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A MicroRNA (mmu-miR-124) Prevents *Sox9* Expression in Developing Mouse Ovarian Cells[1]

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ABSTRACT

In mammals, sex differentiation depends on gonad development, which is controlled by two groups of sex-determining genes that promote one gonadal sex and antagonize the opposite one. *SOX9* plays a key role during testis development in all studied vertebrates, whereas it is kept inactive in the XX gonad at the critical time of sex determination, otherwise, ovary-to-testis gonadal sex reversal occurs. However, molecular mechanisms underlying repression of *Sox9* at the beginning of ovarian development, as well as other important aspects of gonad organogenesis, remain largely unknown. Because there is indirect evidence that micro-RNAs (miRNA) are necessary for testicular function, the possible involvement of miRNAs in mammalian sex determination deserved further research. Using microarray technology, we have identified 22 miRNAs showing sex-specific expression in the developing gonads during the critical period of sex determination. Bioinformatics analyses led to the identification of miR-124 as the candidate gene for ovarian development. We knocked down or overexpressed miR-124 in primary gonadal cell cultures and observed that miR-124 is sufficient to induce the repression of both SOX9 translation and transcription in ovarian cells. Our results provide the first evidence of the involvement of a miRNA in the regulation of the gene controlling gonad development and sex determination. The miRNA microarray data reported here will help promote further research in this field, to unravel the role of other miRNAs in the genetic control of mammalian sex determination.

cell transfection, gene expression, gonad development, microarray, microRNA, miR-124, ovary, regulation of translation, sex determination

INTRODUCTION

The process of sex determination involves mechanisms that prompt the undifferentiated embryonic gonad to follow either of the two alternative developmental pathways, the testis or the ovary. A single gene located on the Y chromosome, the sex-determining region of the Y (*SRY*) gene, is the switch for sex determination in almost all mammals [1–3]. In XY mice, shortly after the expression of *Sry* in somatic cells of the supporting cell lineage of the gonad, testis development begins with the differentiation of these cells as pre-Sertoli cells located in the testis cords that also enclose the primordial germ cells. Several additional cellular events also occur outside the testis cords, including mesonephric cell migration, testis-specific vascularization, and differentiation of Leydig and peritubular myoid cells [see refs. 4 and 5 for reviews]. In females, the genetic pathways for ovary development are largely unknown to date.

Many genes are currently known to be involved in gonadal development [6–8]. Expression of SRY-related HMG-box, gene 9 (*SOX9*) is essential for normal testis development in most vertebrates. Ectopic expression of *Sox9* in the gonads of XX mice leads to a female-to-male sex reversal with the formation of XX testes [9, 10], and XY mice lacking *Sox9* expression develop ovaries, thus showing a male-to-female sex reversal [11, 12]. Hence, *Sox9*, like *Sry*, is necessary and sufficient to trigger testis organogenesis. A positive feedback loop between *Sox9* and fibroblast growth factor 9 (*Fgf9*) is initiated by *Sry*, which results in the up-regulation of *Fgf9* and the repression of wingless-type MMTV integration site family, member 4 (*Wnt4*) in the male gonad [13]. This is crucial for the maintenance of testis development, because *Wnt4* represses the migration of endothelial and steroidogenic cells from the mesonephros to the XX gonad [14], probably by activating the β-catenin signaling pathway. The fact that XY *Fgf9/Wnt4* double mutants developed testes indicates that the primary role of Fgf9 is the repression of *Wnt4* and suggests that the *Sox9*/*Fgf9* positive feedback loop is established through *Wnt4*, thus *Fgf9* repressing a *Sox9* repressor [15].

Ovarian differentiation was assumed to be the default pathway in gonad development. However, several discoveries have shown that the ovarian phenotype, once determined, must be actively maintained throughout life. Although *Foxl2* is required to maintain the ovarian phenotype at the postnatal stage [16], little is known on how ovary differentiation is initiated at the embryonic stage after sex determination. Sex determination is established in terms of a balance between *Fgf9*- and *Wnt4*-expression predominance. It is known that *Sry* tips the balance toward the testis pathway in males, and Wnt signaling may have a similar role in females. R-spondin family member 1 (RSPO1), which, like *Wnt4*, serves to stabilize β-catenin, appears sufficient to block the testicular pathway in some XX humans [17], although this is not the case in mice, where XX *Rspo1* null mutant gonads have a phenotype similar

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to that of *Wnt4* null mutants [18]. Available data suggest that *Rspo1* may cooperate with *Wnt4* to stabilize β-catenin, helping to counteract the establishment of the *Sox9*/*Fgf9* positive feedback loop during mouse ovarian development (see reference [19] for a review). Indeed, mice carrying a constitutive active form of β-catenin show XY female sex reversal [20].

Before sex determination, *Sox9* expression occurs at a basal level in both male and female gonadal primordia, but at 11.5 days postcoitum (dpc), it is up-regulated in males and down-regulated in females [21]. The regulatory action of SRY on *Sox9* has recently been unraveled. SRY, in a synergistic action with NR5A1/SF1, up-regulates *Sox9* in male undifferentiated gonads by binding to a testis-specific *Sox9* enhancer [22]. However, it is not known how *Sox9* is down-regulated in the female gonad. Although *Wnt4* null mutant XX gonads can show transient upregulation of *Sox9* expression [13], null mutations in either the gene for β-catenin (*Ctnnb1*) or *Foxl2* are not sufficient to maintain de-repression of *Sox9* expression in the early XX gonad and neither are compound null mutations involving *Wnt4* and *Foxl2*, or *Rspo1* and *Foxl2* [23, 24] (see also our unpublished data). All this suggests that additional genetic elements other than those currently known are involved in sex determination, especially those required to drive ovary and repress testis differentiation. In this context, several studies have suggested that micro-RNAs (miRNAs) are involved in this process, but this hypothesis has not yet been sufficiently explored.

MicroRNAs are small, noncoding RNAs that regulate the expression of target genes, either by guiding the cleavage of their target mRNAs or by inhibiting their translation [25–28]. miRNAs have been shown to exert post-transcriptional control of genes involved in development and other biological aspects including disease, cell death, cell proliferation and hematopoiesis, in a variety of organisms, including *Caenorhabditis elegans*, flies, mammals and plants [26, 29–34]. Although no miRNA is currently known to play a role in vertebrate sex determination, there is indirect evidence to suggest that miRNA could be involved in the process: several miRNAs show a sexually dimorphic expression pattern in the mouse gonad at 13.5 dpc [35]; and targeted disruption of *Dicer1*, a gene involved in miRNA maturation, has revealed that Dicer protein is necessary to maintain Sertoli cell function [36–38].

