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CICLO XXII

Transcriptional and post-transcriptional mechanisms control meiotic entry in mouse germ cells.

Florencia Barrios Abraham

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Docente Guida/Tutor: Prof. ssa Susanna Dolci

Coordinatore: Prof. Raffaelle Geremia

A mamá, papá e Ignacio

"Un padre e un figlio
con un solo abbraccio
squarciano il tempo
vanno oltre lo spazio"

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CHAPTER 1

INTRODUCTION.

INTRODUCTION.

Why do we study germ cells?

"The germ cell lineage carries the potential for both totipotency and immortality. It forms the fragile link between one generation and the next, and so is of central importance for the survival and evolution of living organisms." (McLaren, 1998)

Overview of germ cell development.

In *Drosophila*, in nematode worms, and in frogs, the germ cell lineage is set aside early in embryonic development due to the existence of cytoplasmic determinants (germ plasm, pole plasm). In mammals, the founder population of gametes, the primordial germ cells (PGCs), are induced to form in the epiblast at the onset of gastrulation as a result of BMP4 signaling from the extraembryonic ectoderm (Lawson and Hage, 1994; McLaren and Lawson, 2005; Ohinata et al., 2009) (Fig.1).

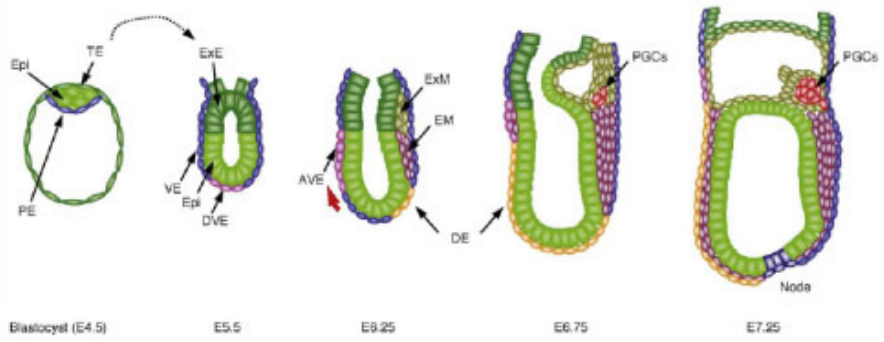

Figure 1. **PGCs specification.**

Schematic representation of early mouse development after 4.5 dpc and the emergence of TNAP-positive PGCs. Just before implantation, the mouse embryo contains three cell lineages: the trophoectoderm (TE), the epiblast (Epi), and the primitive endoderm (PE). The Epi will originate the whole fetus, including the PGCs, while the other two lineages originate the extraembryonic tissues needed for implantation. Signalling from these tissues drives the initial patterning of embryogenesis, including germ cell specification.

To be specified, PGCs sequentially express *Blimp1*, *Prdm14*, and *Stella*, three intrinsic factors that coordinate to repress the somatic mesodermal program, re-acquire the potential pluripotency, and to perform genome-wide epigenetic reprograming (Saitou, 2009a, b). In the murine embryo, by 7.25 days post coitum (dpc), a cluster of about 40 cells with the tipical stainning of alkaline phosphatase (TNAP) activity starts to migrate along the invaginating hindgut towards the genital ridges where they will arrive and settle between 10.5 – 12.5 dpc. During this period, PGCs proliferate at a steady rate every 16 hours irrespective of their sex. The SCF (Kit ligand)/ Kit (Kit receptor) system is of remarkable importance during this period of PGC development to guide migration and promote their survival and proliferation (McLaren, 1998). PGC fate diverges from 13.5 dpc onwards depending on the somatic environment that hosts them: male PGC residing in the fetal testes will enter into mitotic arrest while female PGC inside the ovary will enter meiosis. Female PGCs, now oocytes, will start entering the first meiotic division at 13.5 dpc and progress through leptotene, zygotene, and pachytene stages to arrest after birth as diplotene oocytes inside primordial follicles (McLaren, 1998; Saga, 2008; Western, 2009). Male PGC, from now on referred to as gonocytes, will remain in the G0/G1 cell cycle arrest up to birth and will not enter meiosis before 7 days post partum (7dpp).

Mitotic arrest of male germ cells.

A cell fate choice is undertaken by germ cells when changing the proliferation pattern for a differentiative one. Control of this decision making is critical to safeguard normal development and avoid pathological pathways like for example teratoma formation (Western, 2009).

Cell cycle progression is regulated y the sequential acitivity of various cyclins. The CYCLINS are regulatory subunits that bind, activate and provide substrate specificity for their catallytic partner serine-threonine

kinases, collectively called cyclin-dependent kinases (CDKS). The activity of CYCLIN-CDK complexes is tightly regulated by a complex network of other proteins that function as activators and inhibitors as well as influencing their transcription, sub-cellular localization and degradation.

Murine germ cell's commitment to spermatogenesis rapidly follows somatic sex determination, which involves commitment of the Sertoli cell lineage and organization of the germ cells within the developing testis cords (Western, 2009). At this developmental stage, *Fgf9* expression is required to ensure somatic sex determination and promote germ cell survival in the testes (DiNapoli et al., 2006). By 13.5 dpc gonocytes start entering mitotic arrest, but male germ cell differentiation does not stop. During cell cycle arrest, gonocytes acquire male germ cell specific properties like the establishment of genomic imprinting. The entrance into cell cycle arrest occurs in parallel with the down-regulation of the pluripotency network expressed in proliferating PGCs. This is accomplished both by transcriptional and translational control of gene expression (Western, 2009). A candidate to mediate this features is the RNA-binding protein NANOS2 since in *Nanos2* null mice germ cells do not enter the G0/G1 stage and instead proceed through the M phase of the cell cycle (Saga, 2008; Suzuki and Saga, 2008; Western, 2009) (Fig.2).

