

Discussion

We have used the postmigratory germ cell marker GCNA1 to explore the regulation of primordial germ cell proliferation and differentiation toward gonocytes. First, our results suggest that continuing germ cell differentiation in the period following colonization of the gonad is the result of a cell-intrinsic program already present in 8.5-dpc premigratory germ cells, rather than an inductive program subject to regulatory influences of the fetal gonad. This interpretation is consistent with the observation that ectopic germ cells in 14.5-dpc embryos express GCNA1 irrespective of their sex and in the absence of a well-differentiated gonad (Wang et al., 1997). Second, we have shown that cessation of germ cell proliferation and differentiation temporally overlap, but are not linked. Cultures in which PGC proliferation is either strongly inhibited or stimulated exhibit a similar level of differentiation as control cultures. These observations run counter to a model of gonocyte development in which differentiation occurs only after a fixed number of cell divisions. These results also suggest that premigratory germ cells are not endowed with a differentiation inhibitor that must be reduced below a threshold level prior to differentiation. If PGCs harbored this type of putative inhibitor, then differentiation should be more extensive in the presence of a mitogen as the inhibitor would be more rapidly diluted. Conversely, cultures in which proliferation is inhibited would be expected to differentiate less extensively, as the inhibitor would remain at a high level. Instead, the number of GCNA1-expressing cells is proportional between control cultures and cultures treated with agents that alter PGC proliferation. Third, we have found that agents which promote DNA demethylation or histone acetylation advance the program of germ cell differentiation.

In the presence of 5-azacytidine or trichostatin A, the number of GCNA1-expressing cells can exceed the number of alkaline phosphatase-expressing cells. Several explanations for this observation seem possible. First, while Fig. 6

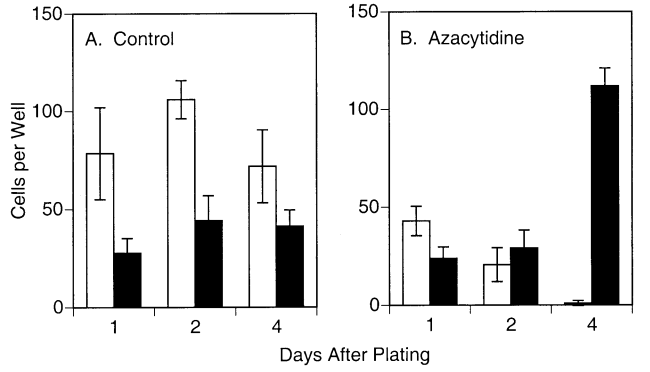

Fig. 5. Differentiation of 10.5-dpc PGCs in the presence of 5-azacytidine. PGCs from 10.5-dpc embryos were seeded onto feeder layers in either control media (A) or media containing 10 μM 5-azacytidine (B). Open bars: Mean and s.d of alkaline phosphatase-positive cells per well. Closed bars: Mean and s.d. of GCNA1-positive cells per well.

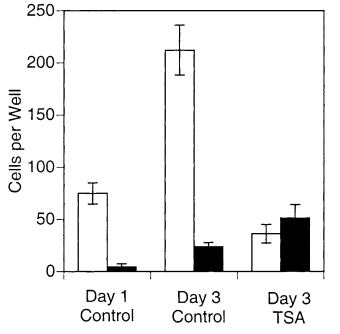

Fig. 7. Differentiation of purified PGCs in the presence of trichostatin A. PGCs from 8.5-dpc embryos were seeded onto feeder layers and the following morning refed either control media (A) or media containing 0.05 μM trichostatin A. Open bars: Mean and s.d of alkaline phosphatase-positive cells per well. Closed bars: Mean and s.d. of GCNA1-positive cells per well.

shows that PGCs are part of the population that behave in this manner, we cannot exclude the possibility that somatic cells of the embryo also express GCNA1 under these conditions. Second, GCNA1 may be a more sensitive probe for germ cells than alkaline phosphatase under these conditions. Third, both TSA and 5-azacytdine are known to be toxic to many cell types. Toxicity of these agents may lead to detection of nuclear GCNA1 in cells that have died or lysed. Selective toxicity may also lead to the preferential loss of undifferentiated cells. Lastly, 5-azacytidine or trichostatin A may extensively accelerate the differentiation of germ cells to include the programmed loss of alkaline phosphatase activity which normally occurs at about 14.5 dpc. In this case, GCNA1 could be detected in germ cells that lack the alkaline phosphatase marker. Additional postmigratory markers may enable us to distinguish among these possibilities.

