Minimal concentrations of retinoic acid induce Stimulated by Retinoic Acid 8 and promote entry into meiosis in isolated pregonadal and gonadal mouse primordial germ cells

Running title: Retinoic acid and meiosis in primordial germ cells

Marianna Tedesco,3 Maria Giovanna Desimio,3 Francesca Gioia Klinger, Massimo De Felici, and Donatella Farini2
Department of Biomedicine and Prevention, Section of Histology and Embryology, University of Rome “Tor Vergata”, Rome, Italy

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2 Correspondence: Donatella Farini; Department of Biomedicine and Prevention, University of Rome “Tor Vergata”, via Montpellier 1, 00133, Rome, Italy. E-mail: donatella.farini@uniroma2.it

3 These authors equally contribute to the work.

ABSTRACT
In the present study, we demonstrate that minimal concentrations of retinoic acid (RA) (≤1 nM), equivalent to the quantity contaminating serum-containing culture media, are sufficient to promote meiotic entry and progression through meiotic prophase I (MPI) stages in isolated 12.5 days postcoitum (dpc) XX and XY mouse primordial germ cells (PGCs) in culture. Similar very low RA concentrations were found to up-regulate or induce stimulated by retinoic acid 8 (Stra8) in such cells, both at mRNA and protein level. In preleptotene/leptotene germ cells, STRA8 was localized in nuclear dots that disappeared at later MPI stages. Besides Stra8, other meiotic genes such as Dmc1 and Rec8 appeared stimulated by RA directly in PGCs with similar concentration-dependent trends. Finally, we found that RA induced Stra8, Sycp3, Dmc1 and Rec8 transcripts promoting meiotic entry in culture also in pregonadal 10.5 dpc PGCs of both sexes. When cultured isolated from somatic cells, such PGCs, however, were unable to progress through MPI stages while they, after entering meiosis, progressed through MPI when cultured within aorta/gonad/mesonephros tissues. We conclude that, beside RA, germ cell intrinsic factors and other exogenous signals from the surrounding somatic cells are probably necessary for meiotic entry and progression in mouse PGCs.

Summary sentence: Minimal retinoic acid concentrations promote entry into meiosis in pregonadal and gonadal PGCs.

Key words: Meiosis, Retinoic acid, Primordial Germ Cells, STRA8

INTRODUCTION
One major decision that germ cells take during embryonic development after reaching the gonadal ridges is to enter meiosis or undergo mitotic arrest in G1/G0. In mammals, this event is independent of the sex chromosome constitution of the germ cells and implies a control by the somatic cells in the developing gonads. In the mouse embryonic ovary, around 13.5 days post coitum (dpc), primordial germ cells (PGCs) shift from mitosis to meiosis entering into the meiotic prophase I (MPI) as primary
oocytes in an anterior to posterior direction [1, 2]. Conversely, at the same time, in the embryonic
testis, XY PGCs exit from the cell cycle and undergo G1/G0 arrest becoming prospermatogonia (for a
review see [3]).

The mechanism/s by which, in mammals, PGCs switch from mitosis to meiosis or undergo cell cycle
arrest have been subject of considerable debate. One of the most debated points has been whether entry
into meiosis is a cell autonomous process, perhaps regulated by an intrinsic molecular clock, or if this
event is actively induced in the ovary and prevented in the testis by surrounding somatic tissue through
a meiosis-inducing (MIS) or preventing (MPS) substance, respectively.

In recent years, results from several laboratories have identified retinoic acid (RA) as the putative long-
sought-after MIS, able to trigger the onset of meiosis in both XX and XY germ cells [4-9]. In the
mouse embryo, around 12.5 dpc, RA appears to be synthesized in the mesonephros of both XY and XX
embryos and to diffuse into the adjacent developing gonad [4]. From 12.5 dpc onward, the ovary also
seems able to synthesise RA [10]. At 11.5 dpc, Cyp26b1, the gene encoding an enzyme that
metabolizes RA, is expressed in the gonadal ridges of both sexes [4]. While thereafter the expression
and the activity of this enzyme disappears in the ovary, they remain high in 13.5 dpc testes, when XY
germ cells are arrested into G0 [4, 5]. Therefore, CYP26B1 could correspond to MPS. In fact, when
Cyp26b1 is ablated or the enzyme activity inhibited in the embryonic testis, mouse PGCs seem to enter
into meiosis [4, 11]. The notion that RA might act as MIS could explain the apparently cell autonomous
entry into meiosis of PGCs of both sexes in ectopic embryonic sites or under certain culture conditions,
which formed the basis of the intrinsic clock theory. In fact, it seems possible that these PGCs enter
meiosis because of exposure to RA present in many embryonic tissues or in the serum which is
normally added to the PGC culture media [12]. Some authors, however, have criticized the role of RA
as MIS observing, for example, that XY mouse PGCs exposed for extended periods in vitro (6-7 days)
to RA or to the CYP26B1 antagonist ketoconazole, undergo apoptosis rather than meiosis [13].
Moreover, against the pro-meiotic action of RA, Kumar et al. [14] studying meiosis in fetal ovary from
mice null for Raldh2 and Raldh3, the enzymes that produce RA, observed that XX PGCs were still
able to enter meiosis. Recent works showing that in the mouse, besides RA also RSPO1/β–catenin
signalling is involved in promoting XX PGC entering into meiosis [15] and that Nodal/activin
signalling is required to suppress meiosis in XY PGCs [16, 17] support the view that the meiosis entry
in mammals is not simply controlled by RA availability.

