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New testicular mechanisms involved in the prevention of fetal meiotic initiation in mice

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ABSTRACT

In mammals, early fetal germ cells are unique in their ability to initiate the spermatogenesis or oogenesis programs dependent of their somatic environment. In mice, female germ cells enter into meiosis at 13.5 dpc whereas in the male, germ cells undergo mitotic arrest. Recent findings indicate that Cyp26b1, a RA-degrading enzyme, is a key factor preventing initiation of meiosis in the fetal testis. Here, we report evidence for additional testicular pathways involved in the prevention of fetal meiosis. Using a co-culture model in which an undifferentiated XX gonad is cultured with a fetal or neonatal testis, we demonstrated that the testis prevented the initiation of meiosis and induced male germ cell differentiation in the XX gonad. This testicular effect disappeared when male meiosis starts in the neonatal testis and was not directly due to Cyp26b1 expression. Moreover, neither RA nor ketoconazole, an inhibitor of Cyp26b1, completely prevented testicular inhibition of meiosis in co-cultured ovary. We found that secreted factor(s), with molecular weight greater than 10 kDa contained in conditioned media from cultured fetal testes, inhibited meiosis in the XX gonad. Lastly, although both Sertoli and interstitial cells inhibited meiosis in XX germ cells, only interstitial cells induced mitotic arrest in germ cell. In conclusion, our results demonstrate that male germ cell determination is supported by additional non-retinoid secreted factors inhibiting both meiosis and mitosis and produced by the testicular somatic cells during fetal and neonatal life.

Introduction

The embryonic gonad has the potential to develop into either a testis or an ovary. In the undifferentiated gonad, the commitment to male or female pathways of the somatic supporting cells depends upon their genetic constitution (Koopman et al., 1991; Lovell-Badge and Robertson, 1990) whereas sex determination of the germ cells depends upon their environment. In the mouse XY gonad, *Sry* expression in somatic cells at 11 days post-coitum (dpc) triggers the differentiation of Sertoli cells leading to the formation of testicular cords at 12.5 dpc (Palmer and Burgoyne, 1991). At this stage, in the interstitial space in between testis cords, steroid-producing Leydig cells differentiate. At 13.5 dpc, peritubular myoid cells surround testis cords. In the XX gonad, no obvious somatic cells differentiation is observed until follicle formation at birth (Pepling, 2006). Germ cell sex is determined between 11.5 and 13.5 dpc; during this period both XX and XY germ cells proliferate actively. At 13.5 dpc, XX germ cells in the ovary initiate meiosis whereas in the testis, germ cells progressively stop proliferating and all have entered the quiescence phase by 15.5 dpc. At this stage male germ cells, also termed "prospermatogonia," express cell cycle inhibitors, multipotency factors and RNA binding proteins such as p63γ, p16/ink4, Oct4 and Nanos2 while their female counterpart do not (Menke et al., 2003; Petre-Lazar et al., 2007; Tsuda et al., 2003; Western et al., 2008). Male germ cells resume their proliferation after the birth, and initiate meiosis a few days later, at about 8 days post-partum (dpp).

Undifferentiated XX and XY germ cells have the potential to follow the male or female pathway. Indeed, undifferentiated XY germ cells outside the testicular environment initiate meiosis (Chuma and

Abbreviations: RA, retinoic acid; GC, germ cell; dpc, days post-conception; dpp, days post-partum; DAB, 3,3'-diaminobenzidine; MIF, meiosis inhibiting factor; MAF, mitotic arrest factor.

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Nakatsuji, 2001; McLaren, 1997; Zamboni and Upadhyay, 1983) and undifferentiated XX germ cells placed in a testicular environment do not initiate meiosis (Adams and McLaren, 2002; Dolci and De Felici, 1990). These experiments suggested the existence of a "meiotic inhibiting factor" (MIF) produced by fetal testis. A current model proposes Cyp26b1 as the MIF (Bowles et al., 2006; Bowles and Koopman, 2007; MacLean et al., 2007). This model is consistent with the observation that XY germ cells initiate meiosis in *Cyp26b1*$^{-/-}$ fetal testis (Bowles et al., 2006; MacLean et al., 2007). Cyp26b1 is a retinoic acid (RA)-degrading enzyme specifically expressed male gonads during fetal life (Koubova et al., 2006). In this model, RA, presumably originating from the adjacent mesonephros or/and adrenal gland, acts as the meiosis inducer. Indeed, RA stimulates the expression of *Stra8*, a gene that is required for initiation of meiosis in the fetal ovary and in the juvenile testis (Anderson et al., 2008; Baltus et al., 2006; Bowles et al., 2006; Koubova et al., 2006). Moreover, in Cyp26b1$^{-/-}$ testes the expression of Nanos2 is decreased and Nanos2 is required for male germ cell differentiation. Indeed, ectopic expressions of Nanos2 in the fetal XX germ cells induced male differentiation and in the fetal testis of Nanos2-/-, male germ cells initiate meiosis albeit several days later than is normal in the XX gonad (Suzuki and Saga, 2008).

Interestingly, several observations suggest the existence of additional testicular pathways that act on the male germ cell differentiation (including meiosis prevention). First, some XY germ cells are committed to the male pathway even when they remain in the mesonephros despite the presence of RA (McLaren, 1984). Moreover, treatment of 11.5 dpc XY gonad with brefeldin A, an inhibitor of secretion and post-Golgi trafficking, has been proven to cause the appearance of meiotic cells in testis cords (Best et al., 2008). Fibroblast growth factor 9 (Fgf9) is expressed in the fetal testis and has been proposed to be the candidate secreted factor opposing RA effects as it inhibits meiosis entry in the 12.5 dpc ovary presumably through the upregulation of Nanos2 (Barrios et al., 2010). Lastly, the existence of a mitotic arrest factor (MAF) actively inducing male germ cell differentiation in the fetal testis is still a mater of debate (for review Ewen and Koopman, 2010).

To further understand male germ cell differentiation in the fetal testis, we used a novel experimental model based on co-culturing undifferentiated XX gonad with a fetal testis; using this model system, we reveal additional mechanisms of inhibition of meiosis and induction of the male pathway. We demonstrate that fetal testis secretes factors that prevent meiosis and induce male differentiation in germ cells.

Materials and methods

Mice

NMRI mice were housed in controlled photoperiod conditions (lights on from 08:00 to 20:00) and were supplied with commercial food and tap water *ad libitum*. Males were caged with females overnight, and the presence of vaginal plug was examined the following morning. Under the assumption that mating occurred between 00:00 and 02:00, the following midday was defined as 0.5 dpc. For accurate staging of 11.5 dpc embryos, the number of tail somites (ts) between the hind limb and the tip of the tail was determined at the time of dissection and only embryos with 18 ± 2 ts were included. Cellular migration was studied using C57BL/6-Tg mice expressing GFP under the control of the β-*actin* promoter (Okabe et al., 1997). Pregnant females were sacrificed by cervical dislocation and their fetuses were removed from uterine horns. The sex of gonads before 12.5 dpc was determined by PCR amplification of *Sry* as previously described (Petre-lazar et al., 2007). All animal studies were conducted in accordance with the guidelines for the care and use of laboratory animals of the French Ministry of Agriculture.

Purification of somatic and germ cells

About 50 gonads from 12.5 or 13.5 dpc fetuses were digested first in 0.25% trypsin–0.02% EDTA (Trypsin/EDTA solution, Sigma-Aldrich, St. Louis, MO) for 5 min, at 37 °C. Trypsin digestion was stopped by adding fetal bovine serum (FBS) to 10% and samples were centrifuged (500 ×*g* for 10 min). Then, the samples were further digested with 2 mg/ml collagenase and 0.02 mg/ml DNase I in HBSS, for 10 min at 37 °C.

