EMBO reports

scientific report

Oct4 is required for primordial germ cell survival

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Previous studies have shown that Oct4 has an essential role in maintaining pluripotency of cells of the inner cell mass (ICM) and embryonic stem cells. However, *Oct4* null homozygous embryos die around the time of implantation, thus precluding further analysis of gene function during development. We have used the conditional Cre/*loxP* gene targeting strategy to assess Oct4 function in primordial germ cells (PGCs). Loss of Oct4 function leads to apoptosis of PGCs rather than to differentiation into a trophectodermal lineage, as has been described for Oct4-deficient ICM cells. These new results suggest a previously unknown function of Oct4 in maintaining viability of mammalian germline.

Keywords: Oct4 function; primordial germ cells; conditional gene targeting; Cre/loxP; apoptosis

EMBO reports (2004) **5**, 1078–1083. doi:10.1038/sj.embor.7400279

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Received 24 August 2004; revised 20 September 2004; accepted 21 September 2004; published online 15 October 2004

INTRODUCTION

The POU domain transcription factor Oct4 shows a remarkable expression pattern in mouse ontogeny. Maternal Oct4 RNA and protein are present in fertilized oocytes until the two-cell stage, and zygotic *Oct4* gene expression starts at the four- to eight-cell stage (Rosner *et al*, 1990; Schöler *et al*, 1990; Yeom *et al*, 1996). During early cleavage, uniform amounts of Oct4 RNA are found in all blastomeres, but the levels decrease in the outer cells of the morula as they polarize and form the trophectoderm. In the 3.5 days postcoitum (dpc) blastocyst, Oct4 RNA and protein levels are low in this epithelial cell layer and become undetectable 1 day later (Schöler *et al*, 1990; Palmieri *et al*, 1994). In contrast, *Oct4* expression is maintained in the inner cell mass (ICM) of the blastocyst. Differential expression of *Oct4* is observed again during embryogenesis, when the ICM differentiates at 4.5 dpc into epiblast (primitive ectoderm, embryonic ectoderm) and hypoblast (primitive endoderm, embryonic endoderm). *Oct4* expression is maintained in the epiblast but, as hypoblast cells differentiate into visceral and parietal endoderm, Oct4 protein levels transiently increase and then decrease to undetectable levels. During gastrulation, *Oct4* expression is progressively repressed in the epiblast and by 7.5 dpc is confined exclusively to newly established primordial germ cells (PGCs; Schöler *et al*, 1990; Yeom *et al*, 1996). PGCs continue to express *Oct4* as they proliferate and migrate to the forming genital ridges. In female PGCs, *Oct4* is repressed by the onset of meiotic prophase I (13–14 dpc) and is then re-expressed after birth, coincident with the growth phase of oocytes. In male embryos, *Oct4* expression persists in germ cells throughout fetal development. After birth, it is maintained in proliferating gonocytes, pro-spermatogonia and later in undifferentiated spermatogonia (Pesce *et al*, 1998a; Tadokoro *et al*, 2002). In addition, embryonic stem (ES) cells, embryonal carcinoma (EC) cells and embryonic germ (EG) cells, pluripotent cell lines derived from the ICM, epiblast and PGCs, respectively, also express *Oct4* as long as they remain undifferentiated (for reviews, see Pesce *et al*, 1998b; Surani, 2001; Donovan & de Miguel, 2003).

This highly restricted, cyclic expression pattern in the germ line, referred to as the 'totipotent cycle', has been taken as an indication that Oct4 may have a role in maintaining the pluripotency and germline potential of pluripotent embryonic cells. Indeed, gene targeting showed that Oct4-deficient embryos survived through the morula stage, but could not form an ICM and also failed to give rise to ES colonies *in vitro* (Nichols *et al*, 1998). These results suggested that differentiation of the ICM into trophoblast cells was due to the absence of Oct4 function, rather than to a reduction in viability or decreased proliferation. Further functional tests involved ES cells whose self-renewal and pluripotent state relied on the inducible expression of *Oct4* (Niwa *et al*, 2000). In these experiments, alteration in *Oct4* expression above or below a twofold threshold level was sufficient to trigger differentiation into hypoblast and trophoblast cells, respectively. These results were taken as an indication that Oct4 may also act in a dosage-dependent manner in the early mouse embryo, during the formation of the first three germ layers. Although a defined level of Oct4 has been demonstrated to be crucial to maintain pluripotency in ICM and ES cells, the molecular read-out required for this important function is still unknown.

