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Essential Role of Sox2 for the Establishment and Maintenance of the Germ Cell Line

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ABSTRACT

Sox2 is a pluripotency-conferring gene expressed in primordial germ cells (PGCs) and postnatal oocytes, but the role it plays during germ cell development and early embryogenesis is unknown. Since Sox2 ablation causes early embryonic lethality shortly after blastocyst implantation, we generated mice bearing Sox2-conditional deletion in germ cells at different stages of their development through the Cre/loxP recombination system. Embryos lacking Sox2 in PGCs show a dramatic decrease of germ cell numbers at the time of their specification. At later stages, we found that Sox2 is strictly required for PGC proliferation. On the contrary, Sox2 deletion in meiotic oocytes does not impair postnatal oogenesis and early embryogenesis, indicating that it is not essential for oocyte maturation or for zygotic development. We also show that Sox2 regulates *Kit* expression in PGCs and binds to discrete transcriptional regulatory sequences of this gene, which is known to be important for PGCs survival and proliferation. Sox2 also stimulates the expression of *Zfp148*, which is required for normal development of fetal germ cells, and *Rif1*, a potential regulator of PGC pluripotency. STEM CELLS *2013;31:1408–1421*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Sox2 is a member of the Sox (SRY-related HMG box) gene family that encodes transcription factors with a single High Mobility Group DNA-binding domain. Sox2 expression is highly conserved among various mammalian species, reflecting an important function in the regulation of embryonic development and in the determination of cell fate [1]. Several lines of evidence indicate that Sox2 may act to maintain or preserve developmental potential in many cell lineages. In fact, besides being one of the key transcription factors for the embryonic stem (ES) cell pluripotency, it has been shown that Sox2 marks the pluripotent lineage of the early mouse embryo. From recent investigations, it has also emerged that Sox2 is one of the transcription factors underlying the derivation of induced pluripotent stem cells [2]. Maternal Sox2 RNA and protein, as for Oct4 [3], are present in fertilized oocytes until the two-cell stage. Zygotic Sox2 RNA, during embryogenesis, is expressed in the morula at 2.5 days postcoitum (dpc), inner cell mass of the blastocyst from 3.5 dpc and, at a later stage, in epiblast cells [4]. Unlike Oct4, however, Sox2 RNA is also expressed by the multipotential cells of the extraembryonic ectoderm (ExE) at 6.5 dpc and later in the ExE portion of the chorion [4]. In both embryonic and extraembryonic lineages, Sox2 downregulation correlates with a commitment to differentiate [4, 5]. After gastrulation, Sox2 is expressed in uncommitted dividing stem and precursor cells of the developing nervous system [6, 7] and sensory placodes, in branchial arches and in the primitive foregut endoderm [4] as well as in primordial germ cells (PGCs) [8]. Postnatally, Sox2 is maintained expressed in the adult brain [9] and in several adult stem and progenitor cells of the epithelial lineage and its function is essential for tissue regeneration and survival in mice [10].

PGCs are the embryonic precursors of the germline, responsible for generating gametes in the adult animal. They arise as a small subset of cells, in the extraembryonic mesoderm (ExM), at around 7.25 dpc identified by high levels of alkaline phosphatase (APase) activity [11]. The transcriptional regulator Blimp1 (B-lymphocyte-induced maturation protein-1, also known as Prdm1) has been identified as a key factor for the specification of PGCs [12, 13]. Gene expression profiling has shown that founder PGCs are characterized by high levels of an interferon-inducible transmembrane protein, fragilis/mil-1/ifitm3 and exclusive expression of a small nuclear

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cytoplasmic shuttling protein, Stella/Pgc7/Dppa3, while their mesodermal neighbors upregulate Hox genes [14]. Sox2 mRNA expression is initially downregulated and then reactivated during germ cell specification, and Sox2 is not expressed in Blimp1 null PGCs [15, 16]. Evidence for Sox2 expression at the protein level in 7.5 dpc embryos has also been shown [16]. Prdm14, another Positive Regulatory-domain containing transcriptional regulator is specifically expressed in nascent PGCs and has been demonstrated to be upstream to Sox2 in these cells [16]. Nevertheless, the role of Sox2 in the nascent population of PGCs and during their differentiation is not yet understood. In this work, we investigated the role of Sox2 during mouse gametogenesis. Our results reveal that Sox2 deletion during the establishment of germ cell lineage impairs PGC development, as previously shown for Prdm14 [16], Oct4 [17, 18], and Nanog [19]. We also found that Sox2 is essential for PGC proliferation in vivo and in vitro, and that it affects the expression of genes which are critical for their development. Our results show that Sox2 is a transcription factor important not only for stem cells but also for the establishment and/or maintenance of the germ cell line before its sexual determination.

Materials and Methods

Mice

The generation of $Sox2^{\beta geo}$ and $Sox2^{loxP}$ mice has been previously described in [4, 20], respectively. $TNAP^{Cre}$ mice were a gift from Dr. D. O'Carroll (EMBL, Monterotondo, Italy). $Blimp1^{Cre}$ mice were obtained from Dr. M. Nussenzweig (Rockfeller University, NY). $Rosa26^{CreERT}$ mice were obtained from Jackson Laboratories (Ann Harbor, http://www.jax.org). $Spo11^{Cre}$ mice have been previously described in [21]. Homozygous YFP-$Rosa26^{loxP/loxP}$ and LacZ-$Rosa26^{loxP/loxP}$ mice were kindly provided by GianGiacomo Consalez (University of San Raffaele, Milan, Italy). Kit^{Egfp} mice have been previously described [22]. For staging embryos, 0.5 dpc corresponded to the day of vaginal plug. Experimental procedures involving mice were approved by the Italian Ministry of Health.

$Sox2^{loxP/lacZ}$, $Sox2^{loxP/+}$, $Sox2^{loxP/+}$;$Blimp1^{Cre}$ embryos did not show any differences in phenotype compared to wt embryos and were all considered equivalent controls with respect to the $Sox2^{loxP/lacZ}$;$Blimp1^{Cre}$ conditional mutants.

Cells and Cultures

ES D3 cell line (kind gift of M. De Felici, University of Rome Tor Vergata, Italy) was maintained in knock-out (KO) medium (Invitrogen) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 20 mM glutamine, nonessential aminoacids (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), 2 mM β-mercaptoethanol (Invitrogen), 1,000 UI/mL leukemia inhibitory factor (LIF) (LIFES, Immunological Sciences), and 15% fetal calf serum (FCS) (Lonza).

PGC isolation from the different genotypes varied according to the developmental stage. To count PGC at 7.5 dpc, embryos were cut in two halves and the posterior part was disaggregated with trypsin-EDTA (Invitrogen). The cell suspension was adhered to poly-L-lysine-coated slides, fixed, and stained for APase. The anterior half of each embryo was used for genotyping. PGCs deriving from 8.5 dpc embryos were obtained by disaggregating the posterior part of each embryo in trypsin-EDTA and plating cell suspensions individually onto mitomycin-C treated Sl4-m220 fibroblasts [23] in KO medium as for ES supplemented with LIF (1,000 UI/mL), basic fibroblast growth factor (bFGF) (25 ng/mL, Immunological Sciences), and stem cell factor (SCF) (10 ng/mL, Immunological Sciences) (PGC medium). Gonads from $Sox2^{loxP/loxP}Rosa26^{Cre\text{-}ERT}$ embryos at 11.5 dpc were isolated, digested with trypsin-EDTA, and 1/10 of the cell suspension was plated onto mitomycin-C-treated Sl4-m220 fibroblasts supplemented with LIF, bFGF, and SCF. All the cultures were fixed after 1 or 3 days and PGCs were identified by APase staining. The remaining embryonic tissues at all the developmental stages were used for genotyping.

4OH-Tamoxifen (Sigma Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) was dissolved in ethanol and then diluted in Dulbecco's modified Eagle's medium to a final concentration of 0.2 μM at the beginning of the culture. Control cultures were treated with diluted vehicle alone.

To count PGC numbers, at the end of cultures, cells were fixed with 4% paraformaldheyde and treated with a mixture of Naphtol AS-MX phosphate (40 μL/mL, Sigma Aldrich) in 1 mg/mL Fast Red FF (Sigma Aldrich) for APase staining to reveal PGCs.

Germ cells from 13.5 dpc embryos for lentiviral infections or for preparing germ cell extracts were obtained from CD-1 mice. To obtain germ cell protein extracts at 13.5 dpc, testes or ovaries were isolated by pricking gonads after a 10 minute incubation in 10 mM EDTA [24].

Germ cells from 12.5 dpc embryos for Sox2 in vitro deletion were isolated from $Sox2^{loxP/loxP}$;$Rosa26^{CreERT}$ embryos and cultured in vitro for 3 days in the absence or presence of 0.2 μM 4OH-Tamoxifen. Germ cells were then recovered by a mouth-operated pipette and processed for semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis.

Postnatal male germ cells (7 dpp) were obtained as previously reported by sequential enzymatic digestion of testes from CD1 albino mice and preplating of cell suspension, [25]. Meiotic and postmeiotic germ cells were obtained from adult testes and purified by elutriation, as previously described [21].

EG (embryonic germ) cells were obtained from 8.5 dpc embryos from $Sox2^{lox/lacZ}$;$Blimp1^{Cre}$ matings as previously described [26] with minor modifications. Briefly, the area corresponding to the base of allantois was cut apart from each embryo and digested in trypsin-EDTA. Each embryonic piece was seeded onto three mitomycin-C-treated Sl4-m220 fibroblasts supplemented with LIF, bFGF, and SCF. Medium was changed daily, and at the fifth day of culture it was replaced by complete KO medium supplemented with 2% FCS, LIF, PD0325901 (Axon, 1 μM), and CHIR 99021 (Axon, 3 μM). Cultures were fixed at the eighth day or replated onto fresh mitomycin-treated mouse embryonic fibroblast feeders.