For purification of SSEA1-positive cells, dispersed cells were incubated with anti-SSEA1 (1/5, anti SSEA1 monoclonal antibody, DSHB, Iowa) in PBS, 0.5% BSA (PB) for 20 min at 4 °C. At the end of the incubation, the cells were centrifuged and washed once with 1 ml of PB. The cells were then incubated with 20 µl of microbead-linked donkey anti-mouse IgM antibody (Miltenyi Biotec, Germany) in 300 µl PB with 2 mM EDTA (PBE) for 15 min at RT. The cells were rinsed once with PBE, and were applied onto an MS^{+} column. The column was rinsed three times with 500 µl PBE to wash out unbound cells, which represented the SSEA1-negative cell fraction. After removal from the magnet, the column was flushed with 1 ml PB, which allowed the collection of the SSEA1-positive cell fraction. Same experimental procedure was used with the SSEA1-negative cell fraction to purify α6-integrin- and p75-NTR-positive cells. α6-integrin is present on Sertoli cells and germ cells (Fröjdman and Pelliniemi, 1994) and p75-NTR is expressed by interstitial mesenchymal cells (Campagnolo et al., 2001). For purification of α6-integrin positive cells, the primary antibody, R-phycoerythrin conjugated monoclonal anti-CD49f (BD pharmingen) was diluted 1/100 in PB, and the secondary antibody, anti-PE microbeads (Miltenyi Biotec, Germany) was diluted 1/25 in PBE. About 88% of the SSEA1-negative, α6-integrin-positive cells were AMH-positive. For purification of p75-NTR positive cells, primary antibody (AB1554, Chemicon, Germany) was used at 1/100 and secondary antibody (goat anti-rabbit IgG, Miltenyi Biotec, Germany) at 1/25. About 90% of the SSEA1-neagtive, p75-positive fraction was 3-BHSD (10%)- or α-SMA-positive (80%). The potential contamination in each fraction was assessed by RT-PCR and immunostaining of AMH, 3-BHSD and α-SMA.

Organotypic culture, culture of germ cells and aggregation of cells

Gonads without their mesonephros were isolated from fetuses under a binocular microscope and kept in culture medium until explantation. Ham F12/DMEM supplemented with glutamax, D-glucose and pyruvate by the manufacturer (1:1, Life Technologies, Inc., Grand Island, NY), was used for all cultures. Hepes (15 mM, Sigma-Aldrich, St. Louis, MO) and gentamicin (0.04 mg/ml, Life Technologies, Inc., Grand Island, NY) were added in this retinoid-free and serum-free medium. For co-culture studies, an 11.5 dpc ovary was placed side by side with fetal or pieces of neonatal testes (from 12.5 dpc to 8 dpp), 11.5 dpc mesonephros or pieces of 13.5 dpc liver on Millicell-CM Biopore membranes (pore size 0.4 µm; Millipore, Billerica, MA). Post-natal testes or liver were cut in pieces similar in size to 13.5 dpc testis. The ovaries were co-cultured 4 days and, in some experiments, all-*trans*-RA or ketoconazole (1 µM or 55 µM respectively, Sigma-Aldrich, St. Louis, MO) was added during the culture period. For conditioned medium studies, an 11.5 dpc ovary was cultured for 4 days on a disc of agar with freshly collected conditioned medium.

SSEA1-positive germ cells from 12.5 dpc testes were cultured during 2 days on a layer of mouse embryonic fibroblasts. Under these conditions about 75% of the isolated germ cells initiate meiosis in control medium. Conditioned media of testis or liver without addition of serum or other factors were used for the culture of germ cells. No XX germ cells were used as most initiated meiosis almost immediately under these conditions.

SSEA1-positive germ cells from 12.5 dpc gonads were mixed with SSEA1-negative (all somatic cells), p75-positive (mesenchymal cells)

or α6-positive (Sertoli cells) cells from 13.5 dpc testis (50 000 SSEA1-positive cells for 400 000 somatic cells). The cell mixtures were centrifuged at 1200 ×*g* for aggregation and cultured on millicell filters for 4 days. To ascertain the origin of SSEA1-positive germ cells from 12.5 dpc gonads, BrdU (5-bromo-2'-deoxyuridine) was injected into the intra-peritoneal space of 11.5 dpc pregnant mice. One day later, gonads were removed and used for SSEA1 purification. Only BrdU-labeled SSEA1-positive germ cells were counted in the aggregates.

Collection of conditioned media

About fifteen 13.5 dpc testes or pieces of 13.5 dpc liver were cultured for 4 days on a disc of agar (1.5%, Sigma, St. Louis, MO) in Ham F12/DMEM (1:1) containing 80 μg/ml gentamicin in a humidified atmosphere containing 95% air–5% CO2. At the end of culture, media were collected and centrifuged for 10 min at 500 ×*g*. For fractionated conditioned media assays, we used separation tubes with 3-kDa and 10-kDa molecular weight cut-off membranes (Nanosep 10 K and 3 K, Pall Corporation, Ann Arbor, MI): conditioned media were first centrifuged in 10-kDa separation tubes, and the lower fraction was centrifuged in 3-kDa separation tubes. The supernatants obtained after centrifugation in 10-kDa and 3-kDa separation tubes were kept and diluted in the culture medium (1/25). Each fraction of medium (<3-kDa, 3-kDa ≪ 10-kDa, >10-kDa) was reused immediately for the culture of the ovaries.

Histology and germ cell counting

Gonads were fixed with Bouin's fluid just after dissection or at the end of the culture. The fixed gonads were dehydrated, embedded in paraffin and cut into 5-μm-thick sections; only one of each 10 serial sections was mounted on glass slides for all tissues. These sections were dewaxed, rehydrated and stained with hematoxilin and eosin. The gonocytes were identified on the basis of their large, spherical nuclei and clearly visible cytoplasmic membrane. Nuclei of prospermatogonia in mitotic arrest contained fine chromatin granules and globular nucleoli, whereas proliferating gonocyte nuclei contained patches of heterochromatin. Meiotic cells displayed marked condensation of the chromatin, forming distinct fine threads with a beaded appearance at the leptotene stage and presenting a characteristic crisscross of coiled chromosome threads at the zygotene stage. Histolab analysis software (Microvision Instruments, Evry, France) was used for counting.

Immunohistochemistry

Five tissue sections *per* gonad were mounted on glass slides, dewaxed, and boiled for 10 min in 10 mM Tris pH 10.6. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 10 min. The sections were then washed with PBS, and blocked for 30 min with 5% normal horse serum (NHS). Slides were incubated overnight at 4 °C with mouse anti-γH2AX antibody (1/500, Euromedex). Bound primary antibody was revealed by incubation with biotinylated horse anti-mouse secondary antibody in 2% NHS then with avidin–biotin–peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories) and finally with DAB to detect peroxidase activity.

Measurement of cell proliferation, BrdU and PCNA

The percentage of cells in S-phase was evaluated by measuring BrdU incorporation, by immunohistochemical methods, using the Cell Proliferation Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's recommendations. BrdU (1%) was either added at the end of the culture period or 3 h before tissue fixation. Briefly, five randomly selected sections were mounted, rehydrated, incubated for 1 h with anti-BrdU antibody and with a peroxidase-linked anti-mouse IgG. Peroxidase activity was detected with DAB. The BrdU incorporation index was determined by blind counting of at least 500 stained and unstained non-meotic germ cells.

For PCNA immunostaining, sections were incubated with mouse anti-PCNA (1/200, Dako) for 1 h at room temperature. To reveal the primary antibody, sections were incubated with anti-mouse biotinylated secondary antibody, then an avidin–biotin–peroxidase complex and finally DAB to detect peroxidase activity.

Immunofluorescence

Commercially available primary antibodies were used for immunofluorescence: anti-Scp3 (working dilution 1/200; Peptide Specialty Laboratories GmbH), anti-γH2AX (1/400) and anti-MVH (1/500, Abcam). After co-culture, gonads or purified germ cells were fixed in 4% paraformaldehyde at 4 °C, washed in PBS, blocked by incubation in 3% BSA supplemented with 0.1% Triton, and incubated overnight with rabbit anti-Scp3, with mouse anti- γH2AX or rabbit anti-MVH antibodies. After 3 washes in PBS/Triton, gonads or germ cells were incubated with donkey anti-rabbit-Cy3-conjugated antibody (1:500), anti-rabbit-FITC-conjugated antibody (1:50) or donkey anti-mouse-Cy3 antibody (1:500). Slides were mounted in Vectashield (Vector Laboratories) and observed with a confocal microscope (Laser Scanning Microscope LSM 5 Pascal, Carl Zeiss).

Reverse transcription and semi-quantitative PCR

At the end of the culture period, total RNA was extracted using the RNeasy mini-kit (Qiagen) and reverse transcription was carried out with the Omniscript kit (Invitrogen), according to the manufacturer's instructions. Stra8, Dmc1 and Scp1 mRNAs were assayed by semi-quantitative PCR as described previously (Trautmann et al., 2008).

