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Msx1 and *Msx2* promote meiosis initiation

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SUMMARY

The mechanisms regulating germ line sex determination and meiosis initiation are poorly understood. Here, we provide evidence for the involvement of homeobox Msx transcription factors in foetal meiosis initiation in mammalian germ cells. Upon meiosis initiation, *Msx1* and *Msx2* genes are strongly expressed in the foetal ovary, possibly stimulated by soluble factors found there: bone morphogenetic proteins Bmp2 and Bmp4, and retinoic acid. Analysis of *Msx1*/*Msx2* double mutant embryos revealed a majority of undifferentiated germ cells remaining in the ovary and, importantly, a decrease in the number of meiotic cells. In vivo, the *Msx1*/*Msx2* double-null mutation prevented full activation of *Stra8*, a gene required for meiosis. In F9 cells, Msx1 can bind to *Stra8* regulatory sequences and Msx1 overexpression stimulates *Stra8* transcription. Collectively, our data demonstrate for the first time that some homeobox genes are required for meiosis initiation in the female germ line.

KEY WORDS: Foetal ovary, Germ cells, Msx, Mouse, Human

INTRODUCTION

During foetal life, male and female gonads differentiate dichotomically from a bipotential and undifferentiated gonad (Kim and Capel, 2006). Whereas the genetics of sexual differentiation in the somatic cells of the gonads have been studied for decades, the genes involved in sexual germ cell differentiation are only now being more widely investigated. The first sign of gonadal sex differentiation in mice occurs from 11.5 days post-coitum (dpc) when somatic supporting cells differentiate in the male gonad (Tilmann and Capel, 2002). Germ cell sex determination occurs shortly after 13.5 dpc; male germ cells enter a quiescent phase whereas female germ cells initiate meiosis (Brennan and Capel, 2004). Shortly after birth, male germ cells resume mitosis whereas in the ovary, oocytes reach the diplotene stage (end of prophase I) (Pepling, 2006). The first testicular meiotic cells are observed at around seven days post-partum (dpp). *Stra8* (stimulated by retinoic acid 8) gene expression is required for meiotic initiation or progression in the foetal ovary as well as in the post-natal testis (Anderson et al., 2008; Baltus et al., 2006; Koubova et al., 2006). Exogenous retinoic acid (RA) has been shown to be able to induce or accelerate meiosis (Bowles et al., 2006; Livera et al., 2000); however, recently, endogenous RA has been proposed to be dispensable for both *Stra8* expression and meiosis induction in the developing ovary (Kumar et al., 2011). In the foetal testis, both *Cyp26b1* expression in the somatic cells and *Nanos2* expression in the germ cells prevent *Stra8* expression and meiosis initiation (Bowles et al., 2006; Suzuki and Saga, 2008). With the notable exception of *Stra8*, very few genes have been demonstrated to be required for the induction of meiosis.

The Msh homeobox (*Msx*: muscle segment homeobox-like, also known as *Hox-7/Msx1* and *Hox-8/Msx2*) gene family encodes homeodomain transcription factors that are important during early foetal development for dorsoventral patterning (Onitsuka et al., 2000; Yamamoto et al., 2001). Three Msx proteins are present in mice whereas only MSX1 and MSX2 are conserved in the human. Msx1 and Msx2 present a functional redundancy, thus, despite being embryonic lethal (Lallemand et al., 2005), double invalidation mutants (dKO) are often used to study the role of Msx during development. *Msx1* and *Msx2* are both expressed during embryogenesis and are principally described in neural tube, tooth and limb development (Ramos and Robert, 2005), and have been implicated in craniofacial development (Alappat et al., 2003). Bone morphogenetic protein (Bmp) and RA signalling pathways regulate Msx gene expression in several tissues (Ramos and Robert, 2005). Interestingly, expression of Bmp2, Bmp4 and Bmp7 increases in foetal mouse ovaries between 11.5 dpc and 13.5 dpc (Ross et al., 2007). Moreover, upregulation of *MSX2* expression has recently been described following BMP4 treatment in the human foetal ovary in organotypic culture, and correlated with an increase in primordial germ cell apoptosis (Childs et al., 2010).

In this study, we demonstrate that *Msx1* and *Msx2* are markedly upregulated in mouse ovaries at the time of meiosis initiation during foetal life. We also show, in organ culture, the stimulation of *Msx1* and *Msx2* expression in foetal gonads by Bmp2, Bmp4 and RA. Moreover, a *Msx1* and *Msx2* double-null mutation results in decreased numbers of female meiotic cells, indicating the necessity for Msx genes in order to initiate germ cell meiosis. Finally, we identified *Stra8* as a potential target of Msx1 and Msx2 in vivo, and confirmed Msx activation of *Stra8* transcription in F9 transfected cells through the direct binding of Msx1 to *Stra8* regulatory sequences.

MATERIALS AND METHODS

Mice and embryos

All animal studies were conducted in accordance with the guidelines for the care and use of laboratory animals of the French Ministry of Agriculture. NMRI mice were kept and coupled under conditions

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previously described (Guerquin et al., 2010). Insertional mutation of mouse *Msx1* by a *lacZ* reporter gene and *Msx2* by a green fluorescent protein (GFP) reporter gene was performed. Single or double homozygous mutant embryos were obtained by NMRI *Msx1*$^{+/-}$ *Msx2*$^{+/-}$ (Bensoussan et al., 2008; Houzelstein et al., 1997) intercrosses. Production of Msx1creERT2 mice by homologous recombination in embryonic stem (ES) cells is described elsewhere (Lopes et al., 2011). The targeted reporter allele was *ROSAmT/mG* as previously described (Muzumdar et al., 2007). The Oct4-GFP mice used were previously described (Yoshimizu et al., 1999).

Collection of mouse foetal gonads and organ culture

Foetal gonads were isolated and cultured as previously described in mice (Guerquin et al., 2010). To bypass the embryo lethality of the double Msx KO (dKO) and to analyse the later development of the ovary, gonads were explanted at 13.5 or 14.5 dpc and cultivated for four or seven days in the presence of 10% foetal calf serum (Sigma-Aldrich). The medium was changed every 48 hours. In some cultures, 5′-bromo-2′-deoxyuridine (BrdU, 1%) was added for the last three hours of culture before fixation.

For gene regulation studies, 11.5 dpc gonads were harvested and the sex of the foetuses was determined using PCR amplification of *Sry* prior to cultures as previously described (Petre-Lazar et al., 2007). Part of the gonads was used as the control and the rest was cultured in the presence of RA 50 nM or 1 μM (Sigma-Aldrich) and/or Bmp4 100 ng/ml or 1 μg/ml (R&D systems) for two days. A set of culture was performed in a similar manner with Bmp2. The vehicle (dimethyl sulphoxide, DMSO) was similarly included in the culture medium of the paired control.

Collection and culture of human foetal gonads

Human foetal gonads were harvested from material available following legally induced abortions in the first trimester of pregnancy and therapeutical termination of pregnancy in the second trimester, i.e. from the 6th until the 15th week post-fertilisation (wpf), in the Department of Obstetrics and Gynaecology at the Antoine Béclère Hospital, Clamart (France) as previously described (Guerquin et al., 2009; Le Bouffant et al., 2010). The Antoine Béclère Ethics Committee approved this study and all women gave their informed consent.

For the study of MSX gene regulation in response to retinoic acid (RA), each human ovary (8-11 wpf) was cut into small pieces and a proportion of these pieces were used as controls; the rest were cultured in the presence of 10% foetal calf serum and RA 1 μM for two weeks, a treatment that induced meiosis (Le Bouffant et al., 2010).