MATERIALS AND METHODS

Animals and Gonadal Cells

Gonads were dissected from Swiss, Parkes, and CD1 outbred mouse embryos at 11.5 and 13.5 dpc and were either fixed or prepared for cell culture. Individual embryos were sexed either by gonad morphology (at 13.5 dpc) or at earlier indifferent stages using sex chromatin staining of amniotic cells [39] and duplex PCR for zinc finger protein 1, Y linked (*Zfy1*) and the gene for myogenin (*Myog*). For purification of the RNA used in microarray experiments, a total of 24 male 11.5 dpc, 19 male 13.5 dpc, 31 female 11.5 dpc, and 18 female 13.5 dpc gonads were unambiguously identified, pooled, and processed (see later discussion). For transfection studies, sexed male and female gonads were pooled, disaggregated both mechanically and enzymatically (125 μg/ml collagenase; Roche) and cultured either in LabTek chamber slides or in 24-well plates, using Dulbecco modified Eagle medium (DMEM; Sigma) with 10% fetal calf serum (FCS, Sigma). Similarly, primary chondrocyte cultures were from mouse embryonic limbs. For in situ hybridization, sexed gonads were fixed overnight in 4% paraformaldehyde and further immersed in 30% sucrose (w/v), embedded in Tissue-Tek optimal cutting temperature compound (Sakura) and sectioned in a cryostat. Mouse housing and handling, as well as laboratory protocols, were approved by the University of Granada Ethics Committee for Animal Experimentation or were carried out under a U.K. Home Office Project License.

MicroRNA Profiling of Mouse Gonads

Total RNA from embryonic gonads at 11.5 and 13.5 dpc was purified using a miRNeasy mini kit (Qiagen) according to the manufacturer's protocol. Further steps were performed by Exiqon miRNA Profiling Service. The quality and integrity of the RNA samples was assessed in a Bioanalyser 2100 (Agilent Technologies), and the concentration was measured with a spectrophotometer (Nanodrop). All samples used for array hybridization showed RNA integrity numbers higher than 9. Micro-RNA samples were labeled using a miRCURY Hy3/Hy5 power labeling kit from Exiquon. Four embryo gonad samples were analyzed: male 11.5 dpc, female 11.5 dpc, male 13.5 dpc, and female 13.5 dpc. Each sample was labeled with Hy3 and double hybridized against a pool of the four samples labeled with Hy5, which was used as a common reference. Hybridizations were performed with an HS400/4800 hybridization station (Exiqon-Tecan). The miRCURY locked nucleic acid (LNA) microarray slides were scanned using a G2565BA microarray scanner system (resolution 10 μm; Agilent Technologies).

Array Slide Quality Control Using Spike-Ins

Hy3 and Hy5 labeling reactions, hybridization, and performance of array experiment were evaluated with RNA controls (spike-in) added at various concentrations, covering the full signal range, to the labeling reactions. A high correlation for both the Hy3 and the Hy5 channels indicated that both labeling and hybridization were successful. Each spike-in control had 32 replicates of capture probes on the array.

MicroRNA Microarray Data Validation

Data from selected micro-RNAs were validated in our laboratory by real-time RT-PCR using the miRCURY LNA micro-RNA PCR system (Exiqon), in a Chromo4 real-time thermocycler (Bio-Rad) according to Exiqon protocols. As primer sets specific for the amplification of miR-124 were not available from Exiqon, microarray data from this micro-RNA were validated using TaqMan micro-RNA assays from Applied Biosystems, according to the manufacturer's procedures. For miR-124, three gene expression quantification experiments were performed with different gonadal RNA samples, and each quantitative (Q)-RT-PCR assay was done in triplicate using the U6 snRNA as a reference. MIAME-compliant data were submitted to ArrayExpress accession: E-MEXP-2252, experiment name: *Expression of miRNAs in mouse embryo gonad during the critical period of sex determination*; specified release date: May 11, 2013.

Identification of Active miR-124 Genes

RT-PCR was used to identify which of the three possible miR-124 precursor transcripts were expressed in the developing gonads. As the predicted amplification products are short sequences, short oligonucleotides (oligos) were designed with the *eprimer3* application from the emboss package, and the alignment temperature of the oligos was increased and adjusted, adding unspecific nucleotides at the 5′ end of each oligo. The primers sequences were as follow: forward 5′-GCC TCT CTC TCC GTG T-3′ and reverse 5′-CCA TTC TTG GCA TTC A-3′ for mmu-miR-124-1; forward 5′-AGA GAC TCT GCT CTC CGT GT-3′ and reverse 5′-CTC CGC TCT TGG CAT TC-3′ for mmu-mir-124-2; and forward 5′-GGC TGC GTG TTC ACA G-3′ and reverse 5′-ATC CCG CGT GCC TTA-3′ for mmu-mir-124-3. One triplicate quantitative (Q)-RT-PCR reaction was carried out using RNA samples from six pooled 13.5 dpc mouse embryonic gonads of each sex.

Bioinformatics

Microarray image analysis was carried out using ImaGene version 7.0 software (BioDiscovery, Inc.). The quantified signals (background correction Normexp, with offset value 10 [40] were normalized by Exiquon miRNA Profiling Service, using the global LOcally WEighted Scatterplot Smoothing (LOWESS) regression algorithm. In order to assess the biological significance of differentially expressed miRNAs, we searched for putative targets in genes involved in sex determination or gonad development, fetching miRNA data from miRbase (http://www.mirbase.org/), gene descriptions from Ensembl (http://www.ensembl.org/), and filtering the results with Gene Ontology (GO) terms (http://www.geneontology.org/) by using Perl scripts (http://www.perl.org/) developed for this purpose. Graphs were drawn and statistics were analyzed with gnu-R software (http://www.r-project.org/).

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Luciferase Assays

HEK293T cells cultured in 24-well plates were cotransfected using Lipofectamine 2000 (Invitrogen), with a miR-124 overexpression plasmid vector (1 μg/well; ref. MmiR3282-MR04; Genecopoeia), and pLuc-*Sox9* 3′-untranslated region (UTR) (0.2 μg/well). Forty-eight hours after transfection, cells were harvested for luciferase assay. Dual reporter assays were performed by measuring both firefly and *Renilla* luciferase activities (LucPair miR dual luciferase assay kit; ref. LPFR-M010; Genecopoeia) according to the manufacturer's protocols.

MicroRNA In Situ Hybridization

In situ hybridization experiments with a miR-124-specific miRCURY LNA micro-RNA detection probe (catalog no. 33007-01; sequence: CTTGGCATT CACCGCGTGCCTTA; provided at 25 μM; Exiqon) were performed. Briefly, 8-μm-long cryosections (four per slide) were hybridized with 30 nM 5′-digoxigenin-labeled LNA probe overnight at 58°C. Signals were revealed using anti-digoxigenin alkaline phosphatase-conjugated antibodies (1:1500 dilution; Roche) and bone morphogenic protein (BMP) purple substrate. In situ hybridization experiments were performed six times with different gonad samples. For negative controls, gonad cryosections were subjected to the entire protocol but the probe was omitted from the hybridization mixture.

