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Impaired meiotic competence in putative primordial germ cells produced from mouse embryonic stem cells

MARIANNA TEDESCO, DONATELLA FARINI and MASSIMO DE FELICI*

Department of Public Health and Cell Biology, Section of Histology and Embryology
University of Rome "Tor Vergata", Rome, Italy

ABSTRACT There are still several unanswered questions and problems about the recently claimed possibility of producing functional germ cells *in vitro* from pluripotent embryonic stem cells (ESCs). In the present paper, we compared by single-cell analysis the capability of putative primordial germ cells (PGCs), produced *in vitro* from ESCs, and that of endogenous PGCs isolated from embryos, to enter and progress through meiotic prophase I. Using a protocol previously reported to be suitable to produce female germ cells from mouse ESC monolayers, we first identified putative PGCs by analysing the expression pattern of several markers such as SSEA1, APase, OCT4, NANOG, MVH and SCP3 of pre-and post migratory PGCs. Next, after isolation of such cells from culture, we tested their meiotic capability. The evaluation at 2-5 days of culture of the number of cells showing meiotic nuclear SCP3 staining in cytospreads showed that it remained nearly constant in the putative PGCs, whereas it increased markedly in endogenous PGCs. Moreover, we observed that in putative PGCs, the nuclear distribution or expression of SCP3 and other meiotic markers such as DMC1, γH2AX and SCP1 were always highly abnormal in comparison to that observed in endogenous cultured PGCs. We conclude that although the formation of cells showing characteristics of PGCs can occur efficiently from ESCs *in vitro*, these cells possess impaired capability to enter and progress through meiotic prophase I.

KEY WORDS: *embryonic stem cell, gametogenesis, meiosis, primordial germ cell*

From the first report by Hübner *et al.* (2003) showing that XY mouse embryonic stem cells (mESCs) are able to produce follicle-enclosed growing oocytes, several studies have described the *in vitro* differentiation of female and male germ cells from mouse and human ESCs (hESC) in either the monolayer or the embryoid bodies (EBs) culture method (for reviews see Hua and Sidhu, 2008; Nagano, 2007; Aflatoonian and Moore, 2006). The experiments carried out to produce germ cells from ESCs are based on years of intense investigations on the *in vivo* and *in vitro* development of mouse primordial germ cells (PGCs), the oocyte and sperm precursors (for reviews see De Felici, 2009; De Felici *et al.*, 2004; De Felici, 2001). In particular the recent progress in elucidating the process of germ line specification in mammals (Ohinata *et al.*, 2009 and references herein) and the possibility to mimic *in vitro* events of later PGC development including entering into meiosis (Farini *et al.*, 2005), have given precious information for the derivation and characterization of germ cells produced from ESCs.

In the mouse embryo, PGC development occurs in about 7 days, from around 5.5 days post coitus (dpc), when the first inductive events of the germ cell lineage occur in the epiblast, to 12.5 dpc when sex differentiation of PGCs into meiotic oocytes in the female and G0-arrested prospermatogoni/gonocytes in male

Abbreviations used in this paper: APase, alkaline phosphatase; bFGF, basic fibroblast growth factor; BLIMP, B-lymphocyte induced maturation; BMP, bone morphogenetic protein; Daz, deleted in azoospermia; Dazl, deleted in azoospermial-like; DMC, disrupted meiotic cDNA; dpc, days post coitum; EB, embryoid body; EG, embryonic germ; eGFP, enhanced green fluorescent protein; epiSC, epiblast stem cvell; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; ePGC, endogenous primordial germ cell; hEG, human embryonic germ; hESC, human embryonic stem cell; KL, kit ligand; IF, immunofluorescence; LIF, leukemia inhibitory factor; MEF, mouse embryonic fibroblast; mESC, mouse embryonic stem cell; MLH, mutL homolog; MVH, mouse VASA homolog; PRDM, PR domain containing; PGC, primordial germ cell; pPGC, putative primordial germ cell; RA, retinoic acid; SMC, structural maintenance of chromosome; STAG, stomalin antigen; SCP, synaptonemal complex protein; SSEA, stage-specific embryonic antigen; SDF, stromal cell-derived factor.

***Address correspondence to:** Massimo De Felici. Dipartimento di Sanità Pubblica e Biologia Cellulare, Università di Roma "Tor Vergata", Via Montpellier, 1, 00173 Roma, Italy. Fax: +39-06-7259-6172. e-mail: defelici@uniroma2.it

