Loss of Synaptonemal Complex Protein-1, a Synaptonemal Complex Protein, Contributes to the Initiation of Follicular Assembly in the Developing Rat Ovary

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In the rat ovary, germ and somatic cells become organized into primordial follicles 48–72 h after birth. Although several genes have been implicated in the control of early follicular growth, less is known about the factors involved in the formation of primordial follicles. Using the method of differential display of mRNAs, we found several genes differentially expressed at the time of follicular assembly. One of them encodes synaptonemal complex protein-1 (SCP1), a core component of the protein complex that maintains recombining chromosomes together during prophase I of the first meiotic division in germ cells. This association, evident during the pachytene stage, ends when chromosomal desynapsis begins in the diplotene stage at the end of prophase I. Oocytes become arrested in the diplotene/dictate stage before becoming enclosed into primordial follicles, suggesting that oocytes must complete meiotic prophase I before becoming competent to direct follicle assembly. We now show that attainment of the diplotene stage results in follicular formation. In developing rat ovaries, SCP1 mRNA expression is confined to oocytes and decreases precipitously within 24 h after birth, preceding the organization of primordial follicles. The premature loss of SCP1, achieved via treatment with an antisense oligodeoxynucleotide targeting SCP1 mRNA, resulted in more oocytes reaching the diplotene stage, as evidenced by a decrease in the number of oocytes containing germ cell nuclear antigen-1 (a nuclear protein whose expression ceases in diplotene) and an increase in the number of oocytes expressing MSY2 (a cytoplasmic Y box protein expressed in oocytes that have become arrested in diplotene). SCP1-deficient ovaries exhibited an increased number of newly formed follicles, suggesting that completion of meiotic prophase I endows oocytes with the ability to orchestrate follicular assembly.

IN RECENT YEARS, substantial efforts have been devoted to identifying the factors and cellular mechanisms involved in the control of ovarian follicular assembly and the initial growth of newly formed primordial follicles. In the rodent ovary, follicles are assembled mostly during the first 72 h of postnatal life (1, 2). The process is a remarkable exercise in cell-cell coordination, because hundreds of primordial follicles are formed within 72 h after birth (2). At birth, oocytes are organized in clusters, but this association changes dramatically during the first 24 h of postnatal life. At this time, pregranulosa cells intercalate between the oocytes, which begin to associate with these cells instead of contacting one another (1, 3). This germ cell-somatic cell association is completed 48–72 h after birth with the isolation of individual oocytes by a single layer of flattened granulosa cells, which, in turn, becomes surrounded by a basal membrane, giving rise to a primordial follicle (1, 4). Once formed,

primordial follicles begin to develop to become primary follicles via the differentiation of the flattened granulosa cells into a cuboidal morphology. Subsequent growth is accomplished by both enlargement of the oocyte and by proliferation of granulosa cells, which results in the formation of additional layers of these cells (1, 4).

Although several factors involved in the control of early follicular growth have been recently identified (5–11), less is known about the molecules that may contribute to follicular formation. Among these molecules, the transcription factor FIGα (factor in the germ line α) stands out as the most important, because in its absence oocytes die, and follicular assembly fails to occur (12). The critical importance of FIGα notwithstanding, other factors, such as anti-Mullerian hormone (10, 13) and the Kit ligand/stem cell factor (8), have also been shown to contribute to the process, the former inhibiting formation of primordial follicles (10, 13), and the latter promoting it (8). Another factor shown to promote primordial follicle formation is bone morphogenic protein-4 (14), a member of the TGF-β superfamily expressed in stromal cells of the developing ovary (14). Interestingly, maternal steroids have also been shown to affect primordial follicle assembly in addition to the transition of primordial to primary follicles (15). This study demonstrated that progesterone delays follicular assembly, and that this inhibition is related to an antiapoptotic effect of the steroid (15). Such a

conclusion is supported by the previous finding that most oocytes undergo apoptosis around the time of birth (16; but see Ref. 17). The overall conclusion that has emerged from these findings is that factors produced by the oocytes are required not only for the growth of ovarian follicles, but also for their formation (18), because the loss of oocytes resulting from genetic mutations (19), pharmacological intervention (20), or genetic manipulation (12) impairs the formation of primordial follicles.

A well-established aspect of oocyte development in rodents is that they enter meiosis and progress through the first meiotic prophase during fetal life, becoming arrested in the last (diplotene) stage, shortly before becoming enclosed by granulosa cells into primordial follicles (21). Until now, no studies have examined the nature of this temporal relationship; thus, the two events have been considered to be coincidental rather than causally related. We now show that the attainment of the diplotene stage of meiotic prophase is accompanied by a dramatic loss of synaptonemal complex protein-1 (SCP1), a core component of the synaptonemal complex (SC) that maintains recombining chromosomes closely apposed during the preceding, pachytene stage (22–24). We also show that the premature loss of SCP1 accelerates attainment of diplotene, which is then followed by follicular formation.

Materials and Methods

Animals

Timed-pregnant Sprague Dawley rats were purchased from B&K Universal (Fremont, CA). Upon arrival at the beginning of the third week of gestation, they were housed under controlled conditions of temperature (23–25 C) and light (14 h of light, 10 h of darkness; lights on from 0500–1900 h), and were given *ad libitum* access to food (Purina laboratory chow, Ralston-Purina, St. Louis, MO) and tap water. Fetal ovaries were collected at 21 d gestation (F21), *i.e.* 24 h before the expected day of birth (which in rats occurs on gestational d 22). Postnatal ovaries were collected 6, 24, 48, and 96 h after birth. The procedures used for animal euthanasia and tissue collection were duly approved by the Oregon National Primate Research Center animal use and care committee.

Differential display of expressed mRNAs

To identify genes that may be differentially expressed at the time of ovarian follicular assembly, we used a differential display kit purchased from GeneHunter Corp. (Nashville, TN), following the procedure recommended by the manufacturer. Total RNA was isolated from ovaries of 21-d-old fetuses and 48 h after birth by the acid phenol extraction method (25). Two hundred nanograms of each RNA were reverse transcribed in parallel reactions using 20 μM each of four deoxynucleotides (dNTPs) and three different one-base anchored oligodeoxythymidine (oligo dT) primers, *i.e.* having an A, C, or G immediately upstream from the 11-nucleotide (nt) oligo dT priming sequence. Upon completion of the RT reaction, 2 μl of each reaction were subjected to PCR amplification using the dNTPs at 2 μM, the same one base-anchored oligo dT primer employed for RT, and one of eight different 13-mer 5' primers provided in the kit (*i.e.* each RNA sample is amplified using a total of 24 primer combinations). GeneHunter provides 10 different kits for the identification of differentially displayed mRNAs. SCP1 was identified in the developing ovary using RNAimage kit 4 (catalogue no. G504). To detect the different PCR products, each 20-μl amplification reaction included 0.2 μl α-[^{33}P]dATP (2000 Ci/mmol; ICN Biomedicals, Inc., Costa Mesa, CA). The PCR parameters used were 30 sec of denaturing at 94 C, 2 min of annealing at 40 C, and 30 sec of extension at 72 C for 40 cycles, followed by 5 min at 72 C. The PCR products were resolved by electrophoresis in 6% denaturing sequencing polyacrylamide gels, using as molecular markers an RNA marker template set (catalog no. 7780, Ambion, Austin,

TX) transcribed in the presence of [^{32}P]UTP under conditions that generate a low specific activity set of five bands (100, 200, 300, 400, and 500 bases) of similar intensity. Upon autoradiographic identification of candidate bands, they were excised from the gel, eluted in water, precipitated with 3 M sodium acetate, and reamplified using the same primers employed for the initial PCR and the dNTPs at 20 μM in the absence of labeled dATP. The sizes of the reamplified products were verified by gel electrophoresis of the ethidium bromide-stained DNA. The appropriate bands were then isolated by gel electroelution-precipitation and cloned into the pGEM-T vector (Promega Corp., Madison, WI). Their nt sequences were determined using AmpliTaq DNA polymerase and a fluorescence-based dideoxy sequencing reaction performed on an Applied Biosystems DNA sequencer model 373A (Foster City, CA). Comparison of the sequences obtained with gene sequences available in the GenBank was carried out using the Basic Local Alignment Search Tool (BLAST) algorithm applied to searches of the nonredundant and expressed sequence tag National Center for Biotechnology Information data bases.