Stimulated by retinoic acid 8 (Stra8) has been proposed as one of the major protein target of RA
promoting the meiotic entry in germ cells [18, 19]. In line with this notion, in XX mice, Stra8
expression was shown to be indispensable for PGCs to engage the meiotic S phase; in its absence, adult
XX are sterile because oocytes are blocked in the preleptotene stage of meiosis [19]. In XY germ cells
after birth, Stra8 appears to be important other than for the onset of meiosis [19, 20], also for meiotic
progression. In fact, in adult testes, even if meiosis seems to begin normally in Stra8⁻/⁻ XY germ cells
they became arrested at later stages of MPI [21].

Although these results give support to an important role of STRA8 during MPI in both XX and XY
germ cells, they indicate that this protein might play different roles in such a process and do not clarify
which such role/s are. The controversies and the many uncertainties concerning the effect/s and
function/s of RA on PGC development and the scant information about the actions of STRA8 at the
beginning of meiosis (for a review see [22]), prompted us to re-evaluate the role of RA in the mitosis/meiosis switch and as inducer of Stra8 in mouse PGCs from pregonadal and gonadal stages.

MATERIALS AND METHODS

PGC isolation and culture

All experiments were carried out in compliance with accepted standards of humane animal care and with the approval of relevant national (Ministry of Welfare) and local (Institutional Animal Care and Use Committee, Tor Vergata University, Rome, Italy) committees. Pregnant CD-1 XX were sacrificed by cervical dislocation at different days post coitum (dpc), and their embryos removed. The Aorta/Gonad/Mesonephros (AGM) region (10.5 dpc), gonadal ridge (GRs; 11.5 dpc) and embryonic ovaries and testis (12.5-14.5 dpc) were dissected from the embryos and freed from the surrounding tissue in Minimal Essential Medium (MEM) containing Hepes-BSA (MEM-Hepes-BSA) (Lonza). Determination of AGM sex was carried out as previously described [23]. For organ culture, AGMs were transferred in D-MEM-F12 (Lonza) with the addition of 0.4 mg/ml BSA, 0.25 mM pyruvate, 0.5 mg/ml N-acetyl-l-cysteine, (Sigma-Aldrich) and maintained under agitation, and changing the medium every day. In most of the experiments, PGCs were purified using the MiniMACS immunomagnetic sorting method [24] and cultured according to a method previously described by Farini and coll. [23]. The culture medium (CM) was D-MEM-high glucose (Lonza), containing non-essential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM glutamine, 0.25 mM pyruvate, N-acetyl-l-cysteine, 75 mg/L penicillin-G, and 50 mg/L streptomycin (Sigma-Aldrich). CM was supplemented with recombinant murine KL (25 ng/ml, Società Italiana Chimici, Italy), mouse bFGF (10 ng/ml, Società Italiana Chimici, Italy), rmCXCL12/SDF1α (10 ng/ml, R&D System) and rhBMP4 (25 ng/ml, R&D System) and forskolin (5μM, Sigma-Aldrich); this medium was named growth factor (GF)-CM. When indicated, 15% Fetal Bovine Serum (FBS; Lonza) was added to the medium and in this case it is indicated as FBS-CM or FBS-GF-CM. Retinoic Acid (RA, Sigma-Aldrich) and the retinoic acid receptor (RAR) antagonist RO 41-5253 (Biomol International) were dissolved in ethanol and stored at -20°C. Ethanol was always added to CM at maximal 1:1000 dilution. Growth factors and compounds were added at the time of seeding and the medium changed every day of culture. Cultures were carried out for the indicated times in a humidified incubator at 37°C and 5% CO2 in air.

Embryonal Stem (ES) and Embryonal Carcinoma (EC) cell culture

E14Tg2a mouse ESCs (kindly provided by Dr. T. Russo, Ceinge Biotecnologie Avanzate, Naples, Italy) were maintained on feeder-free, gelatin-coated plates in the following medium: D-MEM (Lonza) supplemented with 2 mM glutamine, 100 U/ml penicillin/streptomycin, 1 mM sodium pyruvate, 1× nonessential amino acids (all from Invitrogen), 0.1 mM β-mercaptoethanol (Sigma Aldrich), 10% FBS (HyClone Laboratories), and 10^3 U/ml LIFES (Società Italiana Chimici). EC cells P19 (ATCC CRL-1825) were cultured in D-MEM-high glucose (Lonza) supplemented with 2 mM glutamine, 100 U/ml penicillin/streptomycin (Sigma Aldrich) and 10% FBS (HyClone Laboratories).