BrdU Incorporation and TUNEL Assay

5-bromo-2′-deoxyuridine (BrdU) incorporation was performed as previously described in Dolci et al. [27] with minor modifications. Briefly, 8.5 dpc PGCs from $Sox2^{loxP/loxP}$-$Rosa26^{Cre\text{-}ERT}$ or from $Sox2^{loxP/loxP}$ embryos were seeded onto mitomycin-C-treated Sl4-m220 fibroblasts in complete PGC medium and immediately treated with 0.2 μM 4OH-Tamoxifen or with vehicle. After 3 days, BrdU (Life Technologies, Rockville, MD, http://www.lifetech.-com) was added for the last 4 hours of culture and cells were then fixed in 70% ethanol. APase staining was first performed and cells were then treated with 0.1 M HCl for 5 minutes. BrdU was then revealed according to the manufacturer's protocol. For in situ detection of apoptosis (TUNEL), 7.5 dpc embryos were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 24 hours and then dehydrated by increasing concentrations of methanol and stored in 100% methanol at −20°C for 12 hours or until ready for use. Embryos were then rehydrated in decreasing concentrations of methanol in PBST. After three washes in PBST, 50 μL of a labeling solution for the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Roche Applied Science, Basel, Switzerland, http://www.roche-applied-science.com) was added and embryos were incubated at 37°C for 1 hour. Embryos were then washed and stained with DAPI for 1 hour before fluorescence analysis. After individual image capture, each embryo was PCR genotyped.

Lentiviral Transduction and Semiquantitative RT-PCR

Gonads were digested with trypsin-EDTA and cells from four gonads were plated in four-well dishes in KO medium supplemented with 10% FCS. While somatic cells adhered to the plastic, germ cells grew floating in the medium. Cells were infected at the beginning of culture with Sox2-enhanced green fluorescent protein (EGFP) or EGFP lentiviruses and polybrene (1 μg/mL) and cultured for 2 days. K562 cells plated at 20,000 cells per well in four-well chambered slides and transduced 4 hours later with a Sox2-GFP-expressing or GFP-expressing lentiviruses as described previously [20]. After 24 hours, cells were reinfected for additional 24 hours. All the infections were performed at least three times. At the end of the culture, floating cells were harvested, washed from viruses, resuspended in PBS, lysed in Trizol reagent (Invitrogen). RNA was extracted, DNase treated (RQ1 DNAase, Promega, Madison, WI, http://www.promega.com), and reverse transcribed with random hexamer primers using an Invitrogen cDNA reverse transcription kit (with a reverse transcriptase–negative control). For RT-PCR, primer sets are listed in the Supporting Information data. Annealing was carried out at 58°C for all primer sets and 45 cycles were run for all amplification. Actin was amplified at 25 cycles while EGFP and Sox2 at 20 cycles.

Western Blotting

Whole cell extracts (in 10 mM HEPES pH 7.9, 1% Triton X-100, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol, 10 mM β-glycerophosphate, 0.1 mM sodium vanadate, and protease inhibitor cocktail (Sigma-Aldrich) were electrophoresed in polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) (Amersham, Piscataway, NJ, http://www.amersham.com) membranes. Primary antibodies were incubated overnight, and secondary antibodies were incubated 1 hour at room temperature. Signals were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com).

Histology, Immunohistochemistry, and Immunofluorescence

Embryos and tissues were fixed in buffered formalin and paraffin embedded. Serial sections 5 μm thick were obtained at the microtome. For immunohistochemistry, sections were incubated overnight at 4°C with primary antibody diluted in 0.5% BSA in PBS, extensively washed in PBS, and incubated for 1 hour at RT with peroxidase-conjugated secondary antibodies and revealed by 3,3′-Diaminobenzidine (DAB) substrate (Dako Dual Link Envision + / DAB +, Dako, Glostrup, Denmark, http://www.dako.com). Sections were counterstained with hematoxylin and mounted. β-Gal activity on whole testis preparations were performed as previously described [4]. Zygotes and morulas were obtained from $Sox2^{loxP/lacZ}$;$Spo11^{Cre}$ females crossed to CD-1 males.

Cells were fixed in 4% paraformaldehyde, washed in PBS, and permeabilized in 0.5% Triton in PBS. Embryos were incubated in rabbit anti-Sox2 overnight at 4°C and then incubated in secondary Cy3-conjugated antibodies. 4′,6-diamidino-2-phenylindole (DAPI) was used for nuclear counterstaining. Whole mount immunofluorescence of 7.5 dpc embryos was performed according to [28].

Whole mount staining of 7.5 dpc embryos or 10.5 dpc genital ridges for APase activity was performed according to [11] with the exception that fixation of tissues was carried out in 70% ethanol. After microscopy inspections whole mounted embryos at 7.5 dpc were extracted in DNA extraction buffer (10 mM Tris-Cl, 1% SDS, and 1 mg/mL proteinase K mixture) and genotyped. Deconvolution and microscopy inspections were performed on a Leica CTR6000 microscope.

List of Antibodies

Primary antibody used for Western blot analysis were: anti-Sox2 rabbit polyclonal antibody (1:500, AB5603, Chemicon, Temecula, CA, http://www.chemicon.com), anti-actin rabbit polyclonal (1/1,000, A2066, Sigma-Aldrich). Mouse antibody to p63 (1:100, Santa Cruz Biotechnology), mouse antibody to Sox2 (1:50, MAB2018, R&D, Minneapolis, MN, http://www.rndsystems.com), mouse antibody to Oct4 (1:100, Santa Cruz Biotechnology), rabbit antibody to TTF1 (1:100, kind gift from S. Zannini, University of Naples, Italy), rabbit antibody to Lasp1 (1:200, Santa Cruz Biotechnology), mouse antibody to γH2AX (1:10,000, Upstate, Charlottesville, VA, http://www.upstate.com), mouse antibody to VASA (1:100, Santa Cruz Biotechnology), rabbit antibody to Stella (1:200, Abcam, Cambridge, U.K., http://www.abcam.com) were used for immunohistochemistry and immunofluorescence. HRP-conjugated secondary antibodies were used for immunohistochemistry; FITC or Cy3 conjugated secondary antibodies (Santa Cruz Biotechnology) were used for immunofluorescence.

Chromatin Immunoprecipitation

Isolated gonads from 12.5 dpc CD-1 embryos were separated from mesonephroi, cut in several pieces, and snap-frozen in liquid nitrogen. After obtaining about 140 gonads, tissues were immediately crosslinked to DNA by direct addition to the minced frozen gonads of formaldehyde at a 1% final concentration for 10 minutes at 37°C. After sonication, chromatin immunoprecipitation (ChIP) assay was performed according to the Abcam protocol. Protein–DNA complexes were immunoprecipitated overnight in the presence of the specific anti-Sox2 antibody (R&D) or mouse IgGs (Santa Cruz Biotechnology). DNA was purified by phenol/chloroform/isoamylic alcohol extraction and ethanol precipitation, and resuspended in 20 μL of Tris-EDTA (TE) buffer and used directly for PCR. Primers are listed in Supporting Information data.

Statistical Analysis

For continuous variables normally distributed, statistical comparisons between control and treatments were established by carrying out the ANOVA test. When ANOVA test revealed a statistical difference, pair-wise comparisons were made by Tukey's honestly significant difference test and the probability of each presumed "non-difference" was indicated. The Student's t test was used to assess the significance if two comparisons were planned. All experiments were performed at least three times. Values are reported as mean and SD. Asterisks indicate the level of statistical significance (*, $p < .05$; **, $p < .001$).

Results

Sox2 Is Required for PGC Development and Pluripotency

Sox2 is expressed and required to maintain cells of the epiblast in an undifferentiated state [4]. After germ cell specifi cation, Blimp1-positive PGCs specifically regain Sox2 expression as early as 7.25 dpc [29]. Since Sox2 ablation is embryonic lethal, we generated Sox2-conditional knockout mice in the germline. To selectively delete Sox2 in PGCs, we intercrossed $Sox2^{loxP/loxP}$ knock-in mice [20] with $Sox2^{lacZ/+}$; $Blimp1^{Cre}$ mice [12]. These deleters, carrying a BAC transgene in which *Cre* expression is driven by the *Blimp1* (B lymphocyte-induced maturation protein 1) locus, mediate *Cre* excision of floxed targets as early as 7.25–7.5 dpc in PGCs [12]. The early onset of *Cre* activation in Blimp1-positive tissues was evident in compound embryos obtained at 7.5 dpc from EYFP-$Rosa26^{loxP/loxP}$ and $Blimp1^{Cre}$ mice intercrosses (Supporting Information Fig. 1A). The high efficiency of Blimp1-driven Cre activation in the germline was confirmed by β-Gal staining of newborn testes obtained from LacZ -$Rosa26^{loxP/loxP}$ and $Blimp1^{Cre}$ mice intercrosses

(Supporting Information Fig. 1B). Matings between $Sox2^{loxP/loxP}$ and $Sox2^{lacZ/+}$; $Blimp1^{Cre}$ (Fig. 1A) resulted in the birth of live pups at normal Mendelian rates. However, $Sox2^{loxP/lacZ}$; $Blimp1^{Cre}$ mutants were readily recognizable for their microphthalmia and labored breathing which led them to death within a few hours after birth. Examination of perinatal ovaries (17.5 dpc) of $Sox2^{loxP/lacZ}$;$Blimp1^{Cre}$ mice showed a complete depletion of meiotic oocytes, as shown by the absence of γH2AX staining, compared to $Sox2^{loxP/lacZ}$ ovaries (Fig. 1B). Similarly, perinatal testes (17.5 dpc) from $Sox2^{loxP/lacZ}$;$Blimp1^{Cre}$ mice were completely devoid of pro-spermatogonia, as compared to $Sox2^{loxP/lacZ}$ testes (Fig. 1C). Complete absence of germ cells was also observed at 13.5 dpc in both male and female gonads from $Sox2^{loxP/lacZ}$;$Blimp1^{Cre}$ mice (Fig. 1B, 1C).