In situ hybridization

Detection of SCP1 mRNA at the cellular level was carried out essentially as recommended by Simmons *et al.* (26), with the modifications previously reported by our laboratory (27, 28), and using a [^{35}S]UTP-labeled SCP1 cRNA probe transcribed from the SCP1 cDNA sequence obtained from the differential display analysis of fetal d 21 ovaries. The ovaries of different perinatal ages (see above) were fixed by immersion in 4% paraformaldehyde-0.1 M sodium borate buffer (pH 9.5) overnight at 4 C. Thereafter, they were placed in PBS for 24 h at 4 C, transferred to PBS-20% sucrose, and embedded the next day in OCT compound (Miles, Inc., Elkhart, IN) before freezing on dry ice. The hybridization was performed on 14-μm cryostat sections at 55 C for 18–20 h using the conditions and washing stringency previously reported (28). Control sections were incubated with a sense SCP1 cRNA probe transcribed from the same cDNA, but in the opposite direction.

Organ culture and antisense oligonucleotide treatment

The ovaries used to examine the effect of blocking SCP1 synthesis on follicular formation were from 21-d-old fetuses. Ovaries at this age contain no follicles, because follicular assembly in the rat occurs between 48–72 h after birth (1, 2). The glands were dissected under aseptic conditions, placed on sterile lens paper, and cultured on metal grids at the interface of air-culture medium, under an atmosphere of 60% O$_2$, 35% N$_2$, and 5% CO$_2$, as previously described (29, 30). This gas composition was previously shown to maximize the survival of ovaries in organ culture (31). One or two ovaries per well were cultured in 24-well plates, each well containing 750 μl DMEM/Ham's F-12 (50%, vol/vol) medium supplemented with glucose (4.5 g/liter), penicillin (100 U/ml), and streptomycin (100 μg/ml), as previously reported (29, 30). One ovary from each animal was exposed for 4 d to an antisense (AS) oligodeoxynucleotide against SCP1 mRNA (SCP1 AS) and the contralateral ovary was treated with a scrambled (SCR) DNA sequence of identical nt composition (see description of each below). The right and left ovaries from each rat were alternatively used for AS or SCR treatment. The oligonucleotides were used at a concentration of 5 μM, replacing the culture medium with fresh medium containing the same oligonucleotide concentration 48 h after the initiation of culture. At the end of this 96-h treatment, the ovaries were fixed in Kahle's fixative (32), embedded in paraffin, serially sectioned at 6 μm, stained with Weigert's iron hematoxylin, and counterstained with picric acid-methyl blue (5) for morphometric analysis (see below).

The AS used was an 18-mer oligodeoxynucleotide complementary to the sequence 5'-GGC-AGC-ATG-GAG-AAG-CAA-3' encompassing the translation initiation site of the mouse SCP1 gene (GenBank accession no. NM_011516). The SCR sequence used as a control was an 18-mer oligodeoxynucleotide (5'-TTT-ACT-CCG-TTC-GCC-GTC-3'). Analysis of this sequence using the Advanced BLAST algorithm of the National Center for Biotechnology Information showed that it had no homology to any known mammalian DNA sequence. We used a mouse oligodeoxynucleotide because the rat sequence was unavailable at the time. Subsequent release of the rat sequence showed that the mouse oligonucleotide recognizes a sequence upstream of the ATG codon (nt 106–

121 in rat SCP1 mRNA; accession no. NM_012810). Real-time PCR analysis demonstrated that both the mouse and a rat-specific oligodeoxynucleotide targeted to the sequence encompassing the ATG codon in rat SCP1 mRNA (5'-TTG-ACA-ATG-TCC-AGT-CTA-3') were similarly effective in decreasing SCP1 protein levels (see *Results*).

Morphological evaluation

Serial 6-μm ovarian sections were analyzed for follicular development as follows. Every third section (*i.e.* using a periodicity of 18 μm) was imaged on an Axioplan (Carl Zeiss, Jenna, Germany) using a Cool-Snap camera (Roper Scientific, Stillwater, MN). Primordial, primary, and secondary follicles were counted using the manual count feature of MetaMorph (Universal Imaging Co., West Chester, PA). Only follicles in which the nucleus of the oocyte was visible were counted (5, 30, 33). The total number of follicles per ovary was estimated by determining the mean total number of follicles per section after sampling every third section. This mean value was then multiplied by the total number of serial sections collected from each ovary. To classify a follicle in a particular developmental stage, we used the morphological criteria recommended by Hirshfield (34) and Rajah *et al.* (1). According to these criteria, primordial follicles are the initial result of follicular assembly, a differentiation process in which individual oocytes become surrounded by a single layer of flattened epithelial pregranulosa cells, which is, in turn, separated by a basal lamina from a single layer of elongated mesenchymal cells that separates the newly formed follicles from one another. Primary follicles are those in which the epithelial pregranulosa cells surrounding the oocyte have acquired a cuboidal aspect; they represent the first stage of follicular growth. Secondary follicles are those containing two or more layers of granulosa cells solidly surrounding the oocyte, with no signs of antrum formation.

Immunohistofluorescence

The meiotic progression of oocytes was estimated using antibodies to the stage-specific proteins germ cell nuclear antigen-1 (GCNA1) and germ cell-specific Y box protein-2 (MSY2). Although GCNA1 serves as a unique marker of germ cells and oocytes progressing through the zygotene stage of the first meiotic division (17, 35), MSY2 is exclusively expressed in oocytes that have reached the diplotene stage (12, 36). Immunohistochemical detection of GCNA1 and MSY2 was performed on 6-μm, paraffin-embedded sections derived from ovaries fixed by immersion in Bouin's fixative (2 h at room temperature). GCNA1 was detected with a rat monoclonal (IgM) antibody (provided from Dr. G. Ender, University of Kansas, Kansas City, KS) and MSY2 with the polyclonal antibodies mRNP3 and -4 (mRNA binding proteins 3 and 4, gift from Dr. M. T. Murray, Wayne State University, Detroit, MI) prepared in guinea pigs and directed against the immunologically related Y box protein, frog Y2 protein (FRGY2), present in *Xenopus* oocytes (37). These antibodies recognize mammalian MSY2 in Western blots and have been used to identify oocytes in the diplotene stage (12, 36). The ovarian sections were incubated (1 h at 33 C) with undiluted GCNA1 monoclonal hybridoma supernatant, followed by an overnight incubation with mRNP3 and -4 (1:5000). GCNA1 immunoreactivity was developed with Texas Red goat antirat IgM μ-chain-specific antibody (Jackson ImmunoResearch Laboratories, West Grove, PA; diluted 1:250) for 2 h at room temperature, and MSY2 was detected with an Alexa 488-conjugated goat antiguinea pig γ-globulin (Molecular Probes, Eugene, OR; 1:250 dilution; 2 h at room temperature). Control ovarian sections were incubated in the absence of either primary antibody. Fluorescence images were acquired with a Mariannas imaging workstation (Intelligent Imaging Innovations, Denver, CO) using either a $\times$10 N.A. 0.5 Fluar or a $\times$63 N.A. 1.4 PlanApo objective. Images were processed using Photoshop7.0 (Adobe Systems, San Jose, CA).

