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Role of *Mael* in early oogenesis and during germ-cell differentiation from embryonic stem cells in mice *in vitro*

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Summary

In a previous study, we have identified a set of conserved spermatogenic genes whose expression is restricted to testis and ovary and that are developmentally regulated. One of these genes, the transcription factor *Mael*, has been reported to play an essential role in mouse spermatogenesis. Nevertheless, the role of *Mael* in mouse oogenesis has not been defined. In order to analyse the role of *Mael* in mouse oogenesis, the expression of this gene was blocked during early oogenesis in mouse *in vitro* using RNAi technology. In addition, the role of *Mael* during differentiation of embryonic stem cells (ESC) into germ cells *in vitro* was analysed. Results show that downregulation of *Mael* by a specific short interfering RNA disrupted fetal oocyte growth and differentiation in fetal ovary explants in culture and the expression of several germ-cell markers in ESC during their differentiation. These results suggest that there is an important role for *Mael* in early oogenesis and during germ-cell differentiation from embryonic stem cells in mouse *in vitro*.

Keywords: ESC, Germ cells, *Mael*, Oogenesis, RNAi, siRNA

Introduction

Primordial germ cells (PGCs) are the embryonic precursors of gametes. In the mouse, the germ-cell lineage is induced from the proximal epiblast of the egg cylinder at embryonic day 6.5 (E6.5) in response to signalling by bone morphogenetic protein 4 (*Bmp4*) and *Bmp8b* (Zhao *et al.*, 1996; Lawson *et al.*, 1999). PGCs can first be detected at E7.5 as a cluster of alkaline phosphatase-positive cells at the base of the allantois (Ginsburg *et al.*, 1990). From E8.5, founder PGCs migrate through the embryo and arrive at the genital ridge around E10.5, where they become known as gonocytes (McLaren, 2003). After a short period of mitosis, germ cells either cease mitosis and enter meiosis in the female, or progressively arrest in G0/G1 over several days in the male (Childs *et al.*, 2008; Zhou *et al.*, 2010). In mice, the vast majority of oocytes has entered meiosis during embryonic life, and at birth some oocytes are in the transitory stages of prophase (pachytene and early diplotene), while others have entered late diplotene and dictyate in which they apparently remain until meiosis resumes shortly before ovulation (Pedersen & Peters, 1968; Pangas & Rajkovic, 2006).

Germ-cell development in mammals is regulated by transcription factors that ensure appropriate development of PGCs and eventually, oocytes in females and sperm in males. Recently, the transcription factor MAEL has been reported to play an essential role in mouse spermatogenesis (Soper *et al.*, 2008). MAEL is a component of the mouse meiotic nuage and of the PIWI/piRNA pathway implicated in transposon silencing in fruit flies and mice (Costa *et al.*, 2006; Aravin *et al.*, 2009. The *Drosophila* homologue of mouse *Mael*, *maelstrom*, is required for posterior positioning of the microtubule-organizing centre in oocytes (Clegg *et al.*, 2001). In mice, *Mael* expression is restricted to testis and ovary, and is developmentally regulated

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(Bonilla & Xu, 2008). However, the role of *Mael* in mouse oogenesis has not been reported.

In the present study we were interested in the role of *Mael* in mouse oogenesis. The model for this study was based on the culture of fetal ovaries. By using this model, hundreds of fully grown oocytes at the germinal vesicle (GV) stage are obtained from each fetal ovary after 7 days of culture (De Felici, 1991; Bonilla & del Mazo, 2010). In addition, we were interested in analysing the role of *Mael* during differentiation of embryonic stem cells (ESC) into germ cells *in vitro* (Hübner *et al.*, 2003; Toyooka *et al.*, 2003; Clark *et al.*, 2004; Geijsen *et al.*, 2004). The expression of *Mael* was blocked in fetal ovary explants in culture and in ESC during germ line differentiation process using RNAi technology.

Material and methods

Experimental animals

All experimental procedures that involve the use of mice were performed under the regulations established by our Institutional Bioethics Committee on animal care, in accordance with those approved by the National Institutes of Health (Bethesda, MD, USA).

In vitro culture of fetal oocytes

Fetal ovaries were dissected from 17.5 days post-coitum mouse CD-1 fetuses and each ovary was cut into three fragments with fine needles (G 5/8) under a stereomicroscope. Ovary explants were cultured in 1 ml Waymouth medium (Gibco) supplemented with 5% horse serum, 2.5% fetal calf serum (both heat inactivated) and 100 μg/ml penicillin–streptomycin in 4-well dishes (Nunc) as described previously (De Felici, 1991; Bonilla & del Mazo, 2010). Half of the medium was changed every day starting from day 3 of culture, to allow the explants to attach to the bottom of the wells. Each well contained one ovary. The cultures were maintained at 37°C in a humidified incubator under 5% CO_2 in air for 7 days, when the number of oocytes at the GV stage that had a diameter of 40–70 μm grown out of each fetal ovary cultured was analysed.