Histology and germ cell counting

Gonads were fixed with Bouin's fluid or formol immediately after dissection or at the end of the culture. The fixed gonads were dehydrated, embedded in paraffin and cut into 5-μm-thick sections. We mounted one section every five or ten serial sections, from the first to the last section of the gonad, on glass slides. These sections were de-waxed, rehydrated and stained with Haematoxylin and Eosin. Meiotic stages were recognised on the basis of their histological features as previously described (Guerquin et al., 2010). Oocytes (diplotena) were counted and identified on the basis of their large, spherical nuclei and clearly visible cytoplasmic membrane, and oocyte number was estimated as described previously (Guerquin et al., 2009).

Immunohistochemistry

Protocols used have been described previously (Guerquin et al., 2010; Le Bouffant et al., 2010). Briefly, gonads were fixed with 10% formol for Ssea1 (Fut4 – Mouse Genome Informatics), γH2AX (H2afx – Mouse Genome Informatics), Sycp3 and Stra8 staining and Bouin's fluid for p63 (Trp63 – Mouse Genome Informatics) staining and embedded in paraffin. For immunohistochemistry, we used monoclonal anti-Ssea1 [1:5, Developmental Studies Hybridoma Bank (DSHB), Iowa, IA, USA], monoclonal anti-p63 (1:50; 4A4, Santa Cruz Biotechnology), monoclonal anti-γH2AX antibody (1:500, JBW301, Upstate Biotechnology), monoclonal anti-Sycp3 (1:500, Abcam), polyclonal anti-cleaved caspase-3 Asp 175 (1:100, Cell Signaling Technology), or polyclonal anti-Stra8 (1:1000, Abcam). BrdU incorporation was detected using the Cell Proliferation Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's recommendations.

Immunofluorescence

Adult males heterozygous for the recombinant allele *Msx1CreERT2* and homozygous for the *ROSAmT/mG* allele were bred with NMRI females. Pregnant females received two or three intraperitoneal injections of tamoxifen (2 mg/4 mg per injection) at 11.0 and 11.5 dpc, or at 12.0 and 12.5 dpc, or at 13.0, 13.5 and 14 dpc, before being sacrificed at P0. Tamoxifen (Sigma-Aldrich) was dissolved in 0.5 ml ethanol and diluted at 20 mg/ml in sun flower seed oil from *Helianthus annuus* (Sigma-Aldrich). After dissection, enhanced green fluorescent protein (eGFP) fluorescent gonads (and non-fluorescent gonads as negative controls) were fixed overnight in 4% paraformaldehyde (PFA) at 4°C. After being embedded, gonads were cut using a cryostat (Leica). Sections (18 μm thick) were then processed for immunostaining using a polyclonal anti-GFP (1:200; Invitrogen, Carlsbad, CA, USA) and a monoclonal anti-p63 (1:50; 4A4, Santa Cruz Biotechnology) with specific secondary antibody species Alexa Fluor 488- and Alexa Fluor 647-conjugated antibodies (Invitrogen), respectively. Sections were mounted with Vectashield containing DAPI (Vector Laboratories). For Sycp3 detection, the specific primary antibody monoclonal anti-Sycp3 (Abcam) and the secondary Alexa Fluor 594-conjugated antibody were used.

Germ cell purification

Germ cell isolation using Ssea-1 antigen was performed as previously described (Le Bouffant et al., 2010; Pesce and De Felici, 1995). Germ cells were also purified by flow cytometry (FACS) using ovaries from Oct4-GFP mice (Yoshimizu et al., 1999).

Cell culture and transfection

The mouse embryonal carcinoma cell line F9 (DSMZ, Braunschweig, Germany) was transfected with pCIG-GFP or pCIG-GFP-Msx1-HA plasmids by a Lipofectamin transfection assay according to the manufacturer's protocol (Lipofectamin 2000, Invitrogen). Positive transfected cells were isolated by flow cytometry based on GFP fluorescence.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation was performed using the EZ-Magna ChIP kit (Millipore, Billerica, MA, USA), according to the manufacturer's instructions. Twenty-four hours after transfection with pCIG-GFP or pCIG-GFP-Msx1-HA plasmids, F9 cells were fixed with 0.5% PFA for 10 minutes at 37°C. Cross-linking was stopped by adding glycine to a final concentration of 125 mM and the cells were washed with cold PBS. After sonication, chromatin was incubated with magnetic beads conjugated to either 1 μg HA antibodies (ab9110, Abcam, Cambridge) or 1 μg normal rabbit IgG (sc3888, Santa Cruz Biotechnology). The immunoprecipitated chromatin was washed and reverse cross-linked before DNA was extracted using a DNA purification kit (Qiagen). Finally, the immunoprecipitated DNA and the corresponding non-immunoprecipitated DNA (input) were subjected to quantitative PCR amplification for three regions of the *Stra8* promoter and for the *Hspa1b* promoter, known to be bound by Msx1 (Zhuang et al., 2009).

Primers for ChIP analysis were (5′-3′): *Stra8* site A forward TTAATAACTAGGGAGGCCTTTGCA, reverse TCCCCGTGTGCTCC-TACAGT; *Stra8* site B forward GAATGGCAACTAAATTAAAGGCT-GA, reverse TTGCAAACTTTGACCTCTGACAG; *Stra8* site C forward ATTGCTCCTTTCTACCTACCACTTG, reverse CCCTTGCATGT-GAGCCTTCT; *Hspa1b* forward AGTTCTGGACAAGGGCGGA, reverse CCCGCCTCCCTTGAGTAATC; *p65* forward CAGAGCAGA-CACAGCAAATGC, reverse TCATGACCCAACGGTTCTCC. Results are expressed as a percentage of input, showing the proportion of material found in the eluate after immunoprecipitation.

Real-time quantitative PCR

As previously described (Le Bouffant et al., 2010), total RNA from whole gonad pools, Ssea1- or Oct4-purified cells or culture was extracted using the RNeasy Mini-Kit (Qiagen) and reverse transcription was carried out with the Omniscript Kit (Qiagen), according to the manufacturer's instructions. For single gonad analyses, total RNA was extracted using the RNeasy Micro-Kit (Qiagen) and reverse transcription was realised with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

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ABI Prism 7000 system (Applied Biosystems) and SYBR-green labelling were used for quantitative RT-PCR. The Comparative Ct method was used to determine the relative quantities of mRNA, using β-actin or *Ddx4* mRNA as the endogenous reporter. Results are presented as a percentage of the maximum (i.e. with the highest expression being defined as 100%). Each RNA sample was analysed in duplicate. All primers were used at a final concentration of 400 nM. Sequences of oligonucleotides used are given in supplementary material Table S1.

Data analysis

Each data point represents the mean ± s.e.m. of at least three independent experiments. Images show one representative of at least three experiments. Data were analysed using R Commander (R software), by one-way ANOVA followed by the Tukey-Kramer multiple comparisons test, or paired or unpaired Student's *t*-test.

RESULTS

Msx gene expression in foetal and post-natal gonads

The kinetics of *Msx1* and *Msx2* gene expression were analysed in mouse whole gonads, revealing large differences between the ovaries and testes. *Msx1* expression was higher in the mouse foetal ovary compared with that in the testis (Fig. 1A). In the ovary, the maximum expression of *Msx1* was observed at 13.5 dpc, coinciding with meiosis initiation. *Msx2* also peaked in the 13.5 dpc ovary but was already highly expressed at 12.5 dpc (Fig. 1A). No major change to either *Msx1* or *Msx2* expression was observed either in the foetal or in the post-natal testis upon male meiosis initiation (i.e. between 5 and 15 dpp, as defined by *Stra8* and *Spo11* overexpression: supplementary material Fig. S1A). In human foetal gonads (supplementary material Fig. S2A), *MSX1* and *MSX2* were highly expressed in the ovary during meiosis initiation at 14.5 wpf as defined by the expression of the meiotic marker *SPO11* (SPOrulation protein 11).