Peri-implantation lethality of Oct4-deficient embryos precluded determination of its function in germ cells. In the present study, we circumvented this limitation by germ-cell-specific deletion of *Oct4* and show that PGCs undergo apoptosis without Oct4. Therefore, the loss of Oct4 function at different developmental stages and in different cell-type contexts (ICM/ES versus PGC) exerts different physiological effects.

RESULTS AND DISCUSSION

Oct4 locus targeting

To circumvent the peri-implantation lethality of *Oct4* null homozygous embryos (Nichols *et al*, 1998) and to assess Oct4 function in developing germ cells, we applied the conditional Cre/*loxP* gene targeting approach (supplementary Fig 1 online). Conditionally targeted $Oct4^{flox}$ mice were eventually generated and used for subsequent mating with $TNAP^{Cre}$ mice. The latter express Cre recombinase in PGCs due to an insertion of the Cre coding sequence into the *Tissue Non-specific Alkaline Phosphatase* locus. Previously, we evaluated the specificity of this Cre model and found that before 10.5 dpc Cre activity is detected exclusively in PGCs (Lomeli *et al*, 2000). After 10.5 dpc, some embryos showed Cre expression additionally in somatic tissue. However, given that after 7.5 dpc the *Oct4* gene is restricted to germ cells, we presumed that deletion of *Oct4* in somatic cells would be irrelevant and that the phenotype anticipated in PGCs should be cell-autonomous.

Oct4 phenotype in postnatal gonads

To ablate Oct4 function in PGCs through *flox*→Δ biallelic conversion, we set up a multistep mating scheme, as outlined in supplementary Fig 2 online. Postnatal Δ/Δ animals of both sexes did not show any gross behavioural, anatomical or physiological anomalies. However, they were partially or completely infertile, correlating with various degrees of germ cell deficiency in their gonads. In comparison to ovaries of pre-oestral (3-week old) control Δ/+ females, the ovaries of Δ/Δ females contained about half the number of growing follicles. Even more striking was the difference in the number of primordial follicles, with 25–100 times fewer primordial follicles in Δ/Δ ovaries (compare Fig 1A and B). Our interpretation is that compensatory recruitment of primordial follicles into the pool of maturing follicles soon depletes the reservoir. Consequently, 6-week-old Δ/Δ ovaries contained hardly any primordial follicles and very few growing follicles, whereas the Δ/+ females of the same age maintained a large number of ovarian follicles (compare Fig 1E and F). Follicles were mostly absent in Δ/Δ ovaries older than 6 weeks (compare Fig 1G and H). Thus, by the time Δ/Δ females had reached breeding age (~6 weeks), their gonads were essentially free of germ cells, which explains their sterility.

In contrast to the full sterility of all matured Δ/Δ females, the fertility of adult Δ/Δ males was always impaired but varied in severity, even among littermates. Accordingly, histological analyses showed complete or partial depletion of spermatogenic cells in Δ/Δ testes, manifested as germ-cell-free seminiferous tubules with only somatic cells present, alongside unaffected tubules with all stages of spermatogenesis present (Fig 1I,M). The percentage of germ-cell-free tubules varied in different Δ/Δ males from 30% to 100% ($n=7$), correlating with partially or completely impaired fertility of these animals. In contrast to the Δ/Δ females, the phenotypic penetrance in Δ/Δ males did not correlate with age (data not shown). Another visible feature of Δ/Δ testes was a reduction in the diameter of seminiferous tubules lacking germ cells and hyperplasia of the surrounding Leydig cells (Fig 1I,M), similar to other germ-cell-depleted phenotypes. The presence of some surviving spermatogonia in Δ/Δ testes throughout the postnatal period and a few Oct4-positive oocytes in young Δ/Δ ovaries (Fig 1C,K) is consistent with the idea that these cells are descendants of PGCs that escaped Cre-mediated recombination.