MicroRNA Knock-Down and Overexpression in Cultured Cells

Primary gonadal cell cultures were transfected for miRNA inhibition and overexpression experiments. The inhibition of miR-124 in XX embryonic gonad cells was produced with a high-affinity LNA-enhanced probe (antagomir) for specific in vitro knock-down (ref. 139452-04; miRCURY LNA micro-RNA inhibitor 5′-fluorescein-labeled; from Exiqon). As a negative control, we transfected the same type of cells with a different antagomir, specific for miR-144, an miRNA that is not expressed in the female gonad according to our miRNA microarray data. Cells were plated to a density of 50 000 cells per cm^2 in 8-well chamber slides (LabTek) for immunofluorescence studies or in 24-well culture plates for further RNA extraction, in 500 μl of DMEM supplemented with 10% FCS. For transfection with the silencing probes, cells were transfected for 4–6 h after plating with a mixture of 1.5 μl of Lipofectamine 2000 (Invitrogen) in 50 μl of Opti-Mem-I reduced serum medium without antibiotics and 6 μl of probe in 50 μl of Opti-Mem, according to the guidelines of the manufacturer. After 24 h, the medium was replaced with DMEM supplemented with 10% FCS and 15 μg/ml tetracycline. Forty-eight hours later the efficiency of transfection was checked by the fluorescence of the probe. The efficiency of transfection was higher than 90% in all these experiments. Seventy-two hours after transfection, the cells were fixed in 4% formaldehyde or processed for total RNA extraction.

Overexpression of miR-124 was performed using a precursor miRNA expression clone for the mouse *mmu-mir-124-2* gene (ref. MmiR3282-MR04; Genecopoeia). In this case, cells transfected with a precursor miRNA-scrambled control clone (ref. CmiR0001-MR04; Genecopoeia) were used as the negative control. For transfection of primary cultures of embryonic gonad cells with these vectors, we followed a procedure similar to that described previously with some exceptions. Before culture, cells were enzymatically disaggregated with collagenase (250 μg/ml) and then transfected with 1 μg of vector by using an electroporator (Nucleofector II; Amaxa), obtaining a transfection efficiency higher than 60%, which was determined by counting the percentage of fluorescent cells (green fluorescent protein [GFP] was included in the transfection vector). Forty-eight hours after transfections, cells were either fixed in 4% formaldehyde or processed for total RNA extraction.

Both knock-down and overexpression experiments were performed more than five times using 3–4 13.5 dpc pregnant females per experiment, providing approximately 60 embryonic gonads, which were divided into three sets of 20 gonads each. Cells from the first set were treated with the miR-124-antagomir/overexpression vector, those from the second set were treated with the miR-144-antagomir/control vector, and those from the third set remained untreated before fixation or RNA extraction.

Gene Expression Quantification

To check the genetic effects of the miRNA knock-down experiments by Q-RT-PCR, total RNA was extracted with Trizol reagent (Qiagen) after transfection and 300–900 ng total RNA was reverse transcribed using Superscript II (Invitrogen) and random hexamers as primers. RNA samples were purified from both nontransfected and transfected cells with the miR-124-silencing LNA probe. Expression levels of mmu-miR-124 were quantified using TaqMan micro-RNA assays (Applied Biosystems) according to the manufacturer's procedures. Quantification of *Sox9* expression levels was performed using 1-μl aliquots of a 1:10 dilution of the RT reaction as templates in the Q-PCR reactions. Each PCR reaction was run in triplicate, and all experiments were repeated at least three times. All samples were normalized relative to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), using the $2^{-\Delta\Delta CT}$ (Livak) method. The primers for *Sox9* mRNA amplification were forward 5′-CGG AGG AAG TCG GTG AAG A-3′ and reverse 5′-GTC GGT TTT GGG AGT GGT G-3′. For *Gapdh* amplification the primers were: forward 5′-GGC ATT GCT CTC AAT GAC AA-3′ and reverse 5′-TGT GAGG GAG ATG CTC AGT G-3′. The identity of the amplified fragments was confirmed by sequencing in both cases.

SOX9 Protein Detection

Immunofluorescence analyses were performed to check the presence of SOX9 protein in transfected gonadal cells. Cells were cultured in LabTek chamber slides, fixed for 5 min in 4% formaldehyde, washed in PBS, blocked for 1 h at room temperature with 2% BSA in PBS, and incubated overnight with a 1:100 dilution of SOX9-specific rabbit primary antibody (two different antibodies were used, antibody code sc-20095 [Santa Cruz Biotechnology] and code AF3075 [R&D Systems], and the same results were obtained). Slides were then washed in PBS and incubated for 1 h at room temperature with a 1:500 dilution of Alexa Fluor 555 goat anti-rabbit secondary antibody (Invitrogen). Sections were then washed in PBS, incubated in a solution of 100 ng/ml 4′,6-diamidino-2-phenylindole (DAPI) for 30 min at room temperature, washed again in PBS, and mounted (Vectashield mounting medium; Vector Laboratories). Images were obtained with an Olympus BX41 microscope with epifluorescence equipment.

RESULTS

MicroRNA Expression Profiling of Mouse 11.5- and 13.5-dpc Male and Female Gonads

All data from miRNA microarray profiling experiments in the present study are provided in Supplemental Data (Project-Summary.xls; all Supplemental Data are available online at www.biolreprod.org). Among 757 miRNAs included in the miRCURY LNA microarray slides, 71 miRNAs showed differential expression either between sexes or between developmental stages. The expression profiles of these miRNAs are shown in a heat map diagram (Fig. 1), as previously described [41], where rows represent miRNAs and columns represent different samples. This diagram also includes a two-way hierarchical clustering of genes and samples. The miRNA clustering tree, constructed based on $\log_2$ (Hy3:Hy5) ratios (>0.5 SD among the four samples), permits identification of five different gene clusters (Fig. 1, A–E). The miRNAs in clusters A and E are either down-regulated (Fig. 1A) or up-regulated (Fig. 1E) during the transition between the two developmental stages (11.5 and 13.5 dpc) but show no great differences in levels of expression between testes and ovaries at 13.5 dpc. Therefore, these miRNAs are probably not involved in gonadal sex differentiation and thus have little relevance to the current study. In contrast, miRNAs in clusters B, C, and D, show differential expression in a sex-specific fashion at 13.5 dpc but not at 11.5 dpc and are therefore good candidates to be involved in gonadal sex differentiation. Cluster B includes 3 miRNAs that are up-regulated in ovaries and down-regulated in testes at 13.5 dpc, suggesting a potential role in ovarian development. Cluster C contains six miRNAs that are exclusively up-regulated in 13.5 dpc testes, suggesting a role in testis development. On the other hand, cluster D consists of 11 miRNAs up-regulated only in 13.5 dpc ovaries and, therefore, similar to those in cluster B; these miRNAs could be involved in ovarian development.