In order to determine the cell type expressing Msx genes, the expression of *Msx1* and *Msx2* was measured in ovarian and testicular germ cells purified from mouse gonads at 13.5 dpc by magnetic-activated cell sorting (MACS) using antibodies targeting the Ssea-1 (stage-specific embryonic antigen 1) antigen that is specifically expressed in undifferentiated germ cells (Fig. 1B). Both Msx genes appeared to be highly expressed in the female germ cell fraction (Ssea-1) compared with whole gonads, indicating an enrichment of Msx in ovarian germ cells. *Msx1* and *Msx2* gene expression levels were also measured in Oct4 (Pou5f1 – Mouse Genome Informatics)-positive (germ) cells and Oct4-negative (somatic) cells obtained following flow cytometry purification using 13.5 dpc ovaries from Oct4-GFP mice (supplementary material Fig. S1B). Expression of the somatic-specific gene follistatin and the germinal-specific gene *Ddx4* were used to validate the purification. In these purified

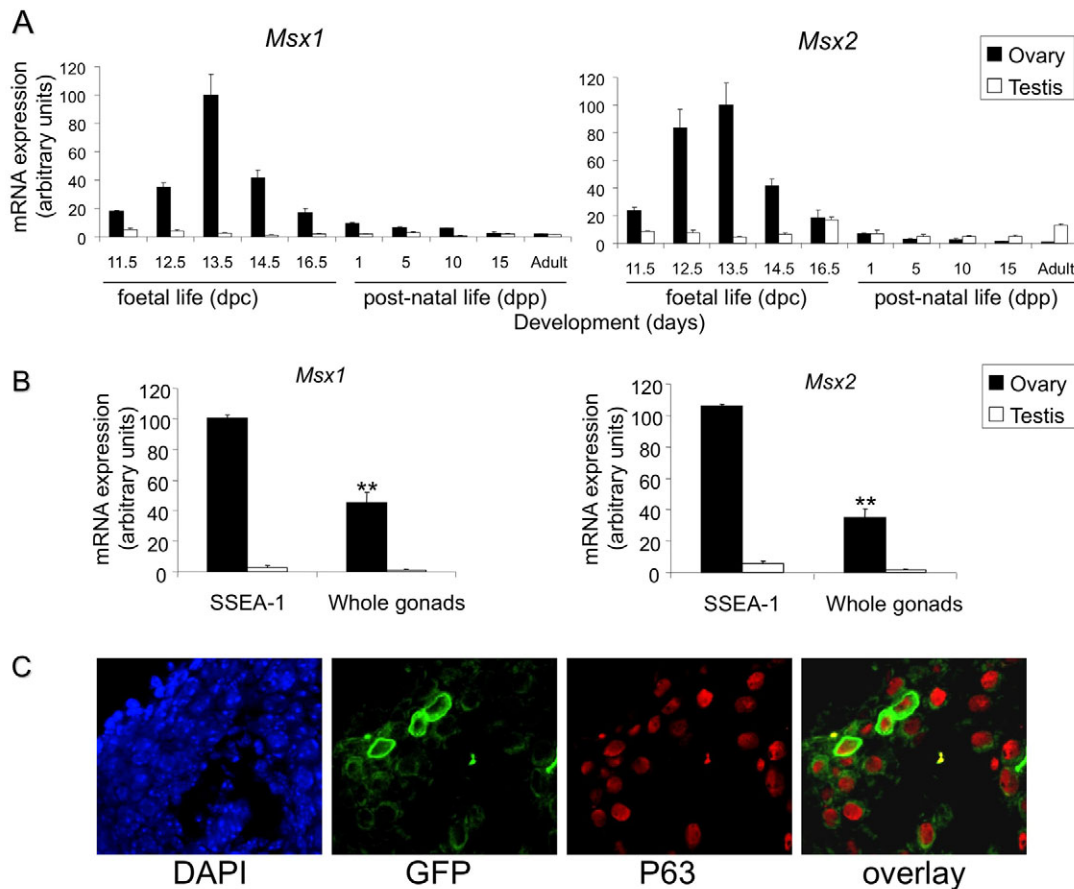

Fig. 1. *Msx1* and *Msx2* gene expression in mouse gonads. *Msx1* and *Msx2* gene expression was measured in mouse ovaries and testes. (**A**) *Msx1* and *Msx2* gene expression was measured using RT-qPCR in whole mouse ovaries and testes. Gonads were harvested at the indicated development stage. (**B**) *Msx1* and *Msx2* gene expression was also measured using RT-qPCR in germ cell purified fractions (Ssea-1). Germ cells were purified at 13.5 dpc by the MACS method, using specific Ssea-1 expression (see Materials and methods). For RT-qPCR, gene (mRNA) expression was normalised to β-actin. (**C**) Immunodetection of GFP reflecting *Msx1* early expression between 13 and 14 dpc (see Materials and methods), and p63, a specific marker for germ cells, in P0 gonads. Error bars represent s.e.m.

fractions, we measured high levels of both *Msx1* and *Msx2* transcript in the germ cells, but poor levels of expression of both genes in the somatic cells. Experiments using Msx1ERT2cre mice with a *ROSAmT/mG* reporter system (see Material and methods), allowed us to study *Msx1* expression through GFP detection. When Cre activation was performed at 13, 13.5 and 14 dpc, at P0, many germ cells expressed both GFP protein and the oocyte-specific marker p63 protein, indicating the expression of *Msx1* in most germ cells at 13-14 dpc (Fig. 1C). In another set of experiments, Cre activation was performed at 11.0 and 11.5 dpc or at 12.0 and 12.5 dpc with 2 or 4 mg tamoxifen per injection. Under these conditions, very few oocytes (~10%) were found expressing the GFP at P0 but this, nevertheless, indicates a weak activity of the *Msx1* promoter as early as 11.5 dpc (data not shown). Surprisingly, not all oocytes expressed GFP when Cre activation was performed at 13, 13.5 and 14 dpc and when *Msx1* expression peaked, probably owing to insufficient levels of Cre (i.e. *Msx1* activated promoter) present in order to obtain sufficient recombinase activity in these germ cells. In very rare cases, somatic cells (data not shown) showed GFP labelling, thus indicating that *Msx1* expression might not be entirely specific to germ cells in the ovary.

RA and Bmp4 upregulate Msx gene expression

RA and Bmps are both known regulators of Msx gene expression in other tissues. As exogenous RA is able to induce meiosis and Bmps are differentially expressed in the foetal gonads and strongly produced in the ovary, we investigated their putative role in Msx regulation. Mouse gonads at 11.5 dpc were cultured for two days with high and low or close-to-physiological doses of RA (1000 and 50 nM, respectively) and/or of Bmp4 (1000 and 100 ng/ml, respectively). At low doses, Bmp4 significantly increased levels of *Msx1* expression in the testis and *Msx2* expression in the ovary (Fig. 2). The low dose of RA had no statistically significant effect but tended to increase levels of *Msx1* expression. At high doses, both Bmp4 and RA treatment induced *Msx1* and *Msx2* mRNA expression (Fig. 2A,B). Similar results were obtained with Bmp2 treatment (supplementary material Fig. S3A). Interestingly, treatment with RA led to increased expression levels of *Msx1* and *Msx2* in F9 cells (data not shown) and *MSX1* in human foetal ovaries (supplementary material Fig. S2B). *MSX2* expression in human foetal ovaries was not significantly increased in response to RA (*n*=6, *P*=0.112). Histological analyses revealed that Bmp4 treatment alone did not alter meiosis initiation in 11.5 dpc mouse ovaries or in testis cultured for two or four days (supplementary material Fig. S4). High doses of RA speeded up the initial stages of meiosis in the ovary and induced meiosis in the testis as previously reported (data not shown) (Guerquin et al., 2010; Trautmann et al., 2008). Investigation into the potential regulation of Bmp genes by RA using qPCR indicated that the expression of *Bmp2*, *Bmp4* and *Bmp7* was not influenced by RA in the ovary explants model (supplementary material Fig. S3B). In order to determine whether RA and Bmp4 cooperate in Msx gene induction, gonads were cultivated for two days with both RA and Bmp4. This experiment revealed no difference in *Msx1* expression whether Bmp4 and RA were added together or alone (Fig. 2A). However, compared with single treatment, co-treatment with Bmp4 and RA did induce stronger expression levels of *Msx2* in the ovary (Fig. 2B).