Proliferating germ cells and somatic cells do not seem to significantly differ in the mechanisms that govern cell cycle (Sorrentino et al., 2007). However, particular cell cycle proteins are expressed in cycling mouse germ cells and not in quiescent germ cells, or vice versa (Western, 2009). Recent findings have shed new light into the mechanisms that underlie the mitotic arrest of gonocytes. Increasing evidence points to an important role of the phosphorylation of the G1-S phase checkpoint retinoblastoma protein 1 (pRB1) in this process (Spiller et al., 2009a; Spiller et al., 2009b; Western et al., 2008). pRB is hyperphosphorilated (inactive) in proliferating PGCs but becomes de-phosphorilated

(activated) in arresting germ cells. In quiescent cells, its expression is down-regulated and eventually abolished. So the transient activation of pRB1 in arresting germ cells may be related to the prevention of the G1/S transition. Its subsequent desapearence suggests that pRB1 activity is not necessary to maintain the quiescent state but just to induce it (Spiller et al., 2009b; Western, 2009; Western et al., 2008) (Fig. 2)

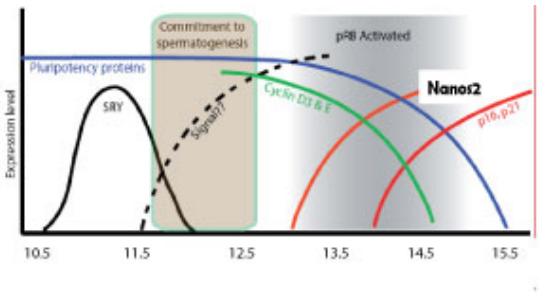

Figure 2. **Overview of fetal male germ cell development.**
Differentiation of the testes initiates in the somatic compartment upon the expression of Sry. After migration and proliferation, gonocytes start entering the mitotic arrest by 13.5 dpc. The male fate is safeguarded by the expression of NANOS2.

Interestingly, in humans, besides pRB1, also other genes suggested to be implicated in the mitotic arrest of gonocytes, are up-regulated in testicular germ cell tumors (TGCTs) (Spiller et al., 2009a; Spiller et al., 2009b; Western, 2009). This is the case for instance of *p63*, *p53*, and *Atm*, whose expression probably reinforces germline integrity controlling the DNA damage checkpoint (Spiller et al., 2009a). Also in line with this, ATM has been shown to be essential to govern cell cycle in pre-meiotic spermatogonia (Takubo et al., 2008).

Spermatogonial stem cell renewal and differentiation.

Two proliferative phases control spermatogonia maintenance and differentiation during postnatal life. The first one occurs when gonocytes exit the cell cycle arrest and resume mitosis after colonizing the basal lamina. This phase is governed by the Ret/GDFα receptor complex expressed by gonocytes which respond to GDNF produced by

the Sertoli cells (Golden et al., 1999; Meng et al., 2000). During this phase gonocytes, and then undifferentiated spermatogonia, expand their own pool while giving rise also to more differentiated spermatogonia. The second proliferative phase, defined as the Kit-dependent one, occurs immediately after that spermatogonia exit from the GDNF dependent proliferation. Spermatogonia start expressing the tyrosine kinase receptor Kit guided by its ligand, KL, produced by Sertoli cells (Besmer et al., 1993; Manova et al., 1993). During this phase, they undergo coordinated rounds of replication giving rise ultimately to preleptotene spermatocytes, the pre-meiotic cells which are committed to enter meiosis.

The spermatogenic niche.

Whole mount studies allowed the topographical description of murine seminiferous tubules (Clermont and Bustos-Obregon, 1968). During adulthood, the seminiferous tubules change in a cyclic manner as a consequence of the ongoing waves of spermatogenesis. Spermatogenesis is the process that leads to the generation of the functional male gamete, the spermatozoa. It starts with the mitotic proliferation of diploid male germ cells, the spermatogonia.

Spermatogonial stem cells (SSCs) are the only stem cells in the body that can be recognized and studied at the cellular level with respect to proliferation and differentiation, and the regulation of these activities. SSCs reside on the basal membrane of the seminiferous tubules and are completely surrounded by Sertoli cells that make up their niche (de Rooij, 2001; Oatley and Brinster, 2006). The first spermatogenic cell type to move away from this position towards the lumen of the tubule are the spermatocytes (de Rooij, 2001). Besides morphological identification of the spermatogonial cell types in normal or disturbed testes, transplantation and pulse-transplantation experiments have been the only methods to functional identify the spermatogenic stem cells (Nakagawa et al., 2007; Yoshida et al., 2007).

Historically, A-single (A_s) spermatogonia are considered the stem cells of spermatogenesis. A_s spermatogonia are single cells that upon mitosis can divide into two new stem cells. A-paired (A_p) produce daughter cells that remain connected by an intercellular bridge and further develop along the spermatogenic line. Mitotic divisions of A_p spermatogonia give rise to chains of 4, 8, 16, and eventually 32, A-ligned (A_l) spermatogonia (de Rooij, 2001; de Rooij and Mizrak, 2008; Huckins, 1971; Oakberg, 1971).