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occur (for a review, see Western, 2009). Elegant recent studies have shown that mouse PGCs are derived from a few precursors located at the proximal posterior epiblast, which around 6.25 dpc express the transcription factor B-lymphocyte induced maturation protein1 (BLIMP1 also known as PR domain containing 1 (PRDM1)) (Ohinata *et al*., 2009 and references herein). PGCs are finally specified at the base of the allantois around 7.5 dpc and move into the gonadal ridges between 8.5 and 11.5 dpc (for reviews, see Hayashi *et al.,* 2007; De Felici, 2009). By 12.5 dpc, the ovary and testis become morphologically distinguishable and PGCs initiate sex-specific development. During migration and for a couple of days after gonad colonization, the somatic status of imprinted genes in PGCs is progressively erased (Szabo and Mann, 1995; Szabo *et al*., 2002; Hajkova *et al*., 2002; Lee *et al*., 2002; Kato *et al*., 1999). Besides the expression of genes and other markers shared from PGCs and ESCs (see below), this epigenetic modification is a valid criterion to distinguish PGCs from ESCs. At 13.5 dpc female PGCs enter meiosis becoming primary oocytes which pass through the meiotic prophase I stages (leptotene/zygotene/pachytene) until around and early after birth become arrested at the diplotene stage. Differently, male PGCs enter mitotic arrest and developed as spermatogonial will be reactivated to initiate spermatogenesis only after birth. Recent studies reported that the decision of meiotic entry or mitotic arrest of post migratory PGCs is regulated by retinoic acid (RA). Male PGCs do not enter meiosis because an enzyme (CYP26b1) expressed in somatic cells in the male gonadal ridge degrades RA. In contrast, the lack of CYP26b1 expression in the embryonic ovary allows PGCs to enter meiosis (for a review, see Bowles and Koopman, 2007). Under various *in vivo* and *in vitro* conditions, male PGCs until 12.5 dpc can be, however, induced to enter meiosis likely if exposed to RA at the right time (for a review, see Kocer al., 2009). This indicates that germ cell sex depends on sex-determining signalling from the environment rather than the sex chromosomes. RA is considered the meiotic inducer also in spermatogonia at later postnatal stages (Pellegrini *et al*., 2008; Anderson *et al*., 2008). Intriguing, RA can stimulate mitotic proliferation of sex undifferentiated PGCs (Koshimizu *et al*., 1995; De Felici, 2000) and prevent mitotic arrest of male prospermatogonia in the fetal testis (Trautmann *et al*., 2008). Moreover, in combination with leukemia inhibitory factor (LIF) and kit ligand (KL), RA favours the formation of embryonic germ (EG) cells from sex undifferentiated PGCs (Koshimizu *et al*., 1997). Thus indicating that germ cell response to RA is strictly developmental stage dependent.

On the basis of the information summarized above and the analyses of germ cell-specific gene expression dynamics and epigenetic methylation status of imprinted loci to identify PGCs, some recent studies have concluded that *bona fide* PGCs can be actually produced both from mouse and human ESCs and that their formation recapitulates many of the crucial developmentally-timed events that occur in the embryo (Wei *et al*., 2008; West *et al*., 2010; Kee *et al*., 2009). The assessment of properly differentiated PGC status requires, however, the demonstration that these cells are able to enter and progress into meiotic prophase I after reaching the premeiotic stage. While the expression of premeioitic markers such as mouse Vasa homolog (MVH) and synaptonemal complex protein 3 (SCP3) or other proteins involved in meiosis (i.e. disrupted meiotic cDNA 1, DMC1, and mutL homolog 1, MLH1) is readily detected in putative PGCs produced form ESCs both at mRNA and protein level, their meiotic capability has been accurately analysed in a few papers only likely for the lack of a suitable method to quantify such process in single PGCs *in vitro*. Novak *et al*. (2006) using XY mouse ESC (mESC) monolayers and a panel of meiosis-specific markers and cytospreads to identify stages of meiotic prophase I found that up to 40% of the cells within colonies grown onto the culture dish or in suspension considered putative PGCs at 14-16 days of culture expressed the synaptonemal complex protein3 (SCP3). However, they also found that these cells did not express other two synaptonemal complex proteins SCP1, SCP2 and some meiotic proteins, such as stromalin antigen3 (STAG3), Rec8 (REC8) and structural maintenance of chromosome1 (SMC1). Moreover, in cytospreads such cells showed highly abnormal distribution of SCP3. Nicholas *et al*. (2009) reported that 1-3% of putative PGCs formed within mouse XX EBs were able to enter into meiosis in culture as evidenced by SCP expression, but showed limited chromosome alignment and meiotic progression. Similar results have been obtained in hESCs. Tilgner *et al*. (2008) found that SCP3 protein detected in stage-specific embryonic antigen 1 (SSEA1) positive putative PGCs formed within hESC monolayers was concentrated in cytoplasmic foci outside the nuclear envelope. Likewise, in cells positive for SCP3 observed throughout differentiating hEBs, the protein was localized predominantly in the cytoplasm (Clark *et al*., 2004). But

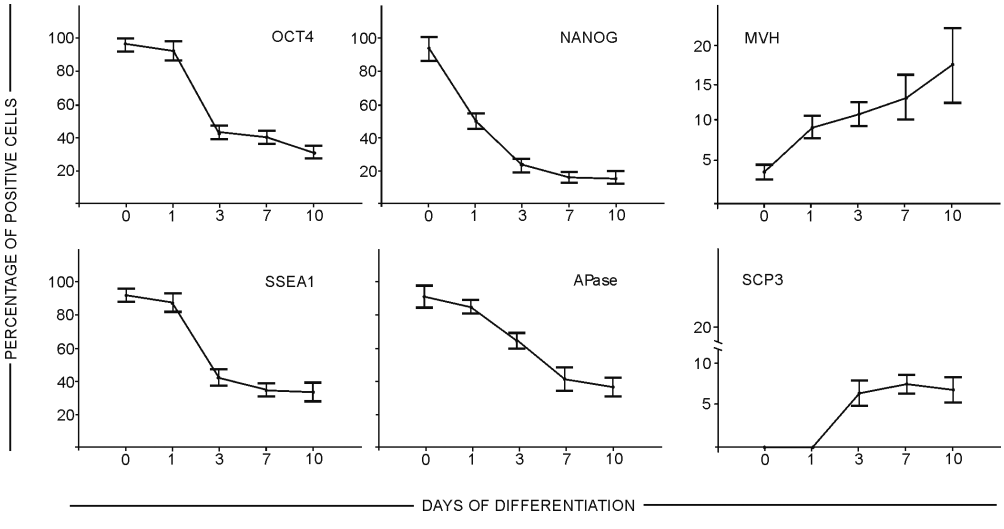

Fig. 1. Expression patterns of various ESC and PGC markers in ESC-D3 cultured for 10 days. *The expression was analyzed using FACS (SSEA1), IF (OCT4, MVH, NANOG, SCP3) or cytochemistry (APase) directly onto the monolayers or on cell samples detached from the monolayer. Similar results were obtained from ESCs cultured for 12 days.*