Msx1 and *Msx2* double-null mutation alters female gonad development

In order to investigate the roles of *Msx1* and *Msx2* during gonad differentiation and meiosis initiation, mouse gonads from *Msx1* and *Msx2* dKO embryos were analysed at 13.5 dpc and at 14.5 dpc, corresponding to the limit of dKO embryo survival. The direct examination of the gonad revealed a pronounced phenotype; the female dKO displayed a substantial difference in shape between right and left ovaries, with the right appearing shorter and thicker, i.e. more rounded, than the left (Fig. 3A). This phenomenon was more pronounced at 14.5 dpc than at 13.5 dpc and was never observed in female gonads from wild-type, heterozygous, or *Msx1*

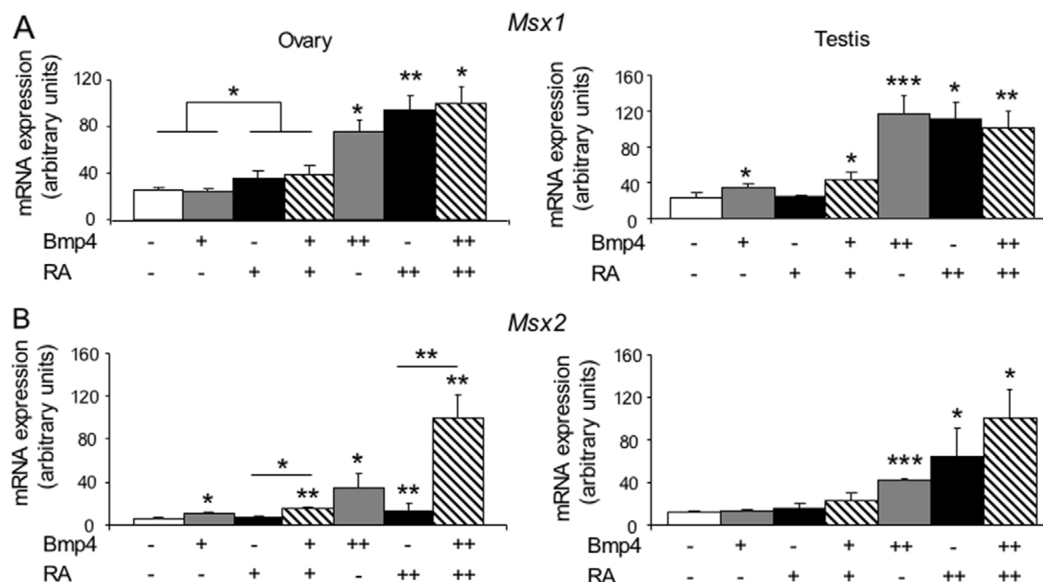

Fig. 2. Bmp4 and RA regulate *Msx1* and *Msx2* gene expression. (**A**,**B**) *Msx1* (A) and *Msx2* (B) gene expression was determined using RT-qPCR in mouse ovary and testis. Explants were performed at 11.5 dpc and cultivated for two days without (white column) or with Bmp4 at 100 ng/ml or 1 μg/ml (+ and ++ on grey columns, respectively) or with 50 nM or 1 μM retinoic acid (RA; + and ++ on black columns, respectively) or with Bmp4 and RA together (striped bar). Gene (mRNA) expression was normalised to *Ddx4*, a gene expressed specifically in germ cells. *$P<0.05$, **$P<0.01$, ***$P<0.001$ in paired Student's *t*-test. Error bars represent s.e.m.

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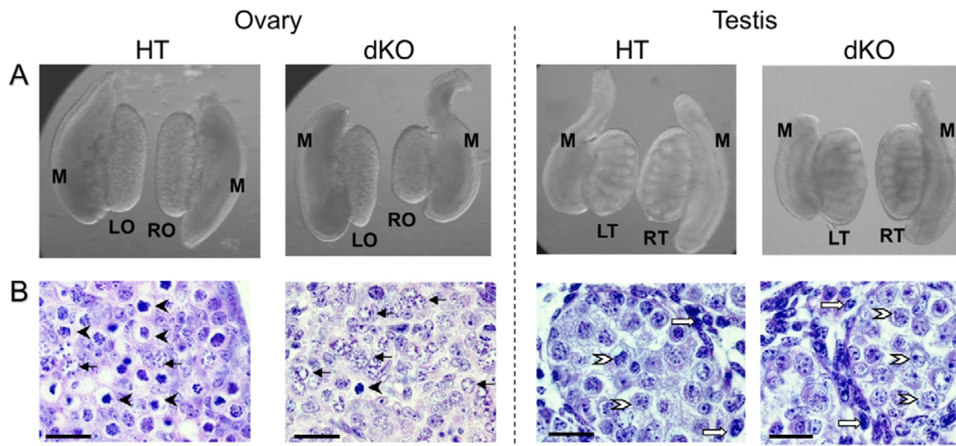

Fig. 3. The *Msx1* and *Msx2* double-null mutation leads to an asymmetric phenotype and meiosis default. (**A**,**B**) Gonads from heterozygous or double knockout mice were obtained at 14.5 dpc and observed under light microscopy (A) or after sectioning with Haematoxylin and Eosin (B). HT, heterozygous; dKO, *Msx1* and *Msx2* double knockout; M, mesonephros; LO, left ovary; RO, right ovary; LT, left testis; RT, right testis. Black arrows indicate oogonia, black arrowheads indicate zygotene stage, white arrowheads indicate pre-spermatogonia and white arrows indicate Leydig cells. Scale bars: 20 μm.

or *Msx2* single knockout mice, whereas it was repeatedly observed in dKO mice (*n*=12). Notably, both left and right male gonads differentiated normally and similarly in male dKO mice (Fig. 3A).

In addition, at 14.5 dpc, the ovaries from *Msx1*- and *Msx2*-null embryos presented considerably fewer meiotic cells when compared with heterozygous embryos. The zygotene stage, which is easily recognised owing to a specific chromatin condensation, appeared less abundant or represented in dKO gonads (Fig. 3B, Fig. 4B; supplementary material Fig. S6). A similar reduction in the number of meiotic cells was observed in both the left and right mutant ovaries. Interestingly, this reduction was not due to a global decrease in the germ cell population as no obvious change in total germ cell number was observed in dKO ovaries when compared with ovaries of other genotypes (Fig. 4A). Furthermore, no increase in germ cell apoptosis, as measured by cleaved caspase 3 staining, could be observed (Fig. 4C). The percentage of cells engaged in meiosis did not vary between wild-type, heterozygous and *Msx1* or *Msx2* single-null mutant ovaries (~80-90%, Fig. 4B) but was significantly decreased in dKO (~23%). These data were confirmed by measuring the percentage of cells stained by the meiotic marker γH2AX; dKO presented 3.5 times fewer γH2AX-positive cells than wild-type ovaries: (20±5% versus 71±9%, *P*=0.004, *n*=3, Fig. 4C). Interestingly, the few meiotic cells observed in dKO ovaries were often retrieved as clusters and mainly from the anterior part of the ovary.