Oct4 phenotype in PGCs

Cre recombinase expression under *TNAP* locus control and consequently $Oct4^{flox}$ allelic excision can occur in germ cells presumably between 7.25 and 15.5 dpc, as suggested by previous studies (Ginsburg *et al*, 1990; Anderson *et al*, 2000; Lomeli *et al*, 2000). Therefore, gamete deficiency in postnatal gonads can be attributed to loss of PGCs within this period of fetal development. To examine this possibility, we stained PGCs for alkaline phosphatase (AP; Ginsburg *et al*, 1990), at different stages of embryonic development. Examination of Δ/Δ embryos showed a PGC population comparable in size to that of Δ/+ embryos at the premigratory (8.5 dpc) and early migratory (9.5 dpc) stages (Fig 2C–F). In contrast, the number of PGCs in Δ/Δ embryos was markedly lower than in Δ/+ littermates in the late migratory (10.5 dpc) and postmigratory (12.5 dpc) phases of PGC development (Fig 2A,B,G–J). Thus our data support the notion that the primary gamete deficiencies observed in adult mice were caused by the loss of PGCs between 9.5 and 10.5 dpc of embryonic development.

The presence of oocytes and sperm in the ovaries and testes of some adult Δ/Δ animals is consistent with the persistence of some postmigratory PGCs in the genital ridges of Δ/Δ embryos (Fig 2G,I). These PGCs may result from either the dispensability of Oct4 function or its function being undisturbed in these cells. We consider the first possibility to be unlikely for two reasons. First, we observed variability in the number of germ cells within genital ridges of the same stage, which reflects variability in Oct4 dependence. Second, $TNAP^{Cre}$ has limited efficiency like most