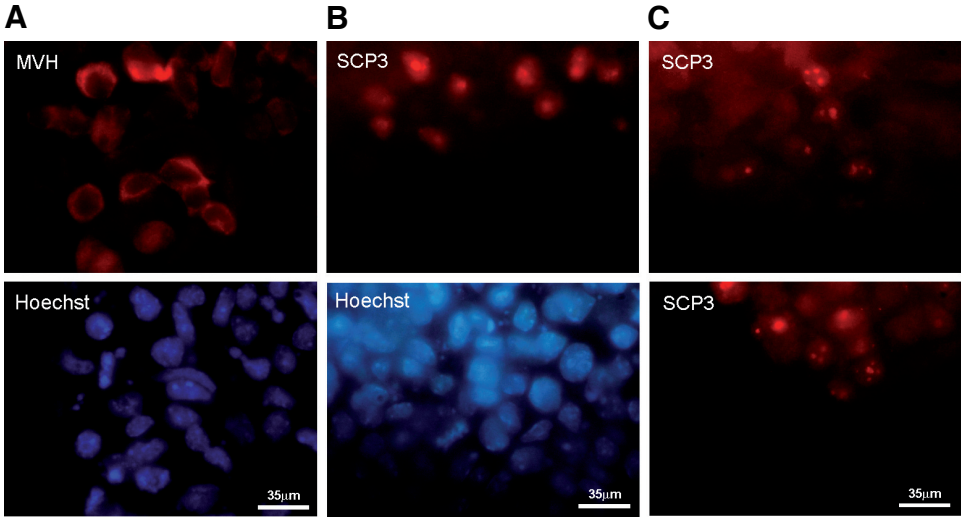

Fig. 2. Representative immunofluorescence for MVH (A) and SCP3 (B,C) performed on ESC monolayers after 10 days of culture. *Nuclear staining with Hoechst is also shown. Similar results were obtained from ESCs cultured for 12 days.*

it has also been reported (West *et al.*, 2008) that when hESCs are cultured for 16 days onto mouse embryonic fibroblast (MEF) monolayers in medium supplemented with basic fibroblast growth factor (bFGF), 90% became positive for MLH1 and SCP3 protein and staining was localized to the nucleus. Finally, in monolayers of XX or XY hESCs cultured for 7 days, Kee *et al.* (2009) reported the presence of putative PGCs with punctuate (resembling preleptotene/leptotene stages) or elongated (considered zygotene/pachytene/diplotene stages) SCP3 nuclear staining in the range from 3 to 27% and 0-10%, respectively, but only after inducing *deleted in azoospermia (Daz), deleted in azoospermia-like (Dazl)* and/or *Boule* over expression.

The lack of crucial information about the meiotic capability of the ESC-derived putative PGCs and the recent development of an *in vitro* culture method in which the majority of the mouse PGCs isolated from the gonadal ridges of 11.5-12.5 dpc embryos enter and progress through meiotic prophase I stages in the absence of somatic cells (Farini *et al.*, 2005), prompted us to precisely evaluate the capability of the putative PGCs produced from mouse ESC monolayers to enter and progress through the stages of meiotic prophase I.

Results

In a first series of experiments, we sought to reproduce the process of PGC formation from mESCs following the protocol used by Hübner and coll. (2003). To this aim, we used the D3-ESC and analyzed the expression pattern of two ESC and PGC markers, the surface antigen SSEA1 by fluorescence-activated cell sorting (FACS) and the enzyme alkaline phosphatase (APase) by cytochemistry (Donovan and de Miguel, 2003). Such analyses were performed from the beginning of culture up to 12 days under spontaneous differentiation of ESCs as monolayers. In addition, during the same period, we studied by immunofluorescence (IF) the expression of the transcription factors OCT4 and NANOG, also expressed both in ESCs and PGCs (Elliot *et al.*, 2007) and of MVH and SCP3, two proteins specific for pre-and meiotic PGCs (Tanaka *et al.*, 2000; Di Carlo *et al.*, 2000). In line with several other works (Hübner *et al.*, 2003; Wei *et al.*, 2008; Nicholas *et al.*, 2009; West *et al.*, 2009; Haston *et al.*, 2009), we observed that the number of cells positive for SSEA1, APase, OCT4 and NANOG undergo a progressive decrease during the culture time although with different kinetics (Figs. 1-2). While at the beginning of culture 90-100% of cells were positive for all markers, at the end of the culture period examined (12 days), only about 20% of the cells expressed these markers. On the contrary, the number of cells expressing MVH increased from less than 5% to approximately 20%. Cells showing SCP3 appeared around 3 days of culture and from then onward represented about 7% of the cultured cells. These analyses together with the results reported in previous works reported above allowed considering the SSEA1+ cells after 7-12 days of culture or a subpopulation within them as putative PGCs.