Gene expression

Total RNA was extracted from the cell samples using the RNasy microkit (Qiagen). Starting from 200-500 ng of RNA, first-strand cDNA synthesis was performed with a quantiTect reverse transcription kit (Qiagen). For quantitative gene expression (qRT-PCR), 10 ng of cDNA was amplified with the KAPA SYBR FAST qPCR kit (Kapa Biosystem) in accordance to the manufacturer's instructions on an ABI PRISM 7300 Sequence Detection System (Apply Biosystem). Cycling was
performed using the default conditions of the ABI 7300 SDS Software 1.3: 2 min at 95°C, followed by
38 cycles of 15 sec at 95°C, 30 sec at 60°C and 30 sec at 70°C. Results were normalized to Mouse vasa homolog (Mvh) or Gapdh levels and the experiments were repeated 3 times. For PCR amplification,
10-30 ng of cDNA and PCR Master Mix (Fermentas) were used as indicated in the manufacturer's
instructions. The primers indicated in Supplemental Table S1 (all Supplemental Data are available
online at www.biolreprod.org) were designed with Primer 3 software and cover different exons.
Densitometric analysis of the bands was performed using Image Quant Software (Molecular Dynamics)
and normalized to Gapdh levels.

Immunostaining
For immunofluorescence in a total cell suspension, AGM, GRs or embryonic gonads were digested for
5 min Trypsin/EDTA solution (Lonza) and mechanically desegregated in a monodispersed cell
suspension. Cells were then cultured for 30 min at 37°C and 5% CO₂ in air in CM and samples of cells
remaining in suspension were collected and transferred onto poly-l-lysine-coated slides and maintained
for 5 min at room temperature (RT) in the same way as for purified PGCs. Cells attached to the slides
were fixed with 4% paraformaldehyde in phosphate-buffered-saline (PBS) (Lonza) for 10 minutes and
then permeabilized for 10 minutes in PBS with 0.1% Triton X-100 (Sigma-Aldrich) at RT. After
1 hour at RT in PBS plus 5% BSA, 1: 400 rabbit polyclonal antibody against STRA8 (Ab49405,
Abcam) and 1:10 rat IgM monoclonal anti-GCNA-1 antibody (specific for gonadal cells, a kind gift
from Dr. G. Enders, University of Kansas, Kansas, USA) in PBS plus 0.5% BSA were added for
overnight incubation at 4°C. Fluorescein isothiocyanate (FITC)-conjugated 1:500 goat anti-rabbit (Alexafluor488 or 568, Molecular Probes) and goat anti-rat IgM (Pierce Endogen) were used as
secondary antibodies for 1 hour incubation at RT. Samples were visualized under a Leica CTR600
microscope with a 40X objective.

Cytospread preparations.
Cells which adhered onto poly-l-lysine-coated slides were incubated in hypotonic solution containing
0.05% Triton X-100 for 10 min at RT and then in 2% formaldehyde with 0.02% SDS for 1 h at 37°C.
After extensive washes with distilled water, spread preparations were frozen at -80°C or directly used
for immunofluorescence analysis. In order to perform SYCP3 and γH2AX immunolabeling or STRA8
localization, cell samples were incubated in PBT (0.15% BSA, 0.1% Tween-20 in PBS) for 60 min at
RT. They were incubated overnight at 37°C with primary antibodies diluted in PBT [1:500 rabbit,
(NB300-231 Novus) or mouse (sc74569 Santa Cruz Biotechnology) anti-SYCP3 antibodies, 1:400
STRA8 antibody, 1:10 anti-GCNA-1 antibody, 1:12000 mouse (05-636 Upstate) anti-phospho-Histone
H2A.X antibody]. Slides were then incubated for 45 min with secondary antibodies (Alexafluor488 or
568, Molecular Probes) diluted to 1:500 in PBT. Nuclei were labelled for 5 min at RT with Hoechst
33349 (1 µg/ml). Samples were visualized under a Leica CTR600 microscope with a 100X objective.
In some experiments, XY germ cells obtained from 10 days postpartum testes [25], P19 EC and
E14Tg2A ES cells were used.

Western blot analyses
12.5 dpc XY PGCs purified from 20 testes and cultured for 24 h in FBS-GF-CM were lysed in 1X SDS
sample buffer. Proteins were separated on 10% SDS-PAGE gels and transferred to PVDF Transfer
Membrane Hybond TM-P (Amersham). The membranes were saturated with 5% non-fat dry milk in
PBS-0.1% Tween20 for 1 h at RT and incubated o/n at 4°C with 1:1000 anti-STRA8 antibody, 1: 500
rabbit (ab13840 Abcam) anti-MVH antibody and 1:1000 rabbit (A2066 Sigma-Aldrich) anti-ACTIN antibody. Secondary anti-rabbit IgGs conjugated to horseradish peroxidase (Amersham) were incubated with the membranes for 1 h at RT, at 1:10000 dilution in PBS-0.1% Tween20. Immunostained bands were detected by the chemiluminescent method (Santa Cruz Biotechnology). Densitometric analysis of the bands was performed using Image Quant Software (Molecular Dynamics) and normalized to ACTIN levels.