Mouse embryonic stem cell maintenance and differentiation

Mouse ESC maintenance was carried out according to the protocol described on the International Gene Trap Consortium (IGTC) website (http://www.genetrap.org/info/protocols/baygenomics/feederES.html). Feeder-independent ESC line ES-E14TG2a (E14) was used. ESC were cultured in ESC culture medium (ES cell medium: Glasgow Modified Minimal Essential Medium (GMEM) medium (Sigma)) supplemented with 2 mM glutamine (Gibco-BRL), 1 mM sodium pyruvate (Gibco-BRL), 1× non-essential amino acids, 10% (v/v) fetal bovine serum (characterized, HyClone), 0.1 mM beta-mercaptoethanol, and 1000 units per ml of leukocyte inhibitory factor (LIF, Chemicon) at 37°C in a humidified 5% CO_2 incubator. ES cells were passaged every 2 days, and the medium was changed on alternate days.

Germ-cell differentiation was based in the generation of embryoid bodies (EBs) from mouse ES cells to induce germ-line differentiation (Keller, 1995; Ling & Neben, 1997). Briefly, 400 cells in 30 μl differentiation medium drops (ESC culture medium without LIF). BMP4 at a final concentration of 50 ng/ml was used at this stage to induce the differentiation of ESC into germ cells (Makoolati *et al.*, 2011); cells were placed onto the inside of the lid of 96-well plates using a 12-multichannel pipette. Each well was half filled with sterile water to maintain humidity. The lid was flipped in a smooth, steady manner to invert and it was placed onto the 96-well plate. The plates were incubated for 5 days at 37°C with 5% CO_2. After 2 to 3 days in culture the EBs had formed. On day 5, the EBs were transferred into 10-cm plates (20 EBs/plate) that contained 10 ml of differentiation medium supplemented with retinoic acid at a final concentration of 2 μM in order to promote germ-cell differentiation (Chen *et al.*, 2012). The plates were incubated for 7 more days at 37°C with 5% CO_2. Analysis of genes expressed in undifferentiated ESC, *Oct4* (Synonym: *Pou5f1*), *Nanog* and *Stella*, the germ-cell markers acrosin (*Acr*), boule-like (*Boll*), deleted in azoospermia-like (*Dazl*), growth differentiation factor 9 (*Gdf9*), mouse vasa homologue (*Mvh*), protamine 2 (*Prm2*), zona pellucida glycoprotein 3 (*Zp3*), and the Sertoli cell marker *Mis* was carried out on day 12 of differentiation.

Transfections

A Stealth short interfering RNA (siRNA; Invitrogen) was designed to *Mael* (NM_175296.4) using the BLOCK-iT RNAi Designer at www.invitrogen.com/rnaidesigner. The sense (5′→3′) strand of the synthetic oligonucleotide duplex was GGCCUUCAGAGAAGCAGAAACUUGU. A siRNA designed to *Dazl* (NM_010021; sense strand CACAACUUCUGAGGCUCCAAAUUCA) was used as a positive control, as its essential role in the differentiation of ESC into pre- and post-meiotic germ cells has been reported (Haston *et al.*, 2009; Kee *et al.*, 2009; Kerr & Cheng, 2010). Medium GC Negative Control RNAi duplex

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Table 1 Sequences of forward and reverse primers used for reverse transcription polymerase chain reaction (RT-PCR) reactions

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	Product size (bp)
β-*Actin*	aagtgtgacgttgacatccg	gatccacatctgctggaagg	347
Oct4	tggagactttgcagcctgag	catactcttctcgttgggaat	620
Nanog	aagtacctcagcctccagca	gaagttatggagcggagcag	399
Stella	cagccgtacctgtggagaacaagag	agccctgggcctcacagctt	455
Acr	cggagtctacacagccacct	gcatgagtgatgaggaggtt	251
Boll	gcggctggaacaatgtatct	ccactgccctggtaaacact	213
Dazl	gccagcactcagtcttcatcag	gttggaggctgcatgtaagt	420
Gdf9	ctgataggcgaggtgagacc	ggaggaggaagaggcagacc	302
Mvh	gctcaaacag ggtctgggaag	ggttgatcagttctcgag	145
Mael	ttccacgaggatttcgattc	tccatacgcttcaaacacca	205
Prm2	gagcgcgtagaggactatgg	acctgcatctcctcctcctt	193
Zp3	cggttgccttgtggatggtc	ttgagcagaagcagtccagc	442
Mis	cgctatttggtgctaaccgtggactt	gcagagcacgaaccaagcga	325

(with no homology in vertebrate transcriptome) was also used in our experiments.