The outcome of our miRNA microarray profiling experiments was validated by Q-RT-PCR. The expression levels of at least 2 representative miRNAs of each cluster, except for

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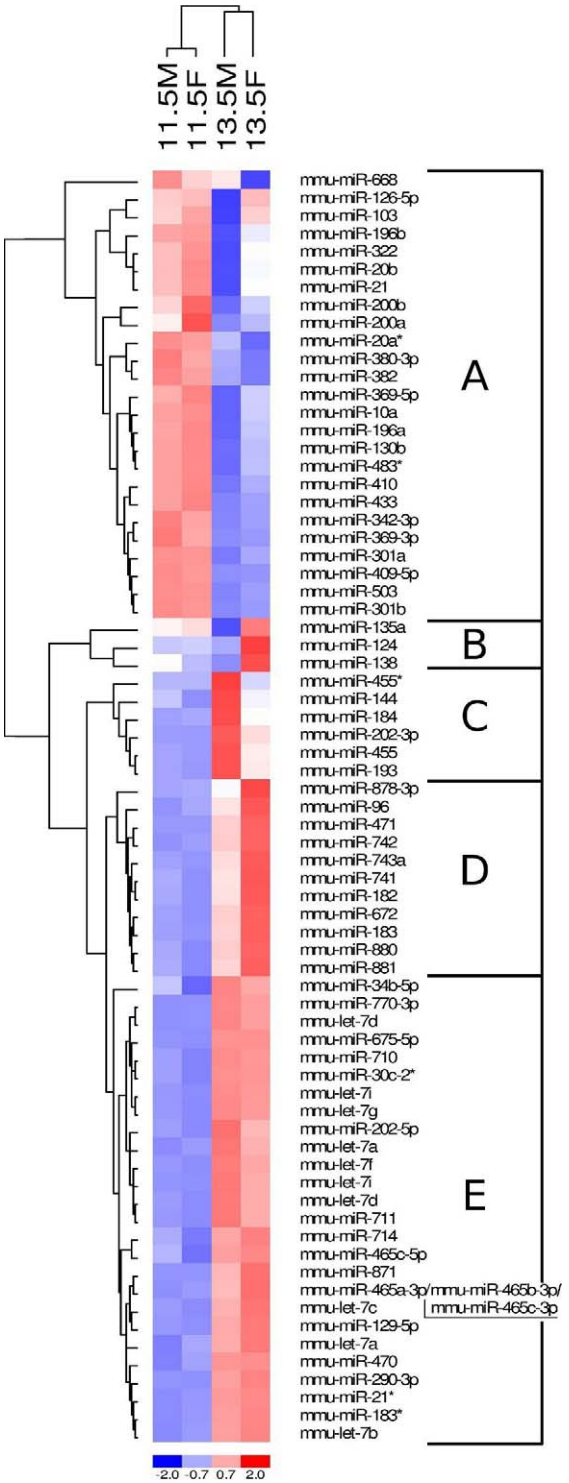

FIG. 1. Heat map diagram showing miRNA expression profiles in embryonic gonads during the period of sex determination. A two-way hierarchical clustering of genes and samples identified five different gene clusters. Clusters A and E include miRNAs showing no sex-specific down-regulation (A) or up-regulation (E) during the transition between 11.5 and 13.5 dpc developmental stages; these genes are thus probably not involved in sex determination. Cluster B contains three miRNAs up-regulated in 13.5-dpc ovaries and down-regulated in 13.5 testes, with a potential role in ovarian development. Cluster C includes 6 miRNAs up-regulated in 13.5 dpc testes but not in 13.5 dpc ovaries, with a possible role in testis development. Cluster D contains 11 miRNAs up-regulated in 13.5 dpc ovaries but not in 13.5 dpc testes, suggesting a role in ovarian development.

cluster E, were measured and compared with the corresponding array expression profiles (Fig. 2A and Supplemental Fig. S1). In general, relative expression levels in microarrays correspond to those in the Q-RT-PCR assays. Although mmu-miR-126-5p and mmu-miR-103 were classified as members of cluster A by the clustering analysis, because differences in expression levels between developmental stages are higher than those between sexes at 13.5 dpc, the differential expression in 13.5 dpc male and female gonads should be taken into account. Thus, we included these miRNAs in the Q-RT-PCR validation set. Our results confirmed that the expression of both miRNAs is significantly higher in females than in males at 13.5 dpc (miR-103 also at 11.5 dpc), suggesting a potential role in ovarian development.

Identification of mmu-mir-124 as a Candidate Gene for Ovarian Development

We performed bioinformatic analyses to investigate the potential biological role of miRNAs that showed sex-specific expression and thus to identify new genetic elements involved in mammalian sex determination. We searched mainly specific miRNA target sequences in the 3′-UTR regions of genes associated with GO terms such as, *sex determination*, *sex differentiation*, *gonad differentiation*, *testis development*, *ovary development*, *gonad development*, *Sertoli cell differentiation*, and *follicle cell differentiation*. Using these criteria, we identified miR-124 as a good candidate gene for ovarian development because several genes involved in sex determination, including *Sox9*, share potential targets of this miRNA in their 3′-UTR regions. Furthermore, miR-124 was included in cluster B (differentially expressed in developing ovaries) and is known to control *Sox9* expression in the brain [42]. These data strongly suggested that miR-124 may play an important role in early steps of ovarian development, and thus, we carried out further analyses of this miRNA.

The functional interaction between miR-124 and *Sox9* was examined in the luciferase reporter assays. We found that miR-124 significantly reduced the activity of the luciferase gene fused to the *Sox9* 3′UTR by more than 70%, supporting the notion that miR-124 can modulate *Sox9* expression by binding to its 3′-UTR (Supplemental Fig. S2). Cheng et al. [42] previously described a similar effect by using a different reporter vector.

miR-124 is Up-Regulated in the Mouse Female Gonad Between 11.5 and 13.5 dpc

In order to confirm the expression data reported by our microarray screening for miR-124, we also performed Q-RT-PCR analyses and in situ hybridization with mouse embryonic gonads. Expression quantification by Q-RT-PCR confirmed that miR-124 is up-regulated in the ovary but not in the testis during the critical period of gonad differentiation between 11.5 and 13.5 dpc (Fig. 2A). According to miRBase, there are three predicted precursor hairpin sequences of mmu-miR-124 in the mouse genome, giving rise to the same mature sequence. The identity of the miR-124 precursor(s) expressed in mouse embryonic gonads was investigated by Q-RT-PCR with sets of primers specific for the three miR-124 pre-miRNAs. Our results revealed that all three miR-124 genes are expressed in 13.5 dpc mouse ovaries (mainly pre-miR-124-2) but not in testes of the same developmental stage, where pre-miR-124-1 is absent and the other two precursors show low expression levels (Fig. 2B). Expression values were always significantly higher in females than in males for the three precursors ($P =$