Relative quantitative RT-PCR

Total RNA was extracted using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH), One microgram of total RNA was annealed to random primers (150 ng; Promega Corp.) and reverse transcribed with 1 μl SuperScript II (200 U/μl; Invitrogen Life Technologies, Inc., Carlsbad, CA). Thereafter, the MSY2 mRNA content of each sample was estimated by relative quantitative PCR using the QuantumRNA 18S

Internal Standard Kit (Ambion, Austin, TX). PCR amplification of the reverse transcribed products was performed using HotStart *Taq* polymerase (Qiagen, Valencia, CA). In the case of MSY2, we used 10 pmol of a forward primer (5'-GCC ACC CCA ACA GCC TAC C-3') corresponding to nt 870–888 in mouse MSY2 mRNA (GenBank accession no. AF073954) and a reverse primer (5'-AGC TGG CTG ATT CCT TCT CA-3') complementary to nt 1334–1353. Each reaction also had 2.5 μl of a mixture of 18S primers/competimers (at a ratio of 3:7). The reaction volume was 25 μl. The PCR conditions used were 15 min at 95 C, followed by 28 cycles of 30 sec at 94 C, 30 sec of annealing at 55 C, and 1-min extension at 72 C, ending with 10 min at 72 C. Equal volumes of the PCRs were electrophoresed on 2% agarose gels stained with ethidium bromide, the gels were scanned electronically, and the images were quantitated using the image analysis Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Real-time PCR

The time course of SCP1 mRNA expression and the ability of SCP1 AS to decrease SCP1 mRNA levels were quantitated by real-time PCR. Total RNA was reversed transcribed using the SuperScript II RT kit (Invitrogen Life Technologies, Inc.) and random hexamer primers, as suggested by the manufacturer. Briefly, 1 μg total RNA was reverse transcribed in a 20-μl volume, and 2 μl of the RT reaction was diluted 1:5 before real-time PCR. Each sample was run in triplicate along with a relative and an absolute standard curve. Relative standard curves were generated by measuring SCP1 mRNA and 18S ribosomal RNA (18S rRNA) content in a serially diluted sample from a pool of fetal (d 21) ovaries. The content of 18S rRNA in each experimental sample was then calculated using as a reference these relative standard curves, and the values obtained were used to normalize the content of SCP1 mRNA in each sample. The primer and probe used to detect 18S rRNA were purchased as a kit (TaqMan Ribosomal RNA Control Reagents Kit, PerkinElmer Applied Biosystems, Foster City, CA). Absolute standard curves were constructed from serial dilutions of SCP1 mRNA (2 ag-200 pg) transcribed *in vitro* using T7 RNA polymerase (38) from a rat SCP1 cDNA template cloned from neonatal ovaries into the pGEM-T plasmid. The threshold cycle number from each sample was referred to the absolute curve to estimate the corresponding RNA content per sample, and each RNA value was then normalized for procedural losses using their respective 18S rRNA values estimated from the relative standard curve. The threshold cycle number was the fractional cycle number at which the fluorescence accumulated to a level 10 times greater than 1 SD from basal values. The SCP1 primers were designed to amplify a 97-bp fragment within the SCP1 mRNA coding region. Both these primers and the fluorescent probe were designed with the help of Primer Express 2.0 software (PerkinElmer Applied Biosystems). The forward primer (5'-AGCTTTTGGGAGAGGTTGAGAA-3') corresponds to nt 2204–2225 in rSCP1 mRNA (GenBank accession no. NM_012810); the reverse primer (5'-TCAGCTATTTTATGTTGGCATCGT-3') is complementary to nt 2277–2300. The internal fluorescent oligodeoxynucleotide probe sequence (5'-CCAAAGCAACAGTTGATGAAGCGTAAAGTT-3') was covalently linked to the fluorescent dye, 6-carboxyfluorescein, at the 5' end and to the quencher dye, tetramethylrhodamine, at the 3' end (PerkinElmer Applied Biosystems). Real-time PCRs were performed in a total volume of 10 μl; each reaction contained 2 μl diluted cDNA or 2 μl standard, 5 μl TaqMan Universal PCR Master Mix (Applied Biosystems), 250 nM of each gene-specific and ribosomal fluorescent probe, 300 nM of each gene-specific primer, and 50 nM of each ribosomal primer. The PCR program used consisted of an initial annealing period of 2 min at 50 C, followed by 10 min of denaturing at 95 C and 40 cycles of 15 sec at 95 C and 1 min at 60 C.

Data analysis

The paired *t* test was used to compare the number of follicles present in SCP1 AS-treated ovaries with the number of follicles detected in the contralateral ovary of the same animal treated with the SCR oligodeoxynucleotide sequence. The unpaired two-tailed *t* test was used to analyze the differences in MSY2 between AS- and SCR-treated groups. Finally, a one-way ANOVA, followed by Duncan's test to compare different groups against a control group, were used to analyze the

postnatal changes in SCP1 and MSY2 mRNA in normal ovaries compared with fetal d 21 glands.

Results

An mRNA containing the sequence of rat SCP1 is differentially expressed around the time of follicular formation

After RT-random PCR amplification of mRNA species expressed in fetal (F21) and postnatal [48 h (PN-48 h)] ovaries, electrophoretic separation of the amplified cDNA species revealed the presence of several differentially displayed bands. One of them, approximately 470 bp in length (Fig. 1A, *arrow*), was selected for additional analysis. Although the overall background of the duplicate lanes derived from fetal ovaries was higher than that of the lanes derived from the postnatal ovaries, this band was selected because it was clearly more abundant in fetal than in postnatal ovaries, in contrast to the numerous other bands seen in the same region of the gel (Fig. 1A). DNA sequencing demonstrated that the
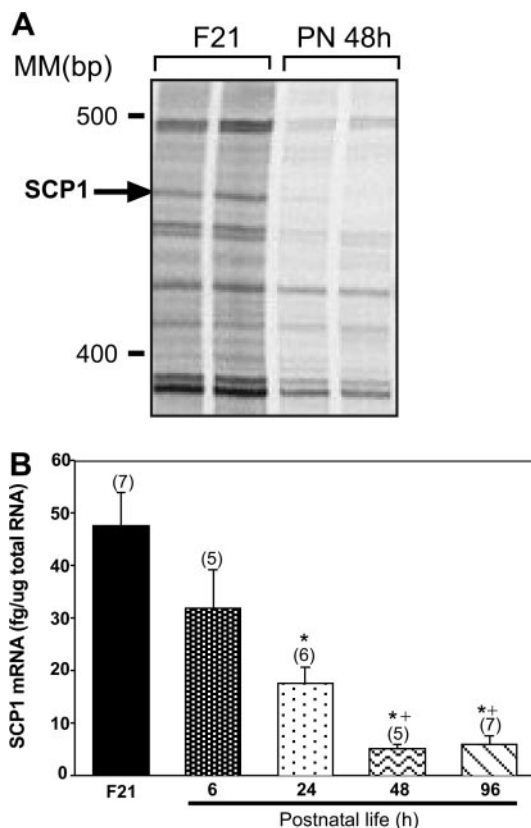
FIG. 1. SCP1 mRNA levels decline in the rat ovary during the hours preceding follicular assembly, which in the Sprague Dawley rat is initiated 36 h after birth (2). A, Autoradiograph showing the portion of a sequencing gel where a differentially displayed band containing the sequence of rat SCP1 mRNA (*arrow*) was detected by differential display analysis. Each PCR was electrophoresed in duplicate. The RNA used for differential display analysis was from rat ovaries collected on F21, *i.e.* 1 d before birth, and PN-48 h. The positions of DNA molecular markers (MM) are shown to the *left* of the gel. B, SCP1 mRNA content in the rat ovary on F21 and at different intervals after birth (6–96 h) as assessed by real-time PCR. *Bars* are the means, and *vertical bars* are the SEM. The *numbers above bars* are the number of animals per group. *, $P < 0.01$ *vs.* F21; +, $P < 0.05$ *vs.* PN-24 h.