Fetal ovary explants from E17.5 fetuses in culture were transfected for 24 h on day 3 of culture, when the ovary explants were attached to the bottom of the 4-well dishes. Complexes that consisted of 40 pmol BLOCK-iT Fluorescent Oligo, Negative Control siRNA, *Mael*-siRNA or *Dazl*-siRNA and 2 μg/ml lipofectamine 2000 (all from Invitrogen) were used for transfections, as recommended by the manufacturer. To assess transfection efficiency, the ovary explants were transfected with 40 pmol BLOCK-iT Fluorescent Oligo. Uptake of the Fluorescent Oligo was visualised by fluorescence microscopy at 16–18 h on treated cells.

ESC (30–40% confluent) were transfected with complexes that consisted of 40 pmol Negative Control RNAi duplex or 40 pmol stealth siRNA and 2 μg/ml lipofectamine 2000. After 16–18 h, ESC were trypsinized, pelleted by centrifugation, and resuspended by trituration into a single-cell suspension in ESC differentiation medium. ESC were then cultured as indicated above to induce their differentiation into germ cells via embryoid body formation. To assess transfection efficiency, ESC were transfected with the BLOCK-iT Fluorescent Oligo. Uptake of the Fluorescent Oligo was visualised by fluorescence microscopy at 6 h after transfection.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from 13.5–18.5 dpc fetal ovaries, neonatal ovaries, fetal ovary explants in culture and ESC using RNeasy Kit (Qiagen) according to the manufacturer's recommendation. The first-strand synthesis was performed using 5 μg RNA, random primers, and reverse transcriptase Superscript II (Gibco-BRL) at 42°C for 1 h. PCR was performed for 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. Gene-specific primers were designed using the Primer3 program (MIT, available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers used in this study to amplify mouse transcripts are listed in Table 1.

Quantitative PCR

All reactions were performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Each PCR reaction was carried out in triplicate in a 25-μl reaction volume using 12.5 μl of 2× SYBR Green master mix, 9.5 μl of water, 2 μl of 3.75–5 μM primer mix and 1 μl of cDNA. Reaction conditions were: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Mouse liver RNA was reverse transcribed and used to construct standard curves. Values for each gene were normalised to the expression levels of *Gapdh*. Real-time PCR product specificity was confirmed by melt curve analysis and gel electrophoresis.

Statistics

Three independent experiments were performed to demonstrate reproducibility. Student's *t*-test was used to evaluate the difference between groups, and differences at $P < 0.05$ were considered to be significant. SAS statistical software (SAS Institute) was used to perform statistical analysis.

Results

Mael expression in fetal and neonatal ovaries

The developmental expression of *Mael* in embryos was analysed during the development of fetal and neonatal

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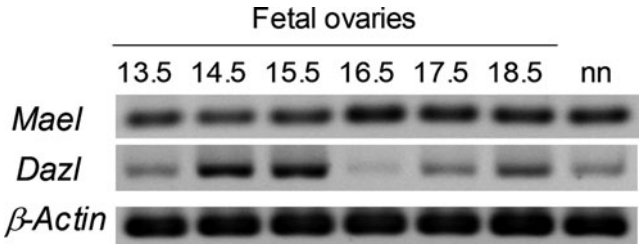

Figure 1 Developmental expression pattern of *Mael* and *Dazl*. Gene expression was analysed by reverse transcription polymerase chain reaction (RT-PCR) using cDNAs prepared from 13.5–18.5 dpc and neonatal (nn) ovaries.

ovaries. *Mael* was highly expressed in 13.5–18.5 dpc and neonatal ovaries. *Dazl* was also expressed in 13.5–18.5 dpc and neonatal ovaries, but its expression was changing during fetal development (Figure 1).

Transfection efficiency in mouse fetal ovary cells and in ESC

Transfection conditions were optimised using cellular internalisation of a fluorescently labelled short RNA duplex (BLOCK-iT Fluorescent Oligo) as an indicator of transfection efficiency. Transfection efficiency was determined by counting the total number of cells per field versus the number of corresponding cells with visible fluorescence uptake and averaging across different fields (300 cells). The transfection efficiency for mouse fetal ovary cells and ESC was routinely observed to be ≥80%, as reported previously (Moreno *et al.*, 2012).

Inhibition of *Mael* expression in mouse fetal ovaries in culture and ESC

siRNA targeting *Mael* and the positive control *Dazl* inhibited their expression by >80% relative to the control after 24 h in mouse fetal ovary cells in culture and ESC, as determined by quantitative (q)RT-PCR analysis (Fig. 2*a* and 2*b*, respectively). Hence we decided to use siRNA to knock down expression of *Mael* in our *in vitro* germ-cell culture systems to determine the role of *Mael* in germ-cell development and differentiation, in particular, in oogenesis.