The proliferation of spermatogonia is controlled in such a way that the number of A_s and A_p spermatogonia remain relatively constant, while increasing the quantity of A_l spermatogonia. It is not clear yet whether spermatogonia stem cell divisions are symmetrical or asymmetrical (de Rooij, 2001). A_l spermatogonia become arrested in the G1-G0 phase of the cell cycle and subsequently, without division, they differentiate into A1 spermatogonia. The A1 spermatogonia enter the S phase, divide into A2 spermatogonia, after which through five subsequent divisions, A3, A4, ln and B spermatogonia and primary spermatocyes, are formed respectively. In total there are 9-11 mitotic divisions during spermatogonial development (de Rooij, 2001) (Fig. 3).

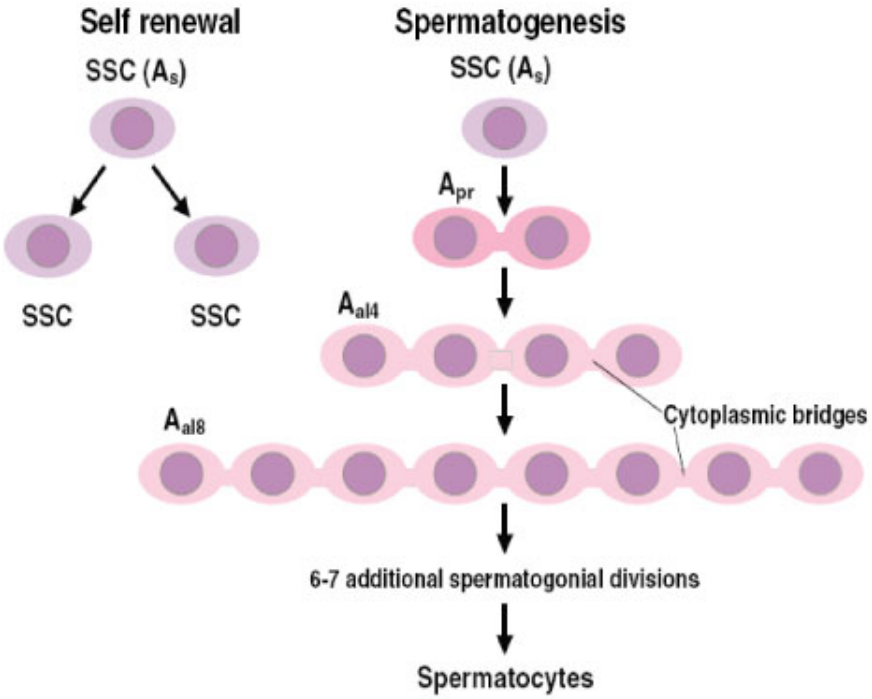

Figure 3. **Classical model for spermatogonial multiplication and stem cell renewal in rodents.** SSCs (purple) can self renew and give rise to two new single cells (A_s spermatogonia), or can differentiate, giving rise to daughter cells that stay together to form a pair of spermatogonia (A_{pr}, pink) that are linked through a cytoplasmic bridge. A_{pr} spermatogonia divide to originate A_{al} that subsequently form a chains of spermatogonia. After 9/10 divisions, spermatocytes form that, after meiotic divisions, give rise to spermatids that will in turn transformate into spermatozoa.

When A_s spermatogonia divides and gives rise to A_{pr} spermatogonia, the daughter cells remain interconnected and subsequently develop in a clonal manner. It has been suggested that the intercellular bridge that connects the daughter cells constitutes a differentiation step, but there is no experimental support that demonstrates whether this is an irreversible step or not (de Rooij, 2001). Moreover, there are no molecular or physiological properties that distinguish between A_s, A_{pr} or A_{al} spermatogonia (de Rooij, 2001; de Rooij and Mizrak, 2008; Oatley and Brinster, 2006; Yoshida et al., 2007). It has been proposed that A_s are the only spermatogonia with stem cell potential, 'the true SSC' (de Rooij, 2001; de Rooij and Mizrak, 2008), but this has been recently challenged (Barroca et al., 2009; Nakagawa et al., 2007). Another event

that has been historically considered as a differentiative step is the transition of A_{al} spermatogonia into A1. The duration of the cell cycle reduces in the later and the pattern of proliferation changes. In contrast to A_s, A_{pr} or A_{al}, the clones of A1-B spermatogonia do not proliferate randomly but are highly synchronized. If A1-B spermatogonia are unable to divide at the right time, they enter apoptosis (de Rooij, 2001).

Recently, a more 'plastic' view of the spermatogenic stem cell compartment has been proposed (Nakagawa et al., 2007; Yoshida et al., 2007). Pulse-labelling of inducibles transgenes, coupled to transplantation experiments, postulated the identification of the 'actual' and 'potential' stem cell compartments of the testes. The 'actual stem cells' would be the ones that retain the labeling even 3 months after the pulse and are able to immediately give rise to all stages of labelled differentiated spermatogenic cell types. The 'potential stem cells' arise from the observation that there is a significant number of cells that possess stem-cell potential but do not self-renew in the normal steady-state spermatogenesis (Nakagawa et al., 2007; Yoshida et al., 2007). According to this model, in the normal rounds of spermatogenesis, actual stem cells supply differentiating cells while maintaining their own population. Potential stem cells function instead as transit amplifying cells and do not take part in the self-renewing pool. In response to actual stem-cell loss or emptied stem-cell niche (either experimental or physiological), the potential stem cells may shift their mode from transit amplification to self-renewal and give rise to new actual stem cells (Nakagawa et al., 2007; Yoshida et al., 2007) (Fig. 4). In accordance with the classical model (de Rooij, 2001), it is still unknown whether cells in these compartmens possess distinct characteristics or whether the difference is gradual and each cell has a variable degree of potential to self-renew and to differentiate (Nakagawa et al., 2007; Yoshida et al., 2007).