Real-time quantitative PCR

A Prism 7000 system (Applied Biosystems) and SYBER-green labeling were used for quantitative RT-PCR. Each RNA sample was analyzed in triplicate. All primers were used at a concentration of 400 nM. For the primers that do not span introns such as *Nanos2*, *p16* and *R-spondin1* (*Rspo1)*, we have performed a control PCR minus RT to exclude genomic contamination of the cDNA samples. Primers for *β-actin* were F: 5'-TGA-CCC-AGA-TCA-TGT-TTG-AGA-3' and R: 5'-TAC-GAC-CAG-AGG-CAT-ACA-GG-3', *Cyp26b1* F: 5'-TGG-ACT-CTC-TCA-TCA-AGG-AGG-T-3'and R:5'-GTC-GTG-AGT-GTC-TCG-GAT-GCT-A, *dosage suppressor of mck1 homolog* (*Dmc1)* F: 5'-GTG-GGT-TCG-AGT-CGG-GAA-T-3' and R: 5'-ATG-TTG-AAA-GGA-GAG-CGG-CA-3', *Nanos2* F:5'-AAT-TCA-GAG-CCG-GAA-GCA-AA-3' and R:5'-TTG-GGC-TCC-TCA-GCT-ACC-TC-3', *Nanos3* F:5'-CGC-CTT-GGA-GGA-GAA-GCA-3' and R: 5'-GCC-ACT-TTT-GGA-ACC-TGC-AT-3', *Oct4* F: 5'-AAC-CAA-CTC-CCG-AGG-AGT-GC-3' and R: 5'-CTA-GCT-CCT-TCT-GCA-GGG-CTT3', *p16* F:5' ACT-CGA-GGA-GAG-CCA-TCT-GG-3' and R:5'-CCA-GTC-TGT-CTG-CAG-CGG-A, *Rec8* F: 5'-CCA-AGG-CCT-GAA-CTC-TCT-TC-3' and R: 5'-ATA-GAA-GAC-CCG-AGA-AAG-CCA-3', *synaptonemal complex protein 3 (Sycp3)* F: 5'-AAA-GAA-ATG-GCT-ATG-TTG-CAA-AAA-3' and R: 5'-TTG-CCA-TCT-CTT-GCT-GCT-GA-3', *Spo11* F: 5'-GTA-TGC-TGA-AAG-TGC-CCA-GGA-3' and R: 5'-CCT-TGG-AAG-TAG-ATA-GCA-CGT-GC-3' and *Mvh* F: 5'-GAA-GAA-ATC-CAG-AGG-TTG-GC-3' and R: 5'-GAA-GGA-TCG-TCT-GCT-GAA-CA-3'. All PCR efficiencies were comprised between 90% and 110%. *Cyp26b1* expression was normalized to *β-actin* expression. *Cyp26b1* expressions of the fetal and post-natal testes (11.5 dpc to 8 dpp), 13.5 dpc liver, 13.5 dpc ovary and 11.5 dpc limb were compared to 13.5 dpc testis. Cyp26b1 expression of p75-NTR- or α-6 integrin-positive cells and the 13.5 dpc dissociated ovarian cells were compared to 13.5 dpc dissociated testicular cell. *Nanos2, nanos3, oct4, p16* expressions were normalized to *mvh* expression and compared between ovaries and testis or between 11.5 dpc XX gonads co-cultured with liver and with testis with

the highest expression being defined as 100%. Additional sequences are provided in supplemental data (Table 1).

Data analysis

Each data point reported represents the mean ± S.E.M. of at least three independent experiments. Images show a representative result for experiments that were all repeated at least three times. The percentage of meiotic or proliferative germ cells was determined by blind counting of at least 500 stained and unstained germ cells. Data were analyzed using Graphpad Instat 3.0, by one-way ANOVA followed by the Tukey-Kramer multiple comparisons test or paired or unpaired Student's *t* test.

Results

Germ cells initiate meiosis in isolated 11.5 dpc XX gonad in culture

XX gonads at 11.5 dpc were separated from the mesonephros and cultured for 4 days in a retinoid/serum free medium. In this culture

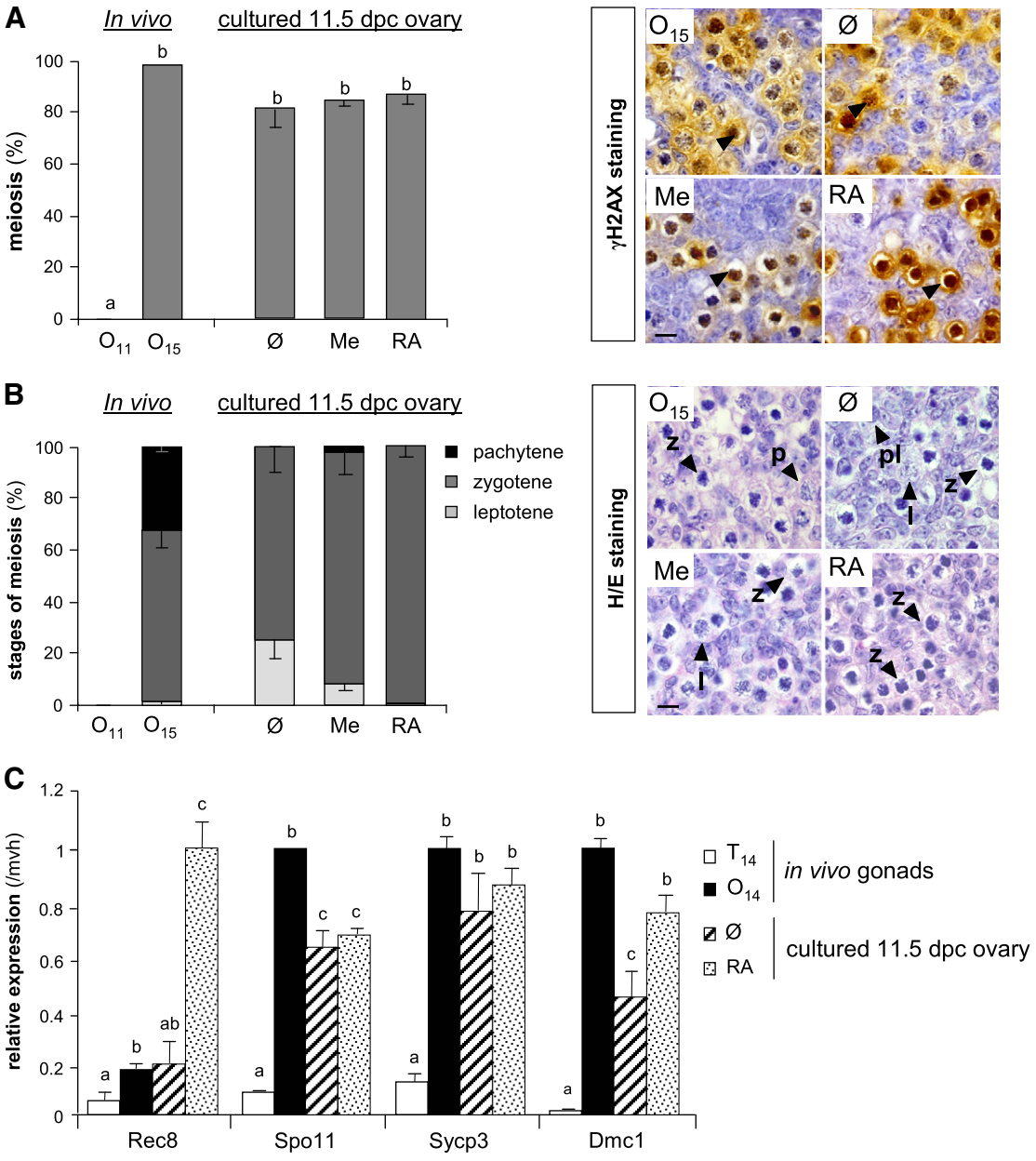

Fig. 1. In culture, 11.5 dpc XX gonads without the mesonephros differentiate normally. Fetal ovaries from 11.5 dpc fetuses were cultured for 4 days alone (Ø), associated with their mesonephros (Me) or with 10^{-6} M of retinoic acid (RA). A. Left panel; Percentage of the meiotic germ cells in gonads in vivo from 11.5 (O11) and 15.5 (O15) dpc fetuses, or in 11.5 dpc cultured ovaries. Means ± SEM of 5–15 values are shown. Right panel; Immunostaining of γ-H2AX on sections from 15.5 dpc ovary or from 11.5 dpc cultured ovaries. A stained germ cell is indicated with a black arrowhead. Bar: 10 μM. B. Left panel; Distribution of meiotic stages in germ cells in gonads in vivo from 11.5 and 15.5 dpc fetuses, or in 11.5 dpc cultured ovaries. Means ± SEM of 5–15 values are shown. Right panel; H/E staining of sections from 15.5 dpc ovaries or 11.5 dpc cultured ovaries. Arrows represent germ cells. pl: preleptotene; l: leptotene; z: zygotene; p: pachytene. Bar: 10 μM. C. Quantitative RT-PCR expression analysis of meiotic markers in 14.5 dpc testis and ovary (in vivo gonads) and in 11.5 dpc ovaries cultured for 3 days in the presence or absence of 10^{-6} M of retinoic acid. All values were normalized against mvh and expressed as percentage of the maximum. Means ± SEM of 3 to 5 values. Different letters represent significantly different values as assessed by ANOVA, with $p<0.05$.