**RA reporter cell line culture**
A line of F9 teratocarcinoma cells, stably transfected with the β-galactosidase gene under control of the RARE from the RARβ gene (S12) (a kind gift of Prof. McCaffery, University of Aberdeen, UK) was used to detect RA concentrations in the culture media. Briefly, the cells were grown at confluence into gelatine-coated 96 well dishes in D-MEM plus 10% FBS. After overnight incubation in D-MEM without serum, the cells were cultured for 24 h in a medium in which RA concentration was to be determined. Following the manufacturer’s instructions, β-galactosidase activity was quantified by a staining kit purchased from Active Motif (cat. 35001).

**Statistical analysis**
All experiments were replicated at least three times. The means were tested for the homogeneity of variance and analysed by ANOVA.

**RESULTS**

Minimal RA concentrations (≤ 1nM) present in FBS-containing culture media are sufficient to induce 12.5 dpc PGCs to enter meiosis and progress through prophase I stages.

We have previously demonstrated that mouse XX and XY PGCs isolated from 11.5 dpc gonadal ridges (GRs) and 12.5 dpc gonads and cultured in a medium containing 15% FBS and a cocktail of soluble growth factors (GFs), namely KL, BMP-4, SDF-1, bFGF and compounds (N-acetyl-L-cysteine, forskolin and RA), were able to enter meiosis and progress through meiotic prophase I (MPI) stages in vitro in the absence of somatic cell support [23]. In the present paper, using the same culture system, we first investigated whether RA was actually required for PGCs to enter and progress through meiosis and in this case at which concentrations. We evaluated the percentage of 12.5 dpc XX or XY PGCs entering the leptotene stage and proceeding through MPI stages during 2 or 4 days of culture in the presence or absence of exogenous RA using double immunostaining for the germ cell marker Germ Cell Nuclear Antigen 1 (GCNA-1) and the meiotic chromosome marker Synaptonemal Complex-3 (SYCP3) in cytospread preparations [23, 26]. Besides cells at leptotene, the entry into meiosis was also assessed by double immunostaining for SYCP3 and γH2AX, a marker of the sites of DNA double-strand breaks occurring between meiotic preleptotene and leptotene [27] (Supplemental Fig. S1). The results showed that after 2 days of culture in FBS-GF-CM or in FBS-GF-CM plus 1 μM RA, the percentage of meiotic cells at leptotene or in other more advanced MPI stages was basically the same (Fig. 1A). The percentage of meiotic cells was, however, significantly lower in XY than XX PGCs (60±8.0 XY vs 80±4.5 XX; p<0.05) (Fig. 1A). Under these culture conditions, but in the absence of FBS after 2 days of culture, the survival of isolated PGCs as expected [23], was quite low (trypan blue assay, around 30%) and none cells at the letpotene stage were observed (data not shown). In order to verify whether the meiotic behaviour of PGCs in FBS-GF-CM could be due to the presence of RA contaminating the serum [15, 28, 29], we added RO 41-5253, a potent antagonist of RA receptors (RARs) [30] to this culture medium. As shown in Fig. 1A, 5 μM RO 41-5253 almost completely
abolished entry into meiosis of both XX and XY PGCs. No evident effect of the antagonist on germ-cell viability was observed (trypan blue assay, not shown).

By analysing the meiotic stages of germ cells cultured up to 4 days in FBS-GF-CM (Fig. 1B), we then observed a higher percentage of more advanced stages of meiosis in XX than in XY cells. In both sexes however, the kinetics of meiotic progression was slower in comparison to the in vivo condition (Supplemental Fig. S2). Like for meiotic entry at 2 days, the addition of 1µM RA did not affect the percentage of germ cells at different meiotic stages (Fig. 1C).

In order to determine the concentration of RA present in FBS-GF-CM additioned with 15% FBS, we used F9-RARE-lacZ cells, a cell line which is stably transfected with RA inducible response element (RARE) driving the lacZ gene [31]. The sensitivity of this reporter is in the range of 10^{-13} and 10^{-7} M RA, with a relatively linear dose-response curve [31]. Using this assay, we confirmed that RA was present in the FBS batch additioned to CM and estimated a concentration of RA around ≤ 1nM both at the beginning or after 24-48 h of PGC culture (Fig. 2).

In vivo and in vitro expression of Stra8 in germ cells and its localization in nuclear dot domains

Stra8 is considered a major target of RA in premeiotic germ cells [18, 32, 33]. Most of the data regarding the control of Stra8 expression by RA in germ cells have been, however, obtained only at RNA level [4, 32, 33]. Therefore, we decided to analyse the expression of STRA8 protein by immunocytochemistry and Western blotting in germ cells in vivo between 12.5 dpc and 16.5 dpc and in vitro following stimulation with different RA concentrations. In line with our previous results [34], immunocytochemistry on single cells showed that while in XY germ cells freshly collected from embryonic gonads STRA8 was undetectable at all stages analysed (Fig. 3B and data not shown), in XX germ cells, it was expressed in a developmental and meiotic stage-dependent manner (Fig. 3). The first faint STRA8 positivity was observed in a few XX PGCs at 12.5 dpc (about 1%), while about 30-40% of 13.5 dpc XX germ cells were clearly STRA8 positive with variable staining intensities (Fig. 3A, B); at 14.5 dpc, the majority of XX germ cells expressed STRA8 (82 ±14%) while at 15.5 dpc, the percentage sharply declined (38 ±12%) to approximate 1% at 16.5 dpc (Fig. 3A).