selected band corresponded to a 498-nt cDNA species identical with rat SCP1 (23), a major protein component of the chromosomal synaptonemal complex, a structure formed between homologous chromosomes during the pachytene phase of the first meiotic prophase (reviewed in Ref. 39).

We next used real-time PCR and a more detailed time course to validate the differential display findings. The results showed that SCP1 mRNA levels decline gradually between F21 and PN-24 h ($P < 0.01$), and then abruptly between 24 and 48 h after birth ($P < 0.05$), remaining at low levels at PN-96 h (Fig. 1B).

SCP1 mRNA is selectively expressed in oocytes, and its expression decreases during the hours preceding follicular assembly

We cloned the SCP1 cDNA obtained from differential display analysis into the riboprobe vector pGEM-T and used this template to generate a cRNA probe to identify by *in situ* hybridization the cellular sites and developmental pattern of SCP1 mRNA expression in the feto-neonatal rat ovary. As shown in Fig. 2A, SCP1 mRNA was strikingly abundant in oocyte clusters present in the fetal ovary before the organization of primordial follicles. In agreement with the decline in SCP1 mRNA levels measured by real-time PCR, the level of expression was still high by 6 h after birth, but decreased dramatically by the end of the first postnatal day (PN-24 h), becoming almost undetectable at the time of follicular assembly (PN-48 h) and remaining low thereafter. Figure 2B illustrates the marked perinatal loss in SCP1 mRNA expression by contrasting the abundance of SCP1 mRNA levels in oocyte clusters of an F21 ovary with the very low or undetectable levels seen in oocytes of an ovary 96 h after birth, a time at which the main wave of follicular assembly had already occurred. Higher magnification images (Fig. 2C) confirmed that SCP1 expression in F21 ovaries is confined to oocyte clusters (*long arrows, left panel*), with no hybridization detected in somatic cells (*short arrows*). The intensity of the signal is dramatically reduced in oocytes after follicular assembly (Fig. 2C, *right panel*, *long arrows*) and, similar to fetal ovaries, is absent in somatic cells (*short arrows*).

Oocytes of the rat ovary progress through the diplotene stage of the first meiotic division within 48 h after birth

Using GCNA1 and MSY2 as molecular markers of meiotic progression, it became apparent that, as previously shown in the mouse (12) and rat (21) ovary, oocytes reach the end of the first meiotic prophase during the early neonatal period. By F21, *i.e.* 1 d before the expected day of birth, most oocytes were GCNA1 immunopositive, and few contain MSY2 (Fig. 3A, *upper left panel*). By 6 h after birth, there were still many GCNA1-positive oocytes, but many more contain MSY2 (Fig. 3A, *upper left middle panel*). In contrast, by 24 h, and particularly at 48 h after birth, most oocytes were MSY2 positive, whereas few still contained GCNA1 (Fig. 3A, *right panels*). Higher magnification images (Fig. 3B) show that in F21 ovaries most oocytes are in clusters and are GCNA1 positive regardless of their content of MSY2 (*left panel*). In contrast,

FIG. 2. Expression of SCP1, a germ cell-specific gene, decreases in oocytes during the hours preceding the formation of primordial follicles in the rat ovary. The mRNA was detected by hybridization histochemistry using a [^{35}S]UTP-labeled SCP1 cRNA transcribed from an SCP1 cDNA template isolated by differential display from fetal rat ovaries collected on F21. A, Hybridization profiles are depicted as darkfield images (*upper panel*), and the regions of the ovary where these signals are localized are shown in brightfield (*lower panels*). Bars, 100 μm. B, The loss of SCP1 mRNA that precedes follicular assembly (F21) persists after the completion of folliculogenesis (PN-96 h). *Bars*, 50 μm. C, Higher magnification images showing the abundance of SCP1 mRNA in oocyte clusters (examples denoted by *long arrows, left panel*) of the rat fetal ovary on F21 and the marked loss in SCP1 mRNA content detected after follicular organization (examples denoted by *long arrows*, *right panel*). Notice the lack of hybridization in elongated somatic cells surrounding the oocyte clusters or follicular granulosa cells (*short arrows*). Bars, 20 μm.
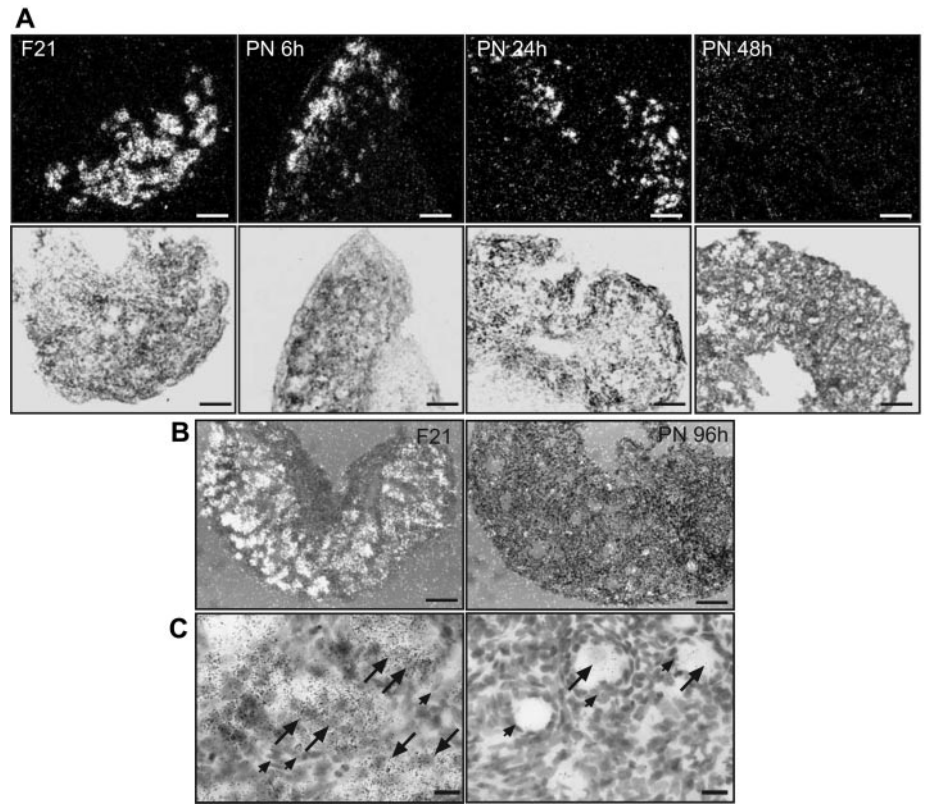
48 h after birth those oocytes already encapsulated into newly formed follicles contain predominantly MSY2, and no or little GCNA1 (Fig. 3B, *right panel*). Preceding follicular assembly, oocytes appear to contain similar levels of GCNA1 and MSY2 (example denoted by *short arrow* in Fig. 3B, *right panel*). Because GCNA1 is only expressed through the pachytene stage of meiotic prophase (12, 35), and MSY2 expression is initiated subsequently (when meiosis has pro-
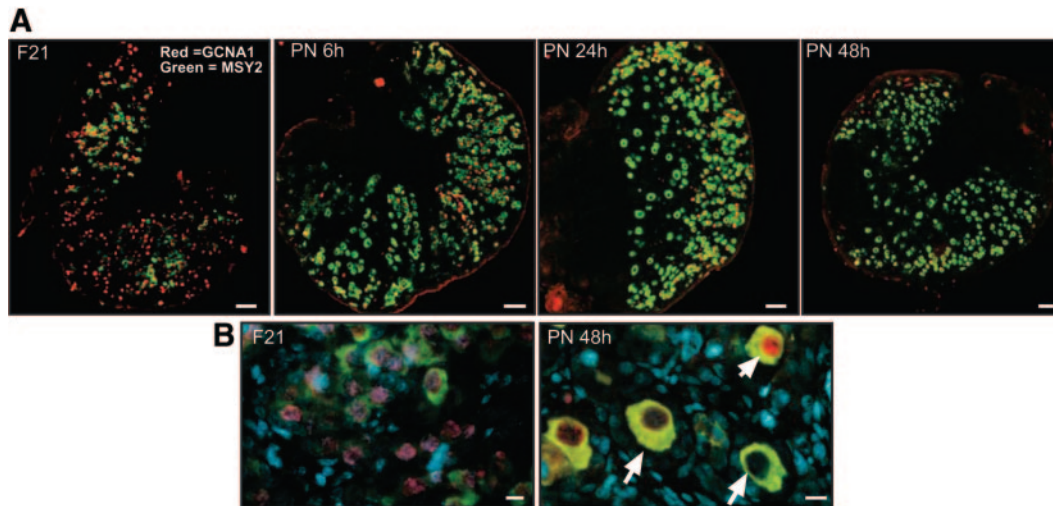