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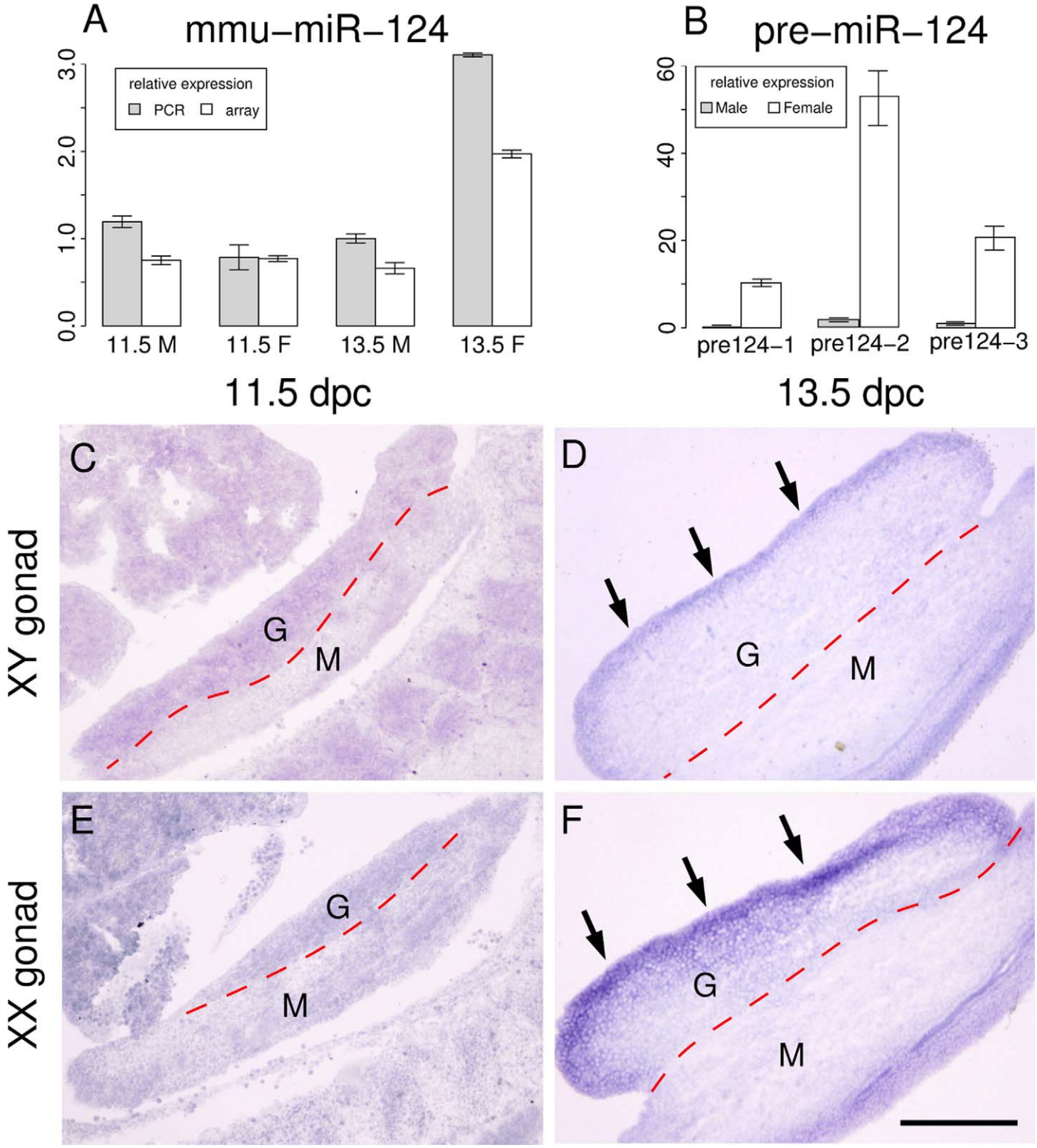

FIG. 2. Spatiotemporal expression pattern of miR-124 in mouse embryonic gonads during the period of sex determination. **A**) Q-RT-PCR (gray bars) and miRNA microarray (white bars) data of miR-124 expression levels in male (M) and female (F) gonads at 11.5 and 13.5 dpc. miR-124 is sex-specifically up-regulated in the 13.5 dpc female gonad. **B**) Q-RT-PCR quantification of the active miR-124 precursors 1, 2, and 3, in male (gray bars) and female (white bars) embryonic gonads. Amplified products derived from all three precursors are mainly detected in the female samples. **C**–**F**) In situ hybridization detection of miR-124 in developing mouse gonads. Hybridization signal is very weak in 11.5 dpc gonads of both sexes (**C** and **E**) and becomes prominent in the coelomic epithelium of the XY gonad and the cortical region of the 13.5 dpc XX gonad (**D** and **F**, arrows in photomicrographs). G, gonad; M, mesonephros**.** Dashed lines mark the border between the gonad and the mesonephros. Bar = 300 μm for all images.

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0.015 for pre-miR-124-1; $P = 0.016$ for pre-miR-124-2; $P = 0.010$ for pre-miR-124-3). In situ hybridization with a miR-124-specific probe showed a faint or very weak expression in both male and female gonads at 11.5 dpc (Fig. 2, C and E). At 13.5 dpc, miR-124 expression was prominently up-regulated in the cortical region of the female gonad, whereas it was sustained, albeit weak, in the coelomic epithelium of the male gonad (Fig. 2, D and F).

Knock-Down of miR-124 Induces Up-Regulation of Sox9 *in 13.5-dpc Embryonic XX Gonadal Cells*

Because the 3′UTR region of *Sox9* contains a miR-124 target sequence, we hypothesize that miR-124 could be responsible for maintaining down-regulation of *Sox9* in the female gonad from the sex differentiation stage onward. To test this hypothesis, we transfected 13.5 dpc embryonic gonadal cells with a specific miR-124-silencing probe, designated antagomir-124 or Ant-124, that antagonizes the action of miR-124. Q-RT-PCR analyses demonstrated a significant reduction of endogenous miR-124 in cells transfected with the antagomir (Fig. 3A). Immunofluorescence analysis revealed that the SOX9 protein was absent in the nuclei of XX embryonic gonadal cells when cells were untreated (Fig. 3B) or transfected with an unrelated antagomir, Ant-144 (Fig. 3C). In contrast, SOX9 expression was observed in XX gonadal cells transfected with Ant-124 (Fig. 3D), such that they look similar to control XY gonadal cells (Fig. 3E). Q-RT-PCR showed a significant increase in *Sox9* mRNA in XX gonadal cells transfected with Ant-124 but neither in untreated XX cells nor in those transfected with Ant-144 (Fig. 3F).

Overexpression of miR-124 Induces Down-Regulation of Sox9 *in Embryonic Chondrocytes but not in XY Gonadal Cells*

Based on our hypothesis, overexpression of miR-124 would be expected to induce down-regulation of *Sox9* in cells expressing this gene naturally. To test this possibility, we transfected both cultured chondrocytes and XY gonadal cells with either a precursor miRNA expression vector containing the mouse *mmu-mir-124-2* gene or a negative control vector containing a scrambled miRNA sequence. The levels of miR-124 increased considerably in cells transfected with *mmu-mir-124-2*, as measured by Q-RT-PCR (Fig. 4A). Immunofluorescence analyses demonstrated that the percentage of SOX9-positive cells was significantly lower in chondrocytes transfected with the *mmu-mir-124-2* expression vector than in those with the control vector. However, this effect was not observed in XY gonadal cells; the percentage of cells expressing SOX9 did not decrease in the presence of the *mmu-mir-124-2* expression vector (Fig. 4, B–F). Consistent with the results of immunofluorescence studies, Q-RT-PCR analyses showed that overexpression of *mmu-mir-124-2* reduces the expression levels of *Sox9* mRNA in chondrocytes, whereas no significant differences were observed in XY gonadal cells (Fig. 4G). Because SOX9 expression is already robust and maintained by several feedback loops in 13.5 dpc mouse embryonic testes, miR-124 might not be able to efficiently overcome SOX9 expression. Therefore, we repeated the same set of experiments using 11.5 dpc XY gonadal cells, where *Sox9* transcription has just started. However, we obtained similar results, confirming the fact that overexpression of miR-124 is not sufficient to affect SOX9 expression in XY gonadal cells (Supplemental Fig. S3).