Effect of Mael knockdown on mouse fetal ovaries cultured in vitro

A mean of 198 oocytes at GV stage and showing a diameter of 40–70 μm had grown out of each fetal ovary after 7 days of culture in the negative control. The knockdown of *Mael* or *Dazl* expression in fetal ovary explants was accompanied by a significant reduction in oocytes at the GV stage to 58 and 60%, respectively (Fig. 3), thus suggesting an important role of *Mael* during the early oogenesis in mouse *in vitro*.

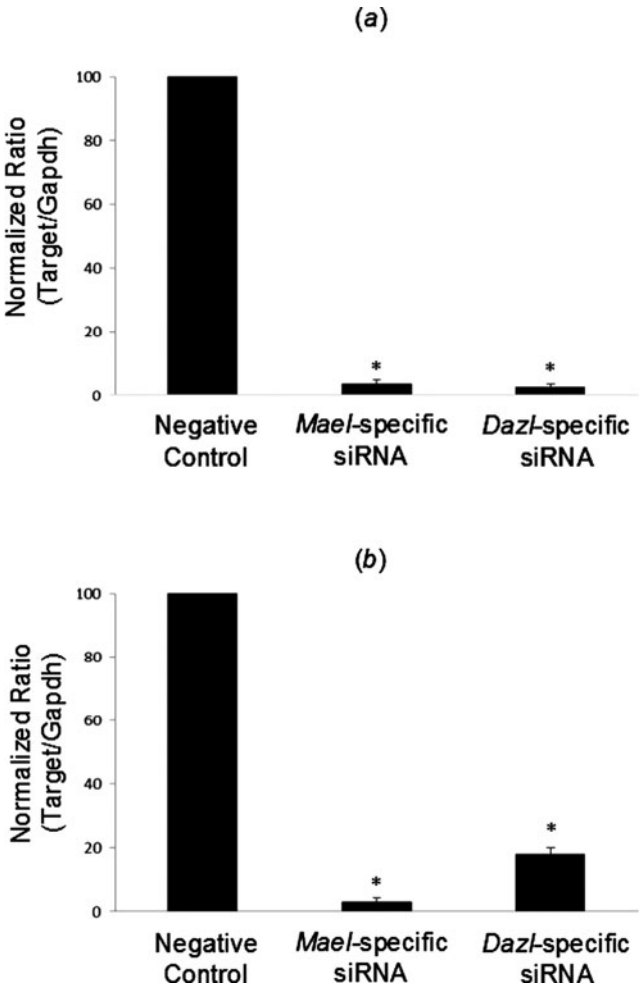

Figure 2 RNAi knockdown of *Mael* in mouse fetal ovaries and embryonic stem cells (ESC). (*a*) *Mael*-specific siRNA and corresponding controls (Negative Control RNAi duplex and *Dazl*-siRNA, used as positive control) were transfected into mouse fetal ovary explants. At 24 h post-transfection, expression of target and internal control genes was analysed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). (*b*) *Mael*-siRNA and corresponding controls were transfected into E14 ESC. At 24 h post-transfection, expression of target and internal control genes was analysed by qRT-PCR. The value for the cells treated with Negative Control RNAi duplex was set to 100%. Expression of *Mael* and *Dazl* is normalised to *Gapdh* and is presented as the fold change in expression relative to negative control. Data are expressed as the mean ± standard error of the mean (SEM). *Statistically significant changes in expression relative to the negative control ($P < 0.05$).

Effect of Mael knockdown on the differentiation of mouse ESC into germ cells

To establish whether *Mael* knockdown disrupts the differentiation of mouse ESC into germ cells, mouse ESC were transfected with *Mael*-specific siRNA

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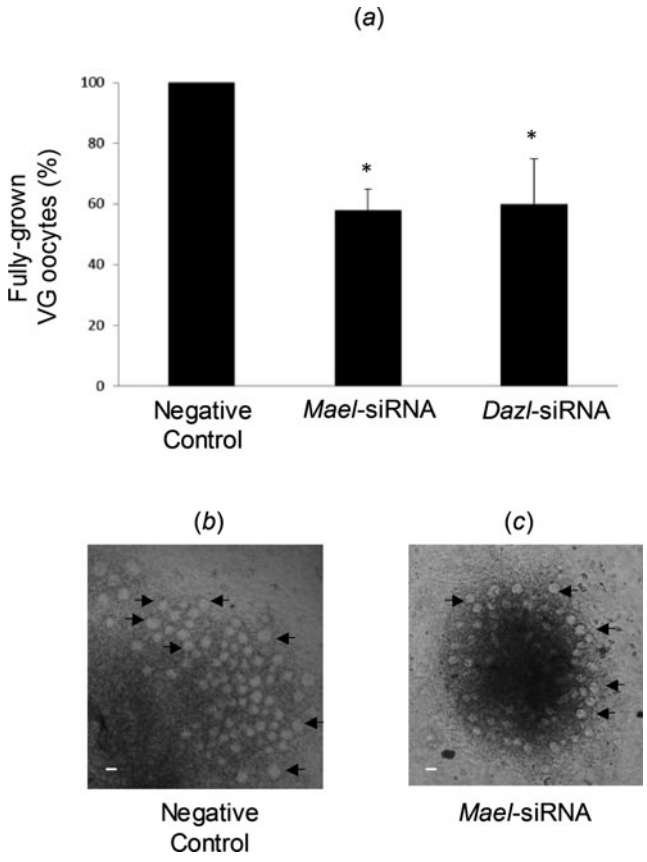