To understand if the germ cell defect occurred earlier, we analyzed embryos at 10.5 dpc, a developmental stage when PGCs are actively migrating toward the genital ridges in the aorta-gonad mesentery (AGM) area. Whole mount APase stainings of $Sox2^{loxP/lacZ}$ and $Sox2^{loxP/lacZ}$; $Blimp1^{Cre}$ genital ridges (Fig. 2A) showed that PGCs are strongly reduced at this stage in mutant embryos. To understand if the few PGCs found in mutants were deriving from cells escaping Sox2 deletion, we performed an immunohistochemical analysis for Sox2 and Oct4 on serial sections of 10.5 dpc embryos. As shown in Figure 2B–2D, several Sox2 and Oct4 positive PGCs were found in $Sox2^{loxP/lacZ}$ embryos actively migrating within the AGM area, while very few PGCs were identifiable within the mutant AGM area. However, since these cells were positive for Sox2, our results suggested that in Sox2 deletion did not occur in all PGCs at this developmental stage.

We then isolated embryos at 7.5 dpc, shortly after PGC specification. Whole mount APase stainings showed a strong decrease of PGCs in the posterior area of $Sox2^{loxP/lacZ}$; $Blimp1^{Cre}$ embryos compared to the $Sox2^{loxP/lacZ}$ embryos (Fig. 3A). We then investigated if PGCs were expressing Stella, which is an early marker of PGC specification. As shown in Figure 3B–3D, a dramatic decrease of Stella positive cells was found in the posterior area which was comparable to that observed by APase staining, suggesting that Sox2 is required during PGC establishment. We did not observe increased apoptosis within the PGC region in mutant embryos compared to controls as judged by nuclear morphology after DAPI staining. Lack of increased apoptosis in the area of PGC specification was confirmed by the analysis of whole mount 7.5 dpc control and mutant embryos after TUNEL staining (Supporting Information Fig. 2).

In order to quantify the entity of PGC loss, each embryo isolated at 7.5 dpc was cut in two halves. The posterior part was disaggregated in trypsin-EDTA and cells were adhered to poly-L-lysine slides. APase staining was performed to identify and count PGCs. As shown in Figure 4A, we detected less than 5% PGCs in $Sox2^{loxP/lacZ}$;$Blimp1^{Cre}$ embryos with respect to control littermates. To understand if the few residual germ cells maintained their proliferative potential, we isolated PGCs at 8.5–11.5 dpc, seeded them onto mitomycin-C-treated Sl4-m220 fibroblasts and cultured them up to 3 days (Fig. 4B, 4C). PGC number was strongly decreased in $Sox2^{loxP/lacZ}$; $Blimp1^{Cre}$ embryos as early as after 1-day of culture, and they did not proliferate after 3 days, as compared to their control littermates.

In 7.5 dpc embryos, Sox2 is expressed in developing PGCs (Supporting Information Fig. 3A–3C). After PGC specification, Sox2 expression remains high up to 12.5–13.5 dpc in germ cells and is then downregulated in both sexes (Supporting Information Fig. 3D–3H; Fig. 5B, and [8]). In the male, Sox2 expression was confined to few gonocytes in 1 days post partum (dpp) testes and absent in 7 dpp and adult testes (Supporting Information Fig. 3F–3H). By Western blot analysis, we found that Sox2 levels decreased from 13.5 to 17.5 dpc in fetal gonocytes, that they were barely detectable at 1 dpp and that it was absent in prepuberal spermatogonia, spermatocytes, and spermatids (Supporting Information Fig. 3I). In the postnatal ovary, Sox2 expression was resumed in primary, growing, and fully grown oocytes (Fig. 5D and [4]).

To understand if Sox2 was essential also during PGC proliferation period, we set up matings between $Sox2^{loxP/loxP}$ and $Sox2^{lacZ/+}$;$TNAP^{Cre}$. These deleters, obtained by knocking *Cre* into the TNAP (tissue nonspecific alkaline phosphatase) locus, mediate *Cre* excision in PGCs between 9.0 and 10.5 dpc [17, 30] (Fig. 1D). Matings between $Sox2^{loxP/loxP}$ and $Sox2^{lacZ/+}$;$TNAP^{Cre}$ resulted in the birth of live pups at normal Mendelian rates, even though all the $Sox2^{loxP/lacZ}$;$TNAP^{Cre}$ mice died within 2 weeks after birth for unknown reasons, possibly due to $TNAP^{Cre}$-driven Sox2 deletion in other tissues essential for postnatal survival, such as neural stem cells [31].

Examination of $Sox2^{loxP/lacZ}$;$TNAP^{Cre}$ prepuberal ovaries (13 dpp) showed complete depletion of oocytes and follicles compared to the normal phenotype of $Sox2^{loxP/lacZ}$ ovaries (Fig. 1E, upper panels). Similarly, perinatal testes (17.5 dpc) from $Sox2^{loxP/lacZ}$;$TNAP^{Cre}$ embryos were completely devoid of pro-spermatogonia (Fig. 1E, lower panels). The germ cell defect was also evident at earlier stages, since we found that the percentage of PGCs was strongly reduced at 11.5 dpc in $Sox2^{loxP/lacZ}$;$TNAP^{Cre}$ gonads compared to $Sox2^{loxP/lacZ}$ gonads (Fig. 4D), confirming that the primary gamete deficiency observed in perinatal and prepuberal animals was caused by the loss of PGCs at 10.5–11.5 dpc.

To understand if Sox2 is also required for PGC proliferation in vitro, we generated $Sox2^{loxP/lox}$;$Rosa26^{Cre\text{-}ERT}$ embryos and cultured 11.5 dpc PGCs for 1 or 3 days. To achieve Sox2 deletion in vitro, we added 0.2 μM 4OH-tamoxifen at the beginning of the culture period. As shown in Figure 4E, 4F, tamoxifen addition did not affect PGC cell number in Cre-negative and in Cre-positive PGCs after 1 day of culture. However, after 3 days from the treatment we found a strong decrease of PGC number in Cre-positive but not in Cre-negative cells. We then carried BrdU incorporation experiments to verify that PGC loss after Sox2 deletion was actually due to defective proliferation. As shown in Figure 4G, we found that both control and Cre-ERT transgenic PGCs were actively incorporating BrdU. After in vitro Sox2 deletion induced by 4OH-tamoxifen Cre-ERT transgenic PGCs showed a severe impairment of DNA synthesis.

Since Sox2 has been shown to be a key player in the pluripotency of ES cells [4], the exclusive Sox2 re-expression in the germ cell line may lead to the reacquisition of potential pluripotency in this lineage. To test if Sox2 was essential for pluripotency, we cultured 8.5 dpc PGCs from different genotypes to obtain EG cells in the presence of the two signaling inhibitors CHIR99021 and PD0325901 [26]. While EG colonies were reproducibly obtained from control littermates we never succeeded in obtaining EG colonies from $Sox2^{loxP/lacZ}$;$Blimp1^{Cre}$ embryos (Fig. 4H), suggesting that Sox2 is also required for reacquisition of PGC pluripotency.

Sox2 Is Required for the Development of Eye and Foregut Endoderm Derivatives

To understand the cause of early postnatal death of $Sox2^{loxP/lacZ}$; $Blimp1^{Cre}$ mutants, we inspected the morphology of their endodermal and neuro-ectodermal derivatives which are known to express both Blimp1 [32] and Sox2 [4] during early embryonic development. Indeed, mutants showed severe