DISCUSSION

Despite the discovery of the mammalian testis-determining gene *SRY* some 20 years ago [1, 2], it is thought that some regulatory elements remain unknown, particularly those involved in determining the ovary. Because miRNAs are involved in many developmental processes, it is reasonable to suspect that they may also have some role in gonad development. Several recent studies strongly suggest that this is the case in mammals [35–38]. Using miRNA microarray technology, we identified 22 miRNAs (including mmu-miR-126-5p, mmu-miR-103, and clusters B, C, and D) that are either up- or down-regulated during the critical period of sexual differentiation in a sex-specific manner, indicating that they could participate in gonadal sex differentiation (Fig. 1). Interestingly, coincident with ovarian cell determination at 13.5 dpc, most of them (16) are up-regulated in the ovary at this stage, suggesting a potential role in ovarian development.

Using a clone-based miRNA amplification profiling method, Takada et al. [35] reported that some miRNAs show a sex-dependent expression pattern in 13.5 dpc mouse gonads. Concerning some of the miRNAs identified by those authors, their results contradict those of our microarray-based screening. Notably, Takada et al. reported that the expression of miR-124 is higher in developing testes than in ovaries [35]. However, our data, particularly for miR-124 expression, have been repeatedly validated by Q-RT-PCR and in situ hybridization, which is not included in studies of Takada et al. In addition, a recent publication reported that miR-202-5p/3p are differentially expressed in the developing testis [43], thus coinciding with our microarray data. In fact, miR-202-3p is included in cluster C and miR-202-5p is in cluster E because the higher expression detected in the testis did not exceed the SD > 0.5 threshold (Fig. 1). This represents further validation of our microarray results.

Several findings support the fact that miR-124 could have an important role during ovarian development. First, our miRNA microarray profiling showed that miR-124 is differentially up-regulated in the XX but not the XY gonad during the transition from 11.5 to 13.5 dpc stages. Second, the computational analysis for the miRNAs selected from our microarray results identified potential targets for miR-124 in the 3′-UTR regions of four genes of the male pathway in both human and mouse: *Sox8* (the SRY-box containing gene 8), *Sox9*, *Dmrt1*, and *AR* (androgen receptor). Third, Cheng et al. [42] showed that the mouse miR-124 regulates adult neurogenesis by repressing SOX9 production in the subventricular zone of the brain [42], raising the possibility that this miRNA could act also in the gonad. Overall, these findings strongly suggested that the main role of miR-124 in the ovary would be to inhibit the production of SOX9 protein.

To verify this hypothesis, miR-124 was knocked down or overexpressed in primary cultures of embryonic gonadal cells. Transfection with an miR-124-specific antagomir induced the ectopic production of SOX9 protein in XX gonadal cells, as well as a significant increase in *Sox9* mRNA/transcript, implying that miR-124 actively represses *Sox9* in female gonadal cells. The increased expression levels of *Sox9* in XX cells transfected with antagomir-124 are lower than those observed in XY gonadal cells, suggesting that SRY-mediated up-regulation of *Sox9* is required to reach the male expression level. In the developing mouse testis, *Sox9* is activated primarily, probably by SF1, in both XY and XX gonads prior to sex differentiation [44]. At the time of sex determination, *Sox9* is up-regulated in the XY gonad by the combined, synergistic action of SRY and SF1 on a testis-specific *Sox9*

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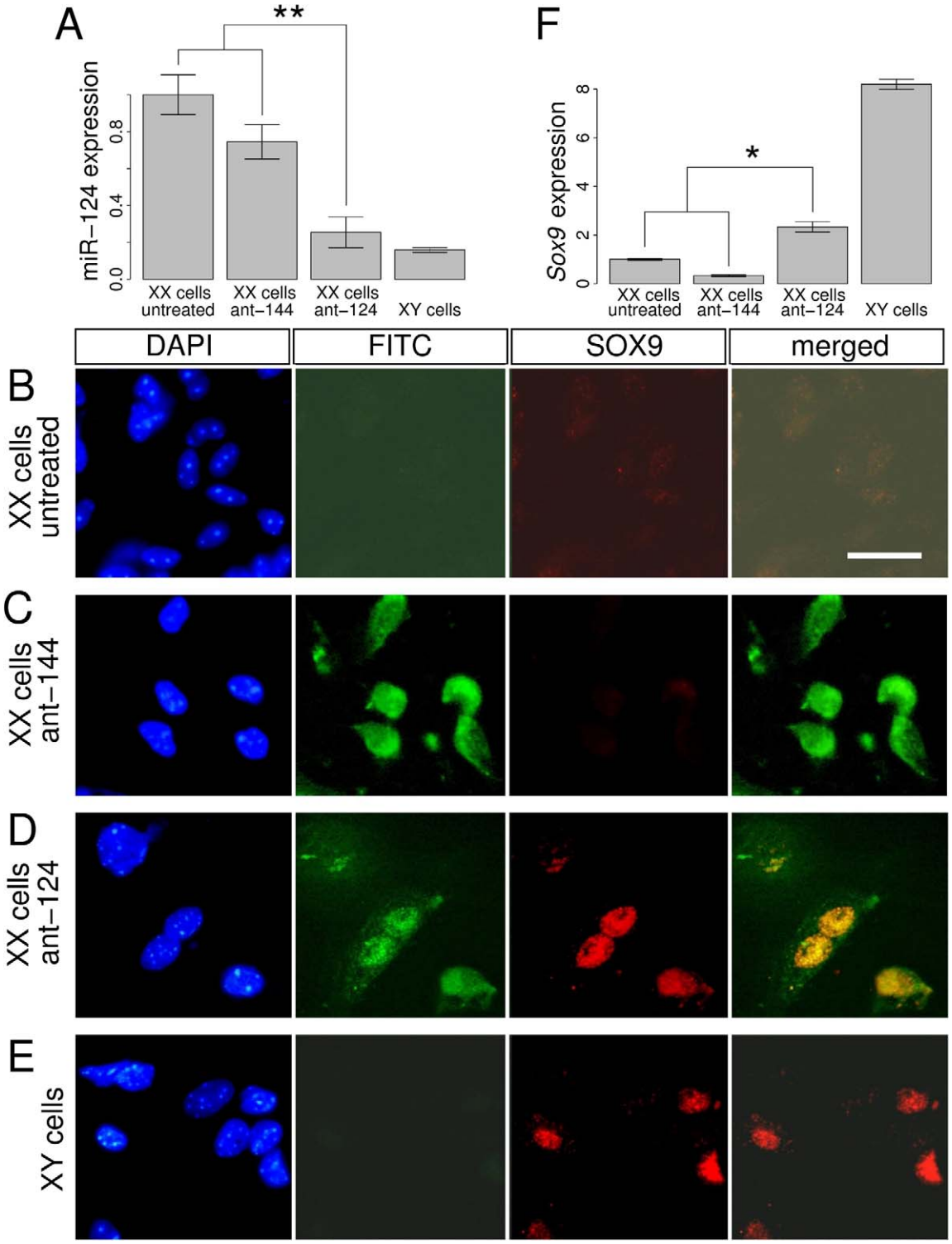