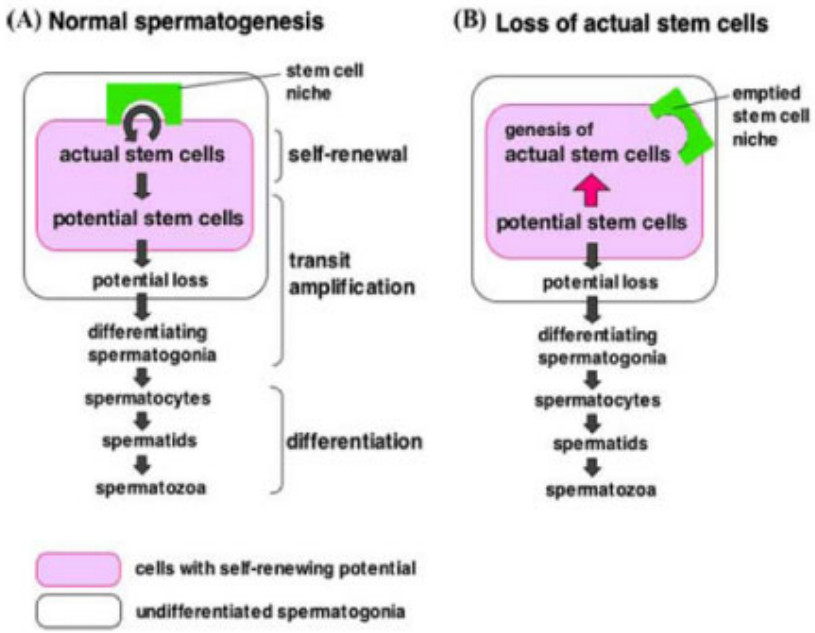

Figure 4. **'Plastic' view' of the spermatogenic stem cell compartment.**
A) In the normal situation, the stem-cell potential is not limited to the cells that actually self-renew in the stem-cell niche (actual stem cells); some of the transit amplyfing cells that do not self-renew may also possess the potential (potential stem cells). B) If actual stem cells are lost, the potential cells switch their mode from transit amplification to self-renewal, originating the newactual stem cells.

The GDNF phase.

Maintaining a balance of stem cell quiescence and activity is a hallmark of a functional niche (Moore and Lemischka, 2006). The balance between self-renewal and differentiation of spermatogonia is tightly controlled both by growth factors secreted from Sertoli cells and by the intrinsic network of transcription factors expressed by spermatogonia. More self-renewal than proliferation would lead to an enrichment of SSCs in the tubules at expenses of spermatogonia differentiation. If differentiation preveals, then the SSCs would deplete themselves, avoiding the generation of new germ cells (de Rooij, 2001).
The master regulator of the maintainance of the stem cell fate in the spermatogenic niche is the Glial cell line-derived neurotrofic factor (GDNF) secreted from Sertoli cells (Meng et al., 2000). A subset of

undifferentiated spermatogonia express the GDNF receptor complex including the ret proto-oncogene (RET) and its co-receptor GDNF familiy receptor alpha 1 (GFRA1) (Golden et al., 1999). *Gdnf*-, *Gfra1*-, and *Ret*-null testes have severe depletion of SSCs a week after birth presumably due to the lack of proliferation of SSCs and their inability to maintain the undifferentiated state (Naughton et al., 2006). The action of GDNF in the maintainance of SSCs is mediated by Akt and Src family kinases (Oatley et al., 2007). GDNF modulates the expression of transcription factors such as *Bclb6, Erm,* and *Lhx1* (Oatley et al., 2006). So far, no putative targets for these transcription factors have been proposed. Curiously, the expression of the pluripotency factors *Oct4* and *Sox2*, which also characterize SSCs, was differentially affected by GDNF being *Sox2* down-regulated and *Oct4* not modulated (Oatley et al., 2006). Moreover, notwithstanding its key role in SSC (see below), *Plzf* expression was not altered either by GDNF (Oatley et al., 2006) supporting the notion that also other growth factors are important to safeguard the SSC niche. In line with this, several findings suggest a critical function of the tryosine kinase receptor of the *Platalet-derived Growth Factor* (PDGF) in guiding, from Leydig cells, gonocytes migration and survival during testes development (Basciani et al., 2008; Gnessi et al., 2000; Nurmio et al., 2007).

PLZF (Promyelocytic Leukemia Zinc Finger, *Zfp145*) is a transcription factor critical for the maintance of SSC fate (Buaas et al., 2004; Costoya et al., 2004; Filipponi et al., 2007). Spontaneous or directed mutagenesis of *Zfp145 locus* leads to a rapid exhaustion of the proliferative spermatogonial compartment causing male sterility (Buaas et al., 2004; Costoya et al., 2004). Although other targets may not be excluded, currently the demonstrated target for PLZF's action in the male germline is the hallmark of differentiating spermatogonia, *Kit* (Filipponi et al., 2007).