The phenotype of such putative PGCs was further characterized by immunoisolation of SSEA1+ cells from D3-ESC monolayers at 7 and 12 days of culture and carrying out immunofluorescence on single cells for OCT4, APase, NANOG, MVH and SCP3. The results showed that at 7 days of culture the majority of the SSEA1+ cells were also APase+ (70%±7) and OCT4+ (60%±5), while only a subset of these cells were NANOG+ (33%±5) and

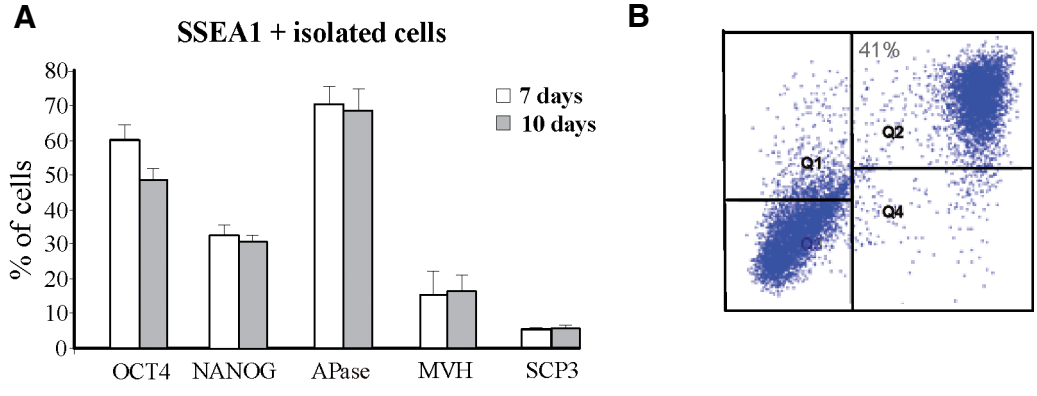

Fig. 3. Characterization of putative PGCs (pPGCs) after 7-10 days of culture. (A) *Expression of OCT4, NANOG, APase, MVH and SCP3 protein in SSEA1+ cells immunoisolated from the ESC monolayer by TG-1 after 7 and 10 days of culture. The evaluation was performed with IF in cell samples attached to poly-l-lysine-coated slides.* **(B)** *A representative FACS analysis of Oct4-GFP ES-OG2+ cells after 10 days of culture for SSEA1 positivity. Note that the most part of gcOct4-GFP+ cells are also SSEA1+ (Q2). Similar results were obtained from putative PGCs isolated from ESCs cultured for 12 days.*

MVH+ (15%±10); less than 10% of the SSEA1+ cells were stained for SCP3 (Fig. 3A). Similar results were obtained at 12 days of culture (not shown).

Parallel experiments carried out under the same culture conditions using the Oct4-GFP ES-OG2 cells used by Hübner and coll. (2003), showed expression pattern of the *gcOct4* transgene similar to that of endogenous OCT4 reported above (not shown) and almost complete coexpression of the transgene and SSEA1 marker after 10-12 days of culture (Fig. 3B).

All these results are consistent with the notion that at 7-12 days of culture around 20% of SSEA1+ putative PGCs are present among cultured ESCs and a significant fraction of them, approximately 10%, express premeiotic MVH+ SCP3+ proteins. We reasoned that by isolating the SSEA1+ or OCT4+ putative PGCs from the culture at 7-12 days and maintaining them under suitable culture conditions previously used with endogenous PGCs immunoselected with antibodies against SSEA1 from gonads (Farini *et al.*, 2005), we should be able to induce those at earlier stages of differentiation (MVH- and SCP3-) to progress at premeiotic stages and those about to begin meiosis (MVH+ and SCP3+) to enter and progress through the meiotic prophase I stages. In fact, the capability to enter into meiosis in response to RA is normally acquired by PGCs around 12.5-13-5 dpc about 7 days after their specification and is associated to MVH and SCP3 expression (Toyooka *et al.*, 2000; Di Carlo *et al.*, 2000). *In vivo*, SCP3 expression is first detected as punctuate nuclear staining at meiotic leptotene. Subsequently, SCP3 localizes along the entire length of the sister chromatids as part of the lateral element of the synaptonemal complex (Di Carlo *et al.*, 2000). PGCs at early stages of differentiation can acquire the capability to enter meiosis following a similar timeline outside the gonadal ridges in culture (Chuma and Nakatsuji, 2001; McLaren and Southee, 1997).

Evaluation of the percentage of cells showing meiotic nuclear SCP3 staining in cytospreads of putative PGCs and endogenous PGCs immunoselected with antibodies against SSEA1 from the ESC monolayers at 7-12 days or from 12.5 dpc gonads, respectively, at the beginning and after 2-5 days of culture in the Farini's medium (Farini *et al.*, 2005) is reported in Fig. 4A. It appeared that while during this culture period the number of SCP3+ cells in endogenous PGCs increased markedly, it remained nearly constant in putative PGCs. Moreover, the nuclear distribution of SCP3 in putative PGCs was always highly abnormal and not clearly associated with chromosomes as instead observed in control PGCs in the most part of which various apparently morphologically normal prophase I stages were recognizable (Fig. 4B). Other markers used to determine entering (DMC1 and γH2AX) and progression (SCP1) into meiosis also showed abnormal or no expression. In particular, putative PGCs in culture showed only a few foci of DMC1 and small spots of cH2AX staining instead of the numerous DMC1 foci and the large, cloud-like regions of γH2AX labelling observed in the nucleus of meiotic PGCs; no SCP1 staining, indicative of the zygotene stage, was detectable in the putative PGGs (Fig. 5).