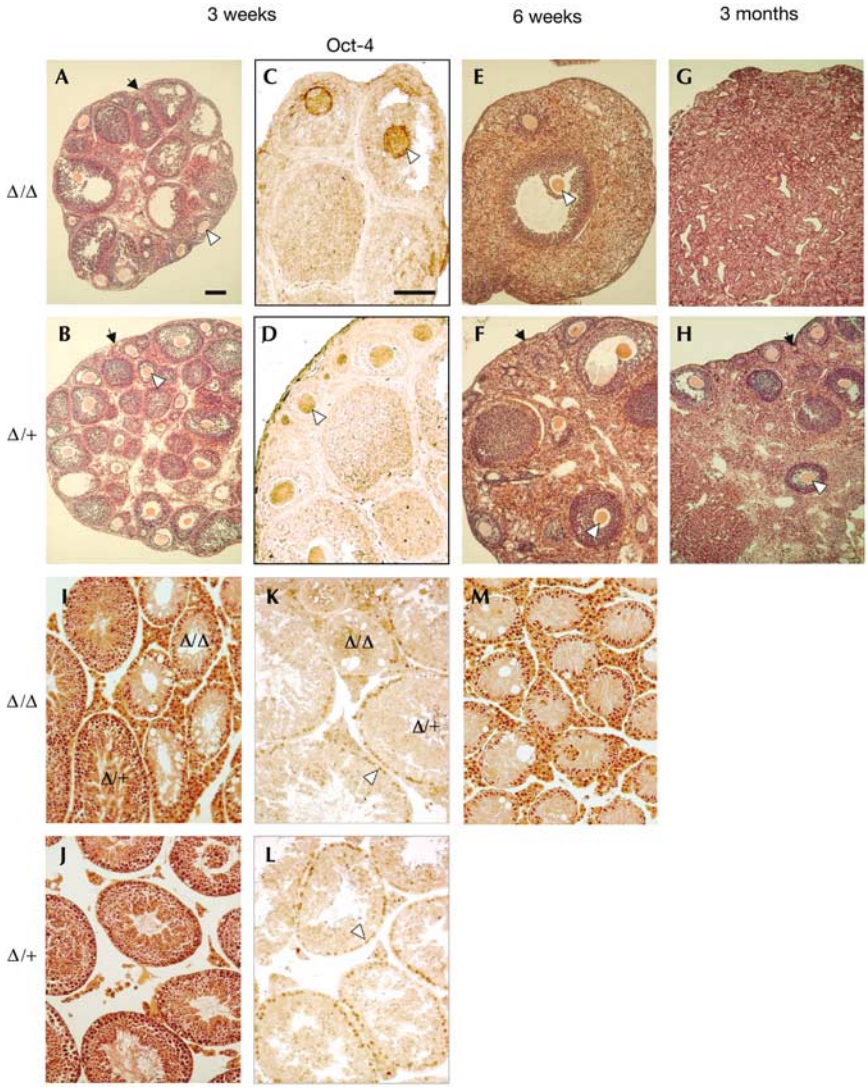

Fig 1 | Oct4 phenotype in adult gonads. Immunostaining with Oct-4 antibody (**C,D,K,L**) and haematoxylin and eosin (**A,B,E–J,M**) of ovaries of indicated ages (**A–H**) and 10-week-old testes (**I–M**), isolated from Δ/Δ (**A,C,E,G,I,K,M**) and the control group Δ/+ (**B,D,F,H,J,L**) mice, is shown. The arrows and arrowheads point to primordial (arrows) and growing (arrowheads) follicles in the ovarian sections. In the testicular sections, representative germ-cell-deficient seminiferous tubules are labelled as Δ/Δ and those harbouring normal spermatogenic cells of all stages as Δ/+; the arrowheads point to Oct4-positive spermatogonia type B. Shown are mildly (70%; **I,K**) and severely (100% of germ-cell-free tubules; **M**) affected testes. Scale bars in (**A**) for (**A,B,E–M**) and in (**C**) for (**C,D**) represent 100 μm.

Cre-expressing models. Previously, we found that on average 60% of 13.5 dpc PGCs in $TNAP^{Cre} \times Z/AP$ crosses underwent Cre-mediated recombination of the floxed reporter allele (Lomeli *et al*, 2000). In the $Oct4^{\Delta/+};TNAP^{Cre/+} \times Oct4^{flox/flox}$ crosses, we find a similar average rate of recombination, inferred from the marked increase in apoptosis seen in 10.5 dpc Δ/Δ embryos that never reached 100% efficiency (see quantitative data in supplementary Figs 3 and 4 online). This latter possibility is further supported by two observations: (1) the presence of Oct4-positive germ cells in young Δ/Δ ovaries (Fig 1C) and in mildly affected Δ/Δ testes (Fig 1K) and (2) the fact that these Δ/Δ males transmit the intact *flox* allele in their sperm. We found this allele at an expected frequency in the progeny by natural backcrossing of mildly affected adult Δ/Δ males and by intracytoplasmic sperm injection (ICSI) of the few spermatozoa recovered from the epididymides of severely affected infertile males into oocytes (data not shown). Therefore, in a subpopulation of PGCs, probably those that survived through embryonic stages (Fig 2G,I) and subsequently gave rise to gametes in adult Δ/Δ animals (Fig 1C,K), Cre recombinase failed to catalyse the excision of the *flox* allele. Taken together, the above data suggest that development of PGCs does not proceed without Oct4.

Fate of Oct4-deficient PGCs

We considered at least four explanations that might account for the loss of PGCs following disruption of Oct4 function. In the first,