FIG. 3. Effects of miR-124 knockdown on *Sox9* expression in 13.5 dpc XX embryonic gonadal cells. **A**) Q-RT-PCR confirmation of miR-124 knockdown; transfection of XX cells with a miR-124-specific antagomir (ant-124) reduced the level of miR-124 similar to that found in XY cells, and significantly lower ($P < 0.01$ in a one-tail Student *t*-test) than in either untreated XX cells or in those transfected with a negative control antagomir (ant-144). **B–E**) Representative photomicrographs of transfected and control cultured cells. Cell nuclei are revealed by DAPI staining (blue). Transfected cells are marked by FITC (green) provided by the silencing vector. The presence of SOX9 protein was detected by immunofluorescence (red). **F**) Q-RT-PCR analysis of the expression levels of *Sox9* in XX gonadal cells subjected to the miR-124 knockdown experiment. *Sox9* expression was significantly increased ($P < 0.05$ in a one-tail Student *t*-test) in XX cells transfected with the ant-124, reaching a level higher than that measured in either untreated XX cells or those transfected with ant-144 but lower than the level observed in XY gonadal cells. All data are means ± SEM from at least three independent experiments. Bar = 10 μm for all images. *$0.01 < P < 0.05$; **$0.001 < P < 0.01$.

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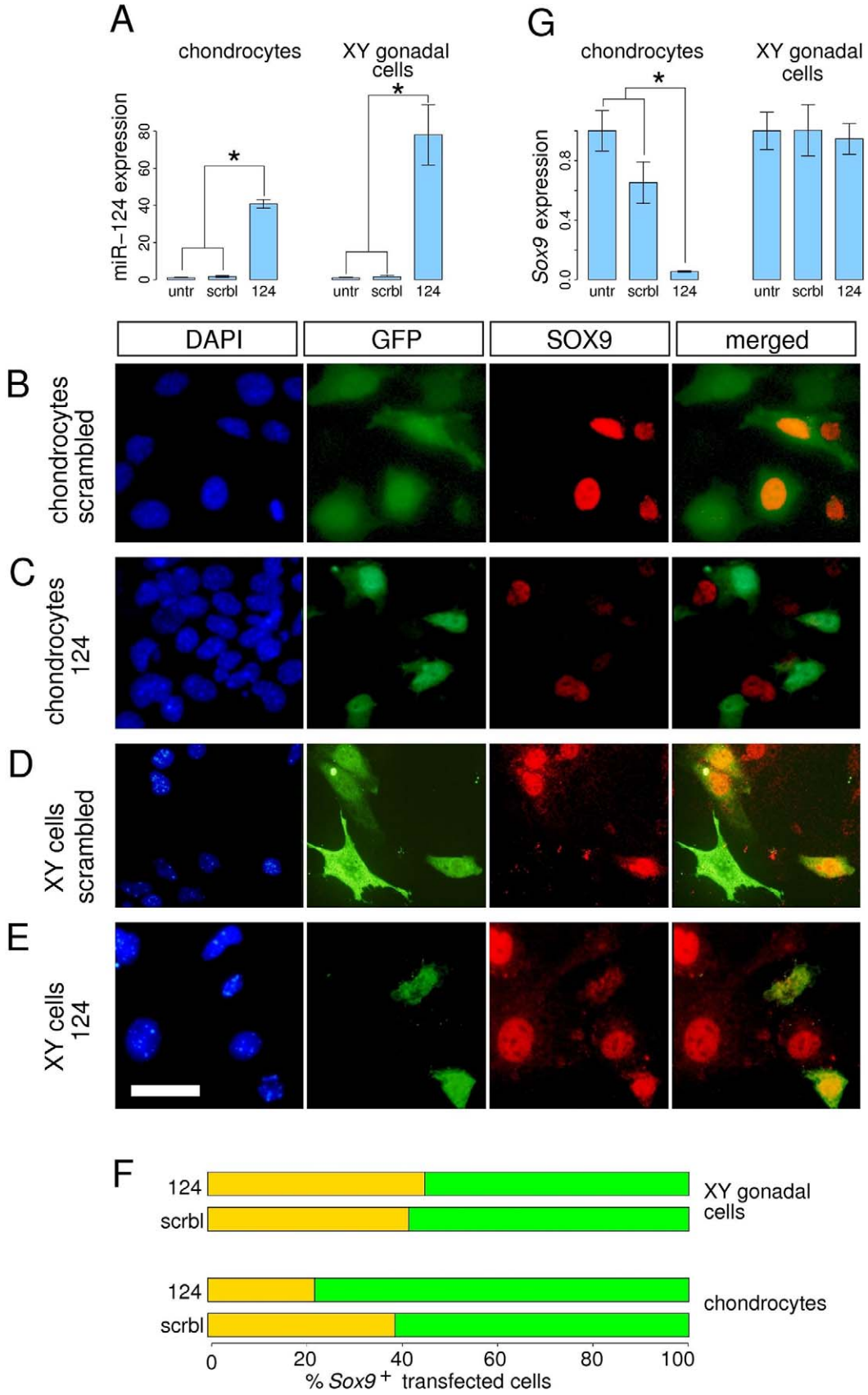

FIG. 4. Effects of miR-124 overexpression in 13.5 dpc embryonic cells expressing *Sox9*. **A**) Q-RT-PCR confirmation of miR-124 overexpression in chondrocytes and XY gonadal cells; in both cases, the cells transfected with the miR-124 expression vector (124) showed significantly increased levels of the miR-124 ($P < 0.05$ in one-tail Student *t*-tests), compared with the control cells (i.e., cells either untreated [untr] or transfected with the scrambled control vector [scrbl]). **B–E**) Representative micrographs of transfected and control cultured cells. Cell nuclei are revealed by DAPI staining (blue). Transfected cells are marked by the GFP provided by the miR-124 expression vector. The presence of SOX9 protein was detected by immunofluorescence

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enhancer (TES) [22], while it is down-regulated in the XX gonad. Afterward, *Sox9* expression is maintained in the developing testis despite the very transient nature of *Sry* expression in mouse pre-Sertoli cells. Several molecular mechanisms contribute to the maintenance of *Sox9* expression, as described earlier. Because XX gonadal cells lack *Sry*, the up-regulation of *Sox9* transcription in XX cells treated with antagomiR-124 may be a secondary consequence of ectopic production of SOX9 protein, presumably through the positive feedback loops. Alternatively, miR-124 could repress both *Sox9* transcription and translation. These data suggest that complete silencing of miR-124 in the XX gonads could be capable of inducing a female-to-male sex reversal in vivo. In this respect, a reasonable approach would be the transfection (or electroporation) of intact gonad explants cultured ex vivo, but we were unable to get positive results after repeated trials using different techniques. These methods are too inefficient to be useful, because it is usually only the outermost cells that take up the DNA. Moreover, although similar numbers of cells in XX and XY gonads were targeted prior to embryonic day 11.5, it is difficult to compare ovaries and testes after they begin to become sexually dimorphic, given differences in size, degree of epithelialization of the external layer, and formation of epithelial testis cords in the last, which further reduce transfection efficiencies. Hence, further research devoted to produce transgenic mice expressing the antagomir is required.