model, germ cells spontaneously initiated meiosis. The first meiotic cells were observed after 2 days of culture as in the 13.5 dpc ovary *in vivo* (sup. Fig. 1A). After 4 days, the percentage of germ cells in meiosis was slightly lower in such ovary cultures than in 15.5 dpc ovary, the equivalent *in vivo* stage, but the difference was not statistically significant (Fig. 1A). This was confirmed by the detection of numerous γ-H2AX cells, a marker of DNA double strand breaks, a hallmark of meiosis. Histological analysis showed that the progression of meiosis *in vitro* was slightly delayed relative to that *in vivo*: 15.5 dpc ovary contained more advanced meiotic stage (pachytene) and fewer early stage (leptotene) cells (Fig. 1B). Culture with the associated mesonephros or addition of retinoic acid (RA) to the culture medium did not increased the percentage of meiotic germ cells but slightly speeded-up meiosis progression (Fig. 1A and B) with more zygotene stages being formed. After 3 days, the meiotic markers, *Rec8, Spo11, Sycp3* and *Dmc1*, were strongly expressed in control cultures though

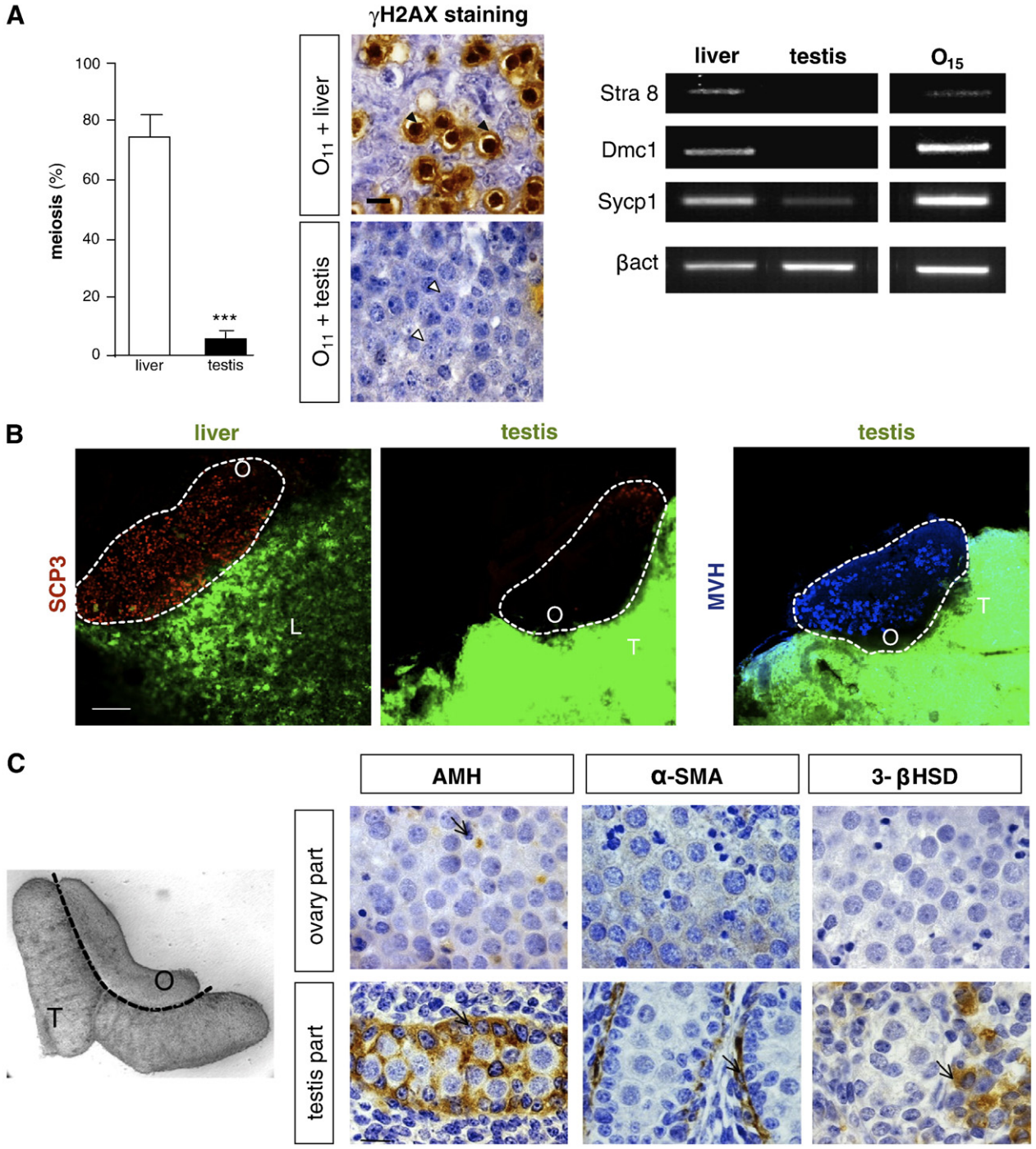

Fig. 2. Fetal testis blocks the entry into meiosis of XX germ cells. Fetal ovaries from 11.5 fetuses were co-cultured for 4 days with 13.5 dpc liver (liver) or 13.5 dpc testes (testis). A. Left panel; Percentage of meiotic germ cells in the co-cultured ovaries. Means ± SEM of 17 values are shown;$^{***}p<0.001$, in an unpaired Student's *t* test. Central panel; Immunostaining of γ-H2AX on sections from 11.5 dpc ovary cultured with liver (O11 + liver) or testis (O11 + testis). A stained germ cell is indicated with a black arrowhead and an unstained germ cell with a white arrowhead. Bars: 10 μm. Right panel: Analysis by RT-PCR of the expression of Stra8, Dmc1 and Sycp1 in 15.5 dpc ovaries (O15) and in the 11.5 dpc ovaries co-cultured with liver or testes. B. Detection by immunofluorescence of Sycp3 (red) and mouse vasa homolog (Mvh) (blue) in the 11.5 dpc ovaries (O) co-cultured with eGFP testes (T) or liver (L). Bar:100 μm. C. Detection of testicular somatic cell markers by immunostaining of AMH, 3-BHSD and α-SMA in the testicular (T) and ovarian (O) parts of the co-culture. Arrows indicate stained cells. Bar: 15 μm.

Spo11 and *Dmc1* expression were lower in comparison to 14.5 dpc ovary (Fig. 1C). RA addition restored the expression of *Dmc1* and increased that of *Rec8*.

Fetal testis prevents meiosis initiation of XX germ cells

To investigate the effects of the testis on meiosis initiation in the ovary, we developed a co-culture model: the 11.5 dpc XX gonad without mesonephros was placed side-by-side with a 13.5 dpc testis and cultured for 4 days (Fig. 2C, black and white photography). Control cultures included using other tissues instead of the testis: placenta, limb, fetal or neonatal ovary, and fetal liver. With all control tissues used, the vast majority of germ cells in the XX gonad initiated and progressed normally through meiosis (Fig. 2A and sup. Fig. 1B). For the rest of this study, 13.5 dpc liver was used routinely as the control. In the presence of liver, germ cells were predominantly at the zygotene stage as observed in the ovary cultured without mesonephros (Fig. 2A). This observation was confirmed by the detection of numerous of γ-H2AX- or Scp3-positive germ cells and meiosis markers including *Stra8*, *Dmc1* and *Sycp1* (Fig. 2A, B). When fetal testes were co-cultured with the 11.5 dpc XX gonad, very few XX germ cells initiated meiosis (Fig. 2A, B). These were mostly localized in the anterior part of the ovary. The lack of meiosis was confirmed by the drastic decrease of γH2AX- and Scp3-positive germ cells (Fig. 2A, B). In the co-culture, *Stra8*, *Dmc1* and *Scp1* mRNAs were almost undetectable (Fig. 2A, right panel). The absence of meiotic markers was not due to a loss of germ cells in these ovaries because the number of germ cells in the ovaries co-cultured with testes was not significantly different from that in co-cultures with liver: respectively 1292 ± 327 and 1549 ± 305 germ cells and MVH-staining was efficiently observed (Fig. 2B).

To ensure that germ cells that did not initiate meiosis did not originate from XY gonads, we performed co-culture with eGFP testes: we did not detect any cell migration from testis to ovary (Fig. 2B). Moreover, we investigated the possible sex-reversal of somatic XX cells into a testicular cell type. No α-SMA or 3-βHSD-positive cells were detected in the ovarian part of the co-culture. In the XX gonad, AMH expression was undetectable except in the border with the testis where rare AMH-positive cells were observed (Fig. 2C).