In order to determine the phenotype of the germ cells that had not initiated meiosis, we analysed the presence of Ssea-1 and Stra8 at 13.5 and 14.5 dpc (supplementary material Fig. S5A; Fig. 4C). At 13.5 dpc, many germ cells were positive for Stra8 (~60%) and almost all expressed Ssea1 in the ovaries from both heterozygous and dKO mice (supplementary material Fig. S5A). At 14.5 dpc, Ssea1 expression had disappeared from most germ cells and more cells expressed Stra8 in the ovaries from heterozygous mice (~80%, Fig. 4C). By contrast, at the same stage in ovaries from dKO mice, most cells still expressed Ssea1 and a striking decrease was observed in the number of Stra8 positive cells when compared with in heterozygous mice. Sycp3 (synaptonemal complex protein 3) staining in the 13.5 dpc ovaries also revealed fewer Sycp3-positive cells in the dKO ovaries (supplementary material Fig. S5B), though at this stage the vast majority of germ cells were still at the pre-meiotic stage as Sycp3 protein was not observed to be loaded on meiotic chromosomes (Fig. 5C). No significant differences were observed in the wild-type, heterozygous, *Msx1* or *Msx2* single knockout mice (data not shown).

Progress and completion of prophase I of meiosis in *Msx1* and *Msx2* dKO

Mouse ovary long-term cultures were performed in order to test the ability of germ cells to either (1) enter into meiosis if they had not already done so by 14.5 dpc, or (2) complete meiosis prophase I if they had already initiated meiosis. Ovaries from 13.5 and 14.5 dpc were cultured for four and seven days, respectively, in order to reach stages equivalent to 17.5 dpc or post-natal day 2. Upon completion of the four-day cultures, a small number of germ cells from the ovaries of dKO embryos were still incorporating BrdU whereas hardly any could be retrieved from wild-type ovaries, indicating that some cells in the dKO were still not engaged in meiosis (Fig. 5A). Interestingly, a massive increase in dKO germ cell apoptosis was also observed at this stage (Fig. 5B). The remaining germ cells in dKO-cultured ovaries expressed Sycp3 protein (Fig. 5C) that was mostly correctly loaded on meiotic chromosomes, as observed in heterozygous and wild-type germ cells, yet with a few cells presenting a nuclear SCYP3 staining similar to that observed in pre-meiotic cells on 13.5 dpc (i.e. dusty or patchy staining).

After seven days in culture, the ovaries from wild-type, heterozygous or *Msx1*-null embryos contained many diplotene oocytes representing the start of follicle formation (Fig. 6A). A small but not significant difference was observed in the *Msx2* knockout. Indeed, *Msx2* knockout ovaries presented considerable variability in oocyte number with three out of six analysed ovaries containing an oocyte population decreased by more than half (~1000 oocytes), whereas the other three contained a normal oocyte number. A very low number of oocytes was observed in ovaries from dKO mice (~7.5 times fewer when compared with wild type). Notably, the number of oocytes appeared to be inversely proportional to their size (Fig. 6B, upper panel) with oocytes from dKO mouse ovaries being larger than those from heterozygous or wild type (12.5 μm for dKO versus 8.9 μm for wild type). Immunostaining for p63, which is specifically expressed in the oocyte at the end of prophase I (Livera et al., 2008), highlighted the small follicle number observed in ovaries from dKO mice (Fig. 6B, lower panel). Interestingly, no pre-meiotic germ cells were retrieved from either wild-type or dKO ovaries after seven days in culture.

Msx proteins regulate *Stra8* gene expression

To investigate further the link between Msx gene expression and meiosis initiation, the expression of markers for germ and somatic cells was measured using qPCR in 14.5 dpc dKO ovaries. We first

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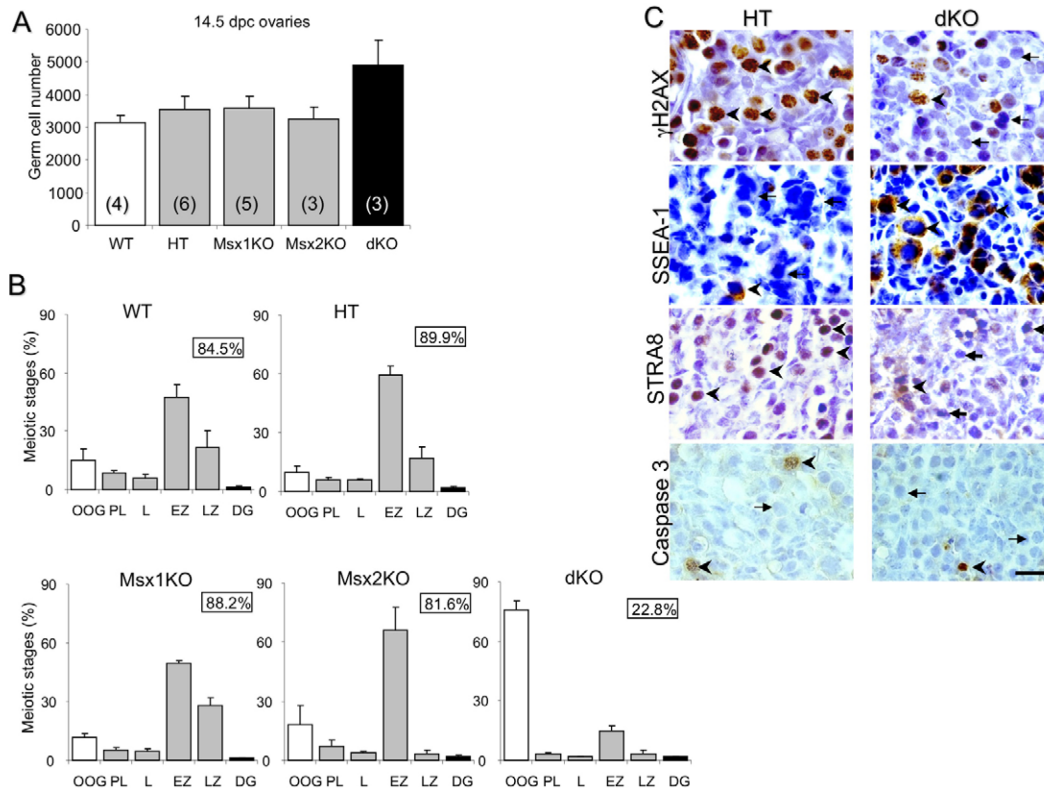

Fig. 4. *Msx1* and *Msx2* involvement in meiosis initiation. (**A**,**B**) Meiosis initiation was measured by counting mitotic and meiotic germ cells in 14.5 dpc ovaries from wild-type, heterozygous or single and double mutant mouse embryos. Global count of the total number of germ cells (A) and detailed count presenting the different percentages of germ cells for each meiotic stage (B) are shown. In A, the number of ovaries studied is given in brackets. In B, the total percentage of meiotic cells is given in the box. Error bars represent s.e.m. (**C**) Immunostaining of meiotic marker γH2AX, undifferentiated germ cell marker Ssea-1, Stra8 or the apoptotic marker active Caspase 3 was performed in heterozygous or mutant ovaries from 14.5 dpc embryos. WT, wild type; HT, heterozygous; Msx1KO, *Msx1* knockout; Msx2KO, *Msx2* knockout; dKO, *Msx1* and *Msx2* double knockout; OOG, oogonia; PL, preleptotene; L, leptotene; EZ, early zygotene; LZ, late zygotene; DG, degenerating germ cell. Arrows indicate negative germ cell staining; arrowheads indicate positive germ cell staining. Scale bar: 20 μm.

investigated whether *Msx1* and *Msx2* double-null mutations induced masculinisation of the female gonad that could in turn prevent meiosis. Expression of male somatic cell markers such as *Cyp26b1* and *Sox9* were analysed but revealed no differences (Fig. 7A; data not shown). We also immunostained for anti-Müllerian hormone (AMH), a member of the transforming factor-β family specifically produced by foetal Sertoli cells during male sexual differentiation, but found none in the dKO ovary (supplementary material Fig. S6B). Female somatic cell markers, such as *Wnt4*, also remained unaltered in ovaries from dKO mutants (data not shown).