Recently, two RNA-binding proteins homologs to *Drosophila* NANOS (*Nos*) that were initially described in embryonic and fetal germ cells (Tsuda et al., 2003) have emerged as markers of SSCs, namely

NANOS2 (Sada et al., 2009; Suzuki et al., 2009) and NANOS3 (Lolicato et al., 2008; Suzuki et al., 2009). Both proteins seem to be important for men fertility (Kusz et al., 2009a; Kusz et al., 2009b) and problably modulate gene expression at the post-transcriptional level (Yamaji et al., 2009). *Nanos3* starts being expressed during the migration and proliferation of PGCs (Suzuki et al., 2008; Tsuda et al., 2003), probably is re-expressed in gonocytes around birth (Yamaji et al., 2009), and is then restricted to proliferating spermatogonia in the postnatal testes (Lolicato et al., 2008; Suzuki et al., 2009). *Nanos2* expression starts in concomitance with gonocyte's mitotic arrest (Tsuda et al., 2003) and is maintained through the postnatal life (Sada et al., 2009). Its conditional deletion deplets the SSC compartment leading to sterility and its overexpression enhances the accumulation of SSCs (Sada et al., 2009). The forced expression of male restricted *Nanos2* in female PGCs has been associated to the inhibition of the spontaneous meiotic entry of the female germline, supporting the idea that NANOS2 has an anti-meiotic function (Saga, 2008; Suzuki and Saga, 2008).

Other factors that have also been associated with the molecular phenotype of SSC are *Oct4* and *Sox2*. These well known transcription factors are essential both for germline development and the maintance of pluripotency in embryonic stem cells. Contrasting data exists on whether the third pluripotency parter, *Nanog*, is expressed (Guan et al., 2006; Seandel et al., 2007) or not (Oatley et al., 2006) in SSCs. A similar situation arise also when considering *Stra8* (Stimulated by Retinoic Acid gene 8) and whether its mRNA is (Guan et al., 2006; Seandel et al., 2007) or is not (Oatley et al., 2006) found in SSC lines. One of the causes for such discrepancies could be the different starting populations used to derive SSC lines with similar developmental potential.

The Kit phase

The cell surphace tyrosine-kinase receptor *Kit* is a fundamental gene for mammalian development. In mice, *Kit* is encoded by the *W* locus

and loss of function mutations in this genomic region are at the bases of pathologies such as severe anemia (affecting hematopoietic stem cells, HSCs), pigmentation defects (through alterations in melanocyte's development), and sterility (due to PGC loss). In humans, gain of function mutations that hyperactivate *Kit* signaling are associated with blood, skin, gastrointestinal, and germ cell tumors. Due to this time and tissue divergent expression pattern, the mechanisms that orchestrate *Kit* expression and activity are subject of active research.

Kit is a longstanding molecular marker of spermatogonia undergoing differentiation (Manova et al., 1993; Manova et al., 1990; Ohta et al., 2000; Packer et al., 1995; Schrans-Stassen et al., 1999; Sorrentino et al., 1991; Vincent et al., 1998; Yoshinaga et al., 1991). During postnatal development of the male germline, *Kit* starts being expressed from 6 days of age onward from type A2 spermatogonia through type B spermatogonia and into preleptotene spermatocytes (Manova et al., 1990; Sorrentino et al., 1991). KIT is critical for survival (Yan et al., 2000), proliferation (Manova et al., 1993), and meiotic entry of spermatogonia (Pellegrini et al., 2008; Vincent et al., 1998).

Regulation of meiotic entry.

Meiosis is a fundamental process for sexual reproduction. Meiosis turns diploid germ cells into haploid gametes. While creating genetic variability it ensures that a new embryo has the right genetic constitution. Alterations in the different steps of the meiotic process determine fertility and congenital pathologies. This is why studying the cellular and molecular biology of meiosis, as a part of germ cell development, is so important.

What drives mammalian germ cells to cease their mitotic divisions and irreversibly commit to enter meiosis is a major topic in the field of reproductive biology (Bowles and Koopman, 2007; McLaren, 1998; Saga, 2008; Wolgemuth, 2006). Studies on model eukaryotes like *Saccharomyces cerevisae* or *Schizosaccharomyces pombe* have shed

pivotal light on the genetics of meiosis and in fact, many genes implicated on this process are evolutionary conserved. However, the molecular nature of the signals that induce cells to enter meiosis, the very first step in triggering the subsequent differentiation, seems to be less well conserved (Bowles and Koopman, 2007; Wolgemuth, 2006).

Substantial debate has been on going to elucidate if the signal that induces meiotic entry is extrinsic or intrinsic to germ cells. The theory supporting the existance of an extrinsic factor, a 'meiosis-inducing substance', that may determine the meiotic entry of fetal oocytes arose after sublte experiments using conditioned media to stimulate the entrance into meiosis of fetal male germ cells (Byskov, 1974; Byskov et al., 1995; Byskov and Saxen, 1976). However, for a long time, the 'meiosis-inducing substance' was not identified. Researcher's attention started to focus on the putative existence of both an intrinsic factor from germ cells or an extrinsic meiosis-inhibiting substance (Bowles and Koopman, 2007). Support to the "intrinsic clock' theory came from studies that mainly used the generation of chimeras between PGC and a variety of somatic tissues as a model to study meiotic entry. It was observed that if PGC mismigrate or are experimentally located in a physological environment different from the gonadal ridges, they enter meiosis irrespective of their genetic constitution. This suggest that timing of entry into meiosis could be determined by the number of mitoses occurring after the germ cell lineage was stablished or after PGCs started migration (McLaren, 2003; McLaren and Southee, 1997). It is important to note that the these experiments (McLaren and Southee, 1997) evaluated meiotic entry and oocyte growth, and genetic constitution was demonstrated to be important for meiotic progression and the formation of functional gametes (Bowles and Koopman, 2007). The 'intrinsic clock' theory has a corollary which is that an overriding mechanism must operate in the fetal testis to actively block entry into meiosis (Bowles and Koopman, 2007). Both theories highlight that testes architecture must be physiologically significant to inhibit meiotic entry of gonocytes (Bowles and Koopman, 2007). Dissociation and

reaggregation of the fetal urogenital ridges may allow germ cells to enter meiosis. This effect is observerd in a restricted developmental window and may be dependent on the experimental procedures used (Dolci and De Felici, 1990; McLaren and Southee, 1997).