Figure 3 Mael knockdown disrupts the growth of oocytes in fetal ovaries in culture. On day 3 of culture, ovary explants were transfected with Negative Control RNAi duplex, *Mael*-siRNA or *Dazl*-siRNA (used as a positive control). (*a*) The number of oocytes at the germinal vesicle (GV) stage and showing a diameter of 40–70 μm grown out of each fetal ovary was analysed on day 7 of culture. Data are presented as the mean ± standard error of the mean (SEM). *When compared with the paired RNAi duplex negative controls, $P < 0.05$. (*b*) Representative fetal ovary explant transfected with Negative Control RNAi duplex. (*c*) Representative fetal ovary explant transfected with *Mael*-specific siRNA. Arrows show fully grown GV oocytes. Scale bar: 50 μm.

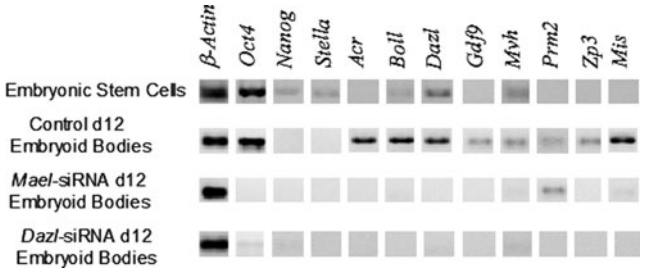

Figure 4 *Mael* knockdown disrupts the differentiation of mouse embryonic stem cells (ESC) into germ cells *in vitro*. ESC were transfected with Negative Control RNAi duplex, *Mael*-siRNA or *Dazl*-siRNA (used as a positive control). ESC were then cultured to induce their differentiation into germ cells via embryoid body formation. Differentiation of mouse ESC into germ cells was carried out on day 12 (d12) of culture by analysis of several germ-cell-specific markers and the Sertoli cell marker, *Mis*. Experiments were carried out in triplicate. Representative gels are shown.

and their response was analysed after 12 days of differentiation. ESC transfected with the Negative Control RNAi duplex were able to differentiate into germ-cell lineage, as indicated by the expression of germ-cell markers *Acr*, *Boll*, *Dazl*, *Gdf9*, *Mvh*, *Prm2*, *Zp3*, and the Sertoli cell marker *Mis* (Fig. 4). Nevertheless, in ESC transfected with *Mael*-siRNA, expression of most of the germ-cell markers was clearly disrupted, thus indicating an essential role of *Mael* for the differentiation of ESC into germ cells *in vitro*. A similar effect was observed when ESC were transfected with *Dazl*-siRNA (Fig. 4).

Discussion

Even when a number of key germ cell-specific genes have been identified, the mechanisms and networks involved in the regulation of germ-cell specification and development remain largely unknown (Thomson *et al.*, 2011). In the present study we were interested in analysing whether *Mael*, which encodes a transcription factor with an essential role in mouse spermatogenesis, is also necessary for mouse oogenesis and germ-cell differentiation from ESC *in vitro*.

The developmental analysis of *Mael* in this study showed that mRNA is detected in ovaries of mouse embryos from 13.5 dpc to 1 day postnatal. A similar expression pattern was found in mouse testes by immunolocalization (Aravin *et al.*, 2009). MAEL was expressed in gonocytes at 14.5, 16.5 and 18.5 dpc.

In order to determine if *Mael* is essential during the early oogenesis in mice *in vitro*, fetal ovary explants in culture were transfected with *Mael*-specific siRNA, as this RNAi strategy has been widely used in gene/protein function in several systems, including cultured hamster ovaries (Wang & Roy, 2006) and recently mouse fetal mouse ovaries (Xu *et al.*, 2011; Moreno *et al.*, 2012).