Regulation of the differentiation program

The development of oligodendrocytes in the perinatal rat brain has been suggested to serve as a model of PGC proliferation (Donovan, 1994; Ohkubo et al., 1996). In clonal cultures containing a source of PDGF, oligodendrocyte precursor cells divide up to eight times and subsequently differentiate postmitotically into oligodendrocytes. In the absence of PDGF, cessation of proliferation and subsequent differentiation occur prematurely. However, differentiation can proceed only in the presence of an effector molecule such as thyroid hormone (Durand and Raff, 2000). PGC proliferation may also be regulated by a timing mechanism. Several laboratories have observed that PGCs explanted into feeder culture increase in number until the time corresponding to about 13.5 dpc in vivo and then decline, suggesting that the proliferative program is already in place by 8.5 dpc. Ohkubo et al. (1996) failed to detect a growth limiting activity of older germ cells upon 8.5-dpc PGCs, nor a growth extending activity of 8.5-dpc cultures on 11.5-dpc cultures, consistent with the notion that proliferation is autonomously timed.

PGC differentiation also appears to be preprogrammed, but by a mechanism that differs from oligodendrocytes in two key respects. First, while oligodendrocyte differentiation occurs postmitotically, PGC differentiation can occur independently of the proliferative state of the cells, indicating that PGCs behave as progenitor cells capable of both differentiation and proliferation. Second, we have not been able to identify an effector molecule for PGC differentiation analogous to the role of thyroid hormone for oligodendrocytes. If an effector molecule does exist, its identity is likely to be obscured by the complex nature of the PGC culture system. At present, expression of GCNA1 is consistent with a stochastic process in which each cell has a probability of undergoing differentiation. Spontaneous, rather than instructive modes of differentiation have been suggested for several cell types, and in some cases probabilistic factors such as cell size have been shown to affect cell fate (Gao and Raff, 1997).

Execution of the differentiation program

Explanted 8.5-dpc PGCs undergo a 2- to 3-day delay prior to increased GCNA1 expression even in the presence of a mitogen. In the embryo, GCNA1 first becomes detectable at 11.5 dpc. Hence, expression in culture mimics that seen in vivo and suggests that a cell timing mechanism regulates differentiation. Although the transcriptional or posttranscriptional requirements for GCNA1 detection are not known, we speculate that expression occurs in two phases. In the first phase, represented by the delay in expression, the regulatory decision to differentiate is made. The second phase, the implementation process, consists in part of genomic demethylation and can be accelerated by 5-azacytidine.

The presence of CpG methylation in fetal germ cells has been studied primarily in relation to the critical role that PGCs play in genetic imprinting and X chromosome reactivation. By 12.5–13.5 dpc, the genomes of both XX and XY germ cells appear to be overwhelmingly hypomethylated at numerous loci, including sites important for distinguishing maternally and paternally imprinted alleles (Davis et al., 2000; Hajkova et al., 2002; Kafri et al., 1992; Lee et al., 2002; Monk et al., 1987; Ueda et al., 2000). Several imprinted genes are biallelically expressed in germ cells by this time, consistent with the notion that methylation could be an important mechanism for silencing imprinted genes in germ cells (Szabo et al., 2002; Szabo and Mann, 1995).

Although the methylation status of earlier migratory phase PGCs is poorly understood, recent evidence indicates the presence of some methylation in these cells. PGCs are first allocated into the extraembryonic mesoderm, which is hypomethylated (Monk et al., 1987; Rossant et al., 1986). However, the methylation status of few individual CpGs has been determined. Certain CpG methylations within the H19 imprinting control region survive a wave of global demethylation early in development (Tremblay et al., 1995). EG cells established from 8.5, 9.5, and 11.5-dpc germ cells also show methylation at particular CpGs (Durcova-Hills et al., 2001; Labosky et al., 1994; Tada et al., 1998). Assuming the process of EG cell establishment faithfully preserves the methylation status of the original germ cell, then there must be mechanisms to either maintain or establish CpG methylation within proliferative and migratory phase PGCs. Lastly, both nuclear transplantation studies (Lee et al., 2002) and RNA analysis (Szabo et al., 2002) indicate that reprogramming occurs in germ cells as they colonize the gonad. We have begun to study the PGC methylation status of genes with an expression pattern similar to GCNA1 in order to better understand molecular mechanisms governing germ cell differentiation. Promoters for several such genes are near CpG-rich regions. Many of these cytosine residues are methylated at 10.5 dpc, but then unmethylated by 13.5