Double staining for STRA8 and SYCP3 in the same cell by the cytospread method made it possible to precisely localize STRA8 in the germ cell nucleus at different meiotic stages. We observed that in XX PGCs at the preleptotene (Supplemental Fig. S3) and leptotene stage, STRA8 was concentrated up to 15 dots per nucleus (Fig. 3C); at the mid zygotene, the number of dots decreased to about 4 per nucleus and disappeared at late-zygotene (Supplemental Fig. S3). Similar results were obtained in cytospreads of XY germ cells isolated from 7 days postpartum (dpp) and 10 dpp testis (data not shown). At any meiotic stages of XX germ cells, STRA8 dots did not colocalize with SYCP3 (Fig. 3C, merge), suggesting that such domains are not localized on the synaptonemal complex. Although, we have yet to colocalize STRA8 dots with known nuclear domains, we observed that such localization is probably unique of meiotic cells. In fact, STRA8 dots were not observed in cytospreads of embryonic stem (ES) cells (E14Tg2A) or embryonal carcinoma (EC) cells (P19) stimulated for 24 h with 1 µM RA in which high levels of STRA8 are concentrated in the nucleus [34] (Supplemental Fig. S3).

In 12.5 dpc XX and XY PGCs cultured for 1 day in FBS-GF-CM, variable intensities of nuclear STRA8 staining in about 70% of germ cells were observed (Fig. 4A, E). This percentage appeared
much higher than that of XX germ cells of comparable in vivo age (13.5 dpc) (around 40%) (Fig. 3A and 4E). To verify whether the minimal RA contaminating FBS-GF-CM could be responsible for such increased/induced \textit{Stra8} expression in cultured XX and XY germ cells, respectively, we carried out RA dose-response experiments in purified 12.5 dpc XY PGCs which were likely not previously exposed in vivo to this compound. \textit{Stra8} expression was evaluated with qRT-PCR and the results normalized against the levels of \textit{Mouse vasa homolog (Mvh)}, a gene which at this stage is specifically expressed in germ cells [35]. As shown in Fig. 4B, FBS-GF-CM alone was able to stimulate \textit{Stra8} expression albeit at significantly lower levels than 1 nM RA, the minimal exogenous RA concentration used in the assay. Maximum stimulation was observed between 100 nM and 1\(\mu\)M RA. Moreover, we confirmed that the stimulatory effect of FBS-GF-CM on \textit{Stra8} expression was due to the presence of RA in the serum, since the addition of 5\(\mu\)M RO 41-5253 to the medium completely abolished such effect (Fig. 4C). On the other hand, when 12.5 dpc XY PGCs were cultured for about 18 h in a medium with GFs but without FBS, no significant stimulation of \textit{Stra8} expression was observed (data not shown).

At protein level, the addition of 1\(\mu\)M RA to FBS-GF-CM induced a marked increase of the intensity of the immunostaining both in 12.5 dpc XX (not shown) and XY PGCs (Fig. 4D), without, however, significantly enhancing the number of STRA8 positive cells (Fig. 4E) nor affecting the localization of the protein in the nuclear dots (data not shown). The increased STRA8 expression in PGCs stimulated by 1\(\mu\)M RA was confirmed by Western blotting (Fig. 4F).

RA is able to stimulate the expression of Sycp3, Dmc1 and Rec8 directly in 12.5 dpc PGCs
In order to verify if, besides \textit{Stra8}, RA is able to stimulate the expression of other meiotic genes in PGCs, we analysed by qRT-PCR the expression of Sycp3, Dmc1, Spo11 and Rec8 in 12.5 dpc XY and XX PGCs cultured for 18 h in FBS-GF-CM alone or supplemented with different RA concentrations. The same analyses were also performed in the presence of the RAR antagonist. The results in Fig. 5 show that both in XX and XY PGCs, RA did not affect the expression of \textit{Spo11} while it stimulated the expression of \textit{Dmc1} and \textit{Rec8} in a dose dependent and RO-415253-inhibitable manner. Interestingly, RA stimulated \textit{Sycp3} expression in a dose-dependent manner only in XY PGCs and the RAR antagonist did not affect such stimulation. All stimulated genes showed responsiveness to RA lower than \textit{Stra8} both in XX and XY PGCs (Fig. 5).