Putative OCT4-GFP+ PGCs isolated from Oct4-GFP ES-OG2 by FACS also showed the same features of impaired meiotic competence (data not shown).

Discussion

While it is unquestionable that it is possible to produce *in vitro* cells with some characteristics of sperm and oocytes both from mouse and human ESCs the precise sequence of the events leading to the germ cell development remains elusive. In addition the efficiency of such process is not clear and the functionality of the produced germ cells is often not proven. In fact so far only two papers reported germ cells derived from mESCs able to produce male germ cells at advanced stages of differentiation and to induce apparently normal fertilization and embryo development after injection into oocytes (Nayernia *et al.*, 2006; Geijsen *et al.*, 2003). Since in the embryo gametogenesis begins with the formation of PGCs it is logical to assume that this process should first occur when ESCs differentiate through the germ line. Several recent papers have actually reported convincing evidence that mouse and human ESCs can spontaneously or after stimulation with specific growth factors, namely bone morphogenetic protein 4 (BMP4), give rise to cells showing phenotypic and molecular characteristics of pre-and post-migratory PGCs (see Introduction).

The results obtained in the present

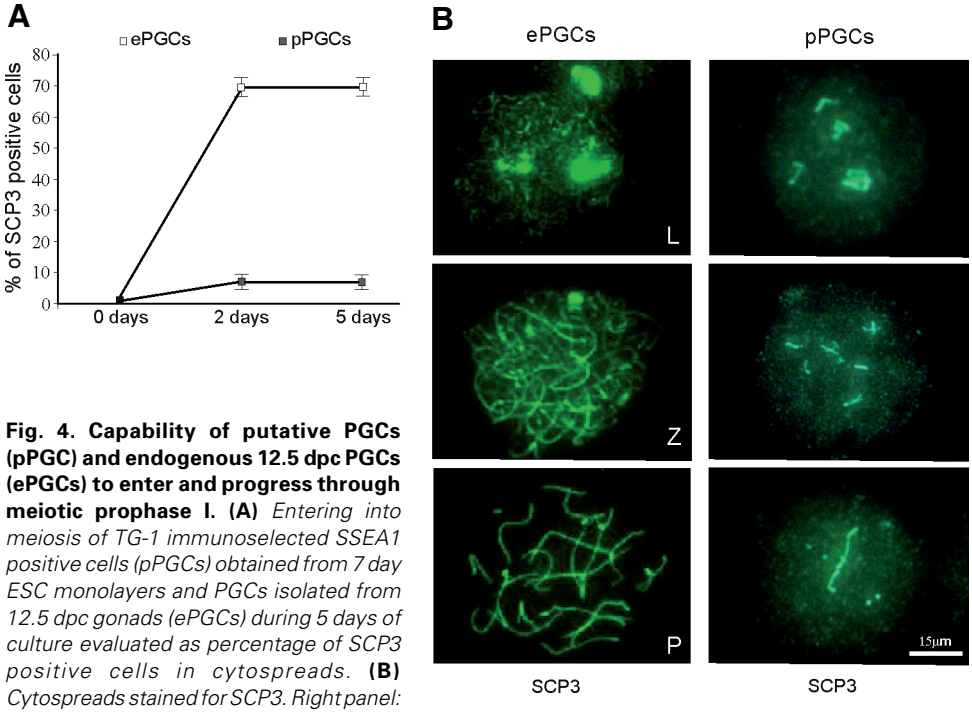

Fig. 4. Capability of putative PGCs (pPGC) and endogenous 12.5 dpc PGCs (ePGCs) to enter and progress through meiotic prophase I. (A) *Entering into meiosis of TG-1 immunoselected SSEA1 positive cells (pPGCs) obtained from 7 day ESC monolayers and PGCs isolated from 12.5 dpc gonads (ePGCs) during 5 days of culture evaluated as percentage of SCP3 positive cells in cytospreads.* **(B)** *Cytospreads stained for SCP3. Right panel: examples of abnormal organization of the synaptonemal complex in pPGCs, left panel: normal synaptonemal complexes in leptotene* (L)*, zygotene (Z) and pachytene (P) prophase I stages in ePGCs. Similar results were obtained from putative PGCs isolated from ESCs cultured for 12 days.*