Furthermore, we have shown that overexpression of miR-124 induces repression of *Sox9* in chondrocytes. However, the reduction of SOX9 expression was not observed in XY gonadal cells, even though the levels of ectopic miR-124 expression were much higher than those of endogenous miR-124 expressed in XX gonadal cells (not shown). The results obtained using 13.5 dpc XY gonadal cells were similar to those observed in 11.5 dpc cells, indicating that the mechanisms driving *Sox9* expression are already robust by 11.5 dpc in developing mouse testes. These findings suggest that the distinct regulatory mechanisms controlling *Sox9* expression between chondrocytes and pre-Sertoli cells determine their different response to the miR-124 overexpression experiments.

In chondrocytes, *Sox9* expression is regulated by BMP and transforming growth factor beta (TGFβ) through a chondrogenesis-specific enhancer (see reference [45] for a review), but no positive feedback regulatory loop has been defined here. Our results indicated that overexpression of miR-124 induces a significant decrease of Sox9 expression at both protein and mRNA levels in chondrocytes, which is consistent with the fact that mammalian miRNAs act predominantly to decrease target mRNA levels [46]. In chondrocytes, however, *Sox9* expression needs to be down-regulated to permit bone formation [47]. This might imply that positive feedback regulation of *Sox9* in chondrocytes will be less robust than in Sertoli cells, where high levels of SOX9 are maintained throughout life. Overall, existing data show that several molecular regulatory mechanisms have been selected to ensure *Sox9* expression in Sertoli cells.

In situ hybridization studies showed that miR-124 is present mainly in the cortical region of the female gonad at 13.5 dpc (i.e., the portion from which the ovarian tissue mostly develops in mammals and other vertebrates [48] and also the region in which the complete inhibition of *Sox9* would be especially necessary in the differentiating female gonad. The fact that *Sox9* is expressed mainly in the medulla of the developing testis is not a valid argument against this hypothesis because this is not the case in developing ovaries, where *Sry* is absent and, thus, *Sox9* is not up-regulated anywhere. Furthermore, it is known that both pre-Sertoli and pregranulosa cells are derived from cells originating in the coelomic epithelium of developing gonads. These coelomic epithelial cells proliferate and migrate into the gonad between 11.0 and 12.5 dpc [49, 50]. Distribution of miR-124-expressing cells in the XX gonad is very similar to that of proliferating cells, shown as bromodeoxyuridine (BrdU)-positive cells by Schmahl et al. [50]. Hence, miR-124 may ensure that *Sox9* is completely silenced in these proliferating XX cortical cells, which might otherwise have the potential to become Sertoli cells. In the coelomic epithelium of the XY gonad, proliferating cells no longer migrate into the gonad at 13.5 dpc, but they might retain a potential to become ectopic *Sox9*-expressing Sertoli cells in the region whose normal fate is to differentiate as the tunica albuginea of the testis. miR-124 could thus secure *Sox9* expression to be silenced in the coelomic epithelium as a back-up mechanism to avoid this possible anomaly.

It is known that Dicer RNase III enzyme plays a crucial role in processing pre-miRNA to produce miRNA. Two different types of conditional *Dicer1* null mutant mice have been developed to study the function of miRNAs in somatic gonadal cells. Papaioannou et al. [36] and Kim et al. [37] used an *Amh-Cre* mouse strain (where the Cre recombinase is driven by the anti-Müllerian hormone gene promoter) to drive the Cre-LoxP-mediated deletion of *Dicer1* specifically in Sertoli cells at 14.5 dpc. Hence, no alteration of *Dicer1* expression was induced in the females derived from these crosses, as *Amh* is expressed only in the Sertoli cells of the testis, shortly after the sex-determination stage. Huang and Yao [38] performed a similar experiment using *Sf1-Cre* mice to delete *Dicer1* at earlier stages in both sexes and found that, whereas testes degenerate postnatally, ovaries appeared to be unaffected until the animals died due to adrenal failure at 5 days postpartum. In both cases, prenatal gonad development appeared to be completely normal. These results led to the surprising conclusion that no miRNA is required for normal ovarian development, but the authors did not provide proof that miRNAs had been completely eliminated and, most importantly, when the miRNA decay eventually took place. Hence, definite experiments of *Dicer* genetic ablation, using more efficient Cre drivers, remain to be done with undifferentiated XX and XY gonads.

We report here the first evidence suggesting that miRNAs play a role in the early steps of ovarian development. We have shown that inhibition of miR-124 in XX gonadal cells results in up-regulation of *Sox9*, indicating that this miRNA is at least partly responsible for *Sox9* repression in the female gonad. Two sets of evidence strongly indicate that *Sox9* must be repressed in the female gonad in order to avoid a female-to-male sex reversal; first, it is known that XX gonads overexpressing *Sox9* develop as testes [9, 10], and second, *Sox9* is down-regulated in the female gonad at the time of sex determination [51, 52]. *Wnt4* plays a role in this down-regulation because *Sox9* is transiently up-regulated in the

(red). **F**) Percentage of transfected chondrocytes and XY gonadal cells that express Sox9. miR-124 transfection (124) induces a significant reduction in the percentage of chondrocytes expressing SOX9, compared with the control (scrbl), but has no similar effect in XY gonadal cells. **G**) Q-RT-PCR measurement of the levels of *Sox9* mRNA in transfected chondrocytes and XY gonadal cells. Consistent with SOX9 immunostaining, overexpression of miR-124 significantly reduced the *Sox9* expression levels in chondrocytes but not in XY gonadal cells compared with either untreated (untr) or transfected cells with the control vector (scrbl). Bar = 10 μm for all images. *$0.01 < P < 0.05$.

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gonads of *Wnt4*$^{-/-}$ null mutant XX mice. However, complete sex reversal is not observed, although the mutant ovary is masculinized, suggesting that an additional ovary-promoting or anti-testis factor(s) may also be involved (see reference [19] for a review). Here we provide evidence that miR-124 is likely to be one such factor reinforcing the ovarian developmental pathway by repressing *Sox9* in the XX mouse gonad at the time of sex determination (Supplemental Fig. S4). Further analysis of the other miRNAs identified in this study will probably permit the discovery of new pieces of the complex puzzle of genes controlling mammalian sex determination.

In conclusion, the sex-specific differential expression pattern of several miRNAs during the critical period of sex determination, along with the identification of potential targets for these miRNAs in the 3′-UTR of master genes that control this process, suggest that miRNAs play critical roles in regulating these genes in mammals. Inhibition of miR-124 in XX gonadal cells induces the ectopic expression of *Sox9*, indicating that this miRNA may be responsible for *Sox9* down-regulation in female gonads at the critical time of sex determination. The ectopic overexpression of miR-124 reduces the levels of *Sox9* expression in chondrocytes but not in XY gonadal cells, demonstrating that the expression of this gene is very robust in the testis since the earliest stages of gonadal differentiation and that different regulatory mechanisms operate for this gene in these two cell types.

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