Mael mRNA levels were reduced significantly by 96.2% in comparison with fetal ovaries transfected with Negative Control RNAi duplex. The knockdown of *Mael* was more efficient than that observed in whole fetal mouse ovaries in culture using siRNA targeting the proliferating cell nuclear antigen (*PCNA*; mRNA levels reduced by 62%) (Xu *et al.*, 2011) and in mouse fetal ovary explants using siRNA targeting the skin–embryo–brain–oocyte transcription factor *Sebox* and the germ cell-specific spermatogenesis

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and oogenesis basic helix–loop–helix transcription factor *Sohlh2* (mRNA levels reduced by 80%) (Moreno *et al.*, 2012).

The number of fully grown oocytes in GV stage was significantly reduced to 58% in fetal ovaries transfected with *Mael*-siRNA, in comparison with fetal ovaries transfected with Negative Control RNAi duplex, thus suggesting that *Mael* plays a relevant role in the early oogenesis in mice *in vitro*. Similar results were obtained with *Dazl*-siRNA (development of fully grown oocytes reduced to 60%), the positive control used in this study due to the essential role of *Dazl* in the differentiation of pre- and post-meiotic germ cells (Haston *et al.*, 2009; Kerr & Cheng, 2010). The fact that we have not observed a complete ablation of oocyte development in our RNAi experiments, even when *Mael* RNA was efficiently knockdown may be due to: (1) transfection of 100% of the cells cannot be achieved, especially in organ- and organ-explant cultures. In our study, cells in fetal ovary explants in culture were transfected efficiently (≥80%), but there are always cells that escape transfection and are able to have a normal development; and (2) even in transfected cells, a complete knockout of gene expression by RNAi cannot be achieved because of the characteristic mechanisms of siRNA action (Martinez et *al.*, 2002). It is often observed that transcription is efficiently knocked down by 24 h post-transfection, although, by 48 h, levels of inhibition are somewhat attenuated (Hough *et al.*, 2006). In our RNAi experiments, oocytes in which *Mael* transcription knockdown was not achieved efficiently could have had an apparently normal development. However, the normal development of oocytes transfected with *Mael*-siRNA is still to be confirmed as, in a previous study with mice lacking *Sohlh2*, it was observed that even when adult female mice are infertile due to lack of ovarian follicles, *Sohlh2*-deficient ovaries can form primordial follicles. But the primordial oocytes are abnormal at the molecular level because they misexpress several germ cell- and oocyte-specific genes, causing their rapid lost; few are present by 14 days of postnatal life (Choi *et al.*, 2008). A similar result was obtained when the role of *PCNA* in the folliculogenesis in mice was studied (Xu *et al.*, 2011). Although more primordial follicles were observed after *PCNA* RNAi, the number of primary follicles decreased, accompanied by downregulation of several key regulators of the transition from primordial to primary follicles. Expression analysis of these key regulators in oocytes transfected with *Mael*-siRNA is being carried out in our laboratory.

As *maelstrom* is required for microtubule organization in *Drosophila* oocytes (Clegg *et al.*, 2001), it will also be very interesting to analyse whether *Mael* is important for microtubule organization in mouse oocytes during the early oogenesis and especially during oocyte maturation, when microtubule organization disruption can be easily assessed as it would impact spindle organization, kinetochore assembly, and microtubule growth or responsiveness (Kim *et al.*, 2008).

Our results also show that *Mael* mRNA levels in mouse ESC transfected with *Mael*-specific siRNA were significantly reduced by 97% in comparison with ESC transfected with Negative Control RNAi duplex. Transfection of ESC with the positive control, *Dazl*-siRNA, resulted in a significant reduction of *Dazl* mRNA by 82%. Downregulation efficiency of the targeted genes was similar to that observed in other studies in which siRNA was used to block the expression of specific genes in ESC (Hyslop *et al.*, 2005; Hough *et al.*, 2006).

Differentiation of male and female germ cells out of the *Mael* knockdown ESC was clearly disrupted. Expression of the male and female germ-cell markers used was very low or undetectable compared with their expression in ESC transfected with Negative Control RNAi duplex. These results indicate a pivotal role of *Mael* in germ-cell differentiation from ESC in mouse *in vitro* and resemble what has been observed in *Drosophila*, where *maelstrom* has been reported to be necessary for proper germline stem cell lineage differentiation (Pek *et al.*, 2009), thus indicating that *Mael* is a phylogenetically conserved regulator of germ-cell development.

Previous studies have shown that fruit fly *maelstrom* and mouse *Mael* genes are necessary for the repression of transposable elements (TE) via a particular class of small RNAs known as piRNAs, for their association with PIWI proteins (O'Donnell *et al.*, 2008; Aravin *et al.*, 2009; Pek *et al.*, 2009). Derepression and mobilization of active TEs has been implicated in gene mutation, abrogation or perturbation of gene expression, and DNA damage (Han & Boeke, 2005; Girard & Hannon, 2008; Soper *et al.*, 2008). Additional studies are necessary to determine if the effects of *Mael* knockdown during the early oogenesis and the germ-cell differentiation observed in the present study are related to transposon derepression.