Recently, by coupling classical embryology to the generation of transgenic mice that allows both gene knock-out and cell tracing experiments, a clear cut in the field has been achieved partially supporting the two previous theories (Baltus et al., 2006; Bowles et al., 2006; Koubova et al., 2006; Lin et al., 2008; Suzuki and Saga, 2008). Sexual dimorphism exists in the timing of meiotic entry since murine female germ cells enter meiosis during fetal life while male germ cells do it postnatally. Notwithstanding this, the mechanisms that trigger meiotic entry seem to be conserved between female and male germ cells (Koubova et al., 2006).

Retinoic acid (RA) has a myriad of physiological roles in embryonic and postnatal development. Since the demonstration that the sterility of vitamin A-deficient (VAD) mice could be reverted by injection of retinol and/or RA (Morales and Griswold, 1987; van Pelt and de Rooij, 1990), RA has gain also a role in this field. Later it was shown that the product of the *Stimulated by retinoic acid gene 8 (Stra8)* was the mediator of RA's effect in meiotic entry: *Stra8* ablation leads to sterility of male mice due to accumulation of premeiotic germ cells (Oulad-Abdelghani et al., 1996). The current model to describe the control of meiotic entry in both female and male germ cells posits that RA acts as an extrinsic meiosis-inducing substance on mitotic germ cells and that STRA8 is the downstream intrinsic factor that determines the last round of premeiotic DNA replication (Bowles et al., 2006; Koubova et al., 2006). During development of the urogenital ridge, RA is produced by the mesonephros of either male or female embryo (Bowles et al., 2006). The translation of *Stra8* instead, is timely divergent among sexes, but in both cases it immediately preceeds the meiotic entry. STRA8 is found in the female germ cells between 12.5 and 16.5 dpc, while in the male germline it is observed for the first time by 8 dpp and

then it holds restricted to spermatogonia during adult life: (Koubova et al., 2006; Oulad-Abdelghani et al., 1996). In the fetal testes, two non soluble factors exist, one extrinsic and one intrinsic to germ cells, to prevent gonocytes from entering meiosis. Within the somatic compartment of the developing genital ridge, there is an enzyme that catabolizes RA in order to avoid precocious entry into meiosis of proliferating PGCs. The expression of this enzyme of the P450 cytochrome group, *Cyp26b1,* becomes restricted to Sertoli cell precursors and interstitial cells by the time of sexual differentiation of the gonad (12.5 dpc) (Bowles et al., 2006; Koubova et al., 2006) (Fig. 5). In concomitance with the start of this bimodal expression of *Cyp26b1*, the RNA binding protein NANOS2 starts being expressed in the male germline to intrinsically suppress the female developmental pathway (i.e. entrance into meiosis) and safeguard the male fate (Saga, 2008; Suzuki and Saga, 2008; Tsuda et al., 2003).

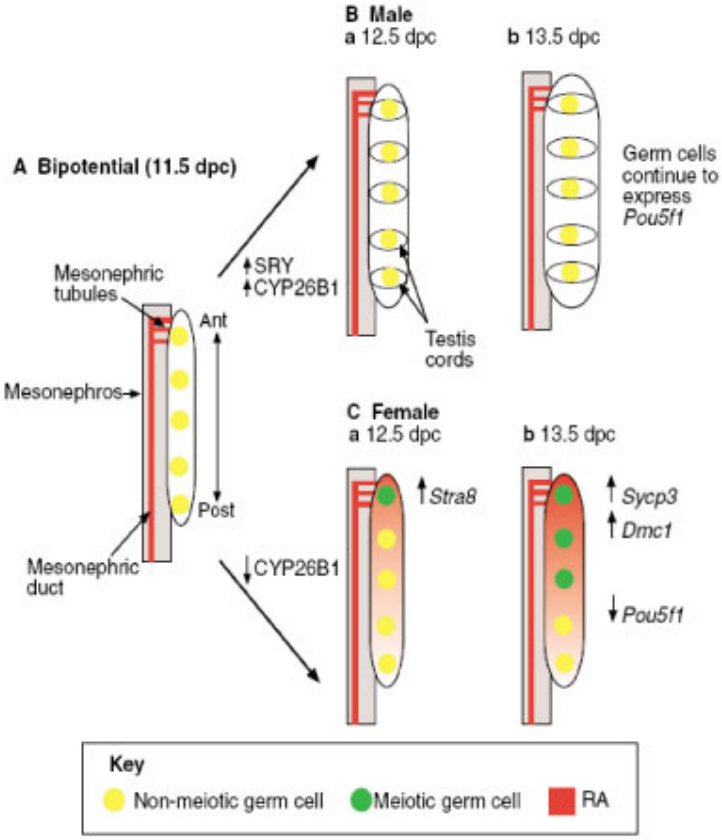

Figure 5. **Regulation of germ cell entry into meiosis in the developing gonadas.**
In the bipotential gonad, by 11.5 dpc, RA is found in the mesonephros of both sexes and also *Cy26b1* is expressed in both sexes within somatic cells. By 12.5 dpc, expression of *Cyb26b1* is found exclusively in the males, and this prevents meiotic entry. In the female, by the same time, *Cyp26b1* dissapears from the somatic compartment and circulating RA induces meiosis in germ cells.