RA induces expression of meiotic genes and promotes entry into meiosis but no meiotic progression in isolated pregonadal PGCs
To evaluate if RA is also able to induce \textit{Stra8} and meiosis also in PGCs before entering into the GRs and, in this case, with which kinetics, we cultured PGCs isolated from 10.5 dpc XX and XY AGM in FBS-GF-CM alone or with 1 \(\mu\)M RA for up to 5 days. At 10.5 dpc, the most part of PGCs is considered pregonadal since they have not entered into the GRs yet [36]. After 1 day of culture, we observed significant levels of \textit{Stra8} transcripts in mixed XX and XY PGCs cultured in FBS-GF-CM and a marked increase of such levels after addition of 1 \(\mu\)M RA to CM (Fig. 6A). At this time, about 30% of PGCs cultured in FBS-GF-CM showed evident nuclear STRA8 staining, that disappeared when RO-415253 was added to CM (Fig. 6B). Moreover, a significant number of PGCs appeared able to enter into meiosis in FBS-GF-CM irrespective of the addition of 1 \(\mu\)M RA but only after 3-5 days of culture. In fact, at these times, about 30% of germ cells were at leptotene stage and positive for \(\gamma\)H2AX (Fig. 6C [a-c]). After entering into meiosis, such PGCs were, however, unable to progress...
further through MPI since other meiotic stages beyond leptotene were not detected up to 5 days of culture. Dots of STRA8 were normally observed in the leptotene nuclei (data not shown). Additional analyses showed that in cultured PGCs, Oct4 gene, known to be down-regulated by RA in stem cells [37] and at the beginning of meiosis in the fetal ovary [1], was rapidly down-regulated (Fig. 6A). Other genes normally associated with gonadal PGCs such as (Mvh [35]; Dazl [38] and meiosis such as Sycp3 [39], Rec8 [40], Dmc1 [41], were expressed in germ cells at significant levels after 5 days of culture (Fig. 6D). In contrast to isolated 10.5 dpc PGCs, XX or XY PGCs of the same age cultured inside AGM for 5 days were able to enter meiosis and to progress up to the zygotene (Fig. 6C [d-f]) and zygotene/pachytene stages (Supplemental Fig. S4), even in CM without FBS, GFs and exogenous RA.

**DISCUSSION**

In the present paper, extending the data by Bowles et al. [42], we report that RA in the range of ≤ 1 nM is able to induce entry into meiosis in vitro by acting directly on isolated PGCs obtained from 10.5 and 12.5 dpc XX and XY mouse embryos. While previous results suggested that by 12.5 dpc XY PGCs are irreversibly committed to cell cycle arrest at G0/G1 [43, 44], our data, in agreement with other studies [9, 45], indicate that at this age, RA is still able to induce meiosis in XY PGCs. The minimal amount of RA able to promote meiosis, was also sufficient to induce significative STRA8 expression in PGCs of both sexes (this paper and Bowles et al., [39], likely for the high affinity binding capability of RARs [46]. In line with Bowles et al. [39], we also show that the induced level of Stra8 transcripts and protein in XY 12.5 dpc PGCs was comparable to that of XX PGCs of the same age. We found that the minimal RA concentration exerting such promeiotic effect and Stra8 stimulation in cultured PGCs may contaminate the culture medium from the added FBS in which isolated PGCs are maintained when cultured for prolonged period. Thus explaining the apparent spontaneous entering of mouse PGCs into meiosis when cultured in serum containing medium [43] or other not defined culture conditions reported in other works [44]. Intriguing, we found that the culture in FBS-GF-CM for 1 day induced STRA8 in the most part of 12.5 dpc PGCs of both sexes (around 70%). This, however, did not accelerate the timing of the meiotic entry (as evaluated by the number of cells positive for γH2AX and at leptotene stage) nor synchronize the meiotic progression in the germ cells of both sexes, during the subsequent days of culture. This was particularly evident in 12.5 XX PGCs in which after 1 day of culture, the number of cells expressing STRA8 was significantly higher than that of PGCs of comparable in vivo age (13.5 dpc) (around 70% vs 30-40%) but no increasing meiotic rate and synchronization of the meiotic stages after 2-4 days of culture was observed. The precocious induction of STRA8 in 12.5 dpc XX PGCs in culture might be due to the loss of the anterior-posterior ovarian gradient of RA flowing from the mesonephros in the embryo [4]. Increasing RA concentration in the culture medium enhanced STRA8 expression in single 12.5 dpc PGCs after 1 day of culture without changing the number of positive cells.

Taken together, these results indicate that very low concentrations of RA are sufficient to promote meiotic entry in mouse PGCs by inducing slight levels of STRA8 only after the acquisition of germ cell meiotic competence. In other words, anticipating the expression of STRA8 or increasing the level of the protein do not anticipate the beginning of meiosis in incompetent germ cells. Changes in epigenetics, chromatin structure and expression of cell cycle controlling factors have been described to precede entering into meiosis in PGCs and can represent components of such meiotic competence [47-49]. The finding that minimal RA concentrations needed to stimulate the expression of Stra8 and other meiotic genes as well, at levels sufficient to induce XY PGCs to begin meiosis supports the notion that
the prevention of this crucial process in such cells is not solely regulated by RA availability. Interestingly, recent results show that in the mouse embryo, Polycomb Repressive Complex 1 (PRC1) bound to Stra8 promoter antagonizes RA signalling preventing premature induction of its expression and entry of PGCs into meiosis [50]. Moreover, additional mechanisms must be present in the fetal testis to prevent meiosis such as the inhibitory action of FGF9 and Nodal/activin [16, 17, 42, 51], the expression of NANOS2 [52] and other proteins that have yet to be identified and characterized [53].