dpc (unpublished observations). Together, these results indicate that PGCs undergo extensive demethylation as they colonize the gonad. Our data suggest that this demethylation is part of the process by which continuing germ cell differentiation is coordinated. Although mechanisms are unclear, reductions in DNA methylation are known to lead to differentiation of several cell types (Eden and Cedar, 1994; Reik et al., 2001). PGCs appear to undergo genomic demethylation between 10.5 and 12.5 dpc (Hajkova et al., 2002), which may not only contribute to genetic reprogramming, but may also provide a means for PGCs to effect their own differentiation. Elucidation of the molecular mechanisms underlying this demethylation may provide insight into how the germ cell differentiation program is executed.

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References

Bendel-Stenzel, M.R., Gomperts M., Anderson R., Heasman J., Wylie, C., W., 2000. The role of cadherins during primordial germ cell migration and early gonad formation in the mouse. Mech. Dev. 91, 143.

Chang, D., Calame, K., 2002. The dynamic expression pattern of B lymphocyte induced maturation protein-1 (Blimp-1) during mouse embryonic development. Mech. Dev 117, 305.

Cooke, J.E., Godin, I., Ffrench Constant, C., Heasman, J., Wylie, C.C., 1993. Culture and manipulation of primordial germ cells. Methods Enzymol. 225, 37–58.

Coucouvanis, E.C., Jones, P.P., 1993. Changes in protooncogene expression correlated with general and sex-specific differentiation in murine primordial germ cells. Mech. Dev. 42, 49–58.

Coucouvanis, E.C., Sherwood, S.W., Carswell Crumpton, C., Spack, E.G., Jones, P.P., 1993. Evidence that the mechanism of prenatal germ cell death in the mouse is apoptosis. Exp. Cell Res. 209, 238–247.

Davis, T.L., Yang, G.J., McCarrey, J.R., Bartolomei, M.S., 2000. The H19 methylation imprint is erased and re-established differentially on the parental alleles during male germ cell development. Hum. Mol. Genet. 9, 2885–2894.

De Felici, M., Dolci, S., Pesce, M., 1992. Cellular and molecular aspects of mouse primordial germ cell migration and proliferation in culture. Int. J. Dev. Biol. 36, 205–213.

Di Carlo, A., Travia, G., De Felici, M., 2000. The meiotic specific synaptonemal complex protein SCP3 is expressed by female and male primordial germ cells of the mouse embryo. Int. J. Dev. Biol. 44, 241.

Donovan, P.J., 1994. Growth factor regulation of mouse primordial germ cell development. Curr. Top. Dev. Biol. 29, 189–225.

Donovan, P.J., Stott, D., Cairns, L.A., Heasman, J., Wylie, C.C., 1986. Migratory and postmigratory mouse primordial germ cells behave differently in culture. Cell 44, 831–838.

Durand, B., Raff, M., 2000. A cell-intrinsic timer that operates during oligodendrocyte development. Bioessays 22, 64–71.

Durcova-Hills, G., Ainscough, J., McLaren, A., 2001. Pluripotential stem cells derived from migrating primordial germ cells. Differentiation 68, 220–226.

Eden, S., Cedar, H., 1994. Role of DNA methylation in the regulation of transcription. Curr. Opin. Genet. Dev. 4, 255–259.

Enders, G.C., May, J.J., 1994. Developmentally regulated expression of a mouse germ cell nuclear antigen examined from embryonic day 11 to adult in male and female mice. Dev. Biol. 163, 331–340.

ffrench Constant, C., Hollingsworth, A., Heasman, J., Wylie, C.C., 1991. Response to fibronectin of mouse primordial germ cells before, during and after migration. Development 113, 1365–1373.

Fujiwara, T., Dunn, N.R., Hogan, B.L., 2001. Bone morphogenetic protein 4 in the extraembryonic mesoderm is required for allantois development and the localization and survival of primordial germ cells in the mouse. Proc. Natl. Acad. Sci. USA 98, 13739–13744.