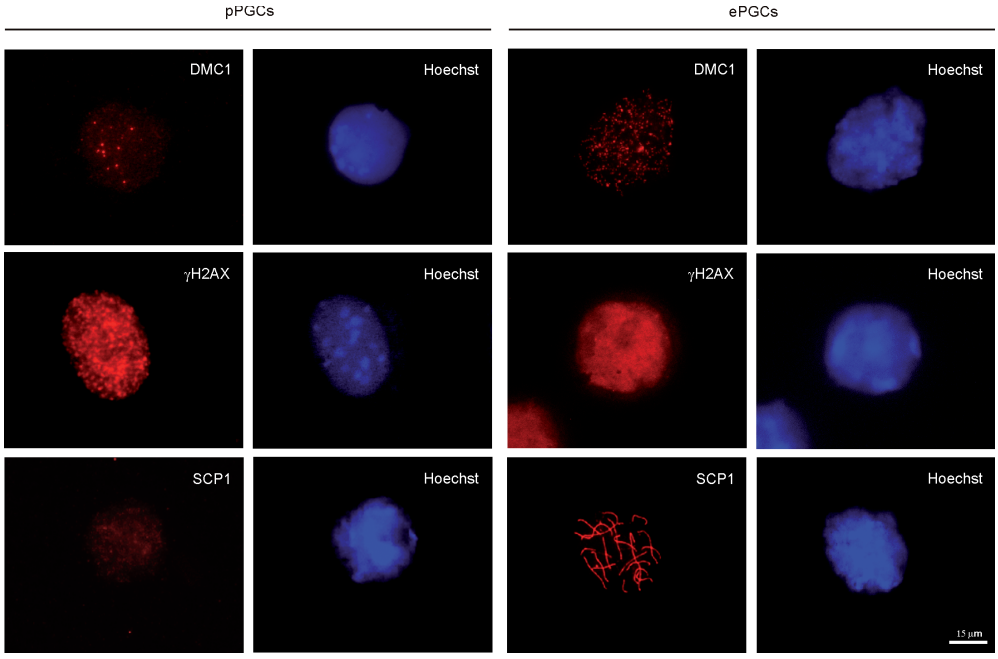

Fig. 5. Cytospread staining for DMC1, cH2AX and SCP1 in putative PGCs (pPGCs) isolated from ESCs after 10-12 days of culture and endogenous PGCs (ePGCs) in culture. *Note abnormal DMC1 and cH2AX staining and absence of SCP1 staining in pPGCs in comparison to ePGCs as described in the text.*

paper are in accord with the reports that putative PGCs can arise from ESC monolayers after relatively short time of culture reflecting timing of PGC formation from the epiblast (Wei *et al.*, 2008; West *et al.*, 2010; Kee *et al.*, 2009). Since the formation of putative PGCs in culture is likely asynchronous it is difficult to properly follow the differentiated status reached by single cells. For this reason, we decided to isolate cells expressing markers of early and pre-meiotic PGCs at 7-12 days and to evaluate their capability to enter and progress through meiotic prophase I stages using cytospreads immunolabeling for meiotic markers including the synaptonemal complex proteins SCP1 and SCP3 and the recombination proteins DMC1 and γH2AX. The results showed that, at this time, putative PGCs originated from cultured ESCs are basically meiotic incompetent or showed severe meiotic anomalies. So far the meiotic competence of ESC-derived putative PGCs has been little investigated likely for the lack of a suitable assay to analyse and quantify such event in comparison to endogenous PGCs isolated from the embryo. Here we report that when exposed to a cocktail of growth factors including RA, considered to be the physiological inducer of meiosis in PGCs (for a review, see Bowles and Koopman, 2007), putative PGCs spontaneously produced from ESC monolayers have a much lower capability to enter and progress through meiotic prophase I *in vitro* in comparison to endogenous PGCs. As reported in the Introduction, human putative PGCs formed in ESC monolayers appear to possess a quite variable meiotic capability ranging from about 3 to 20% but only after over expression of specific genes (Kee *et al.*, 2009). Moreover, we observed in line with previous works carried out in mouse (Novak *et al.*, 2006; Nicholas *et al.*, 2009) and in human ESCs (Clark *et al.*, 2004; Tilgner *et al.*, 2008), a severe impairment in the capability of such putative PGCs to organize a normal meiotic synaptonemal complex. From these and the present results we can speculate that the ability of PGCs to respond to the meiotic induction by RA requires the acquisition of "meiotic competences" not properly or rarely achieved after their formation in the ESC culture systems employed so far. It is known, that endogenous PGCs can acquire such capability outside the gonads following a precise timeline in culture covered by the culture periods used in the present work (Chuma and Nakatsji, 2001).This suggests defective or incomplete PGC differentiation from ESCs under a variety of different culture conditions. In line with this notion, micro-array analyses have shown that many "PGC signature" genes (i.e. *Dazl*, *Mvh* and *developmental pluripotency-associated protein 2, Dppa2*) were not fully up-regulated in putative PGCs obtained from *Mvh-LacZ* or *Mvh-GFP* knock-in mouse ESCs (Mise *et al.*, 2008).

Despite the present results, we cannot exclude that some cells able to enter meiosis were undetected in our assay or will enter meiosis after longer period in culture or if transferred into a favourable environment. In this regard, strong evidence exists that both mouse and human ESCs can give rise to haploid germ cells resembling spermatocyte/spermatides or even tailed sperm after relatively long culture time (mouse:Toyooka *et al.*, 2003; Geijsen *et al.*, 2003; Najernia *et al.*, 2004 and 2006; Kirkis *et al.*, 2007; Yu *et al.*, 2009; human: Tilgner *et al.*, 2008; Aflatoonian *et al.*, 2009) Thus suggesting that PGCs produced in ESC culture more frequently differentiate towards male rather female germ line characterized by entering into meiosis only at later postnatal developmental stages. Considering only the more convincing results obtained in the mouse, it appears that efficient differentiation of the ESC-derived germ cells through advanced spermatogenic stages requires transplantation into a favorable testicular environment and does not necessitate stage-matched testicular cells. Interestingly, these characteristics also belong to endogenous PGCs which from as early 8.5 dpc have been reported to colonize the testis of infertile newborn mice and enter spermatogenesis (Chuma *et al.*, 2005).