The mechanism by which RA induces the expression of *Stra8* and the entrance into meiosis is mediated by RA receptors (RAR) in both female and male germ cells (Koubova et al., 2006; Pellegrini et al., 2008). Although the major mediator of RA's effect, up to now, is STRA8 (see above), the existance of other mediators, intrinsic or extrinsic to germ cells, is not excluded (Bowles and Koopman, 2007). In line with this, it has been demonstrated that RA significantly induces the expression of *Kit* in spermatogonia (Pellegrini et al., 2008; Zhou et al., 2008); and this effect is paralled by an increase in meiotic entry which is enhanced

by the co-stimulation with Kit ligand in long term cultures (Pellegrini et al., 2008). Moreover, meiotic entry of female germ cells is accompained by the modulation of a variety of genes, and this is inhibited by RAR antagonists (Bowles et al., 2006; Bowles and Koopman, 2007; Koubova et al., 2006). It cannot be rulled out the posibility that this effect of RA is mediated directly or indirectly by STRA8, since it has been shown that STRA8 is able to bind to chromatin in the male germline (Mark et al., 2008) and modulate gene activity within an heterologous system (Tedesco et al., 2009).

Besides vertebrate's germline, no other tissue express *Stra8* nothwithstanding that RA is widespread throughout the embryo (Bowles and Koopman, 2007; Oulad-Abdelghani et al., 1996). This may be due to a particular epigenetic configuration of the germline (Bowles and Koopman, 2007; Seki et al., 2005). In line with this, it has recently been shown that the expression of *Stra8* is sensitive to the modulation of histone deacethylase activity (Wang and Tilly, 2010).

The function of STRA8 has not been described yet, but is necessary for premeiotic DNA replication and progression of the meiotic prophase both in the female and the male germline (Anderson et al., 2008; Baltus et al., 2006; Mark et al., 2008). In both female and male *Stra8* null mice, there are germ cells that escape from the prevailing phenotype by the time of meiotic commitment but are anyhow lost later (Baltus et al., 2006; Mark et al., 2008). These cells have been conceptually related to the undifferentiated "poised" PGC which, irrespective of their sex, may enter meiosis if adequately stimulated during a precise time-window (Bowles and Koopman, 2007; McLaren and Southee, 1997; Saga, 2008). Nothwithstanding that male PGCs normally do not enter meiosis, they show chromosome condensations suggestive of meiotic entry (McLaren and Southee, 1997) and expression of meiotic markers at the protein level (Di Carlo et al., 2000). This developmental window in which germ cells are competent to enter meiosis irrespective of their genetic constitution may now be

characterized at the molecular level by the expression of the RNA binding protein DAZL *(Deleted in azoospermia–like)*. *Dazl* is expressed in proliferating PGCs and post-natal spermatogonia and this seems to be a prerequisite for RA – mediated induction of *Stra8* and meiotic commitment (Lin et al., 2008).

AIM OF THE THESIS.

We have studied two kinds of factors that are related to the proccess of meiotic entry.

Considering the RNA-binding protein NANOS2 as a determinant of SSC fate, we wanted to investigate how RA and FGF9 balance stemness and differentiation of spermatogonia. The outcomes of these studies are presented as the second and third chapters of this Thesis: the work by (Pellegrini et al., 2008) and our recently accepted paper in *Journal of Cell Science*.

To unravel the molecular network that governs *Kit* expression and activation, and how this affects germ cell development has historically been the central interest of our group. We now aim to characterize the role played by two germline-specific transcription factors in the regulation of *Kit* expression. These studies are currently on going and are thus presented as *Manuscript in preparation* in the fourth chapter of this Thesis: *"The role of Sohlh transcription factors on spermatogonia differentiation"*.

CHAPTER 2

ATRA and KL promote differentiation toward the meiotic program of male germ cells.

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Report

ATRA and KL promote differentiation toward the meiotic program of male germ cells

Manuela Pellegrini,[1] Doria Filipponi,[1] Manuele Gori,[1] Florencia Barrios,[1] Francesca Lolicato,[1] Paola Grimaldi,[1] Pellegrino Rossi,[1] Emmanuele A. Jannini,[2] Raffaele Geremia[1] and Susanna Dolci[1,*]

[1]Department of Public Health and Cellular Biology; University of Rome 'Tor Vergata'; Rome; [2]Department of Experimental Medicine; Università dell'Aquila; L'Aquila, Italy

Key words: kit, retinoic acid, meiosis, spermatogenesis

While it is known that Retinoic Acid (RA) induces meiosis in mouse female fetal gonads, the mechanisms which regulate this process during spermatogenesis are poorly understood. We show that the All trans RA derivative (ATRA) and Kit Ligand (KL) increase meiotic entry of postnatal mouse spermatogonia in vitro without synergism. Competence to enter meiosis is reached by spermatogonia only at the stage in which they undergo Kit-dependent divisions. Besides increasing Kit expression in spermatogonia, ATRA also upregulates KL expression in Sertoli cells. Both ATRA and KL increase the expression of Stimulated by Retinoic Acid Gene 8 and Dmc1, an early meiotic marker. A specific Kit tyrosine kinase inhibitor prevents the increase in the number of meiotic cells induced by both the two factors, suggesting that they converge on common Kit-dependent signalling pathways. Meiotic entry induced by ATRA and KL is independent from their ability to affect germ cell viability, and is mediated by the activation of PI3K and MAPK pathways through Kit autophosphorylation. ATRA-induced phosphorylation of the two downstream kinases is mediated by a non-genomic mechanism.