Fujiwara, Y., Komiya, T., Kawabata, H., Sato, M., Fujimoto, H., Furusawa, M., Noce, T., 1994. Isolation of a DEAD-family protein gene that encodes a murine homolog of Drosophila vasa and its specific expression in germ cell lineage. Proc. Natl. Acad. Sci. USA 91, 12258–12262.

Gao, F.B., Raff, M., 1997. Cell size control and a cell-intrinsic maturation program in proliferating oligodendrocyte precursor cells. J. Cell Biol. 138, 1367–1377.

Garcia-Castro, M.I., Anderson, R., Heasman, J., Wylie, C., 1997. Interactions between germ cells and extracellular matrix glycoproteins during migration and gonad assembly in the mouse embryo. J. Cell Biol. 138, 471–480.

Ginsburg, M., Snow, M.H., McLaren, A., 1990. Primordial germ cells in the mouse embryo during gastrulation. Development 110, 521–528.

Hahnel, A.C., Rappolee, D.A., Millan, J.L., Manes, T., Ziomek, C.A., Theodosiou, N.G., Werb, Z., Pedersen, R.A., Schultz, G.A., 1990. Two alkaline phosphatase genes are expressed during early development in the mouse embryo. Development 110, 555–564.

Hajkova, P., Erhardt, S., Lane, N., Haaf, T., El-Maarri, O., Reik, W., Walter, J., Surani, M., 2002. Epigenetic reprogramming in mouse primordial germ cells. Mech. Dev. 117, 15.

Jones, P.L., Veenstra, G.J., Wade, P.A., Vermaak, D., Kass, S.U., Landsberger, N., Strouboulis, J., Wolffe, A.P., 1998. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat. Genet. 19, 187–191.

Juttermann, R., Li, E., Jaenisch, R., 1994. Toxicity of 5-aza-2′-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. Proc. Natl. Acad. Sci. USA 91, 11797–11801.

Kafri, T., Ariel, M., Brandeis, M., Shemer, R., Urven, L., McCarrey, J., Cedar, H., Razin, A., 1992. Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line. Genes Dev. 6, 705–714.

Koshimizu, U., Watanabe, M., Nakatsuji, N., 1995. Retinoic acid is a potent growth activator of mouse primordial germ cells in vitro. Dev. Biol. 168, 683–685.

Kuramochi-Miyagawa, S., Kimura, T., Yomogida, K., Kuroiwa, A., Tadokoro, Y., Fujita, Y., Sato, M., Matsuda, Y., Nakano, T., 2001. Two mouse piwi-related genes: miwi and mili. Mech. Dev. 108, 121–133.

Labosky, P.A., Barlow, D.P., Hogan, B.L., 1994. Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. Development 120, 3197–3204.

Lawson, K.A., Dunn, N.R., Roelen, B.A.J., Zeinstra, L.M., Davis, A.M., Wright, C.V.E., Korving, J.P.W.F.M., Hogan, B.L.M., 1999. Bmp4 is required for the generation of primordial germ cells in the mouse embryo. Genes Dev. 13, 424.

Lee, J., Inoue, K., Ono, R., Ogonuki, N., Kohda, T., Kaneko-Ishino, T., Ogura, A., Ishino, F., 2002. Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells. Development 129, 1807–1817.

Manova, K., Bachvarova, R.F., 1991. Expression of c-kit encoded at the W locus of mice in developing embryonic germ cells and presumptive melanoblasts. Dev. Biol. 146, 312–324.

Matsui, Y., Zsebo, K., Hogan, B.L., 1992. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 70, 841–847.

McLaren, A., 1984. Meiosis and differentiation of mouse germ cells. Symp. Soc. Exp. Biol. 38, 7–23.

Molyneaux, K.A., Stallock, J., Schaible, K., Wylie, C., 2001. Time-lapse analysis of living mouse germ cell migration. Dev. Biol. 240, 488–498.

Monk, M., Boubelik, M., Lehnert, S., 1987. Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. Development 99, 371–382.

Nan, X., Ng, H.H., Johnson, C.A., Laherty, C.D., Turner, B.M., Eisenman, R.N., Bird, A., 1998. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature 393, 386–389.

Ohkubo, Y., Shirayoshi, Y., Nakatsuji, N., 1996. Autonomous regulation of proliferation and growth arrest in mouse primordial germ cells studied by mixed and clonal cultures. Exp. Cell Res. 222, 291–297.