FIG. 3. Meiotic stages in perinatal ovaries identified by the immunohistochemical visualization of GCNA1 and MSY2, two stage-specific markers, during the hours preceding and accompanying follicular assembly. A, Low magnification images illustrating the decrease in GCNA1 content (*red*) and the concomitant increase in MSY2 abundance (*green*) that occur in oocytes before follicular formation. *Bars*, 50 μm. B, Higher magnification images showing the abundance of GCNA1 and the relatively lower content of MSY2 in oocyte clusters of a fetal (F21) ovary (*left panel*) and the predominance of MSY2 in oocytes of newly formed follicles at PN-48 h (*arrows*). The *short arrow* in the *right panel* points to an isolated oocyte not yet surrounded by pregranulosa cells to form a primordial follicle and showing a similar abundance of GCNA1 and MSY2. Cell nuclei, stained with Hoechst, are in *blue*. Bars in B, 10 μm. GCNA1, a germ cell nuclear protein expressed in oocytes before they reach the diplotene stage of the first meiotic division (35), was detected with a monoclonal antibody raised in rats, and the reaction was developed to a red color with Texas Red-conjugated goat antirat μ-chain-specific IgM. MSY2, a cytoplasmic protein of the Y box family expressed in oocytes that have reached the diplotene/dictyate stage (36), was detected with a polyclonal antibody raised in guinea pigs, and the reaction was developed to a green color with an Alexa 488-conjugated goat antiguinea pig IgG.

gressed to the diplotene stage) (36), the expression profiles observed indicate that in the rat ovary, oocytes attain the diplotene stage during the hours preceding the initiation of follicular assembly.

An AS oligodeoxynucleotide against SCP1 mRNA reduces SCP1 mRNA content in feto-neonatal ovaries

Ovaries explanted on F21 in organ culture and treated for 4 d with an SCP1 AS complementary to either mouse or rat SCP1 mRNA exhibited significantly ($P < 0.003$) lower levels of SCP1 mRNA than control ovaries treated with SCP1 SCR, as assessed by real-time PCR (Fig. 4). Because of the difficulty of obtaining a sufficient amount of protein from feto-neonatal ovaries for Western blot analysis, we used dispersed seminiferous tubules from adult male rats to determine whether the AS treatment reduces SCP1 protein levels. The results of two independent experiments showed that both rat and mouse SCP1 AS decreased SCP1 protein content by about 70% (data not shown).

Appearance of MSY2 expression that characterizes attainment of the diplotene stage of first meiosis division is advanced by SCP1 AS-mediated inhibition of SCP1 synthesis

Consistent with the immunohistofluorescence results described above, measurement of MSY2 mRNA content in fetal (F21) and neonatal (PN-48 and -96 h) ovaries showed that expression of the MSY2 gene increases during this period (Fig. 5A). *In vitro* treatment of F21 ovaries with SCP1 AS for 24, 48, and 96 h increased MSY2 mRNA levels at all time points examined, with the greatest change occurring after