In summary, evidence of an essential role of *Mael* during the early oogenesis and the differentiation of ESC into germ cells in mouse *in vitro* is presented. Unravelling of the molecular mechanisms underlying these processes is essential for a better understanding of the genetics of infertility.

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References

Aravin, A.A., van der Heijden, G.W., Castaneda, J., Vagin, V.V., Hannon, G.J. & Bortvin, A. (2009). Cytoplasmic compartmentalization of the fetal piRNA pathway in mice. *PLoS Genet.* **5**, e1000764.

Bonilla, E. & del Mazo, J. (2010). Deregulation of the Sod1 and Nd1 genes in mouse fetal oocytes exposed to mono-(2-ethylhexyl) phthalate (MEHP). *Reprod. Toxicol.* **30**, 387–92.

Bonilla, E. & Xu, E.Y. (2008). Identification and characterization of novel mammalian spermatogenic genes conserved from fly to human. *Mol. Hum. Reprod.* **14**, 137–42.

Chen, W., Jia, W., Wang, K., Zhou, Q., Leng, Y., Duan, T. & Kang, J. (2012). Retinoic acid regulates germ cell differentiation in mouse embryonic stem cells through a Smad-dependent pathway. *Biochem. Biophys. Res. Commun.* **418**, 571–7.

Childs, A.J., Saunders, P.T. & Anderson, R.A. (2008). Modelling germ cell development *in vitro*. *Mol. Hum. Reprod.* **14**, 501–11.

Choi, Y., Yuan, D. & Rajkovic, A. (2008). Germ cell-specific transcriptional regular sohlh2 is essential for early mouse folliculogenesis and oocyte specific gene expression. *Biol. Reprod.* **79**, 1176–82.

Clark, A.T., Bodnar, M.S., Fox, M., Rodriquez, R.T., Abeyta, M.J., Firpo, M.T. & Pera, R.A. (2004). Spontaneous differentiation of germ cells from human embryonic stem cells *in vitro*. *Hum. Mol. Genet.* **13**, 727–39.

Clegg, N.J., Findley, S.D., Mahowald, A.P. & Ruohola-Baker, H. (2001). Maelstrom is required to position the MTOC in stage 2–6 *Drosophila* oocytes. *Dev. Genes Evol.* **211**, 44–8.

Costa, Y., Speed, R.M., Gautier, P., Semple, C.A., Maratou, K., Turner, J.M. & Cooke, H.J. (2006). Mouse MAELSTROM: the link between meiotic silencing of unsynapsed chromatin and microRNA pathway? *Hum. Mol. Genet.* **15**, 2324–34.

De Felici, M. (1991). *In vitro* culture systems form germ cells from mouse embryo: primordial germ cells and oocytes. In: Mazo, J.D. (ed.) *Reproductive Toxicology: In Vitro Germ Cell Developmental Toxicology from Science to Social and Industrial Demand*. New York: Plenum Press, pp. 41–50.

Geijsen, N., Horoschak, M., Kim, K., Gribnau, J., Eggan, K. & Daley, G.Q. (2004). Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature* **427**, 148–54.

Ginsburg, M., Snow, M.H. & McLaren, A. (1990). Primordial germ cells in the mouse embryo during gastrulation. *Development* **110**, 521–8.

Girard, A. & Hannon, G.J. (2008). Conserved themes in small-RNA-mediated transposon control. *Trends Cell Biol.* **18**, 136–48.

Han, J.S. & Boeke, J.D. (2005). LINE-1 retrotransposons: modulators of quantity and quality of mammalian gene expression? *Bioessays* **27**, 775–84.

Haston, K.M., Tung, J.Y. & Reijo Pera, R.A. (2009). Dazl functions in maintenance of pluripotency and genetic and epigenetic programs of differentiation in mouse primordial germ cells *in vivo* and *in vitro*. *PLoS One* **4**, e5654.

Hough, S.R., Clements, I., Welch, P.J. & Wiederholt, K.A. (2006). Differentiation of mouse embryonic stem cells after RNA interference-mediated silencing of OCT4 and Nanog. *Stem Cells* **24**, 1467–75.

Hübner, K., Fuhrmann, G., Christenson, L.K., Kehler, J., Reinbold, R., De La Fuente, R., Wood, J., Strauss, J.F. 3rd, Boiani, M. & Schöler, H.R. (2003). Derivation of oocytes from mouse embryonic stem cells. *Science* **300**, 1251–56.

Hyslop, L., Stojkovic, M., Armstrong, L., Walter, T., Stojkovic, P., Przyborski, S., Herbert, M., Murdoch, A., Strachan, T. & Lako, M. (2005). Downregulation of NANOG induces differentiation of human embryonic stem cells to extraembryonic lineages. *Stem Cells* **23**, 1035–43.