Next, we used 12.5 dpc ovaries co-cultured with fetal testis or liver. The testis similarly prevented meiosis initiation in the 12.5 dpc ovary. However, about half of the germ cells in the XX gonad escaped the testicular inhibitory effect and entered into meiosis normally (sup. Fig. 2).

Testicular conditioned medium prevents the entry into meiosis

We observed no migration of testicular cells in the ovarian part of the co-culture model (Fig. 2C). Moreover, the few meiotic germ cells observed in the ovarian part of the co-culture were not observed near the testes. We therefore investigated the effect of putative secreted factors present in testicular conditioned media on the initiation of meiosis in the ovary. XX gonads from 11.5 dpc fetuses were cultured with retinoid/serum free media conditioned by 13.5 dpc liver as a control. In this condition, $87 \pm 8.7\%$ of germ cells initiated meiosis whereas conditioned medium of 13.5 dpc testes decreased meiosis initiation: only $27 \pm 11\%$ of germ cells entered meiosis (Fig. 3A). There was a similar decrease in the percentage of γ-H2AX-positive germ cells in the ovaries cultured in medium conditioned by testes (Fig. 3A). Moreover, we did not observed any significant modification of the germ cell number in the ovaries cultured with testicular conditioned medium compared to the control ovaries (1371 ± 288 with liver conditioned medium versus 1028 ± 191 with testicular conditioned medium).

No somatic sex reversal in the ovary was observed when investigated by Q-PCR the expression of male somatic markers in the ovaries cultured with conditioned media of testes. We did not observe any increase of the expression of *Sox9* and *Fgf9* in the ovaries cultured with testicular conditioned medium in comparison to the control ovaries. The expression of female somatic markers such as *Wnt4*, *Follistatin (Fst) and Adam19* was maintained in the same conditions (sup. Fig. 3A). The expressions of *R-spo1* and *Fst* were mildly decreased in cultured ovaries in comparison with *in vivo* ovaries and testicular conditioned medium did not decrease further their expression. Surprisingly testicular conditioned medium increased slightly *Fst* expression. Moreover, we did not detect any AMH, 3-βHSD or α-SMA positive cells in these ovaries by immunostaining (sup. Fig. 3B).

Next, we studied the effect of testicular conditioned medium on meiosis initiation of purified SSEA1-positive germ cells. Germ cells were isolated from 12.5 dpc male gonads instead of 11.5 dpc gonads in order to obtain sufficient cells and as part of these cells can still be induced toward meiosis at this stage (Barrios et al., 2010). After 2 days of culture, these isolated germ cells cultured on mouse embryonic fibroblasts in control medium or liver conditioned medium rapidly initiated meiosis while in presence of testicular conditioned medium, we observed a significant decrease in the percentage of meiotic cells revealed by γ-H2AX/MVH double staining (sup. Fig. 4).

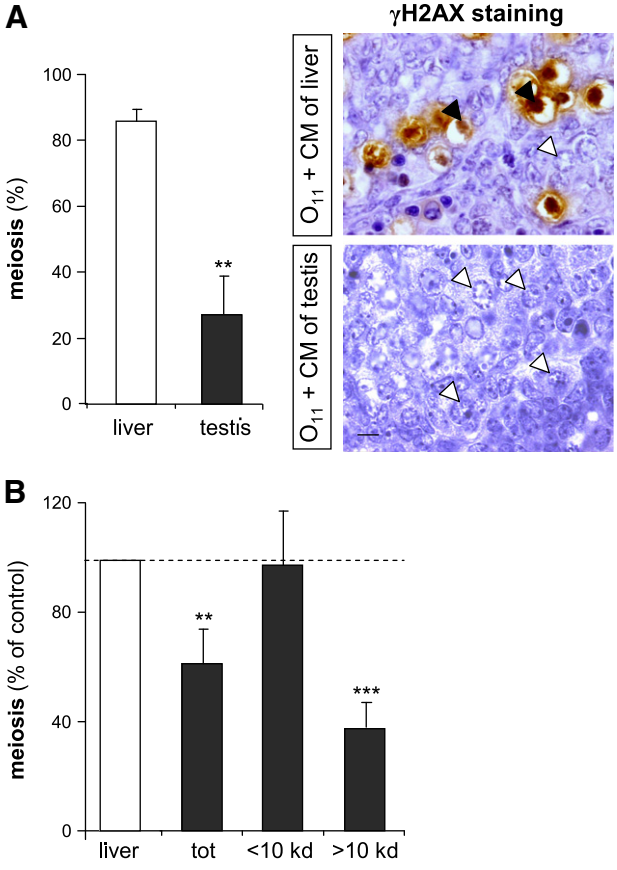

Fig. 3. Testicular diffusible substance blocks the entry into meiosis of XX germ cells. Retinoid/serum free conditioned media were obtained after 4 days of culture of 13.5 dpc testes or liver and were used for the culture of ovaries from 11.5 dpc fetuses. A. Left panel; percentage of meiosis in the ovaries cultured with conditioned media from liver or testes. Means ± SEM of 7 values; $^{**}p<0.01$, in an unpaired Student's *t* test. Right panel: Immunostaining of γH2AX in cultured ovaries with conditioned medium (CM) from liver or testes. Black arrowheads indicate stained germ cells and white arrowheads unstained germ cells. B. Fractions of conditioned media from liver or testis were obtained by centrifugation in Nanosep columns with a cut-off of 10 kDa. Each fraction of conditioned medium was used separately for the culture of 11.5 dpc ovary (< or > 10 kDa). The histograms represent the percentage of meiotic germ cells in the ovary at the end of culture. Data are expressed as a percentage of control values (controls were each fraction of conditioned media from liver). Means ± SEM of 3 to 7 values. $^{***}p<0.001$, $^{**}p<0.01$ in a paired Student's *t* test.

When testing the stability of the meiotic inhibitory activity, we observed that freeze–thaw cycles, boiling (10 min) and trypsin-treatment of conditioned media fully abolished the effect of testicular conditioned media on meiosis initiation in the XX gonad (data not shown). We also used molecular weight cut-off experiments to investigate the nature of the factor(s) involved in this inhibition of meiosis. Conditioned media were separated into three fractions: under 3 kDa, above 10 kDa (>10kDa) or between 3 kDa and 10 kDa. When these three fractions were pooled (tot), the meiotic inhibitory activity of testes was restored (albeit slightly diminished presumably due to the handling). Only the >10 kDa fraction of testicular conditioned medium inhibited the initiation of meiosis (Fig. 3B).

Cyp26b1 expression is not correlated with testicular inhibition of meiosis

To study the ontogenesis of the meiotic inhibitory activity of the testes, we co-cultured 11.5 dpc XX gonad with fetal testes from 12.5 dpc (data not shown) to 14.5 dpc fetuses or from 1 or 8 dpp neonates (Fig. 4A). After 4 days in culture, in the ovaries co-cultured with 12.5, 14.5 and 1 dpp testes, germ cells did not initiate meiosis. In the presence of 8 dpp testes most germ entered into meiosis in the ovaries as illustrated by the percentage of the γH2AX-positive germ cells.

Cyp26b1 has been has been suggested to be the MIF. In this model of co-culture, we could consider that Cyp26b1 strongly expressed in the testes might create a RA-depleted area that prevents the meiosis initiation in the nearby ovary. We therefore studied Cyp26b1 expression ontogenesis to investigate a possible correlation between its expression and the ability of the developing testis to prevent meiosis initiation. Cyp26b1 expression in fetal testes was higher than that in the fetal ovary and was substantially higher in 8 dpp post-natal testes (Fig. 4B). To investigate the role of Cyp26b1 in our co-culture model, we co-cultured 11.5 dpc XX gonad and 13.5 dpc testes in the presence of ketoconazole, an inhibitor of Cyp26b1. Even in presence of ketoconazole or RA, the fetal testes prevented significantly the meiosis initiation in the ovarian part of the co-cultures (Fig. 4C). However, we observed that the meiosis prevention was incomplete in the presence of RA or ketoconazole when compared to co-cultures performed in control medium (Fig. 4C).

Fetal testis triggers the male differentiation of XX germ cells

In our co-culture model with fetal testis, XX germ cells that had not initiated meiosis looked like XY quiescent gonocytes (prospermatogonia). To study the ability of the testis to triggers XX germ cell differentiation towards the male pathways, we measured the proliferation of germ cells in co-cultured ovary by BrdU incorporation. In the ovary co-cultured with liver, non-meiotic germ cells proliferated actively (49.7 ± 3.5% of non meiotic germ cells, equivalent to 12% of total germ cells, were BrdU positive). In the ovary co-cultured with fetal testis, the percentage of BrdU-positive germ cells was significantly lower, with an almost complete arrest of XX germ cell proliferation (Fig. 5A). Interestingly, in ovaries cultured with testes-conditioned medium, there was no significant change of the XX germ cell proliferation (Fig. 5B).