How our observations may conciliate with the studies reporting the formation of oocyte-like cells in the ESC culture? None of these studies clearly documented the presence of meiotic PGCs preceding the appearance of the oocyte-like cells, nor the functionality of these latter. Moreover, the frequency of formation of the oocyte-like cells was very low or not reported. In fact, although oocyte-like cells enclosed within follicle-like structures were observed by Hübner and coll. (2003) after about 1 month of culture of D3-ESCs, the frequency, the meiotic stage and functional data concerning these cells were not provided. Lacham-Kaplan *et al.* (2006) reported that EBs cultured in the conditioned medium derived from newborn mouse testis germ cells developed ovarian

structures containing unspecified number of putative oocytes; the oocyte-like cells were surrounded by one to two layers of flattened cells and did not have a visible zona pellucida; oocyte-specific markers, such as *Figα* and *Zp3*, were expressed by the ovarian structures. The same two papers cited above showing the formation of tailed sperm reported the presence of oocytes-like cells in their mouse ESC culture systems (Kirkis *et al.*, 2007 and Yu *et al.*, 2009). Outgrowth of 4 day EB cells containing putative PGCs onto newborn ovarian granulosa cells formed a few oocyte-like cells 25μ in size after 10 days of culture (Qing *et al.*, 2007). These cells expressed some oocyte specific genes such as *Figα*, *Gdf9* and *Zp1-3*, but showed abnormal SCP3 staining in the cytoplasm. In ESCs stably transfected with the promoter region of the mouse *Gdf9* coupled to enhanced green fluorescent protein (eGFP) and cultured on feeder cell layers, large eGFP-positive cells, surrounded by a structure resembling a zona pellucida appeared transiently and very rapidly on the first days of culture. However, in EB cultures, eGFP-positive cells appeared transiently and then reappeared in regional clusters after a relatively long culture of 30–45 days (Salvador *et al.*, 2008). Finally, putative PGCs obtained from 21 day EBs reaggregated with newborn ovarian tissues and transplanted under the kidney capsule of recipient mice give rise to oocytes enclosed in primary follicle at very low frequency (0.023%) (Nicholas *et al.*, 2009). In humans, as far as we know, only two papers reported the occasional formation of oocytes-like cells from ESCs (Chen *et al.*, 2006; Aflatoonian *et al.*, 2009).

The lack of evidence of proper beginning and progression through meiotic prophase I by putative PGCs and the available information about the characteristics of the oocyte-like cells produced *in vitro* raise doubts that continuous female gametogenesis can be correctly reproduced in the present ESC culture systems. In fact, even PGCs isolated from the embryo as early as 10.5 dpc although able to enter and progress through meiotic prophase I outside the gonad are unable to reach the growing oocyte stage (Chuma and Nakatsuji, 2001; Farini *et al.*, 2005). This can be achieved by isolated fetal oocytes providing availability of the growth factor kit ligand (KL) and subsequently of follicular cell support (Klinger and De Felici, 2002). Moreover, several studies indicate that synchronized interactions between oocytes and ovarian somatic cells are important to ensure normal ovogenesis (Qing *et al.*, 2008 and references therein).

In conclusion, we postulate that the formation of oocyte-like cells reported in ES culture may bypass the normal entering and progression of the putative PGCs through meiotic prophase I and is occasionally induced only when a contemporary differentiation of follicular-like cells from the surrounding ESCs occurs.

Finally, two recent papers reported that putative PGCs can be formed *in vitro* with high efficiency from epiblast and epiblast stem cells (EpiSCs), the cell type from which endogenous PGCs originate (see Introduction) (Ohinata *et al.*, 2009; Hayashi and Surani, 2009). Such PGCs appear to posses gene expression and epigenetic signature very close to endogenous migratory PGCs. While the meiotic competence of the epiblast-derived cells were not investigated, EpiSC-derived PGCs after 7 days of culture form undetermined number of SCP3+ cells when co-cultured with 12.5 dpc ovarian cells and oocyte-like after about 40 days of culture at low frequency (around 0.02%).

All together these results demonstrate a robust spontaneous or inducible formation of *bona fide* PGCs from ESCs but a relatively low or defective formation of germ cells in the culture dishes at later stages of differentiation mainly in the female germ line. Here we show that such defect may originate from abnormal or incomplete *in vitro* PGC specification relatively to the acquisition of the meiotic competence at the time proper of the female germ line. Recent papers indicate that epigenetic status (Wang and Tilly, 2010) and elevated expression of certain genes (i.e. *Dazl*) (Kee *et al.*, 2009; Lin *et al.*, 2008; Linher *et al.*, 2009) is crucial for the meiotic commitment of PGCs. Such processes could be altered in putative PGCs. One of the major challenges for future studies will be the identification of the molecular basis of the PGC meiotic competence and the compounds and/or conditions able to induce such competence in PGCs produced from ESCs or epiblast cells *in vitro*.

Materials and Methods

ESC culture

The XY ESC-D3 (ATCC) and the Oct4-GFP ES-OG2 (a kind gift of Prof. H. Shöler, Max Plank Institute, Monster) lines were maintained on mitomycin C-treated MEFs in 0.1% gelatin-coated tissue culture plates in Dulbecco's modified Eagle's medium (D-MEM) containing 4.5 g/l glucose, 100 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 0.25 mM pyruvate, *N*-acetyl-l-cysteine, 75 mg/L penicillin-G, 50 mg/L streptomycin (Sigma) supplemented with 15% fetal bovine serum (Invitrogen) and 1,000 U/ml LIF (ESGRO). Cultures are trypsinized and expanded at a ratio of 1:7 on fresh feeder cells every 48 h, when the colony size reaches approximately 80 to 100 μm in diameter.