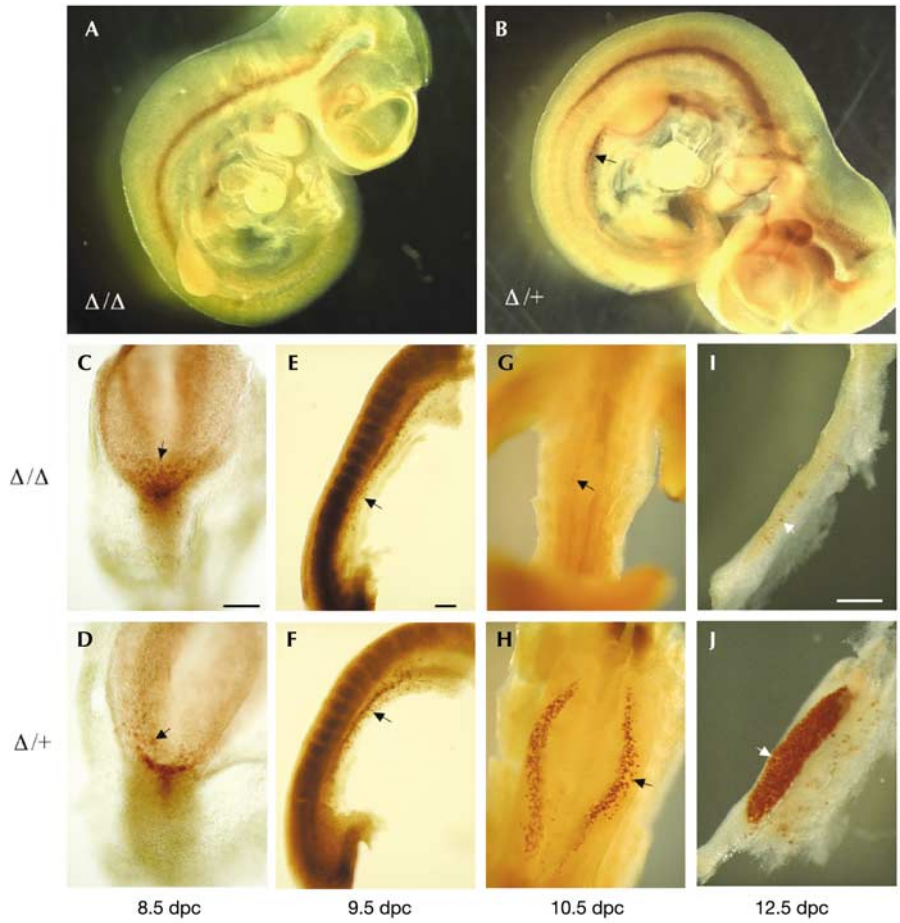

Fig 2 | Oct4 phenotype in PGCs. Alkaline phosphatase (AP) staining of Δ/Δ (**A,C,E,G,I**) and Δ/+ embryos (**B,D,F,H,J**) is shown. The 8.5 dpc (**C,D**) and 9.5 dpc (**E,F**) embryos in both groups show no difference in the number of PGCs (arrows) that are located at the base of allantois within the hindgut pocket (8.5 dpc) and, subsequently, in the hindgut endoderm (9.5 dpc). As opposed to the further increase in the number of PGCs in 10.5 dpc Δ/+ embryos (**B,H**), the Δ/Δ littermates show a marked decrease in the number of PGCs (**A,G**), which is further apparent in 12.5 dpc genital ridges (**I** versus **J**). Photographs were taken from the ventral (**C,D,G,H**) or lateral (**A,B,E,F**) sides of embryos. Scale bars in (**C**) for (**C,D**) and in (**E**) for (**E,F**) represent 100 μm, and scale bar in (**I**) for (**I,J**) represents 200 μm.

somatic differentiation would be analogous to the trophoblast differentiation seen in Oct4-deficient ICM and ES cells (Nichols *et al*, 1998; Niwa *et al*, 2000). However, other than into pro-oogonia or gonocytes, differentiation fates have not been reported for mammalian PGCs. Accordingly, we were not able to detect the expression of early (Cdx-2) or mature (TROMA-1) trophoblast markers in Δ/Δ embryos between 9.5 and 10.5 dpc within the dorsal mesentery, which is the site of PGC migration (data not shown). Our results further argue against a second scenario, where Oct4 loss of function could interfere with PGC migration by disrupting their ability to receive a chemotactic signal and/or migrate towards the developing genital ridges. Whereas ectopic PGCs are known to undergo apoptosis in wild-type and mutant mouse models (Stallock *et al*, 2003), we did not observe any difference in the distribution of PGCs between genotypes, and all of the apoptotic PGCs that we analysed were isolated from the dorsal mesentery. Third, we hypothesized that Oct4 loss in PGCs might trigger premature meiosis, due to the observation that Oct4 is downregulated in female PGCs on their entry into meiosis and coincides with a massive wave of cell death in the fetal ovary at 13.5 dpc (Pesce *et al*, 1998a). However, as we did not observe clear nuclear signs of meiosis in Δ/Δ PGCs before 13.5 dpc (data not shown), we excluded the third possibility as well.