Although in the present paper, we present no data clarifying the role of STRA8 in meiosis, our new observation that the protein is concentrated in nuclear dots specifically during preleptotene/early zygotene stages identifies the period of its crucial action in the nucleus and evidences structural STRA8 domains which are typical of meiosis. In fact, STRA8 dots were not observed in the nucleus of stem cells such as ES and EC cells in which nuclear STRA8 localization is strongly induced by RA [34]. Moreover, dots were not localized in double-strand DNA breaks present at the first stages of prophase I. In fact, the dots were still present in meiotic germ cells obtained from Spol1−/− mice in which the meiotic DNA breaks do not form [54] (data not shown). Likewise, STRA8 dots were not associated to leukemia nuclear bodies (PML) or Cajal bodies (CB) (data not shown) which are known to contain splicing machinery, transcription elongation factors and histone–modifying enzymes [55]. All these negative results do not allow us so far to associate such STRA8 domains to any known structure or defined processes.

So far it has yet to be established if besides Stra8, RA is able to induce other meiotic genes in PGCs. Here we show that RA also exerts a dose-dependent stimulatory effect on Dmc1 and Rec8 transcripts in both XX and XY 12.5 dpc PGCs. This effect is dependent by the RAR activity because it was affected by the antagonist treatment. There are currently no data on the transcriptional control as well as the presence of canonical RARE elements in the promoter of these two genes. Regarding Sycp3, RA is able to stimulate its expression in XY but not in XX PGCs in which Sycp3 is already expressed at high levels. Furthermore, in XY PGCs the Sycp3 stimulation by RA was not dependent by the RAR activity because RO-415253 didn’t change the gene expression levels. This can be explained by the alternative ways by which RA may control transcription including binding to other nuclear receptors [56] or non-genomic effect [57].

Finally, we found that RA was able to induce Stra8 both at mRNA and protein levels and meiotic entry in a significant number (about 30%) of XX and XY 12.5 dpc PGCs isolated from 10.5 dpc AGM, a stage when the most part of PGCs have yet to reach the gonadal ridges [36]. This observation clearly indicates that PGC responsiveness to RA in term of Stra8 induction is present in pregonadal PGCs and is therefore independent of the gonadal environment and sex. Such precocious Stra8 expression, however, did not accelerate the kinetics of meiotic entry since the first leptotene stages in such cultured PGCs were observed only after three days of culture, thus suggesting the need to acquire germ cell meiotic competence as discussed above. Interestingly, pregonadal PGCs although able to engage meiosis and to express genes typical of gonadal germ cells such as Dazl and Mvh and a number of meiotic genes such as Sycp3, Dmc1 and Rec8, after 5 days of culture, appeared unable to progress beyond the leptotene stage. In line with our results, Yokobayashi et al. [50] found that in mouse embryos deficient for PRC1, Stra8 and other meiotic genes, including Rec8 and Sycp3, are precociously activated in XX PGCs and these cells are unable to complete meiosis.
All these results indicate that entering and progression through MPI likely require the function of distinct and successive regulatory factors. Moreover, the observation that 10.5 dpc PGCs of both sexes cultured within the AGM were instead able, during the same culture period, to enter and progress through meiosis like PGCs isolated from 11.5-12.5 dpc (this paper and [23]), suggests that during this time, the somatic environment provides PGCs with the factors necessary to acquire proper meiotic progression capability. Although we did not address the reason of entering into meiosis of XY PGCs within AGM even in the absence of exogenous RA, it is likely that under such culture conditions testis differentiation, in particular the formation of testicular cords, is defective (our unpublished observations and [58]).

The responsiveness of pregonadal mitotic PGCs to RA in term of STRA8 induction could cause cell cycle deregulation in such cells. In this regard, in previous studies, we and others [59, 60] have shown that RA is a potent mitogen for pregonadal mouse PGCs obtained from 8.5-10.5 dpc embryos and one of the factors contributing to their transdifferentiation into the pluripotent and tumorigenic embryonal germ (EG) cells [61, 62]. Interestingly, it has been proposed that dysfunction of the mitotic-meiotic switch, with cells aberrantly co-expressing functions pertinent to both states could provide the genetic instability which causes germ cell tumour development [63, 64]. Further studies are needed to clarify whether a relationship between the inability of the isolated pregonadal PGCs after RA stimulation to enter but not to progress through meiosis and the apparently contemporary mitogenic and transdifferentiation effect of RA on such cells exists.