Osterlund, C., Tohonen, V., Forslund, K.O., Nordqvist, K., 2000. Mage-b4, a novel melanoma tumor antigen (MAGE) gene specifically expressed during germ cell differentiation. Cancer Res. 60, 1054–1061.

Pesce, M., De Felici, M., 1995. Purification of mouse primordial germ cells by MiniMACS magnetic separation system. Dev. Biol. 170, 722–725.

Pitman, J.L., Lin T.P., Kleeman, J.E., Erickson, G.F., L., MacLeod, C.L., 1998. Normal reproductive and macrophage function in Pem homeobox gene-deficient mice. Dev. Biol. 202, 196.

Reik, W., Dean, W., Walter, J., 2001. Epigenetic reprogramming in mammalian development. Science 293, 1089–1093.

Richards, A.J., Enders, G.C., Resnick, J.L., 1999a. Activin and TGF beta limit murine primordial germ cell proliferation. Dev. Biol. 207, 470.

Richards, A.J., Enders, G.C., Resnick, J.L., 1999b. Differentiation of murine premigratory primordial germ cells in culture. Biol. Reprod. 61, 1146.

Rossant, J., Sanford, J.P., Chapman, V.M., Andrews, G.K., 1986. Undermethylation of structural gene sequences in extraembryonic lineages of the mouse. Dev. Biol. 117, 567–573.

Rucker, E.B., Dierisseau, P., Wagner, K.U., Garrett, L., Wynshaw-Boris, A., Flaws, J.A., Hennighausen, L., 2000. Bcl-x and Bax regulate mouse primordial germ cell survival and apoptosis during embryogenesis. Mol. Endocrinol. 14, 1038–1052.

Szabo, P.E., Hubner, K., Scholer, H., Mann, J.R., 2002. Allele-specific expression of imprinted genes in mouse migratory primordial germ cells. Mech. Dev. 115, 157–160.

Szabo, P.E., Mann, J.R., 1995. Allele-specific expression and total expression levels of imprinted genes during early mouse development: implications for imprinting mechanisms. Genes Dev. 9, 3097–3108.

Tada, T., Tada, M., Hilton, K., Barton, S.C., Sado, T., Takagi, N., Surani, M.A., 1998. Epigenotype switching of imprintable loci in embryonic germ cells. Dev. Genes Evol. 207, 551.

Tam, P.P., Snow, M.H., 1981. Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos. J. Embryol. Exp. Morphol. 64, 133–147.

Toksoz, D., Zsebo, K.M., Smith, K.A., Hu, S., Brankow, D., Suggs, S.V., Martin, F.H., Williams, D.A., 1992. Support of human hematopoiesis in log-term bone marrow cultures by murine stromal cells expressing the membrane-bound and secreted forms of the human homolog of the steel gene product, stem cell factor. Proc. Natl. Acad. Sci. USA 89, 7350–7354.

Tremblay, K.D., Saam, J.R., Ingram, R.S., Tilghman, S.M., Bartolomei, M.S., 1995. A paternal-specific methylation imprint marks the alleles of the mouse H19 gene. Nat. Genet. 9, 407–413.

Ueda, T., Abe, K., Miura, A., Yuzuriha, M., Zubair, M., Noguchi, M., Niwa, K., Kawase, Y., Kono, T., Matsuda, Y., Fujimoto, H., Shibata, H., Hayashizaki, Y., Sasaki, H., 2000. The paternal methylation imprint of the mouse H19 locus is acquired in the gonocyte stage during foetal testis development. Genes Cells 5, 649–659.

Wang, D.H., Ikeda, Y., Parker, K.L., Enders, G.C., 1997. Germ cell nuclear antigen (GCNA1) expression does not require a gonadal environment or steroidogenic factor 1: examination of GCNA1 in ectopic germ cells and in Ftz-F1 null mice. Mol. Reprod. Dev. 48, 154–158.

Ying, Y., Liu, X.M., Marble, A., Lawson, K.A., Zhao, G.Q., 2000. Requirement of Bmp8b for the generation of primordial germ cells in the mouse. Mol. Endocrinol. 14, 1053–1063.

Ying, Y., Zhao, G.Q., 2001. Cooperation of endoderm-derived BMP2 and extraembryonic ectoderm-derived BMP4 in primordial germ cell generation in the mouse. Dev. Biol. 232, 484–492.

Yoshida, M., Kijima, M., Akita, M., Beppu, T., 1990. Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J. Biol. Chem. 265, 17174–17179.