Second, we studied the expression of *Stra8* and *Nanos2*, two major genes defined as crucial for induction or repression of meiosis, respectively. *Nanos2* transcript level did not vary but we did detect a decrease in *Stra8* expression (Fig. 7A), in agreement with the results of Stra8 immunostaining. To examine the role of Msx in *Stra8* regulation, *Stra8* expression was studied following transfection of F9 cells with pCIG-Msx1-HA or pCIG alone. In pCIG-Msx1-HA transfected cells, *Stra8* was upregulated in comparison with control cells (Fig. 7B). A similar result was obtained when the HA domain was added to the C-terminal or N-terminal region (data not shown). Using a chromatin immunoprecipitation assay following F9 cell transfection with Msx1-HA (*n*=4), we investigated whether Msx1 could efficiently bind the *Stra8* promoter, using the *Hspa1b* promoter as a positive control (Msx1 having previously been shown to bind to this region). A region of the *p65* promoter, devoid of any Msx binding site, was used as a negative control. Based on the presence of Msx binding sites in the *Stra8* promoter, seven regions were analysed, spanning from 4 kb upstream of the first exon to 6 kb into the first intron. Our results revealed three distinct homeobox binding sites in the *Stra8* regulatory sequence (Fig. 7C). The regions displaying significant Msx binding activity were termed site A (–3849; –3800 bp); site B (–1687; –1636 bp); and site C (+2219; +2269 bp). Interestingly, site A contains an Msx binding motif that is conserved in human, rat and mouse (supplementary material Fig. S7).

DISCUSSION

Msx proteins are required for the correct development of many organs, including the limbs, teeth and neural crest (Lallemand et al., 2005; Ramos and Robert, 2005). Here, we showed for the first time the importance of *Msx1* and *Msx2* in the developing ovary and, in particular, for the crucial step of meiosis initiation.

Both genes are expressed during foetal life in ovarian germ cells with *Msx2* expression appearing shortly before *Msx1*, suggesting an early role for *Msx2* in ovarian development. *Msx* gene expression correlates with female foetal meiosis initiation;

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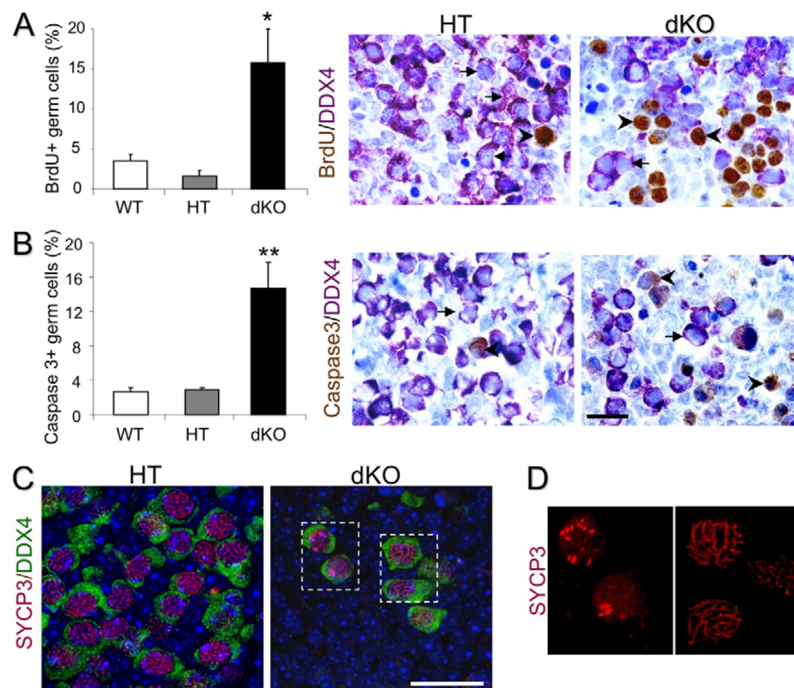

Fig. 5. Msx1- and Msx2-deficient germ cell destiny after meiosis initiation. Ovaries from heterozygous or double knockout mice were obtained at 14.5 dpc and cultivated for four days. (**A**) To study germ cell proliferation, double immunostaining against specific germ cell markers Ddx4 and BrdU showing proliferation in the ovary were performed (right panels) and double-stained proliferating germ cells were counted (left panel). (**B**) To study apoptosis, double immunostaining against the specific germ cell marker Ddx4 and the apoptotic cell marker Caspase3, were performed (right panels) and double-stained apoptotic germ cells were counted (left panel). Arrows indicate Dxd4-positive cells and arrowheads indicate BrdU- and caspase3-positive cells in A and B, respectively. (**C**) The number of meiotic germ cells was studied at this stage and double immunostaining using antibodies specific to the germ cell marker Ddx4 and to the meiotic marker Sycp3 were performed. (**D**) Magnification of the boxed areas in C. Error bars represent s.e.m. Scale bars: 20 μm.

however, Msx genes are not activated when male meiosis begins in the post-natal testis. On the contrary, as previously reported (Anderson et al., 2008; Mark et al., 2008) levels of *Stra8* and other meiotic markers increase sharply in the mouse post-natal testis over the studied period. This suggests a specific role for Msx in female meiosis. *Msx1* and *Msx2* are also expressed in the human foetal ovary, in accordance with a recent report identifying expression of *MSX* in the human ovary (Childs et al., 2010), indicating a conserved role for Msx in female mammalian germ cells. Cell-sorting experiments, using Ssea-1 or Oct4-GFP, revealed high expression levels of *Msx1* and *Msx2* in mouse germ cells. The Msx1ERT2cre mice-ROSAmT/mG reporter system confirmed that, at 13.0 dpc, *Msx1* is expressed in germ cells.

Msx1 and *Msx2* double-null mutations prevented meiosis initiation in most germ cells in the 13.5-14.5 dpc ovary, as demonstrated by the decrease in the number of *γH2AX*- and *Sycp3*-positive cells. Germ cells that had not initiated meiosis did not initiate the male pathway either, as no increase in *Nanos2* was observed in dKO ovaries. These cells remained blocked in an undifferentiated state, as shown by Ssea-1 antigen detection, and most died shortly after as shown by the increased apoptotic rate in cultured ovaries. Cells which initiated meiosis at 14.5 dpc in the dKO ovaries progressed normally throughout prophase I of meiosis and reached the diplotene stage in long-term cultures. This indicates that Msx might be required solely for the initiation of meiosis but not for progression through prophase I of meiosis. Contrastingly, two findings suggest that Msx genes might also facilitate later meiotic progression. First, in some of the ovaries from *Msx2* knockout mice, we observed a decrease in the number of oocytes reaching the diplotene stage, whereas no defect was observed at the time of meiosis initiation. Second, in the dKO condition, the decrease in the percentage of oocytes reaching the diplotene stage was more pronounced than the decrease in germ cells initiating meiosis at 14.5 dpc. However, this observation should be treated with caution as this Msx phenotype did appear to vary.