FIG. 5. MSY2 mRNA content increases in the rat ovary during perinatal development, and this increase can be induced prematurely by inhibition of SCP1 synthesis. A, Ovarian MSY2 mRNA content, estimated by semiquantitative PCR, increases significantly at PN-48 h, remains elevated after completion of follicular assembly (PN-96 h). B, Treatment of F21 ovaries in organ culture with SCP1 AS (5 μM) increases MSY2 mRNA levels as early as 24 h after initiation of the treatment, with the effect being more pronounced after 96 h. C, *In vitro* treatment of F21 ovaries with SCP1 AS decreases the fraction of oocytes expressing the meiotic prediplotene marker GCNA1 and increases the fraction of oocytes containing the diplotene stage marker MSY2. *, $P < 0.05$; **, $P < 0.01$ [*vs.* MSY2 mRNA content of F21 ovaries (A) or *vs.* SCR-treated control (B and C)]. *Bars* are group means, and *vertical bars* represent the SEM. *Numbers above bars* are the number of animals per group.
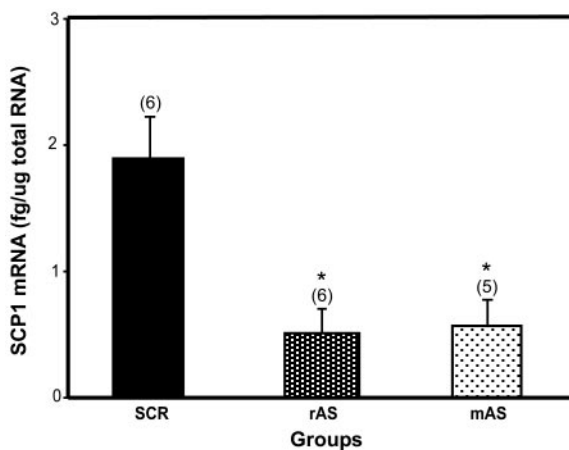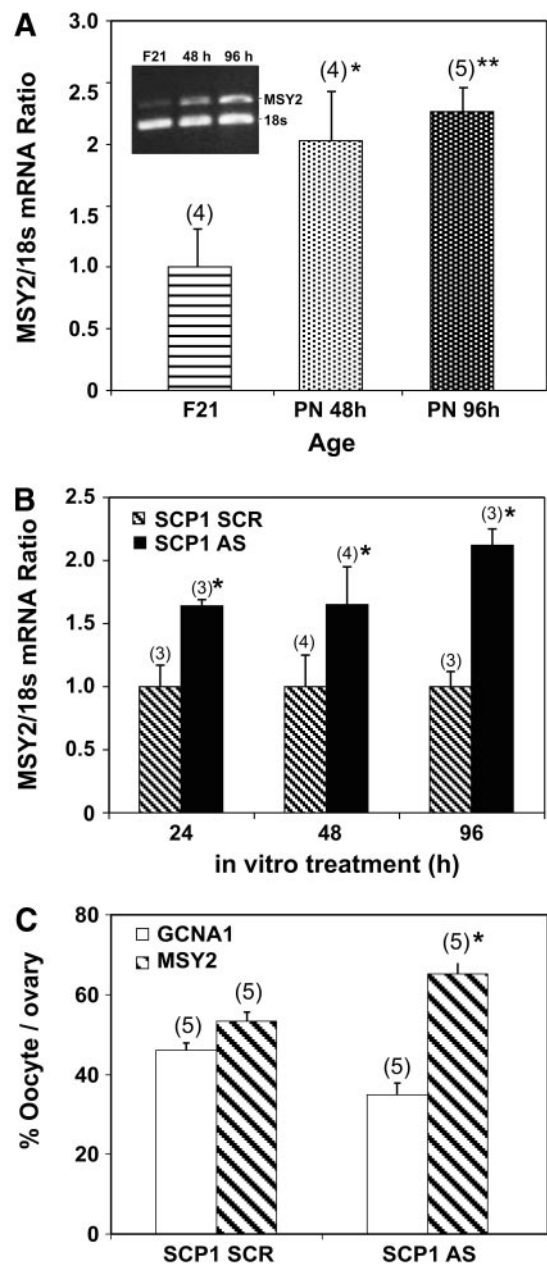
FIG. 4. Inhibition of SCP1 gene expression in the rat ovary using AS directed against either rat or mouse SCP1 mRNA (see text for details). The ovaries were removed on F21 and placed in organ culture for 4 d in the presence of either an SCP1 AS or a SCR DNA sequence with the same base composition. For each animal, one ovary was treated with SCP1 AS, and the contralateral ovary was treated with SCR. At the end of this treatment, RNA was extracted from individual ovaries, and the SCP1 mRNA content was measured by real-time PCR. *Bars* are the means, and *vertical lines* are the SEM. *Numbers above bars* are the number of animals per group. Notice that both ODNs were equally effective. *, $P < 0.003$ *vs.* SCR control. rAS, AS against rat SCP1 mRNA; mAS, AS against the mouse sequence.

96 h of exposure (Fig. 5B). To determine whether these changes in mRNA content were accompanied by similar changes in the number of oocytes expressing MSY2 protein,

F21 ovaries were exposed in organ culture to SCP1 AS for 96 h. The contralateral ovary from each animal was treated with SCP1 SCR. As shown in Fig. 5C, the ovaries treated with SCP1 SCR had approximately equal numbers of oocytes expressing GCNA1 and/or MSY2, indicating that under the *in vitro* conditions used, a subpopulation of oocytes progresses toward the end of the meiotic prophase, as would have occurred in most oocytes had the ovaries been left *in situ*. Ovaries treated with SCP1 AS showed a reduction in the number of GCNA1-positive oocytes and a corresponding increase in the proportion of oocytes expressing MSY2 (Fig. 5C). The images depicted in Fig. 6 illustrate these changes. In addition, they show that although most oocytes in SCP1 SCR-treated ovaries are in transition (*i.e.* express both GCNA1 and MSY2), a majority of oocytes in SCP1 AS-treated ovaries have a reduced GCNA1 content and increased MSY2 expression (Fig. 6A). Examination of the ovaries at higher magnification (Fig. 6B) showed that oocytes expressing mostly GCNA1 in SCP1 SCR-treated ovaries are not contained within follicles (*left panel*). In contrast, oocytes showing loss of GCNA1/increased MSY2 expression in SCP1 AS-treated ovaries are usually enclosed within newly formed follicles (Fig. 6B, *right panel*, examples denoted by *arrows*). These findings indicate that inhibition of SCP1 synthesis accelerates attainment of the diplotene stage, and that oocytes in this stage are usually enclosed within follicles.

SCP1 AS-dependent inhibition of SCP1 synthesis increases the rate of follicular formation in fetal ovaries

A 96-h *in vitro* exposure of F21 ovaries in organ culture to SCP1 AS increased the number of primary follicles compared with that in contralateral ovaries treated with SCP1 SCR (Fig. 7A). Although the number of primordial follicles was also increased, the change did not achieve statistical significance, probably because of a rapid transition of the newly formed primordial follicles into the primary stage. The overall number of follicles detected in the SCP1 AS-treated ovaries was significantly greater than that in SCP1 SCR-treated glands (Fig. 7B).

The microphotographs shown in Fig. 8 illustrate these results. The ovaries treated with the SCP1 SCR (Fig. 8, *left panel*) exhibit the immaturity typical of fetal ovaries at this stage of development, *i.e.* the presence of many naked oocytes organized in clusters surrounded by pockets of mesenchymal cells (*arrowheads*). However, it is also clear that, as shown by others (40), follicular assembly proceeds in organ culture in the absence of external inputs, as evidenced by the presence of both primordial (*short arrows*) and primary (*long arrows*) follicles. The glands treated with SCP1 AS (Fig. 8, *right panel*) showed more primordial (*short arrows*) and primary (*long arrows*) follicles than the SCP1 SCR-treated controls, indicating that completion of the first meiotic prophase induced by a reduction in SCP1 expression results in an increased rate of follicular formation.

Discussion

The present study demonstrates that expression of SCP1, an oocyte-specific gene required for chromosomal pairing during the first meiotic division of germ cells (22, 41), decreases strikingly during the hours antedating the organization of primordial follicles in rat ovary. Because loss of SCP1 normally occurs at the end of the first meiotic prophase (41, 42), these results provide quantitative evidence for the concept that most oocytes reach the end of prophase I around the time of birth (17). We also show that a premature re-
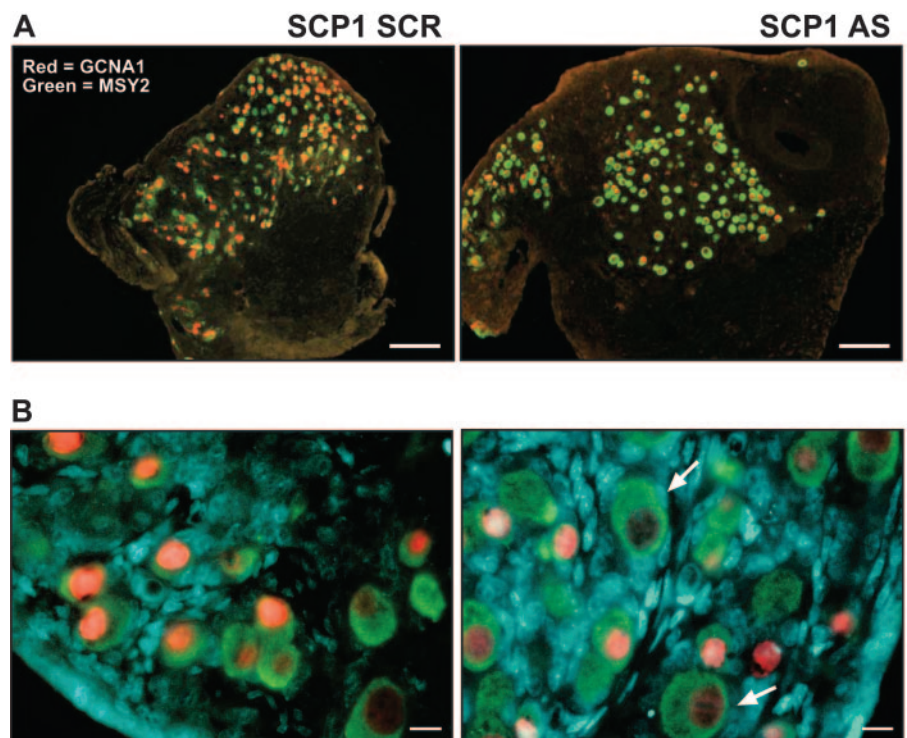