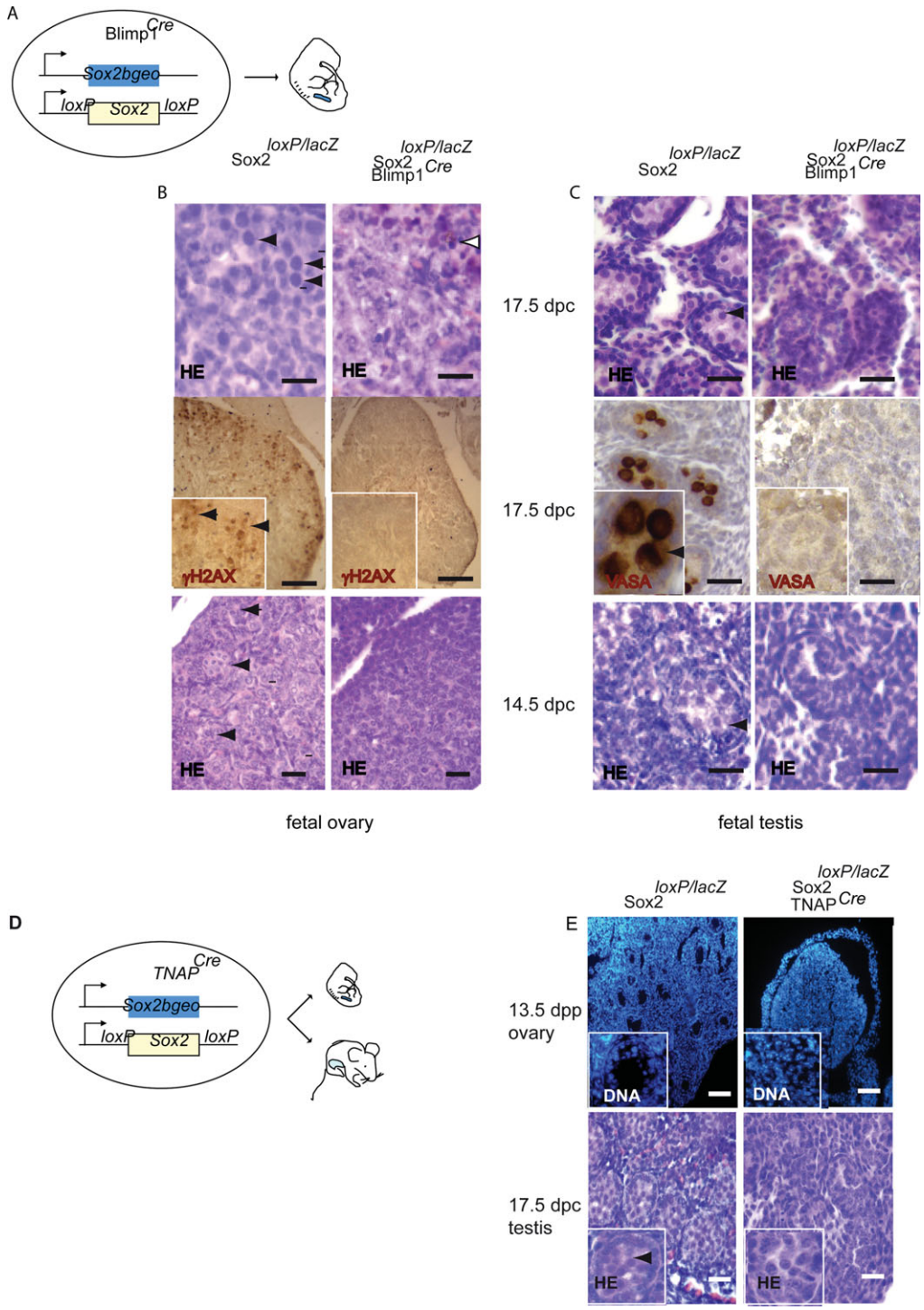

Figure 1. Absence of male and female germ cells in Sox2 conditional mutants. **(A):** Schematic representation of the genotype of conditional knockout mice, in which one Sox2 allele is replaced by *lacZ* (β-geo) and the second Sox2 allele is flanked by two *loxP* sites. Sox2 deletion is driven by Blimp1^{Cre} in nascent 7.5 dpc primordial germ cell. Phenotype was inspected in fetal gonads. **(B):** Upper panels: HE staining of ovaries from control Sox2$^{loxP/lacZ}$ (left) and Sox2$^{loxP/lacZ}$;Blimp1^{Cre} (right) 17.5 dpc mice. Middle panels: γH2AX staining for meiotic oocytes in control Sox2$^{loxP/lacZ}$ (left) and Sox2$^{loxP/lacZ}$; Blimp1^{Cre} (right) in 17.5 dpc ovaries. Insets represent a $\times$40 magnification. Lower panels: HE staining of ovaries from control Sox2$^{loxP/lacZ}$ (left) and Sox2$^{loxP/lacZ}$; Blimp1^{Cre} (right) 13.5 dpc mice. **(C)**: Upper panels: HE staining of control Sox2$^{loxP/lacZ}$ (left) and Sox2$^{loxP/lacZ}$; Blimp1^{Cre} (right) 17.5 dpc testes. Middle panels: Vasa staining for pro-spermatogonia in control Sox2$^{loxP/lacZ}$ (left) and Sox2$^{loxP/lacZ}$; Blimp1^{Cre} (right) 17.5 dpc testes. Insets represent a $\times$40 magnification. Lower panels: HE staining of control Sox2$^{loxP/lacZ}$ (left) and Sox2$^{loxP/lacZ}$;Blimp1^{Cre} (right) 13.5 dpc testes. **(D):** Schematic representation of the genotype of conditional knockout mice, in which Sox2 deletion is driven by TNAP^{Cre} PGCs in 9.5–10.5 dpc. Phenotype was inspected both in fetal and postnatal gonads. **(E):** Upper panels: DAPI staining of 13 dpp ovaries from control Sox2$^{loxP/lacZ}$ (left) and Sox2$^{loxP/lacZ}$;TNAP^{Cre} (right) mice. Bottom panels: HE staining of 17.5 dpc testes from control Sox2$^{loxP/lacZ}$ (left) and Sox2$^{loxP/lacZ}$;TNAP^{Cre} (right) mice. Insets represent a $\times$40 magnification. Scale bar = 50 μm. Black arrowheads point to germ cells in control ovaries and testes. White arrowheads point to somatic cells undergoing apoptosis in mutant ovaries, due to the lack of germ cells. Abbreviations: Blimp-1, B-lymphocyte-induced maturation protein-1; dpc, days postcoitum; HE, hematoxylin-eosin.

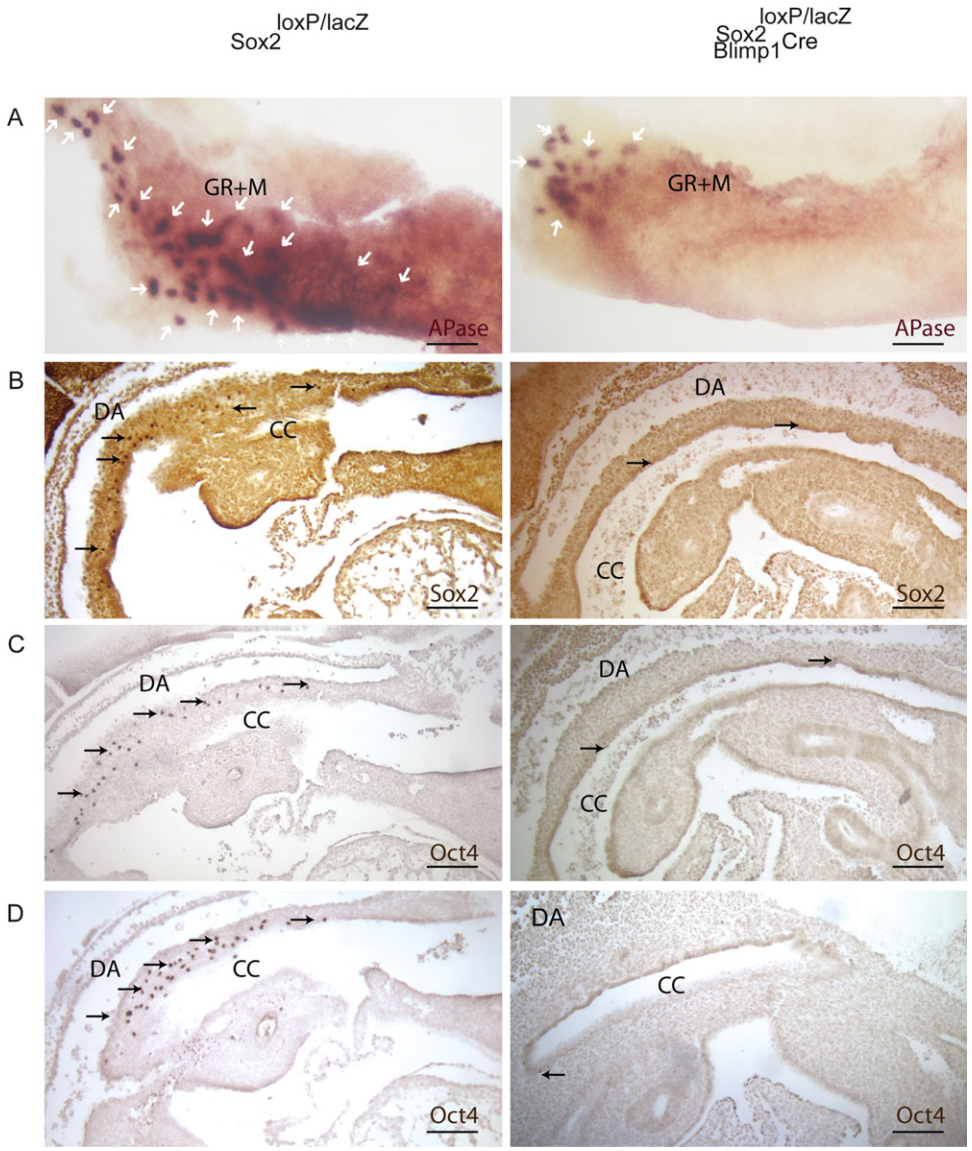

Figure 2. Strong reduction of primordial germ cells (PGCs) in Blimp1^{Cre} Sox2 conditional mutants at 10.5 days postcoitum (dpc). **(A):** APase staining on whole mount preparations of GR + M at 10.5 dpc in Sox2$^{loxP/lacZ}$ (left panel) and Sox2$^{loxP/lacZ}$;Blimp1^{Cre} (right panel). White arrowheads point to positive PGCs. Scale bar = 50 μm. **(B):** Immunohistochemical analysis of Sox2 expression in serial sections from Sox2$^{loxP/lacZ}$ (left panel) and Sox2$^{loxP/lacZ}$;Blimp1^{Cre} (right panel) embryos at 10.5 dpc. Scale bar = 100 μm. **(C):** Immunohistochemical analysis of Oct4 expression in serial sections from Sox2$^{loxP/lacZ}$ (left panel) and Sox2$^{loxP/lacZ}$;Blimp1^{Cre} (right panel) embryos at 10.5 dpc. Scale bar = 100 μm. **(D):** Immunohistochemical analysis of Oct4 expression in further serial sections from Sox2$^{loxP/lacZ}$ (left panel) and Sox2$^{loxP/lacZ}$;Blimp1^{Cre} (right panel) embryos at 10.5 dpc. In (B–D), arrows point to positive PGCs. Scale bar = 100 μm. Abbreviations: APase, alkaline phosphatase; Blimp1, B-lymphocyte-induced maturation protein-1; CC, coelomic cavity; DA, dorsal aorta; GR + M, genital ridges and mesonephroi.

defects of stomach, lung, and eye development, confirming the role of Sox2 in the development of foregut endoderm and retina [33–36]. Sox2$^{loxP/lacZ}$;Blimp1^{Cre} mutant eyes showed a strong reduction of the lens vesicle at 12.5 dpc, while at later stages (17.5 dpc), also the retina was severely impaired (Supporting Information Fig. 4). Gross morphology of the stomach was normal (Supporting Information Fig. 5A), however, histological analysis showed complete absence of forestomach differentiation, with glandular epithelium replacing the stratified squamous keratinized epithelium, as also shown by lack of p63 expression (Supporting Information Fig. 5D, 5E). Lasp1, a protein expressed in gastric glands [37] has been shown to be downregulated by Sox2 in ES cells [38]. Indeed, we found that Lasp1 was barely detectable in control Sox2$^{loxP/lacZ}$ gastric glands, but it was strongly upregulated in Sox2$^{loxP/lacZ}$; Blimp1^{Cre} mutants (Supporting Information Fig. 5H–5K). Sox2$^{loxP/lacZ;}$ Blimp1^{Cre} mutant lungs showed retarded bronchial branching at 12.5 dpc, as judged by the decreased number of bronchi per section (Supporting Information Fig. 6). Labeling for TTF1 showed only a few bronchial epithelial cells positively stained in mutant lungs compared to controls (Supporting Information Fig. 6G, 6H). Alveoli were not expanded and the bronchial tree was underdeveloped, explaining perinatal lethality of these mutants.