Similarly, it is interesting to point out that, although being obvious, the phenotype of the dKO was incomplete as some germ cells did manage to initiate and complete meiosis prophase I. A likely explanation for this is the possible redundancy observed between Msx genes; indeed, ovaries from *Msx1* knockout and *Msx2* knockout mice presented no defect in meiosis at 14.5 dpc, indicating that both genes can fully compensate for one another. We observed very weak expression of *Msx3* in the mouse female germ cells at 13.5 dpc and this expression did not increase in the dKO condition (data not shown). Thus, in mice, *Msx1*, *Msx2* and *Msx3* might share similar meiosis initiation regulatory functions. Alternatively, it has been proposed that Msx genes interact with other homeobox genes such as *Lhx2* (Bendall et al., 1998), which share the same expression pattern in gonads (R.L.B. and G.L., unpublished data). Other homeobox genes, such as Rhox genes, have been shown to be differentially expressed at the time of germ cell sex determination (Daggag et al., 2008), leading us to postulate that they might interact with Msx genes, and in some cases compensate for *Msx1* and *Msx2* invalidation.

Msx1 and Msx2 are transcription factors that are able to induce or repress gene expression (Hayashi et al., 2006; Zhuang et al., 2009). Ovaries from dKO mice showed significantly decreased *Stra8* expression at 14.5 dpc but not at 13.5 dpc. This is most likely to indicate that Msx proteins are not required for the initial induction of *Stra8* expression in the mouse foetal ovary but rather for maintaining or increasing *Stra8* expression. Interestingly, in F9 cells, our experiments indicated that *Stra8* could be a target of Msx proteins; indeed overexpression of *Msx1* increased *Stra8* expression and Msx1 was found to bind the *Stra8* promoter. Although we cannot exclude the implication of a co-factor because Msx often interacts with other proteins in order to repress or induce gene expression (Newberry et al., 1997; Shetty et al., 1999), our in vitro experiments allow us to assume reasonably that *Stra8* is probably directly regulated by Msx1. We thus propose that the default meiosis observed in the absence of functional Msx1 and Msx2 is caused partially by a

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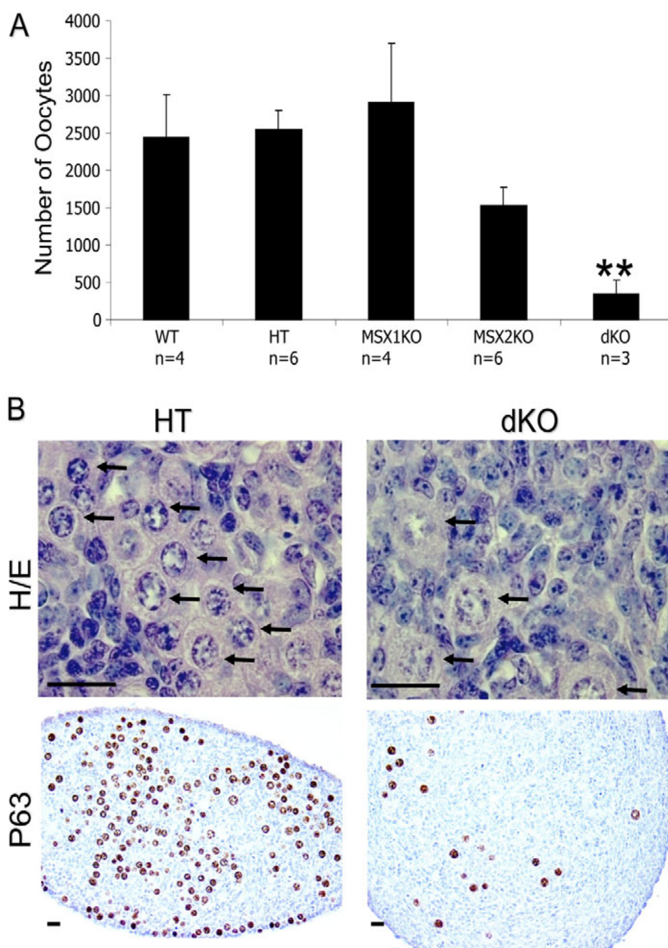

Fig. 6. *Msx1* and *Msx2* invalidation does not fully prevent meiosis completion. (**A**,**B**) Ovaries were explanted at 14.5 dpc and cultivated for seven days allowing oocytes to complete prophase of meiosis I. Diplotene oocytes were counted (A) as observed with Haematoxylin and Eosin staining (B, upper: H/E) and stained with p63 antibody (B, lower), which is specifically expressed in oocyte nuclei at this stage (B). Black arrows highlight oocyte-forming follicles. WT, wild type; HT, heterozygous; Msx1KO, *Msx1* knockout; Msx2KO, *Msx2* knockout; dKO, *Msx1* and *Msx2* double knockout. Scale bars: 20 μm. Error bars represent s.e.m. ***P*<0.01 in paired Student's *t*-test.

defect in *Stra8* upregulation. However, involvement of additional Msx target genes could exist. To date, very little data is available concerning *Stra8* regulation: Dmrt1 controls *Stra8* activation in the foetal ovary and represses it in the post-natal testis (Krentz et al., 2011; Matson et al., 2010). Interestingly, the phenotype of the Msx dKO was very similar to that recently described in the foetal ovaries of *Dmrt1* mutant mice with both a decrease in *Stra8* expression, though more pronounced in the *Dmrt1* mutant than in the Msx dKO, and a decrease in the number of follicles. Contrasting with the G1 arrest described in female germ cells in the foetal *Stra8* mutant ovaries, we did observe a small number of germ cells still proliferating at a stage equivalent to 17.5 dpc (13.5 dpc + 4 days of culture) in the Msx dKO. This might be due to Msx dKO leading to only a moderate decrease in *Stra8* expression and no alteration in the initial *Stra8* induction in pre-meiotic cells, which would result in a phenotype different from germ cells fully deficient in Stra8. Another possibility that cannot be ruled out is that Msx proteins might alter the expression of additional genes in female germ cells and *Stra8* might not be the only gene controlled by Msx proteins. This last hypothesis is sustained by the fact that a lack of Sycp3-positive germ cells is already observed at 13.5 dpc when no major decrease in Stra8-positive cells was noticed.

Finally, in this study we also analysed the regulatory factors RA and Bmps, which are known to induce Msx gene expression in other systems, as well as being differentially produced during gonadal development. Bmp4 is required, during early development of the mouse embryo, for primordial germ cell generation (Lawson et al., 1999) and is later expressed, along with Bmp2 and Bmp7 (Ross et al., 2007), at 12.5 dpc in the ovary. *Msx1* and *Msx2* are clearly stimulated by Bmp2 and Bmp4 and represent the first Bmp-regulated factors during the gonad sex determination period in both mice (this study) and human (Childs et al., 2010). This might explain the sex-specific expression of Msx genes identified in the developing gonads. Although RA has been shown to promote meiosis (Bowles et al., 2006; Koubova et al., 2006), meiosis can occur without RA signalling (Kumar et al., 2011). Existing literature concerning the role of RA in Msx gene regulation appears to be contradictory (Brown et al., 1997; McGonnell et al., 2001; Shen et al., 1994; Yokouchi et al., 1991). In this study, *Msx1* and *Msx2* were both overexpressed after RA treatment in the foetal gonads. We hypothesise that exogenous RA might, at least in part, act indirectly to induce Msx gene/protein expression that, in turn, would maintain *Stra8* expression. Interestingly, in contrast to RA, Bmp4 alone is insufficient to either accelerate meiosis in female gonads or induce meiosis in male gonads. This probably indicates that Msx genes are required for the correct differentiation in most of the germ cells in the foetal ovary but that Msx gene/protein expression is probably not sufficient to induce meiosis. Childs et al. (Childs et al., 2010) correlated *MSX* induction in response to BMP4 to an increase of apoptosis in foetal human female germ cells whereas we observed no significant reduction of apoptosis in Msx dKO mice. A likely explanation of this discrepancy is that early human foetal germ cells are unable to initiate meiosis prior to 10 wpf and when these cells are forced to differentiate in response to RA they undergo apoptosis (Le Bouffant et al., 2010) whereas mouse foetal germ cells initiate meiosis (Bowles et al., 2006; Trautmann et al., 2008). One may thus hypothesise that a similar process occurred in response to BMP4 in human ovary.