FIG. 6. Representative examples of the changes in the fraction of oocytes expressing the meiotic markers GCNA1 and MSY2 caused by inhibition of SCP1 synthesis. F21 ovaries were placed in organ culture and were treated for 96 h with SCP1 AS or SCP1 SCR (5 μM). A, Low magnification images illustrating the overall shift in the number of oocytes expressing GCNA1 or MSY2, a change quantitated in Fig. 5C. *Orange-yellow* cells contain similar levels of GCNA1 and MSY2. B, Higher magnification images demonstrating that oocytes expressing mostly GCNA1 in SCP1 SCR-treated ovaries are prefollicular (*left panel*), whereas those containing mostly MSY2 in SCP1 AS-treated ovaries are already encapsulated into a follicular structure (examples denoted by *arrows*). The markers were visualized by immunohistofluorescence as outlined in Fig. 3, and the reactions were developed to either a *red* (GCNA1) or a *green* (MSY2) color. Cell nuclei, stained with Hoechst, are shown in *blue*. *Bars* in A, 100 μm; *bars* in B, 10 μm
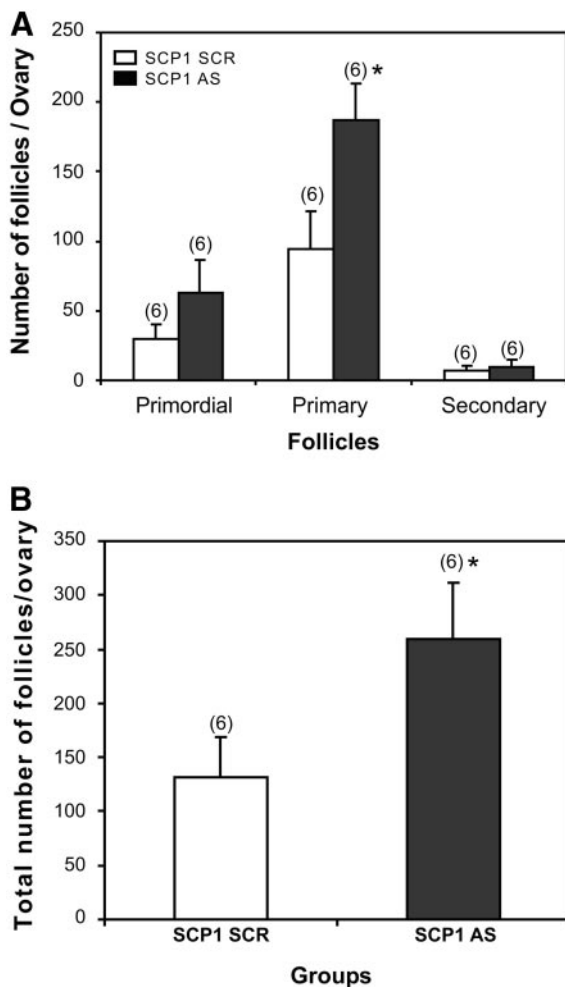
FIG. 7. Blockade of SCP1 synthesis in F21 ovaries via administration of an AS oligonucleotide against SCP1 mRNA (SCP1 AS) accelerates follicular formation. A, Number of follicles in different stages of development. B, Total number of follicles per ovary. One ovary from each of six animals was exposed for 4 d in organ culture to SCP1 AS (5 μM), which is directed against the region encompassing the translation initiation site of SCP1, and the contralateral ovary was treated with the same dose of an SCR DNA sequence of identical base composition. *, $P < 0.02$ *vs.* SCR-treated group.

duction in SCP1 expression, achieved via administration of an antisense oligonucleotide to fetal ovaries in organ culture, increases the number of oocytes reaching the end of prophase I and results in a greater number of newly formed follicles after 4 d of treatment. Although our results do not clarify the exact mechanism by which a loss in SCP1 expression leads to increased folliculogenesis, they do suggest that reaching the diplotene stage at the end of meiotic prophase I allows the oocyte to engage granulosa cells in the bidirectional communication process required for follicular organization.

Mammalian germ cells proliferate mitotically even after they arrive at the gonadal ridge (43). In rodents, proliferation stops by embryonic d 13.5 and the oogonia become haploid by virtue of a single round of DNA replication, followed by two rounds of chromosomal division (41, 44). These divisions are known as meiosis I and II. The first meiotic division has

a long prophase, which is divided into five sequential stages: leptotene, zygotene, pachytene, diplotene, and diakinesis. During the course of these stages, the chromosomes replicate originating sister chromatids. The sister chromatids of each homologous chromosome then pair (synapse) with their homologous partner and finally dissociate (desynapse). Pairing is initiated at the zygotene stage with the formation of a ribbon-like structure known as the SC. The SC maintains the sister chromatids together during the pachytene stage. Dissolution of the complex defines the end of pachytene and the beginning of diplotene; in this phase the homologous chromosomes separate and become transcriptionally active. It is commonly accepted that in rodents, oocytes reach diplotene around the time of birth and enter the late diplotene stage (diffuse diplotene, most commonly known as dictyate) shortly thereafter. They remain arrested in this stage until the time of ovulation (43).

The SC is a proteinaceous structure with a tripartite configuration consisting of two axial (lateral) regions, one along each sister chromatid, and a central element that connects the lateral elements via an array of transverse filaments (39). Although SCP1 is a major component of these filaments (22, 24), SCP2 and SCP3, two other major protein components of the SC, form part of the axial/lateral elements (39, 44). Of importance for the interpretation of the present results is the fact that initial disassembly of the SC at diplotene coincides with the loss of SCP1, but not SCP3, from the complex (42).

Traditionally, completion of meiotic prophase I with the attainment of the diplotene stage has been considered to be coincidentally, but not functionally, related to the organization of primordial follicles. Our results indicate the existence of a causal relationship between these two events and suggest that attainment of diplotene results in the activation of an as yet to be identified oocyte-to-somatic cell signaling pathway that favors the organization of primordial follicles. The striking loss of SCP1 expression observed in oocytes during the hours preceding the initiation of follicular assembly demonstrates a tight temporal correlation between completion of the first meiotic prophase and the initiation of follicular assembly. A recent study using DNA arrays to profile the expression of genes at the time of follicle formation in the mouse ovary also showed that expression of the SCP1 gene decreases before the time of follicular assembly (45). By showing that inhibition of SCP1 expression with antisense oligonucleotides results in early attainment of the diplotene stage and the assembly of more follicles, our results suggest that completion of meiotic prophase I is functionally coupled to the initiation of follicular assembly.