Sox2 Is Expressed in Postnatal Oocytes but Is Not Essential for Oogenesis Nor for Maternal to Zygotic Transition

Sox2 expression has been found in primary, secondary, and fully grown oocytes [4] as well as in zygotes (Fig. 5B–5D,

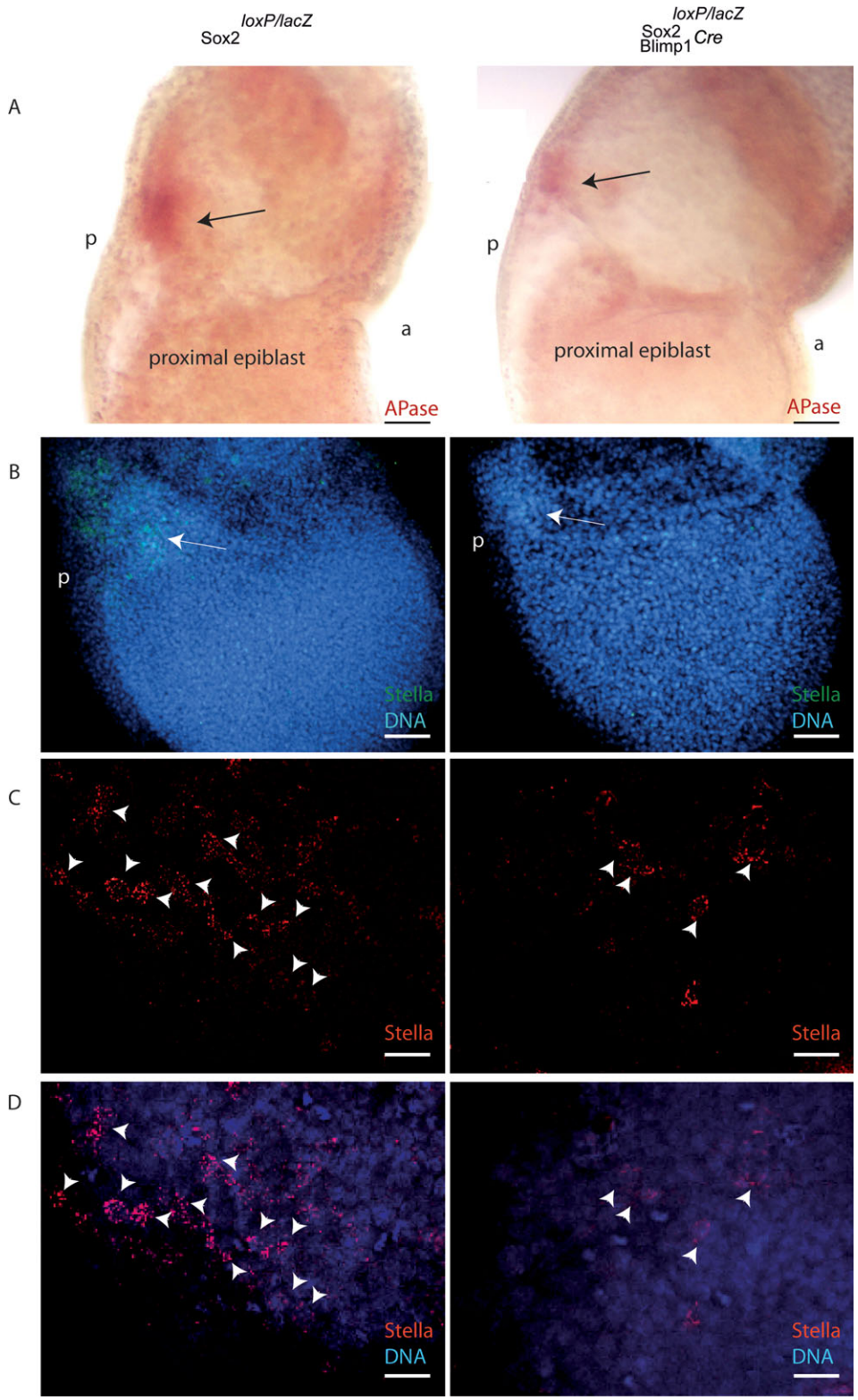

Figure 3. Strong reduction of primordial germ cells (PGCs) in Blimp1^{Cre} Sox2 conditional mutants at the time of their specification (7.5 dpc). **(A):** APase staining on whole mount preparations of 7.5 dpc Sox2$^{loxP/lacZ}$ (left panel) and Sox2$^{loxP/lacZ}$;Blimp1^{Cre} (right panel) embryos. Arrows indicate APase positive cells in the extraembryonic mesoderm close to the posterior area of proximal epiblast (a, anterior; p, posterior). Scale bar = 100 μm. **(B):** Low magnification immunofluorescence analysis of Stella expression (green) in whole mount preparations of from Sox2$^{loxP/lacZ}$ (left panel) and Sox2$^{loxP/lacZ}$;Blimp1^{Cre} (right panel) embryos at 7.5 dpc, merged with DAPI staining (blue). Arrows indicate Stella positive cells in the extraembryonic mesoderm close to the posterior (p) area of proximal epiblast. Scale bar = 100 μm. **(C):** High magnification immunofluorescence analysis of Stella expression (red) in different control (left panel) and mutant (right panel) embryos at 7.5 dpc. Arrowheads indicate Stella positive cells. Scale bar = 25 μm. **(D):** Same image of (C) merged with DAPI staining (blue). Abbreviations: a, anterior; APase, alkaline phosphatase; Blimp1, B-lymphocyte-induced maturation protein-1; p, posterior.