ESC differentiation

In order to induce germ cells differentiation of ESCs the protocol described in Hübner *et al.* (2003) was employed. Briefly the ESCs were grown in medium without LIF and MEFs. The ability of MEFs to reattach faster to a tissue culture plate than ESCs was used for MEF removal. After two round of preplating, the cell suspension contains about 98% ES cells and only about 2% MEFs. The ES cells were plated at a density of 1 to $2.5x10^4$ cells/cm^2 in gelatinized 6 cm tissue culture plates in ESC medium without LIF. The medium was replaced on day 3, if the cultures were confluent. Over the next few days the cell layer overgrows, and substantial cell death took place. The medium was then changed every day and care was be taken to remove as many dead cells and debris as possible.

Isolation and culture of putative PGCs

Putative-PGCs (pPGCs) obtained in culture were isolated at different days of differentiation following MiniMACS immunomagnetic method (De Felici and Pesce, 1995). Briefly, the cells were resuspended in D-MEM and TG-1 antibody, which recognize SSEA1 epitope, (1:300, generously provided by P. Donovan, University of California, USA); incubation was carried out with continuous agitation at 4°C. After 45 min, super paramagnetic micro beads suspension conjugated with monoclonal mouse IgM antibody (Miltenyi Biotech) was added. Incubation was carried out for 15 min at 4°C with continuous agitation as described above. Cells were than pipetted on top of an AS Column (Miltenyi Biotech) and placed in a MiniMACS separation unit (Miltenyi Biotech). The column was washed 3 times with D-MEM in the magnetic field and the effluent was collected as SSEA1 negative cells. To elute cells retained by the column (SSEA1-labeled pPGCs), this was removed from the separation unit, placed on a Eppendorf tube, and flushed out with 1 ml of D-MEM using the plug supplied with the column. Isolated pPGCs were seeded onto a Transwell Falcon cell culture polyethylene terephtalate (PET) membrane filter (Falcon) inserted into a 24-well plate tissue culture dish in a total of 0.9 ml

(0.2 ml and 0.7 ml above and below the membrane, respectively) of high glucose D-MEM (Gibco), containing non-essential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM glutamine, 0.25 mM pyruvate, 75 mg/L penicillin-G, 50 mg/L streptomycin (Sigma), 15% fetal calf serum (Gibco), recombinant mouse KL or stem cell factor (SCF), human bFGF, mouse stromal cell-derived factor 1 (SDF1) and human BMP4 (R&D System) and *N*-acetyl-l-cysteine, forskolin and RA (Sigma) (Farini's medium). Growth factors and compounds were added at the time of seeding at the concentration reported in Farini *et al*. (2005) and changed every day of culture (2 or 5 days). Cultures were carried out in a humidified incubator at 37°C and 5% CO_2 in air.

Flow cytometry

Culture plates of differentiating ES-D3 and Oct4-GFP ES-OG2 cells were EDTA-trypsinized 5 min at 37°C, washed and resuspended in PBS with 0.4% bovine serum albumin (PBS-BSA). After filtration through a 40μ cell strainer to remove aggregates, single cell suspensions were incubated for 30 min on ice in PBS-BSA containing monoclonal antibody against the surface protein SSEA1 conjugated with allophycocyanin (APC) or with an APC-isotype control and then rinsed twice in PBS-BSA. FACS analyses or GFP+ cell sorting were performed using Becton Dickinson FACScalibur and FACScan flow cytometers (Becton Dickinson).

Immuno- and APase staining

IF was performed on fixed adherent cells or trypsinized single cells detached from the cell monolayers and left to adhere to poly-l-lysine-coated slides before fixation. All samples were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature and then permeabilized for 10 minutes in PBS/0.1% Triton X-100. After 1 hour in PBS/5% BSA, the cells were incubated overnight at 4°C with the primary antibody to OCT4 (1:300, mouse monoclonal IgGs, SantaCruz), NANOG (1:200, rabbit polyclonal IgGs, SantaCruz) MVH (1:300, rabbit polyclonal IgGs, AbCam), DMC1 (1:200, rabbit polyclonal IgGs, Santa Cruz), γH2AX ($1:10^4$, mouse monoclonal IgG1s,Upstate), SCP1 (1:100, goat polyclonal IgGs, Santa Cruz) or SCP3 (1:100, rabbit polyclonal IgGs, SantaCruz). The next day cells were rinsed three times in PBS and incubated with the appropriate TRITC or FITC conjugated secondary antibodies (1:500 in PBS, AlexaFluor, Molecular Probes) for 45 min in the dark. For nuclear staining, fixed cells were incubated in PBS for 10 min with Hoechst 33349 dye (1 μg/ml). Cells with only secondary antibodies staining were negative controls. APase staining was performed by cytochemistry as reported in Farini *et al*. (2005).

The meiotic prophase I stages were determined by characteristics patterns of SCP3 immunolabeling in cytospreads as described in Farini *et al*. (2005).

Statistical analysis

All experiments were replicates at least three times. The means were tested for homogeneity of variance, and analyzed by ANOVA. The level of significance was set at $P < 0.05$ and $P < 0.01$.

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