Because programmed cell death, apoptosis, is the only fate ascribed to PGCs that leave the germ lineage in response to genetic mutations or missing cues from the somatic environment (for reviews, see McLaren, 2000; Zhao & Garbers, 2002; Tres *et al*, 2004), we assessed whether it may also account for PGC loss following Oct4 withdrawal. To this end, histological analyses of sections of the aorta–gonad–mesonephros (AGM) region of 10.5 dpc Δ/Δ embryos were performed. Immunostaining for the Oct4 protein again confirmed its loss by Cre-mediated recombination of the *flox* locus (compare Fig 3A and B). Importantly, near the genital ridge, we observed compact clusters of cells with dark, fragmented nuclei, which is a hallmark of apoptosis (data not shown). These clusters showed co-staining with the stage-specific

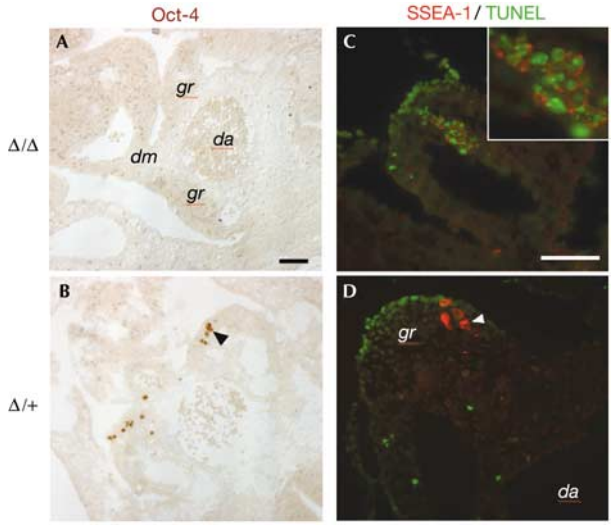

Fig 3 | Apoptosis of *Oct4*-deficient PGCs. Labelling with Oct-4 antibody (**A,B**), and double TUNEL (green)/SSEA-1 antibody (red) staining (**C,D**) of the Δ/Δ (**A,C**) and control Δ/+ (**B,D**) 10.5 dpc embryo sections are shown. Significant reduction in Oct4 protein staining can be seen in the Δ/Δ sections. Shown in (**C,D**) is the AGM region of the same embryos as in Fig 2G,H. Note a strong TUNEL signal in the nuclei of the same clustered cells showing residual SSEA-1 staining on the cell membrane (**C** and inset). The SSEA-1 signal was notably weaker than that from viable PGCs of the +/Δ embryos (**D**) yet sufficiently strong to be distinguished from the background. Scale bars in (**A**) for (**A,B**) and in (**C**) for (**C,D**) represent 50 μm. The inset is a twofold magnification of the PGC/apoptotic area in (**C**). Arrowhead, PGC; *da*, dorsal aorta; *dm*, dorsal mesentery; *gr*, genital ridge.

embryonic antigen 1 (SSEA-1), a marker of migratory PGCs, and with TUNEL staining (Fig 3C and inset), implying that they represent *bona fide* PGCs undergoing apoptosis. We additionally confirmed the result as well as quantified PGC loss in Δ/Δ embryos, using fluorescence-activated cell sorting analyses (supplementary Figs 3 and 4 online).

In sum, our results show that the ablation of *Oct4* expression in PGCs impairs their maintenance starting by 10 dpc. In fact, lack of Oct4 resulted in a massive (as high as 70%; supplementary Figs 3 and 4 online) wave of premature apoptosis in PGC before their colonization of the developing gonadal ridges, contributing to postnatal gonads depleted of pro-spermatogonia and oocytes. Although *Oct4* and *TNAP* are coexpressed in PGCs during their initial formation in the proximal epiblast at 7.25 dpc, the phenotype is not evident until 10 dpc. A possible explanation for this finding is that Cre recombinase requires 1 or 2 days after the onset of its expression to reach a critical threshold level to catalyse recombination *in vivo*. In agreement with this, Cre excision activity in crosses between the $TNAP^{Cre}$ and the Cre reporter *Z/AP* mice was first detected at 9 dpc (Lomeli *et al*, 2000). Therefore, the $Oct4^{flox}$ allele may remain intact until 9 dpc. The subsequent degradation of Oct4 mRNA and protein may further delay the manifestation of the Oct4 phenotype until about 10 dpc, when the majority of Δ/Δ PGCs showed features of apoptosis. An alternative explanation is that Oct4 function is dispensable in premigratory and early migratory PGCs but becomes essential by 10 dpc, when PGC proliferation is highest (Tam & Snow, 1981; Gomperts *et al*, 1994). An inducible Cre model might help to discriminate between the two possibilities by precise control of the timing of recombination in the $Oct4^{flox}$ locus.