Next, we investigated the expression of germ cells markers of male differentiation in co-cultured XX gonad. *In vivo*, at 14.5 dpc, the expression of each Oct4, Nanos3, Nanos2 and $p16^{Ink4}$ is substantially higher in the testis when compared with the ovary (Fig. 5C). Interestingly both Nanos2 and Nanos3 expressions peaked at 14.5 dpc in the testis and are inhibited by RA-treatment (sup. Fig. 5B). In the ovarian part of the co-culture with liver, the level of expression of all these markers was similar to that in 14.5 dpc ovary. In ovary co-cultured with testis, the expression of all of these markers was higher (Fig. 5C). To determine if the presence of RA might prevent this testicular effect on the XX germ cell differentiation, we co-cultured ovary with liver or testes in presence of RA (sup. Fig. 5C). RA-treatment decreased about two-fold the expression of the most part of these markers in the ovarian part of the co-cultures with liver and with testes as it did in 12.5 dpc testes. Nonetheless the expression of these markers was always significantly increased in the ovarian part of the co-culture with testes in comparison to the co-culture with liver.

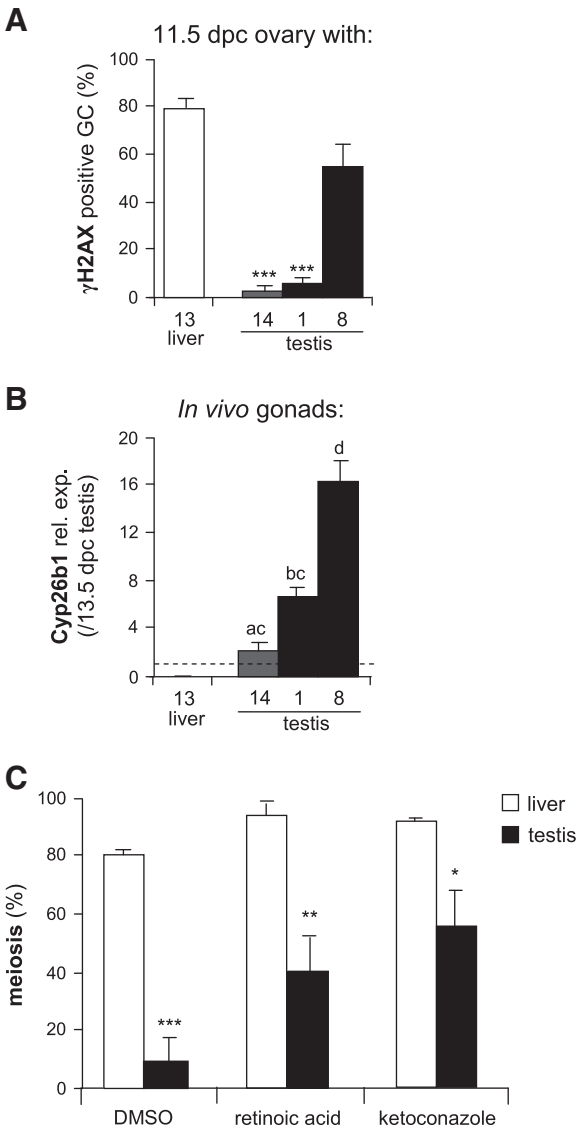

Fig. 4. Fetal and neonatal testes inhibits the initiation of meiosis in the XX gonad. Fetal ovaries from 11.5 dpc fetuses were co-cultured for 4 days with 13.5 dpc liver , 14.5 dpc fetal testes (14), 1 dpp (1) or 8 dpp (8) neonatal testes. A. Percentage of γ-H2AX-positive germ cells in the co-cultured ovaries. Means ± SEM of 7–15 values $^{***}p<0.001$ in an unpaired Student's *t* test. B. Cyp26b1 expression relative to that in 13.5 dpc testis in 13.5 dpc liver, ovary, and testes from 14.5 dpc to 8 dpp. All values were normalized against β-actin. Different letters represent significantly different values in ANOVA comparisons, with $p<0.05$. C. 11.5 dpc ovaries were co-cultured with 13.5 dpc liver or testis in the presence of DMSO, 10^{-6} M retinoic acid or 55 μM ketoconazole. After 4 days of culture, the percentage of meiotic germ cells was determined. Means ± SEM of 7 values; $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$, in an unpaired Student's *t* test.

Testicular somatic cells prevent meiosis and promote male germ cell differentiation

To determine the cellular origin of the testicular activity preventing meiosis, we agglomerated SSEA1-positive germ cells from 12.5 dpc ovary with total somatic cells, enriched population of Sertoli cells or interstitial mesenchymal cells (Leydig cells and peritubular cells)