Kee, K., Angeles, V.T., Flores, M., Nguyen, H.N. & Reijo Pera, R.A. (2009). Human DAZL, DAZ and BOULE genes modulate primordial germ-cell and haploid gamete formation. *Nature* **462**, 222–5.

Keller, G.M. (1995). *In vitro* differentiation of embryonic stem cells. *Curr. Opin. Cell Biol.* **7**, 862–9.

Kerr, C.L. & Cheng, L. (2010). The dazzle in germ cell differentiation. *J. Mol. Cell Biol.* **2**, 26–9.

Kim, K.H., Kim, E.Y. & Lee, K.A. (2008). SEBOX is essential for early embryogenesis at the two-cell stage in the mouse. *Biol. Reprod.* **79**, 1192–201.

Lawson, K.A., Dunn, N.R., Roelen, B.A., Zeinstra, L.M., Davis, A.M., Wright, C.V., Korving, J.P. & Hogan, B.L. (1999). Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes Dev.* **13**, 424–36.

Ling, V. & Neben, S. (1997). *In vitro* differentiation of embryonic stem cells: immunophenotypic analysis of cultured embryoid bodies. *J. Cell Physiol.* **171**, 104–15.

Makoolati, Z., Movahedin, M. & Forouzandeh-Moghadam, M. (2011). Bone morphogenetic protein 4 is an efficient inducer for mouse embryonic stem cell differentiation into primordial germ cell. *In Vitro Cell Dev. Biol. Anim.* **47**, 391–8.

Martinez, J., Patkaniowska, A., Urlaub, H., Lührmann, R. & Tuschl, T. (2002). Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* **110**, 563–74. McLaren, A. (2003). Primordial germ cells in the mouse. *Dev. Biol.* **262**, 1–15.

Moreno, D.L., Salazar, Z., Betancourt, M., Casas, E., Ducolomb, Y., González, C. & Bonilla, E. (2012). Sebox plays an important role during the early mouse oogenesis *in vitro*. *Zygote* **18**, 1–5.

O'Donnell, K.A., Burns, K.H. & Boeke, J.D. (2008). A descent into the nuage: the maelstrom of transposon control. *Dev. Cell* **15**, 179–81.

Pangas, S.A. & Rajkovic, A. (2006). Transcriptional regulation of early oogenesis: in search of masters. *Hum. Reprod. Update* **12**, 65–76.

Pedersen, T. & Peters, H. (1968). Proposal for a classification of oocytes and follicles in the mouse ovary. *J. Reprod. Fertil.* **17**, 555–7.

Pek, J.W., Lim, A.K. & Kai, T. (2009). *Drosophila* maelstrom ensures proper germline stem cell lineage differentiation by repressing microRNA-7. *Dev. Cell* **17**, 417–24.

Soper, S.F., van der Heijden, G.W., Hardiman, T.C., Goodheart, M., Martin, S.L., de Boer, P. & Bortvin, A. (2008). Mouse maelstrom, a component of nuage, is

essential for spermatogenesis and transposon repression in meiosis. *Dev. Cell* **15**, 285–97.

Thomson, M., Liu, S.J., Zou, L.N., Smith, Z., Meissner, A. & Ramanathan, S. (2011). Pluripotency factors in embryonic stem cells regulate differentiation into germ layers. *Cell* **145**, 875–89.

Toyooka, Y., Tsunekawa, N., Akasu, R. & Noce, T. (2003). Embryonic stem cells can form germ cells *in vitro*. *Proc. Natl. Acad. Sci. USA* **100**, 11457–62.

Wang, C. & Roy, S.K. (2006). Expression of growth differentiation factor 9 in the oocytes is essential for the development of primordial follicles in the hamster ovary. *Endocrinology* **147**, 1725–34.

Xu, B., Hua, J., Zhang, Y., Jiang, X., Zhang, H., Ma, T., Zheng, W., Sun, R., Shen, W., Sha, J., Cooke, H.J. & Shi, Q. (2011). Proliferating cell nuclear antigen (PCNA) regulates primordial follicle assembly by promoting apoptosis of oocytes in fetal and neonatal mouse ovaries. *PLoS One* **6**, e16046.

Zhao, G.Q., Deng, K., Labosky, P.A., Liaw, L. & Hogan, B.L. (1996). The gene encoding bone morphogenetic protein 8B is required for the initiation and maintenance of spermatogenesis in the mouse. *Genes Dev.* **10**, 1657–69.

Zhou, G.B., Meng, Q.G. & Li, N. (2010). *In vitro* derivation of germ cells from embryonic stem cells in mammals. *Mol. Reprod. Dev.* **77**, 586–94.