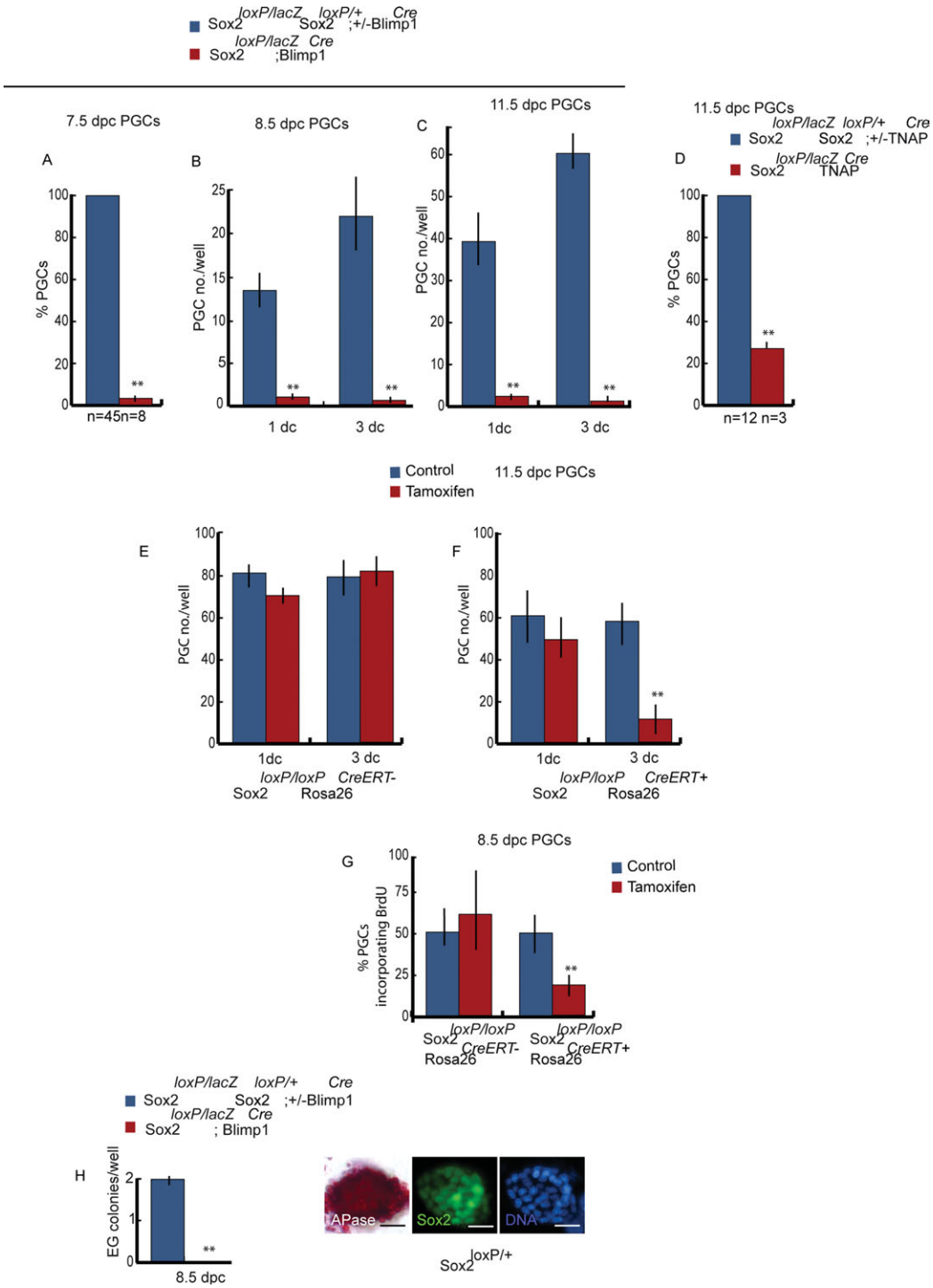

Figure 4. Sox2 deletion impairs PGC proliferation. **(A):** Percentage of PGCs in Sox2$^{loxP/lacZ}$;Blimp1^{Cre} 7.5 dpc embryos compared to control littermates. Data represent the analysis of eight litters in which at least a null mutant/litter was present; *n* indicates the number of embryos used for quantitation. **(B):** Number of PGCs from 8.5 dpc Sox2$^{loxP/lacZ}$; Blimp1^{Cre} embryos and from control littermates after 1 and 3 days of culture. **(C):** Number of PGCs from 11.5 dpc Sox2$^{loxP/lacZ}$;Blimp1^{Cre} gonads and control gonads after 1 and 3 days of culture. **(D):** Percentage of PGCs in Sox2$^{loxP/lacZ}$; TNAP^{Cre} 11.5 dpc embryos compared to control littermates. PGCs were counted from at least three litters in which at least a null mutant was present; n indicates the number of embryos used for quantitation. **(E):** Number of PGCs from Sox2$^{loxP/loxP}$ 11.5 dpc embryos, recovered after 1 or 3 days of culture in the presence of 4OH-tamoxifen compared to untreated cells. **(F):** Number of PGCs from Sox2$^{loxP/loxP}$;Rosa26^{CreERT} 11.5 dpc embryos, recovered after 1 or 3 days of culture in the presence of 4OH-tamoxifen compared to untreated cells. Occurrence of Sox2 deletion in vitro was verified in each well by PCR analysis. **(G):** Percentage of BrdU incorporating PGCs from 8.5 dpc Sox2$^{loxP/loxP}$ or Sox2$^{loxP/loxP}$;Rosa26^{CreERT} embryos in control and 4OH-tamoxifen treated cultures. **(H):** EG colony numbers obtained from PGCs isolated from 8.5 dpc Sox2$^{loxP/lacZ}$; Blimp1^{Cre} embryos and from control littermates. A representative EG control colony stained for APase is shown on the right while no EG colonies could ever be obtained from mutant PGCs. Bar = 25 μm. All experiments were repeated at least four times. Asterisks indicate the level of statistical significance. Abbreviations: APase, alkaline phosphatase; Blimp1, B-lymphocyte-induced maturation protein-1; BrdU, 5-bromo-2′-deoxyuridine; dpc, days postcoitum; EG, embryonic germ; PGC, primordial germ cell.

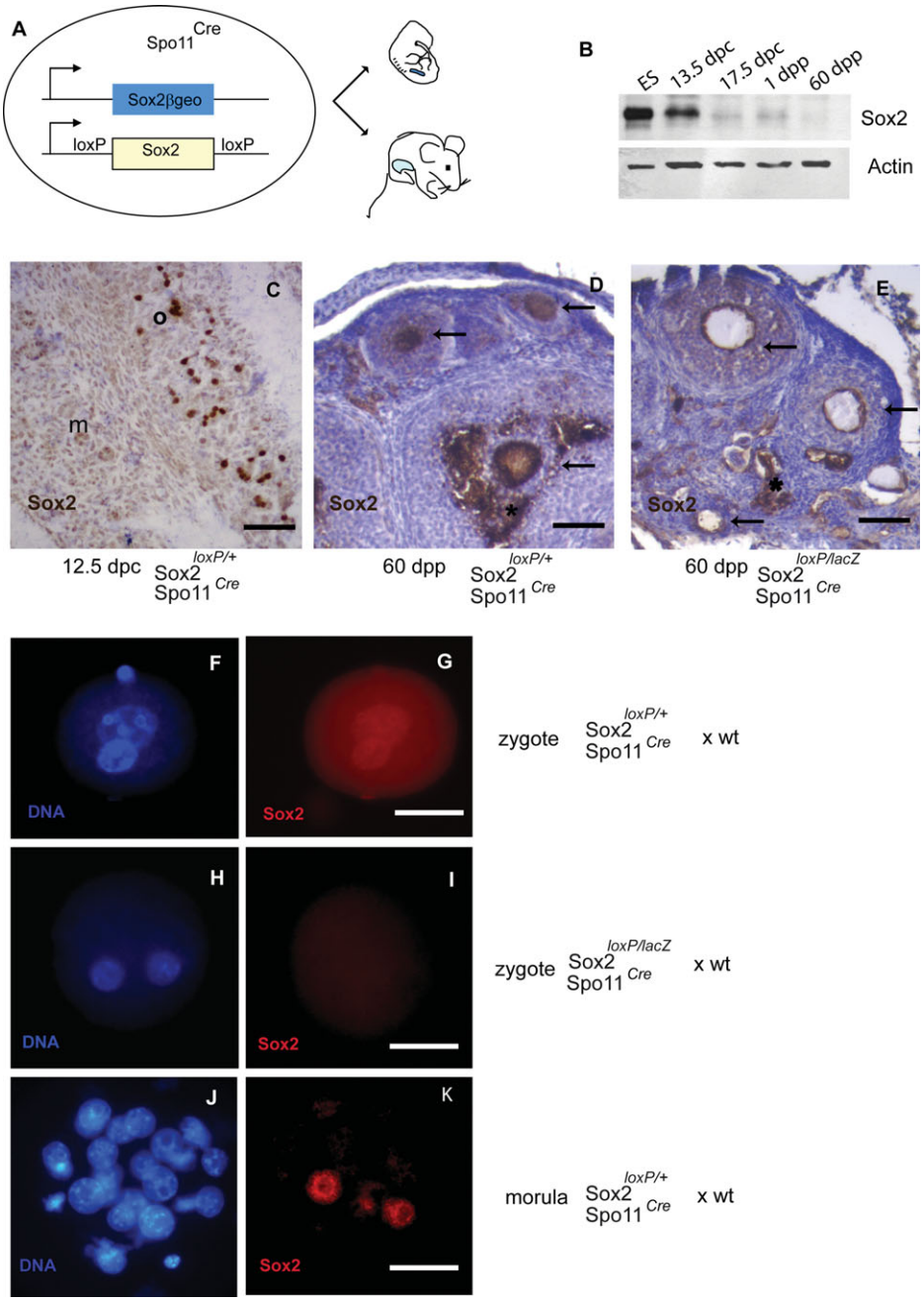

Figure 5. Sox2 is not required for postnatal oogenesis and early embryonic development. **(A):** Schematic representation of the genotype of conditional knockout mice, in which deletion of the second Sox2 allele is driven by Spo11^{Cre} in 13.5–15.5 dpc oocytes. Phenotype was inspected both in fetal and postnatal ovaries. **(B):** Western blot analysis of Sox2 levels in wt ovaries at different developmental ages compared to ES cells. **(C):** Sox2 detection in a control ovary at 12.5 dpc. **(D):** Sox2 detection in a control adult ovary at 60 dpp (arrows point to Sox2 positive oocytes; asterisks indicate areas of aspecific peroxidase staining). **(E):** Sox2 detection in a adult Sox2$^{loxP/lacZ}$; Spo11^{Cre} ovary at 60 dpp (arrows point to Sox2 negative oocytes; asterisks indicate areas of aspecific peroxidase staining). Scale bars = 100 μm. **(F–K):** DAPI staining (F, H, J) and immunofluorescence analysis (G, I, K) of Sox2 expression in a control zygote (F, G), in a zygote derived from a Sox2$^{loxP/lacZ}$; Spo11^{Cre} oocyte fertilized by wt sperm (H, I) and from control morula (J, K). Scale bars = 50 μm. Abbreviations: dpc, days postcoitum; dpp, days post partum; ES, embryonic stem; m, mesonephros; o, ovary.

5F, 5G). To understand if Sox2 might play a role also in oocyte development or in the initial phases of embryogenesis, before the initiation of embryonic transcription, we set out to delete Sox2 specifically in meiotic germ cells. To this end, we crossed Sox2$^{loxP/loxP}$ with Sox2$^{lacZ;/+}$;Spo11^{Cre}, a deleter which expresses *Cre* in pachytene spermatocytes of the postnatal testis and in lepto-zygotene oocytes of fetal ovaries [21] (Fig. 5A). We obtained Sox2$^{loxP/lacZ}$;Spo11^{Cre} compounds at the expected Mendelian frequency, indicating that this genotype is not embryonic lethal. Gonads from six males and six females were analyzed for the presence of germ cells. Both testis and ovary from Sox2$^{loxP/lacZ}$; Spo11^{Cre} mice showed normal morphology as compared to the control littermates, suggesting that Sox2 was not essential for spermatocyte

differentiation or for oocyte growth (Supporting Information Fig. 7A–7D). By immunocytochemistry $Sox2^{loxP/lacZ}$ growing oocytes as well as fully grown oocytes were positively stained by an anti-Sox2 antibody (Fig. 5D). Maternally derived Sox2 was also present in zygotes (Fig. 5F, 5G) while the embryonic form was resumed in some cells at the morula stage (Fig. 5J, 5K). $Sox2^{loxP/lacZ}$;$Spo11^{Cre}$ oocytes and zygotes deriving from fertilization with wt sperm were completely negative (Fig. 5E, 5H, 5I). After crossing mutant females to wt males, we found that they gave normal progenies and transmitted the deleted allele with the expected Mendelian frequency (Supporting Information Fig. 7E, 7F).