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FIGURE LEGENDS

FIG. 1. Minimal RA concentrations contaminating FBS are sufficient to promote entry and progression through meiotic prophase I (MPI) in cultured PGCs. A) XX and XY 12.5 dpc PGCs were cultured in FBS-GF-CM with or without 1μM RA and in the presence or not of the RA receptor antagonist RO-41-5253. Cytospreads for SYCP3 and GCNA1 labelling were performed after 2 days of culture. PGCs cultured in FBS-GF-CM showed chromosome assembly typical of early MPI irrespective of addition of RA to the medium. The percentage of meiosis in XY PGCs was significantly lower than in XX PGCs. Bars indicate the mean percentage ±SEM of three scores performed in three independent experiments on a total of at least 200 cells. *P ≤0.05. RO-41-5253 is able to inhibit meiosis in PGCs of both sexes and in both culture conditions. B) Stages of MPI after identification by SYCP3 labelling (red) in GCNA-1-labeled germ cells (green) in chromosome cytospreads from 16.5 dpc ovary. L, leptotene; Z, zygotene; P, pachytene. Bar = 5 μm. C) The progression of PGCs through MPI stages is not affected by exogenous RA. Stages of MPI in XX and XY germ cells were evaluated after 2 and 4 days of culture in FBS-GF-CM with or without the addition of 1 μM RA to the culture medium. Bars indicate the mean percentage ±SEM of three scores performed in three independent experiments on a total of at least 200 cells.

FIG. 2. Detection of RA activity in FBS-CM compared to different exogenous doses of RA. Monolayer of F9 RARE-LacZ-expressing cells were cultured for 24 h in CM with or without 15% FBS or in CM with an increasing amount of RA. Cells were then stained for β-galactosidase activity. The presence of RA in serum is confirmed by the inhibitory action of RO-41-5253 added to the FBS-containing cultures. Original magnification x25.

FIG. 3. Expression of STRA8 in PGCs at different developmental stages. A) Percentage of XX STRA8-positive PGCs isolated from gonads at different developmental ages. Average values ±SEM were obtained from three different experiments. B) Immunocytochemical staining for STRA8 in XX and XY PGCs isolated from 13.5 dpc gonadal ridges. The identity of PGCs was confirmed by GCNA1 staining. Bar = 25 μm. C) STRA8 localization in PGCs nuclear dots (arrows) in 14.5 dpc XX germ cells of the leptotene (L) stage. Bar = 5 μm.

FIG.4. Expression of STRA8 in cultured PGCs and its regulation by RA. A) Immunocytochemical staining for STRA8 protein in XY PGCs purified from 12.5 dpc testes and cultured for 24 h in FBS-GF-CM. At the end of the culture, cells were fixed and stained with the STRA8 antibody. Bar = 25 μm. B) 12.5 dpc XY germ cells were isolated and cultured in GF-CM with or without serum or in GF-CM containing different concentrations of RA for 18 h. qRT-PCR for the expression of Stra8 was carried out in triplicate. Bars indicate the mean ±SEM, n = 3 biological replicates; Mvh was used as the normalization control. *p ≤0.05. **p ≤ 0.01. Normalization to Gapdh was also carried out with similar results. C) 12.5 dpc XY PGCs were cultured for 18 h in GF-CM with or without FBS and RO-415253 (5 μM) and qRT-PCR analysis for Stra8 expression was performed as indicated above. D) 12.5 dpc XY PGCs were cultured for 24 h in FBS-GF-CM with 1 μM RA and labelled for STRA8. Bar = 25 μm. E) 12.5 dpc XX and XY PGCs were cultured for 16-18 h in FBS-GF-CM with or without 1 μM RA and
immunostained with STRA8 antibody before counting in 25 random fields. F) Lysates obtained from 12.5 dpc XY PGCs purified from 20 testes and cultured for 24 h in FBS-GF-CM with or without RA were probed with STRA8 and MVH antibodies. Actin expression was used as a loading and normalization control. Quantified data were shown under each panel.

**FIG. 5.** Induction of meiotic genes by RA in PGCs. XY (○) and XX (●) germ cells were isolated from testes and ovary at 12.5 dpc and cultured for 18 h as indicated. qRT-PCR was used to test the expression of meiotic genes \((Dmc1, Spo11, Rec8, Sycp3, Stra8)\). Each point represent mean ± SEM. n=3. * p ≤ 0.01.

**FIG. 6.** STRA8 expression and entry into meiosis in 10.5 dpc cultured PGCs. A) RT-PCR analysis of Stra8, Oct4 and Gapdh expression in PGCs purified from 10.5 dpc AGM region and cultured as indicated for 18 h (ddH2O, negative control water blank for PCR amplification). Quantified data were shown under each panel. B) Immunocytochemical staining for STRA8 protein in XY PGCs purified from 10.5 AGM and cultured for 24 h in GF-CM with or without 15% FBS or with RO-415253 (5 μM). At the end of the culture, cells were fixed and stained with the STRA8 antibody. Bar = 25 μm. C) SYCP3 (green) and γH2AX (red) immunolocalization in cytospreads from XY isolated 10.5 dpc PGCs cultured for 5 days in FBS-GF-CM with 1 μM RA (a-c) or XY PGCs obtained from AGM after 5 days of culture (d-f). Bar = 10 μm. D) Expression of the indicated genes in the mixed purified 10.5 dpc PGCs cultured for 5 days in FBS-GF-CM with or without 1 μM RA.
Figure 1
Figure 4

A

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B

![Graph showing STRA8 expression levels](Graph.png)

C

![Graph showing STRA8 expression levels](Graph.png)

D

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E

![Bar graph showing percentage of STRA8 positive PGCs](BarGraph.png)

F

![Western blot analysis](WesternBlot.png)