Although Msx genes are clearly involved in the meiosis initiation process, this is unlikely to explain the left-right gonad asymmetry in Msx dKO. A similar phenotype has been observed in *Rspo1* KO mice (Chassot et al., 2008; Tomizuka et al., 2008) in which the smaller right gonad was characterised as being a late ovotestis. Somatic marker (*Sox9*, *Cyp26b1*, *Amh* and *Wnt4*) expression in the dKO ovary, however, indicated an absence of somatic cell masculinisation and permitted us to conclude that the small right gonad in Msx dKO was not in fact an ovotestis. The Rspo1/Wnt/β–catenin signalling pathway has recently been identified as a master regulator of ovarian development. Msx genes are also described as being regulated by the Wnt/β-catenin signalling pathway and this regulation is increased by synergic Bmp signalling (Hussein et al., 2003). Msx proteins are thus potentially involved in two major signalling pathways involved in germ cell determination: Wnt and Bmp.

In conclusion, we have demonstrated in this study the role of *Msx1* and *Msx2* in gonadal development and their conservation and requirement for germ cell sex determination and female meiosis.

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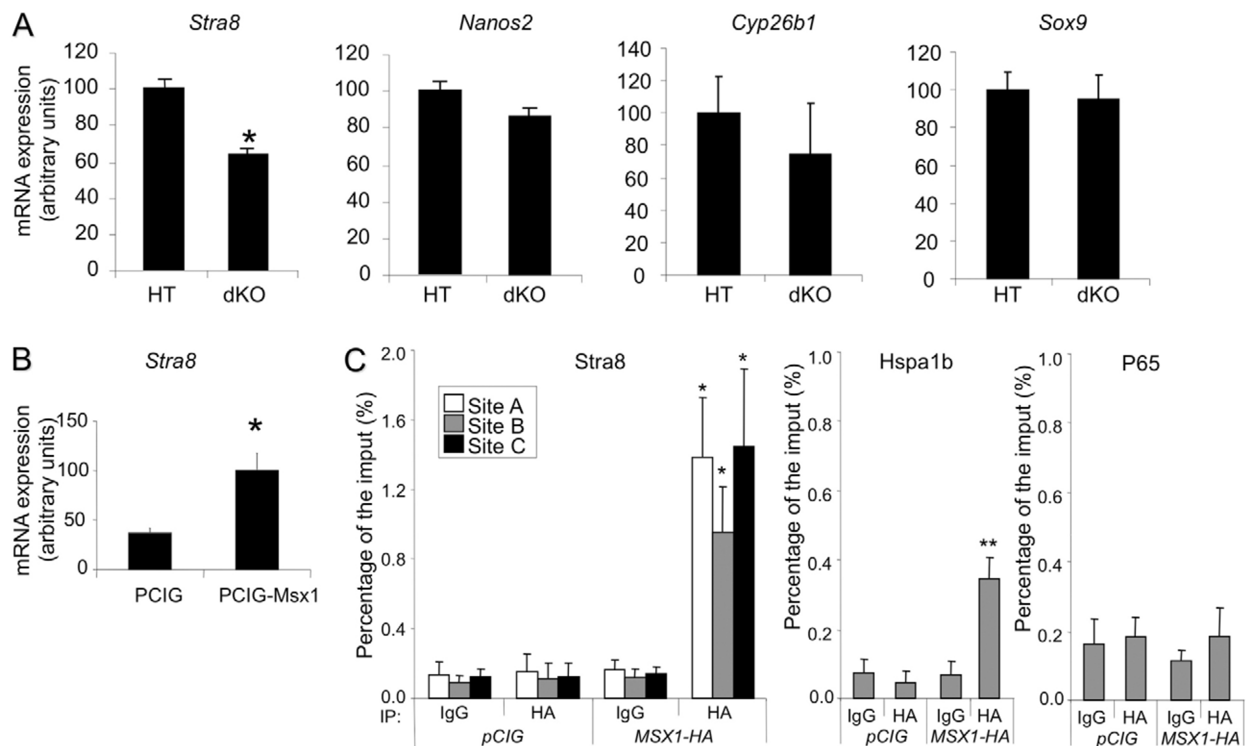

Fig. 7. In vivo and in vitro regulation of *Stra8* expression by Msx proteins. (**A**) Germ cell markers (*Nanos2*, *Stra8*) and somatic cell markers (*Cyp26b1*, *Sox9*) were analysed using RT-qPCR in the ovaries of heterozygous or *Msx1* and *Msx2* double knockout embryos at 14.5 dpc. (**B**) *Stra8* gene expression was analysed by RT-qPCR in F9 cells transfected with pcig or pcig-Msx1-HA plasmid construct. (**C**) ChIP experiment was performed with F9 cells transfected with pCIG-Msx1-HA or pCIG alone, on the *Stra8* promoter region (left panel), on the *Hspa1b* promoter region (middle panel) and on the *P65* promoter region (right panel). HT, heterozygous; dKO, *Msx1* and *Msx2* double knockout. Gene (mRNA) expression was normalised to β-actin for somatic genes and *Ddx4* for genes expressed in germ cells (*Nanos2* and *Stra8*). Error bars represent s.e.m. **P*<0.05, ***P*<0.01, in paired Student's *t*-test.

Taken together, these data lead us to postulate a central role for these Msx proteins in the control of female germ cell lineage differentiation. *Msx1* is an early gene implicated in germ cell differentiation and able to regulate *Stra8* expression. It will be of great interest to investigate further the transcriptional cascade triggered by homeobox genes in foetal germ cells during the sex determination period.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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Table S1. qPCR primer sequences

Gene	Primer sequence (5′-3′)
Mouse *Msx1*	F: TCTCGGCCATTTCTCAGTCG
	R: AGAGCATCTTCTGGCAGCTTG
Mouse *Msx2*	F: CATAAAAGCATCCCCCTCCC
	R: AGGAGCAGAGTTGGCACCAC
Mouse *Bmp2*	F: GCTTCTTAGACGGACTGCGG
	R: GCAACACTAGAAGACAGCGGGT
Mouse *Bmp4*	F: AGCCCGCTTCTGCAGGA
	R: AAAGGCTCAGAGAAGCTGCG
Mouse *Bmp7*	F: CCAAAGAACCAAGAGGCCC
	R: GCTGCTGTTTTCTGCCACACT
Mouse *Sox9*	F: GGCAAGCTCTGGAGGCTG
	R: CCTCCACGAAGGGTCTCTTCT
Mouse *Nanos2*	F: AATTCAGAGCCGGAAGCAAA
	R: TTGGGCTCCTCAGCTACCTC
Mouse *Actb* (β-actin)	F: GCCCTGAGGCTCTTTTCCAG
	R: TGCCACAGGATTCCATACCC
Mouse *Stra8*	F: GCCTGGAGACCTTTGACGA
	R: GGCTTTTGGAAGCAGCCTTT
Mouse *Ddx4*	F: GAAGAAATCCAGAGGTTGGC
	R: GAAGGATCGTCTGCTGAACA
Human *MSX1*	F: AGTTCTCCAGCTCGCTCAGC
	R: GGAACCATATCTTCACCTGCGT
Human *MSX2*	F: TGGATGCAGGAACCCGG
	R: AGGGCTCATATGTCTTGGCG
Human *SPO11*	F: CTGTTGCTGTGCCATCGAATA
	R: CATCTGTAACTAAATTCCGAATTCCTT
Human *ACTB* (βACTIN)	F: TGACCCAGATCATGTTTGAGA
	R: TACGGCCAGAGGCGTACAGC