Sox2 Binds *Kit* and *Tal1* Regulatory Regions in PGCs and Upregulates Kit, Rif1, and Zfp148

Since Sox2 deletion affects early germ cell development, we reasoned that it might regulate germ cell genes essential for their survival and proliferation. One of these genes is *Kit*, which encodes the tyrosine-kinase receptor for Kit Ligand (Kitl), essential for PGCs development [27]. Sox2 overexpression in ES cells has been shown to induce high levels of *Kit* mRNA, as evaluated by microarray analysis [38]. In this study, it was also shown that Sox2 induced downregulation of *Tal1*, a mesodermal gene which regulates erythroid differentiation in early hematopoietic stem cells. To understand if Sox2 might regulate *Kit* and *Tal1* mRNA levels also in PGCs, we infected 13.5 dpc PGCs in vitro with a lentiviral bicistronic vector containing *Sox2* and *Egfp* ORFs. We found that *Kit* mRNA was upregulated in Sox2-infected germ cells as compared to Egfp-infected control cells (Fig. 6A), notwithstanding the relatively low efficiency of infection (less than 30%, Fig. 6C). *Tal1* was not expressed in Egfp- or in Sox2-infected PGCs (Fig. 6A). To verify if Sox2 exerted a repressive effect on *Tal1* expression, we infected a leukemic cell line K562, which expresses *Tal1* and found that Sox2 actually downregulated it, whereas *Kit* was strongly upregulated also in this cell type (Fig. 6B). Besides *Kit*, we found that also *Rif1* and *Zfp148* were upregulated by Sox2 overexpression in PGCs (Fig. 6A). Notably, these two genes are highly expressed in PGCs [39] and have been found to be potential Sox2 targets in ES cells [40, 41]. *Smad1* and *GM114*, two other genes expressed in PGCs [42, 43], were not modulated by Sox2 transduction (Fig. 6A).

We found three ACAAAG consensus sequences within the 6.9 kb of the *Kit* promoter and one within the first intron (Fig. 6D). To evaluate whether Sox2 was able to interact in vivo with these *Kit* regulatory regions in PGCs, it was immunoprecipitated from formaldehyde crosslinked chromatin obtained from 12.0 dpc whole gonads. By PCR, we found that only the ACAAAG sequence within the *Kit* first intron was significantly and reproducibly enriched in Sox2 immunoprecipitated chromatin (Fig. 6F). We also found that Sox2 interacted with two ACAAAG consensus sequences (Fig. 6E) found within the *Tal1* 5′-flanking sequences (Fig. 6G). On the contrary, we were not able to find any reproducible Sox2 binding to *Zfp148* or to *Rif1* regulatory regions by ChIP analysis (data not shown).

In order to confirm that the genes found to be regulated by Sox2 overexpression in PGCs are physiological targets of the endogenous gene, we deleted Sox2 in vitro by treating with 4OH-tamoxifen PGCs isolated from 12.5 dpc $Sox2^{loxP/lox}$;$Rosa26^{Cre\text{-}ERT}$ embryos. After 48 hours from 4OH-tamoxifen addition germ cells were collected and analyzed by semiquantitative RT-PCR. As expected Sox2 was efficiently downregulated after 4OH-tamoxifen addition, but we also observed reproducibly downregulation of Kit, Rif1, and Zfp148 expression, whereas Nanog expression appeared to increase and expression of Oct4, Blimp1, and Prdm14 did not change (Fig. 6H, left panels). We did not observe any adverse effect of 4OH-tamoxifen addition on PGC survival, since the levels of Sox2 or Kit expression were not affected in $Sox2^{loxP/lox}$ control PGCs (Fig. 6H, right panel).

Dramatic Decrease in the Expression of a Reporter Gene Driven by *Kit* Regulatory Sequences in Sox2-Deleted PGCs

We generated compound $Sox2^{loxP/lacZ}$;$Blimp1^{Cre}$;Kit^{Egfp} embryos. In Kit^{Egfp} transgenic animals, expression of the reporter EGFP is driven by *Kit* regulatory regions of the promoter and the first intron (corresponding to the genomic region depicted in the scheme of Fig. 6D), which have been previously shown to be specifically expressed in forming PGCs in the ExM [22]. As shown in Figure 7, in control embryos at 7.5 dpc, PGCs can be easily identified by the expression of the EGFP transgene driven by *Kit* transcriptional regulatory sequences, whereas, in Sox2 conditionally deleted mutant embryos we found a dramatic reduction or even complete absence of labeled cells. These in vivo data confirm the deleterious effect of Sox2 deletion on *Kit* transcription in nascent PGCs, and the importance of Sox2 at the time of PGC specification.

Discussion

In this study, we show that Sox2 plays an essential role in the establishment and maintenance of the germ cell line before its sexual determination. We found that deletion of Sox2 during their specification strongly affects PGC numbers. Indeed, by using $Blimp^{Cre}$ deleters, we found that the germ cell-less phenotype was already evident at around 7.5 dpc, when PGCs are first identified [11]. At this developmental age, we found a strong reduction of APase positive and Stella-expressing PGCs, suggesting that Sox2 is required at the time of their specification. The few residual PGCs found between 7.5 and 10.5 dpc in these mutants probably reflect a noncomplete Sox2 excision by $Blimp^{Cre}$ deleters. However, this deletion becomes almost complete at later stages, as demonstrated by the absence of germ cells in 13.5–17.5 dpc gonads. By using $TNAP^{Cre}$-mediated gene deletion, which occurs between 9.5 and 10.5 dpc, we also found that *Sox2* deleted germ cells of both sexes are severely reduced at 11.5 dpc, during their proliferative period. An important role of Sox2 in PGC proliferation is further confirmed by the decrease of PGC numbers in $Rosa26^{Cre\text{-}ERT}$ mediated *Sox2* deletion in vitro. The reduction of PGC numbers is mainly due to a reduction of DNA synthesis as indicated by BrdU incorporation experiments and not to an increase of apoptosis. Although *Sox2* is re-expressed in postnatal oocytes, we now show that its deletion during meiosis by Spo11-driven Cre expression does not affect female fertility nor early embryonic development.

Sox2 levels are low in PGC precursors at 6.75 dpc but they are progressively upregulated in the subsequent stages [15, 16]. Since Sox2, which forms a heterodimeric complex with Oct4, has been shown to be a key player in the pluripotency of ES cells [4], Sox2 expression in the germ cell line may lead to the reacquisition of potential pluripotency in this lineage. Indeed, it has been proposed that testicular teratoma susceptibility might be linked to the lack of *Sox2* repression by DMRT1 in prospermatogonia [44]. In agreement with this hypothesis, we never succeeded in obtaining EG colonies from $Sox2^{loxP/lacz}$-$Blimp^{Cre}$ embryos. However, the lack of

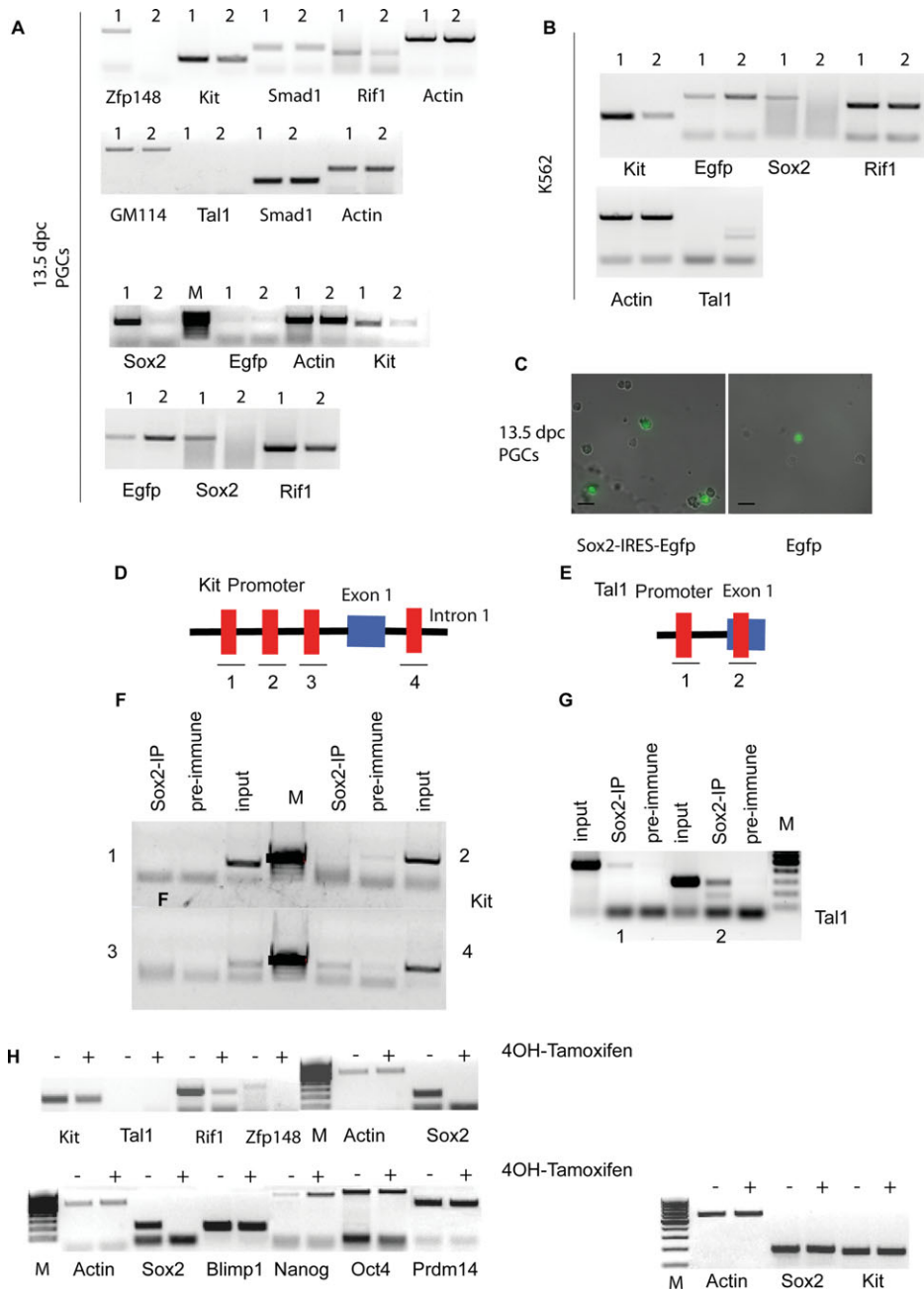

Figure 6. Sox2 upregulates the expression of genes essential for PGC development. **(A):** Representative semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis for the reported genes in 13.5 dpc PGCs infected with a lentiviral vector overexpressing Sox2 and Egfp (1) or Egfp alone (2). Different individual experiments are shown. Analysis for the expression of the genes of interest was repeated at least three times and gave similar results. **(B):** Representative semiquantitative RT-PCR analysis for the reported genes in K562 leukemic cell line infected with a lentiviral vector overexpressing Sox2 and Egfp (1) or Egfp alone (2). Analysis for the expression of the genes of interest was repeated at least three times, with similar results. **(C):** Fluorescence images of 13.5 dpc PGCs infected with a lentiviral vector overexpressing Sox2 and Egfp (left) or Egfp alone (right). **(D, E):** Schematic representation of Sox2 potential binding sites (ACAAAG) in Kit (D) and Tal1 (E) transcriptional regulatory regions. **(F, G):** Representative semiquantitative PCR analysis after chromatin immunoprecipitation (ChIP) carried out on 12.5 dpc wt gonads. Primers used for Kit (F) and Tal1 (G) amplifications correspond to those numbered in (D) and (E). ChIP analysis was performed three times with reproducible results. **(H):** Representative semiquantitative RT-PCR analysis for the reported genes in 12.5 dpc PGCs from $Sox2^{loxP/loxP}$;$Rosa26^{CreERT}$ embryos in vitro cultured in the absence (−) or presence (+) of 4OH-Tamoxifen. Left panels show the analysis on Cre-Ert positive PGCs, the right panel shows the analysis on Cre-Ert negative PGCs. Analysis for the expression of the genes of interest was repeated three times, with similar results. Abbreviations: dpc, days postcoitum; PGC, primordial germ cells.