The germ cell phenotype in Δ/Δ males did not seem to progress with age. On the contrary, the germ cell deficiency in Δ/Δ females was exacerbated by age. The population of resting oocytes, already impaired at the prepubertal age, was further depleted during the first ovulatory cycles and/or by subsequent ovarian atresia. This difference might be due to the fact that spermatogenesis relies on self-renewing stem cells, whereas the oocyte pool is mainly established during fetal development.

Speculations

Apoptosis is the only fate ascribed to PGCs that leave the germ lineage in response to genetic mutations or missing cues from the somatic environment (for reviews, see McLaren, 2000; Zhao & Garbers, 2002; Tres *et al*, 2004). In line with these observations, ablation of Oct4 in migratory PGCs promotes their apoptosis. This is a remarkably distinct function in germ cells from its previously demonstrated role as a gatekeeper of pluripotency in ICM cells and their cultured counterparts, ES cells (Nichols *et al*, 1998; Niwa *et al*, 2000). However, our data do not preclude the potential superimposition of several pathways. For example, loss of Oct4 might initially trigger PGCs to differentiate into somatic cell types other than trophoblast (see above), and then undergo apoptosis due to a lack of appropriate survival signals. These two phenomena may also serve as different solutions to the same problem. The *Oct4* gene is downregulated whenever lineages split off from the germ line, namely into the trophoblast and hypoblast, and in each of the three somatic lineages (Pesce *et al*, 1998b). As PGCs are not known to exit the germ line spontaneously by differentiation, the forced loss of Oct4 function in our experimental model may establish a conflict that can be only resolved by apoptosis. In more specific terms, however, the different physiological read-outs of Oct4 deficiency in ICM cells and PGCs should reflect distinct sets of target genes and/or partner proteins recruited by Oct4 at the two different stages of germline development.

The Fgf/Frfr, LIF/gp130/Jak/Stat3 and Steel/c-kit/AKT/mTOR/Bax transduction pathways have been shown to operate in migratory PGCs to promote their growth and survival (for reviews, see McLaren, 2000; De Miguel *et al*, 2002; Zhao & Garbers, 2002; Stallock *et al*, 2003). Whether and how Oct4 interacts with the elements of these pathways in PGCs remains to be determined. This is not a trivial task, however, considering scant PGC number, their short lifespan *in vitro* and the lack of a suitable long-term culture model. The EG cells, despite their PGC origin, are more closely related to the ES cells in both growth factor dependence and developmental capacity (for reviews, see Surani, 2001; Donovan & De Miguel, 2003). Accordingly, the EG cells we established from migratory 8.5 dpc $Oct\text{-}4^{flox/flox}$ PGCs differentiated into trophoblast rather than having underwent apoptosis, after Oct4 function had been abolished by an inducible Cre (data not shown). The recent demonstration of retroviral transduction of PGCs might provide a feasible approach for designing future experiments to investigate potential pathways downstream of *Oct4* (De Miguel *et al*, 2002).

Conclusion

Given these two essential roles of Oct4 in maintaining pluripotency in early embryonic cells and viability in PGCs, unravelling other possible functions of Oct4 has become a highly relevant pursuit. Specifically, comparing differential gene expression in Oct4 null and wild-type PGCs, as well as with ES cells, could identify potential Oct4 target genes involved in PGC survival. This knowledge should help us to understand how Oct4 exerts its pleiotropic effects during mouse development, as well as to draw general principles that define the commonalities and differences between ES cells and germ cells.

METHODS

Targeting vector design, ES cell electroporation, genotyping procedures and mouse experimentation are described in the legend to supplementary Fig 1 online. Histological procedures are described in supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS

We thank P.-O. Angrand and F. Stewart for the pHC-Cre and pNPK-CreAR3 plasmids, S. Schlatt for advice on adult germ cells, D. Groff, A. Leu, V. Koehlin, M. Volkov and R. Turek for excellent technical assistance, A. Malapetsas for editing the manuscript and lab members for useful discussions. This work was supported by National Institutes of Health (NIH) grants RO1HD42011-01 to H.R.S. and RO1-HD-44066-01A1 to K.J.M., the Marion Dilley and David George Jones Funds and the Commonwealth and General Assembly of Pennsylvania to H.R.S. and K.J.M., and by Max-Planck Gesellschaft to A.T. J.K. was supported in part by the NIH Medical Scientist Training Program (T32-GM07170).

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