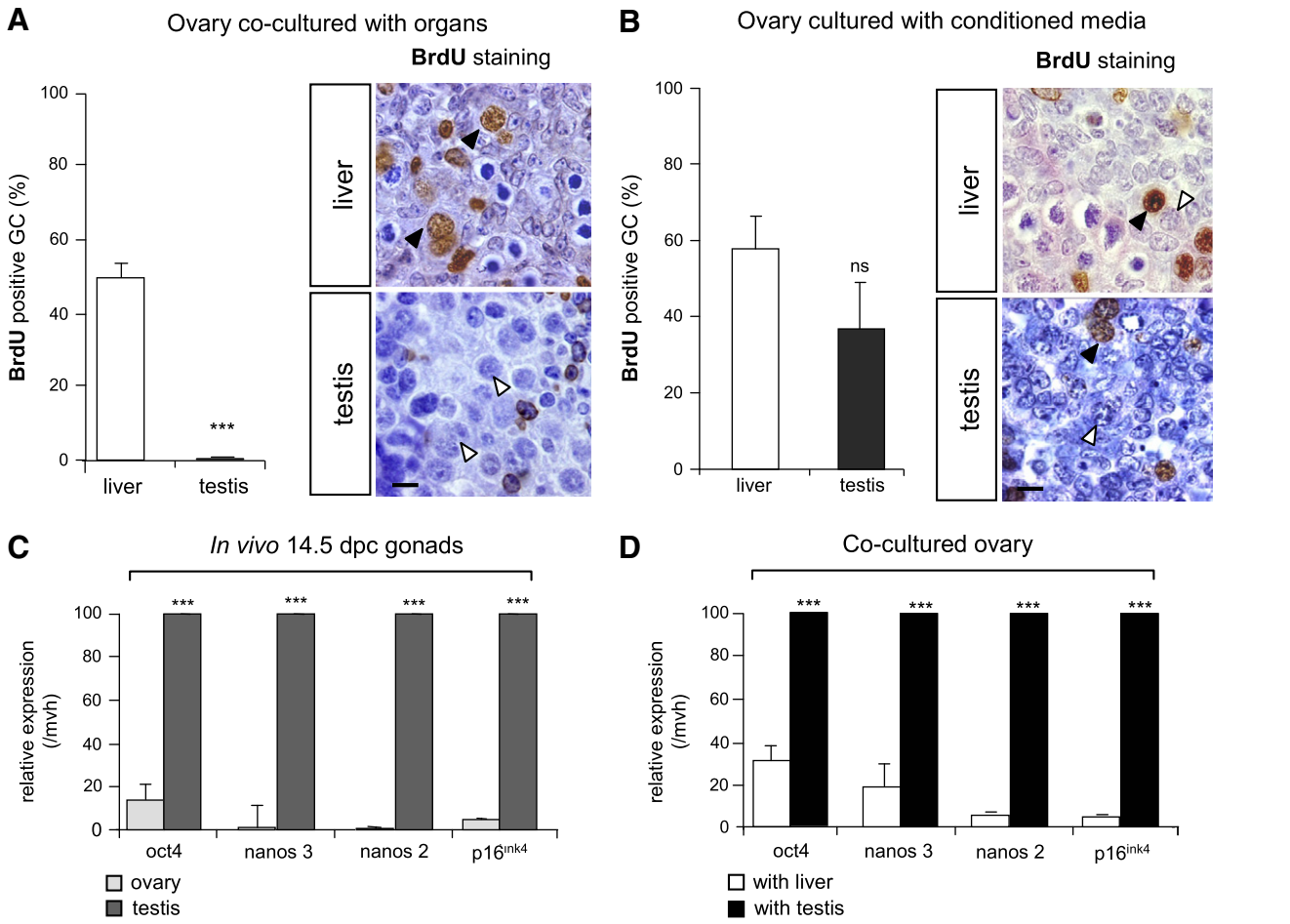

Fig. 5. Fetal and neonatal testes trigger XX germ cell differentiation towards the male pathways. A. 11.5 dpc ovaries were co-cultured with 13.5 dpc liver or testes during 4 days. Left panel; Percentage of proliferative germ cells in the ovarian part determined by BrdU incorporation. Meiotic germ cells were excluded from this counting. Means ± SEM of 6 values. Right panel; Immunostaining of BrdU in ovaries co-cultured with liver or testes. B. 11.5 dpc ovaries were cultured in presence of conditioned media of liver or testes for 4 days. Left panel: percentage of the non meiotic BrdU positive germ cells in the ovaries cultured with conditioned media from liver or testes. Means ± SEM of 4 values. Right panel: Immunostaining of BrdU in cultured ovaries with conditioned medium from liver or testis. For A and B; Black arrowheads indicate stained germ cells and white arrowheads indicate unstained non-meiotic germ cells. Bar represent 10 μm. C. Quantitative RT-PCR expression analysis of male differentiation markers in 14.5 dpc testis and ovary (*in vivo* gonads). D. Quantitative RT-PCR expression analysis of male differentiation markers in the ovarian part of ovaries co-cultured with testis or liver. For C and D, all values were normalized against mvh. Means ± SEM of 5 values; $^{***}p<0.001$, in an unpaired Student's *t* test.

from 13.5 dpc testes. α6-integrin expressed in Sertoli cells is used to enrich these cells (α6-positive cells, Fröjdman and Pelliniemi, 1994). P75-neurotrophin expressed in interstitial mesenchymal cells is used to enrich these cells (p75-positive cells, Campagnolo et al., 2001). Total somatic cells from 13.5 dpc ovaries were used to form control agglomerate. XX germ cells associated with ovarian somatic cells entered into meiosis as expected (67.8 ± 4.8% in meiosis; Fig. 6A, ovary). XX germ cells associated with total testicular somatic cells were mostly prevented from initiating meiosis: only 25.7 ± 7.5% of the germ cells entered meiosis (testis). Similarly, purified p75- (interstitial cells) and α6-positive (Sertoli cells) cells each efficiently prevented XX germ cells from initiating meiosis (Fig. 6A). As for the 12.5 dpc XX germ cells, 12.5 dpc XY germ cells also initiated meiosis in the ovarian environment and were fully prevented from entering meiosis by either p75-positive cells or α6-positive cells (sup. Fig. 6A).

To determine whether testicular cells also inhibit XX germ cell proliferation, we assessed the percentage of PCNA-positive germ cells in the agglomerate. As *in vivo*, non-meiotic XX germ cells placed in ovarian environment proliferated actively whereas in a testicular environment, the percentage of proliferative XX germ cells decreased sharply (Fig. 6B). In presence of p75-positive cells, XX germ cell proliferation was inhibited as in the somatic testicular environment. In contrast, in presence of α6-positive cells, the percentage of PCNA-positive germ cells remained high. Similar results were also obtained with 12.5 dpc XY germ cells (data not shown). Interestingly, XX germ cells looked like XY prospermatogonia (characterized by the presence of nucleoli and fine chromatin granules in the nuclei) in the presence of total testicular somatic cells or p75-positive cells but not with α6-positive cells; this observation correlated with the decrease in SSEA1 expression in the XX germ cells placed in the p75-positive cells, whereas in the α6-positive cells SSEA1 expression remained very similar to that in the ovarian environment (Fig. 6C).

Cyp26b1 expression was determined in each purified population of testicular cells. P75-positive cells expressed Cyp26b1 similarly to total dissociated testicular cells; α6-positive cells expressed three times as much Cyp26 b1 as p75-positive cells (sup. Fig. 6B).

Discussion

In this study, we developed an original model in which meiosis is inhibited and mitotic arrest is induced in germ cells from undifferentiated XX gonad co-cultured with fetal testes. This model revealed that the process of germ cell male differentiation is more complex than previously suspected and not only requires RA degradation but also involves additional diffusible factors produced by the fetal testis.

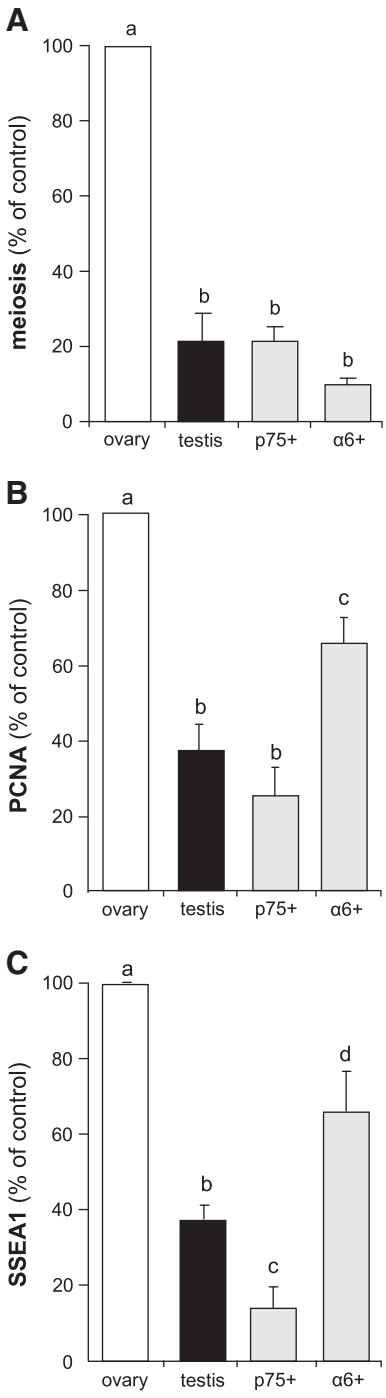

Fig. 6. Identification of the testicular cell type responsible of meiotic inhibition and germ cell mitotic arrest. SSEA-1-positive germ cells were purified from 12.5 dpc ovaries and aggregated with total somatic cells from 13.5 dpc gonads (ovary or testis) or purified somatic cells (p75- or α6-positive cells) from 13.5 dpc testis, and aggregates were cultured for 4 days. A. Percentage of meiotic germ cells. B. Percentage of PCNA-positive germ cells in the aggregates. C. Percentage of SSEA1-positive germ cells in the aggregates with germ cells from 12.5 dpc testis and ovary. Means ± SEM of 4 to 8 values. Different letters represent significantly different values in ANOVA comparisons, with $p<0.05$.

Mesonephric RA is dispensable for female germ cell entry into meiosis in vitro

RA presumably originating from the mesonephros has been proposed as the meiosis inducing factor (Bowles et al., 2006; Koubova et al., 2006). Surprisingly, we observed that in culture conditions devoid of any retinoids and in the absence of mesonephros germ cells differentiate and initiate meiosis in the ovary. This indicates that the mesonephros is dispensable for meiosis initiation. However one cannot conclude about RA involvement as we cannot rule out the eventuality that a small amount of RA is produced within the fetal ovary itself based on our experiments. On the other hand, the addition of a large amount of exogenous RA (10^{-6} M) did not increase nor did it induce earlier meiotic initiation in the 11.5 dpc cultured ovary. RA addition sped up meiotic progression in the ovary. Though similar observations were already reported in the rat (Livera et al., 2000), this challenges the idea that mesonephric RA is required to induce meiosis after 11.5 dpc in the mouse ovary. Altogether these observations allowed us to use XX gonads without the mesonephros as a biological test to investigate the role of diffusible factors produced by the fetal testis on germ cells differentiation. In this *in vitro* system germ cells differentiation appears similar to what is observed *in vivo* though some molecular markers such as *Dmc1, Spo11, Fst* and *Rspo1* were mildly decreased in cultured ovaries.

Meiotic inhibiting factors are secreted by the developing testis

It has been long known that XX and XY germ cells have the potential to follow either the male or the female pathway according exclusively to their somatic environment (Adams and McLaren, 2002; Chuma and Nakatsuji, 2001; Dolci and De Felici, 1990; Zamboni and Upadhyay, 1983) suggesting the existence of a "Meiosis Inhibiting Factor" (MIF, Byskov and Saxen, 1976). Using our co-culture model, we demonstrated that the testes secrete factors that prevent the initiation of meiosis, and for the first time, we define the temporal windows when the developing testis produces the MIF. In our model, this meiotic inhibitory activity was detected as early as 12.5 dpc, when male somatic cells differentiate and form the testis cords, and disappears at 8 dpp, when male meiosis begins.