EG colonies in mutant PGC cultures might be simply due to the severe reduction of proliferative potential.

After *Sox2* reactivation, several PGC-specific genes have been shown to be consistently upregulated after 7.25 dpc, while somatic mesodermal genes are repressed [29]. Among these genes, *Kit* has been shown to be upregulated in Sox2 overexpressing ES cells [38]. We found that *Kit* is upregulated by Sox2 overexpression also in PGCs, while it is

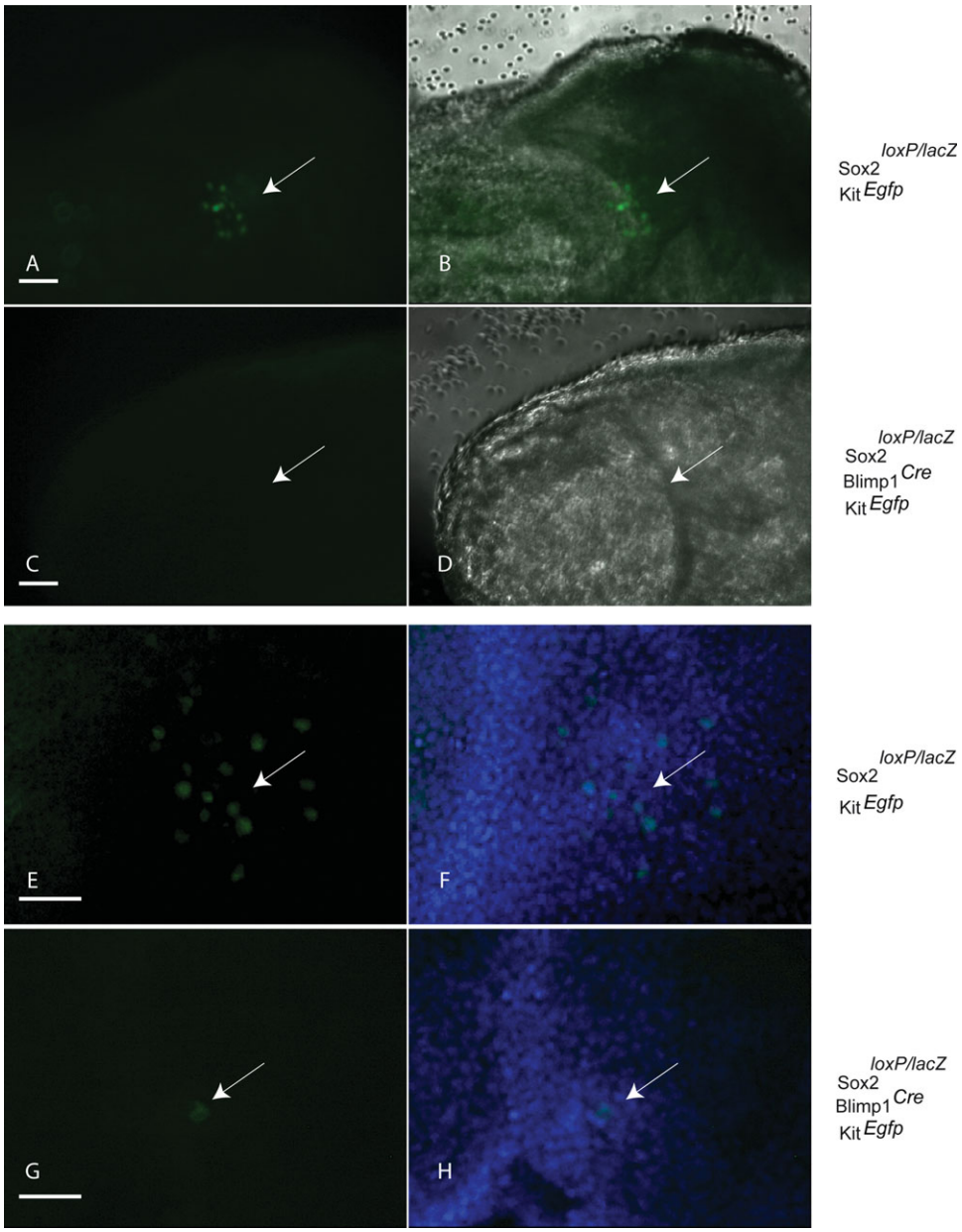

Figure 7. Decreased expression of a reporter gene driven by *Kit* regulatory sequences in Sox2-deleted primordial germ cells (PGCs). EGFP fluorescence in $Sox2^{loxP/lacZ}$; Kit^{Egfp} embryos (**A, B, E, F**) and in $Sox2^{loxP/lacZ}$; $Blimp1^{Cre}$, Kit^{Egfp} (**C, D, G, H**) at 7.5 dpc. (B) and (D) show merged fluorescence with bright-field images. (F) and (H) show merged fluorescence with DAPI staining. Arrows indicate the posterior area of proximal epiblast in which EGFP positive PGCs are found. Scale bar = 50 μm. Abbreviation: Blimp1, B-lymphocyte-induced maturation protein-1.

downregulated upon Sox2 deletion in vitro. *Kit* is subjected to tight transcriptional control, because it is essential not only for germ cells but also for hematopoietic stem cell and melanocyte development. Indeed, a variety of transcription factors are known to regulate *Kit* expression in hematopoiesis and melanogenesis [45], but the mechanisms that modulate *Kit* expression in the germline are probably multiple. While postnatally Sohlh1 and Sohlh2, two bHLH transcription factors, are essential for *Kit* expression in spermatogonia and possibly in oocytes, they are not required during PGCs differentiation [46]. To date little is known on how the transcription of the *Kit* gene during early germ cell development in both sexes is turned on. We have identified a region within the first *Kit* intron, which is specifically bound by Sox2 in PGCs. Notably, the first *Kit* intron was previously shown to be essential for *Kit* expression in these cells as well as in hematopoietic precursors [22, 47]. Since Kit signaling is essential for survival and proliferation of germ cells [25, 27, 48], we can hypothesize that one of the mechanisms by which *Sox2* ablation affects PGCs is through downregulation of *Kit* transcription. Although it is generally accepted that Kit is not strictly required for the establishment of the germline [49], more recent work has shown that PGCs are dependent on Kitl expression from the surrounding environment as early as at the time of their specification [50]. Moreover, by using an EGFP reporter gene driven by *Kit* regulatory sequences, we now show that the Kit locus is transcriptionally active at the time of PGC specification. Kit regulatory regions driving EGFP expression include the intronic region which is specifically bound by Sox2 in PGC chromatin. Nascent PGCs are

specifically identified by this reporter at 7.5 dpc, and a dramatic decrease in EGFP expressing PGCs is observed in Sox2 conditionally deleted embryos at this stage, confirming in vivo the requirement of Sox2 for *Kit* transcription in PGCs. However, besides Kit, misexpression of other Sox2 targets might contribute to the strong reduction of PGC number that we observe in 7.5 dpc mutant embryos.

We also found that the promoter region and the first exon of *Tal1*, a mesodermal gene which controls embryonic blood formation, are bound by Sox2 in PGCs. When overexpressed in a hematopoietic cell line, Sox2 induces downregulation of *Tal1* raising the hypothesis that Sox2 can repress mesodermal gene expression in PGCs and eventually block a hematopoietic fate in these cells. However, Sox2 deletion in 12.5 dpc PGCs does not result in Tal1 expression even though we cannot exclude that it might occur at earlier developmental stages.

We found that two other genes highly expressed in PGCs, *Zfp148* and *Rif1*, are positively regulated by Sox2 in these cells. *Zfp148* heterozygosity causes PGC loss from 11.5 to birth [51], indicating that it is required for the normal development of fetal germ cells, while Rif1 is potentially involved in maintaining ES cell pluripotency both in mice [52] and in humans [41].

Even though SOX2 has been reported not to be expressed in human PGCs [53], it is expressed in human intratubular germ cell carcinoma in situ [54], and in embryonal carcinoma [8], which are thought to be derived from PGCs. Moreover, human PGCs have been found to express SOX17 [55], which is a strictly related transcription factor showing the same DNA binding properties of SOX2 and interchangeable partner specificity [56].

Conclusions

Up-to-date few genes have been demonstrated to be essential for the establishment of the germ cell line: *Blimp1* [12], *Prdm14* [16], *Oct4* [18] and *AP2-γ* [57]. Our present data show for the first time that also *Sox2*, at least in mice, is required for PGC development, both during their specification period and during their proliferative phase which precedes their sexual differentiation, through the regulation of genes which control their proliferation, their cellular identity as well as their potential pluripotency.

Acknowledgments

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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