We used two stage-specific molecular markers to monitor the progression of the first meiotic prophase in the rat ovary: GCNA1 and MSY2. GCNA1 is a nuclear protein abundantly expressed in germ cells and oocytes progressing through the zygotene stage of the first meiotic division (17, 35). When the chromosomes are decondensed by desynapsis signaling the end of the pachytene stage and initiation of the diplotene stage, GCNA1 expression is considerably reduced (17, 35). In contrast, MSY2 is a cytoplasmic protein that belongs to the Y box family of RNA-binding proteins (36) and is expressed in oocytes that have reached the diplotene stage (12, 36). Consistent with these observations, our results show that the
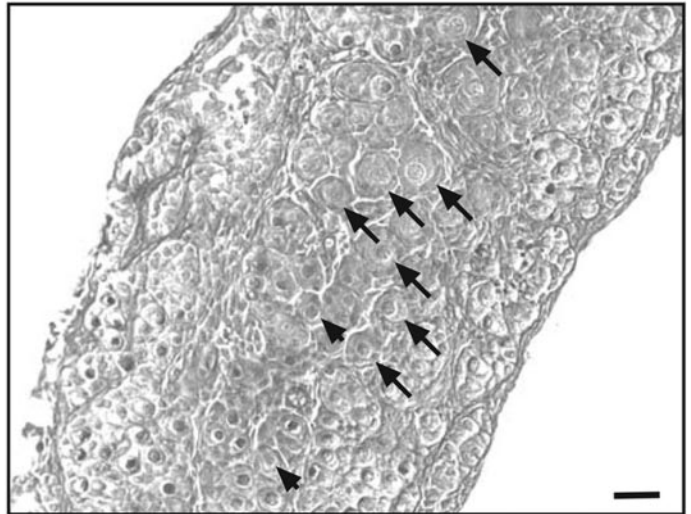
SCP1 SCR
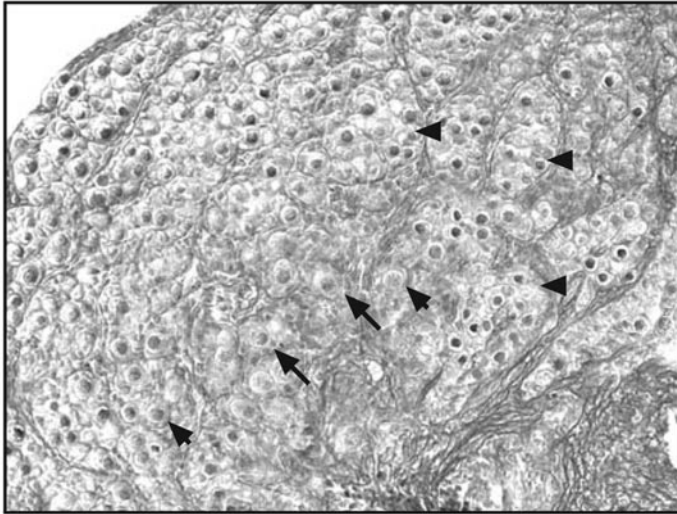
SCP1 AS

Fig. 8. Morphological aspect of F21 ovaries exposed for 4 d in organ culture to either SCP1 AS to reduce SCP1 synthesis or to SCP1 SCR. The *left panel* depicts the region of a control SCP1 SCR-treated ovary containing primordial (*short arrows*) and primary (*long arrows*) follicles. The microphotograph also illustrates the immaturity of SCP1 SCR-treated ovaries, which show a predominance of naked oocytes mostly organized in clusters surrounded by pockets of mesenchymal cells (*arrowheads*). The *right panel* depicts an ovary exposed to SCP1 AS. Oocyte clustering appears diminished, and both primordial (*short arrows*) and primary follicles (*long arrows*) are readily detected. *Bar*, 50 μm.

fraction of oocytes expressing MSY2 increases markedly during the initial 48 h after birth, coinciding with a loss of GCNA1. Similarly, the content of MSY2 mRNA increases after birth, indicating that MSY2 expression in oocytes is at least in part controlled at the transcriptional level. In keeping with this view, MSY2 mRNA levels increase after birth in the *in situ* ovary, and SCP1 AS-induced loss of SCP1 expression in cultured ovaries causes a premature increase in MSY2 gene expression. This increase appears to be caused by more oocytes completing the first meiotic prophase, because MSY2 became detectable, and GCNA1 undetectable, in a significantly greater fraction of oocytes from SCP1 AS-treated ovaries than in control gonads treated with SCP1 SCR. In keeping with the concept that most AS effects in tissues other than the nervous system are mediated by ribonuclease H (which cleaves RNA/DNA heteroduplexes) (46), SCP1 AS treatment resulted in a reduction of SCP1 mRNA abundance. Although this reduction was incomplete, it was accompanied by an approximately 2-fold change in the fraction of oocytes showing increased MSY2/reduced GCNA1 levels and in the number of follicles detected after treatment in organ culture. Future experiments using more effective gene-silencing strategies will be required to determine whether more complete suppression of SCP1 expression results in a more pronounced effect on oocyte development and follicle assembly.

The mechanisms by which the loss of SCP1 at the completion of meiotic prophase I facilitate the assembly of primordial follicles are not known. Two possibilities deserve consideration. One is that the effect of SCP1 AS on follicle number is related to an increased oocyte survival. Under these conditions, more oocytes would be available for follicular assembly, so that more follicles would be formed. Conceptually, this situation might be construed as similar to

that resulting from the lack of the oocyte transcription factor FIGα. In the absence of FIGα, oocytes die, and follicles fail to form (12). However, it is unclear which event occurs first (3, 12), *i.e.* do follicles fail to form because the oocytes are dead or do the oocytes die because primordial follicles fail to form? Although we did not quantitate the rate of oocyte apoptosis in SCP1 AS- and SCP1 SCR-treated ovaries, neither the use of MSY2/GCNA1 as markers of meiotic progression (Fig. 6) nor a standard histological staining (Fig. 8) provided any overt indication that such a difference in oocyte survival between the two groups actually exists. Although it has been postulated that programmed cell death of oocyte clusters is limited to the first 24–48 h after birth (16), others have contested such interpretation (17) and showed that loss of oocytes occurs continuously throughout meiotic prophase (between gestational d 13.5 and 24 h after birth). In this latter study, the number of oocytes reaching early diplotene was found to increase dramatically within 24 h after birth without an appreciable change in the apoptosis rate, *i.e.* an observation entirely consistent with our findings. There is evidence, however, that oocyte apoptosis is important for follicular formation, because progesterone reduces the rate of primordial follicle formation by inhibiting oocyte apoptosis (15). Additional studies are obviously required to resolve this issue.

The second explanation, which we favor, is that the transcriptional/translational activity of the oocyte changes upon reaching the end of meiotic prophase I (47), and that this change affects the production of molecules (48, 49) required for oocyte-granulosa cell communication at the time of follicular assembly (50). We have observed that follicular assembly is accompanied by a reduction in cadherin-11 expression in oocytes and that this loss also occurs in ovaries

in which follicular assembly was prematurely set in motion by inhibition of SCP1 expression (unpublished observations). Because cadherin-11 is a homophilic adhesion molecule (51), it is possible that the dissociation of oocyte clusters that precedes the encapsulation of individual oocytes into follicular structures (1) might result, at least in part, from the loss of cadherin-11-dependent oocyte-oocyte adhesiveness. Additional experiments are required to test this hypothesis and identify other relevant molecules that contribute to the process.

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