The high molecular weight of the MIF was demonstrated by fractionation of conditioned media. This indicates that the MIF is likely secreted proteins. This hypothesis is confirmed by the fact that freeze thaw steps, boiling, and trypsin-treatment, abolish the inhibitory effect on meiosis initiation of testicular conditioned media. Moreover, the absence of testicular cell migration in the XX gonad in the co-culture model and the lack of masculinization of somatic cells in the XX gonad are consistent with the MIF being secreted proteins. This is also in agreement with a recent publication highlighting the secretory role of Sertoli cell lines in inhibition of meiosis (Best et al., 2008).

Mitotic arrest factors and meiotic inhibiting factors can be dissociated

We demonstrated that, in addition to preventing meiosis, the developing testis induced male differentiation in XX germ cells. Indeed, germ cells in XX gonad co-cultured with fetal testis presented typical features of prospermatogonia. In the co-culture model, the fetal testis induced XX germ cell entry into mitotic arrest and this inhibition of proliferation is correlated with the up-regulation of the expression of the cell cycle inhibitor P16/ink4. Moreover, XX germ cells also expressed Oct4 and Nanos2 indicating that XX germ cells in our co-culture model are committed to differentiate towards the male pathways, like XY germ cells *in vivo*. Importantly, several evidences suggest that the two effects of the developing testis (preventing meiosis and inhibiting proliferation) rely on at least two different secreted factors: the MIF and the MAF (mitotic arrest factor). Previously, Adams and McLaren evidenced that 11.5 and 12.5 dpc XX germ cells were prevented from initiating meiosis and differentiate as prospermatogonia when placed in recipient 12.5 dpc male urogenital ridges (Adams and McLaren, 2002). We obtained similar results using 12.5 dpc XX germ cells and 13.5 dpc testicular somatic cells and refined this study by demonstrating that P75-positive cells (interstitial cells: mostly peritubular and Leydig cells) but not α6-positive cells (Sertoli cells) inhibited proliferation although both cell-

types prevented meiosis. Testicular conditioned medium only inhibited the initiation of meiosis whereas the close presence of the testis (i.e. in co-culture) inhibited both meiosis and proliferation. We propose that the MIF is more stable or act at long range and that the MAF act only in close proximity.

Meiotic inhibiting and mitotic arrest factor(s) remain to be identified

Unfortunately the weak stability of the secreted factors inhibiting meiosis and the absence of the mitosis preventing activity in conditioned media did not allow us to demonstrate exactly how many factors are acting and more than two factors may be involved. Recently, Fgf9, a testicular secreted protein, has been shown to prevent meiosis initiation in 12.5 dpc XX germ cells (Barrios et al, 2010). Ffg9 is produced by Sertoli cells and thus could be one good candidate for being the MIF here described. However we also reported that the MIF was produced both by interstitial (p75+) and Sertoli cell (α6+), while Fgf9 is produced only within testicular cords (Schmahl et al., 2004), thus it is likely that the MIF is composed of several factors and that some remained to be identified.

The nature of the MAF is more elusive as it appeared to be produced by the interstitial cells in our aggregate cultures. RA excess has been proven to prevent mitotic arrest in the developing testis (Trautmann et al., 2008). However, Cyp26b1 is produced by both interstitial and Sertoli cells in the developing testis; its expression being about three times higher in Sertoli cells. Thus if Cyp26b1 was the only factor corresponding to the MAF, Sertoli cells should also inhibit germ cell proliferation though we observed no such effect. Additional investigations will be required to identify all the factors that compose the MIF and MAF and to determine which are physiologically relevant.

MIF acts directly on undifferentiated germ cells

Recently, R-spo1/Wnt4/β-catenin pathway has been shown to induce female differentiation of the XX gonad and meiosis initiation (Maatouk et al., 2008; Chassot et al., 2008; Naillat et al., 2010). However the invalidation of R-spo1 or Wnt4 in the XX gonad also induces the early appearance of steroidogenic cells and we observed no such cells in the ovarian part of our co-culture model suggesting that this pathway was unlikely to be altered. Moreover other somatic testicular markers (Sox9, AMH, αSMA, Fgf9) were not induced in the XX gonad when meiosis was inhibited by conditioned medium and female somatic markers were not decreased neither. Lastly, testicular conditioned medium prevented significantly the meiosis initiation in the isolated undifferentiated germ cells indicating that the MIF produced by the testis act directly on undifferentiated germ cells.

Male germ cell sex determination is a multi-step process

Cyp26b1 has been proven to be required to prevent meiosis initiation in the fetal testis (Bowles et al., 2006) and later RA degradation has been proven to be required for male germ cell mitotic arrest (Trautmann et al., 2008). In this regard, the production of additional testicular factors (MIF and MAF) with similar activities appears intriguing. However, we bring forward evidence that Cyp26b1 alone is not sufficient and that the testis uses supplementary measures to prevent meiosis. First, the testis secretes proteins preventing germ cell differentiation towards the female pathway while Cyp26b1 is a member of the CYP450 family all of which are cytoplasmic enzymes and not diffusible (Nelson, 1999). Second Cyp26b1 expression is not correlated with meiosis prevention in our co-culture model: Cyp26b1 is abundant in the 8 dpp testis and 8 dpp testis co-cultured with ovaries did not inhibit meiosis in XX germ cells. These findings suggest that two distinct pathways of prevention of meiosis are present in the fetal testis: a local RA depletion by Cyp26b1 and secretion of the MIF by the somatic environment.

We considered two hypotheses to explain the meiosis initiation observed in the Cyp26b1$^{-/-}$ testes during fetal life: first, the secretion of the testicular substances that prevent meiosis requires RA degradation by Cyp26b1; second, both MIF and Cyp26b1 being required simultaneously to inhibit meiosis. Accordingly with the first hypothesis: RA has previously been shown to alter testicular somatic cell secretions (Livera et al., 2002). The second hypothesis is sustained by the absence of correlation between Cyp26b1 expression and the testicular meiotic activity in our experiments and our observation that some XX germ cells did not enter into meiosis in the ovary co-cultured with testes in the presence of RA. Eventually, both hypotheses may be

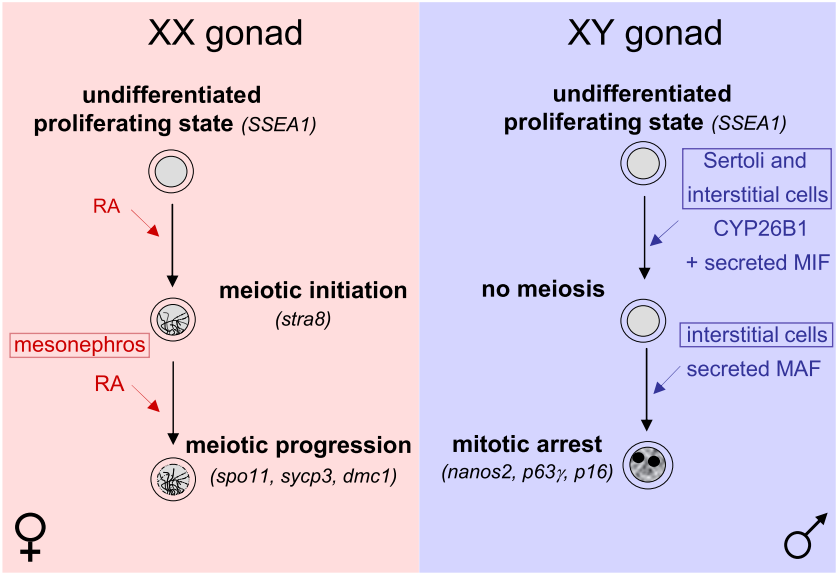

Fig. 7. Proposed model of the action of the fetal testis on the differentiation of germ cells. At 11.5 dpc, germ cells remain undifferentiated and bipotential. These cells express undifferentiated markers such as SSEA1, and proliferate actively. In the fetal ovary (XX gonad), the germ cells express Stra8 and initiate meiosis. Later, retinoic acid (RA) produced by the adjacent mesonephros promotes meiosis progression. In the fetal testis (XY gonad), somatic cells (interstitial and Sertoli cells) secrete the MIF (Meiosis inhibiting factor), a substance that prevents meiosis initiation. Second, the fetal testis secretes additional factor(s)–called MAF (Mitotic arrest factor)–that promote(s) the male pathways in the non-meiotic germ cells, defined by the mitotic arrest of these cells and the expression of male markers (such as nanos2).

true simultaneously and additional work will be required to definitively elucidate whether the production of the MIF and/or MAF depends upon RA status.

Conclusion

Our various findings indicate that the establishment of the male pathway in fetal germ cells is a complex and multi-step process which requires the degradation of RA by Cyp26b1 and also additional mechanisms dependant of the secretion of testicular substances: the MIF and the MAF (Fig. 7). The identification of these secreted factors will be a challenging goal in the coming years.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2010.08.002.

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