These data suggest that RA may control the timing of meiosis by influencing both the somatic and the germ cell compartment of the postnatal testis through the activation of the KL/Kit system.

Introduction

Spermatogenesis is the cyclic differentiative process of male germ cells, in which mitotic, meiotic and spermiogenetic phases orderly occur. During the mitotic phase, spermatogonia proliferate and continuously self-renew to give rise to two sub-populations of germ cells: the differentiated germ cells and the stem cells. One current model proposes that the A single (As) stem cells either renew themselves or divide into paired (Apr) daughter cells that remain connected by an intercellular bridge.[1,2] Apr spermatogonia divide further to form long chains of aligned (Aal) cells, which then generate the differentiating A1–A4, Intermediate, and Type B spermatogonia. This population of spermatogonia starts expressing the receptor-coupled tyrosine kinase Kit and becomes responsive to Kit Ligand (KL)[3] before entering the prophase of meiosis I, in which spermatocytes are formed. During meiosis I homologous chromosomes of spermatocytes align and are kept together tightly by a meiosis-specific nucleoprotein structure known as the synaptonemal complex, which is essential for the homologous recombination process.

Retinoids have been shown to be essential for spermatogenesis progression in human and mice.[4] Much of the role of retinoids has been studied using animal models kept on a diet deficient on vitamin A (VAD) or absent on vitamin A derivatives.[5] These animals are sterile because the seminiferous tubules contain only undifferentiated Kit negative spermatogonia, indicating a role of vitamin A in spermatogonia differentiation.[5] More recently it has been shown that the vitamin A derivative retinoic acid (RA) (either as all-trans or as 9-cis retinoic acid) promotes the expression of Stimulated by Retinoic Acid Gene 8 (Stra8), a key regulator of mammalian meiosis,[6] and Kit expression in undifferentiated spermatogonia.[7,8] RA functions inside the nucleus recognising two different classes of retinoid receptors. Both classes (RARs and RXRs) consist of three types of receptors, α, β and γ, encoded by distinct genes[9] and transduce RA signal by binding directly to retinoic acid responsive elements (RARE). During post-natal development, each RAR is detected predominantly in a specific cell type of the seminiferous epithelium: RAR_α in Sertoli cells, RAR_β in round spermatids and RAR_γ in A spermatogonia.[10] RAR_α gene targeting specifically in Sertoli cells (RAR_α $Ser^{-/-}$) showed germ cell apoptosis and seminiferous epithelium dysfunctions related to the disruption of Sertoli cells cyclical gene expression, which preceded testis degeneration.[11] Deletion of RAR_β or RAR_γ, on the contrary, do not cause primary testis defects.[10,12]

It has been recently shown that RA produced by mesonephroi causes entry into meiosis of germ cells in the ovary, while its effect is prevented in the fetal testis by the action of the retinoid-degrading enzyme CYP26B1.[13,14] It has been proposed that a critical role in inducing meiosis in both female and male[6,15,16] is played by the RA-stimulated Stra8 gene; however it remains to be elucidated if RA regulates other meiosis promoting genes and which are the precise mechanisms that orchestrate meiotic entry in the postnatal testis.

The *Kit (White spotting locus)* gene, encoding the transmembrane receptor for Kit Ligand (KL) regulates proliferation, survival and/or

*Correspondence to: Susanna Dolci; Department of Public Health and Cell Biology; University of Rome Tor Vergata; Via Montpellier 1; Bldg. E Nord; Rome 00133 Italy; Tel.: +390672596252; Fax: +390672596268; Email: dolci@uniroma2.it

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migration of stem cells for gametogenesis,[17,18] haematopoiesis[19] and melanogenesis.[20] In postnatal germ cells, Kit is expressed specifically in differentiating spermatogonia and is downregulated at the time of meiotic entry.[3,21,22] The role of Kit/KL in the maintenance and proliferation of germ cells has been highlighted by a mouse model with a point mutation of *Kit* that eliminates the PI3K docking site Y719F.[23,24] While PGC proliferation in both sexes is not compromised during embryonic development, Kit(Y719F)/Kit(Y719F) males are sterile due to the lack of spermatogonia proliferation during the prepuberal period and an arrest of spermatogenesis at the premeiotic stages.

In this paper we show that both the retinoic acid derivative All trans Retinoic acid (ATRA) and KL can regulate meiotic entry of isolated mouse spermatogonia cultured in vitro by activating common signal transduction pathways.

Results

ATRA and KL increase meiotic cells in cultures of isolated mouse spermatogonia. It has been recently shown that ATRA induces meiosis in fetal germ cells in organ-culture systems,[13,30] however, it is not clear whether it may also influence meiosis in isolated postnatal germ cells. To define the developmental age at which spermatogonia from the CD1 strain start the expression of Kit, the molecular event that precedes entry into meiosis, we took advantage of the p18 transgenic line[25] which has been expanded for more than eight generations on a CD1 background. This transgenic line express EGFP under the control of c-kit promoter and first intron and show specific expression only in spermatogonia of the prepuberal testis.[26] Figure 1 shows that EGFP expression, and thus Kit, was absent at 1 dpp (Fig. 1A), appeared at 4 dpp in few small spermatogonial foci within the seminiferous tubules (Fig. 1B), and was spread at 7 dpp in discrete segments of the testicular tubules (Fig. 1C), showing the characteristic "wavy" expression pattern of mouse spermatogenesis. Having established the temporal pattern of Kit expression, we chose 7 dpp as the developmental age in which Kit was uniformly represented within all the tubules.

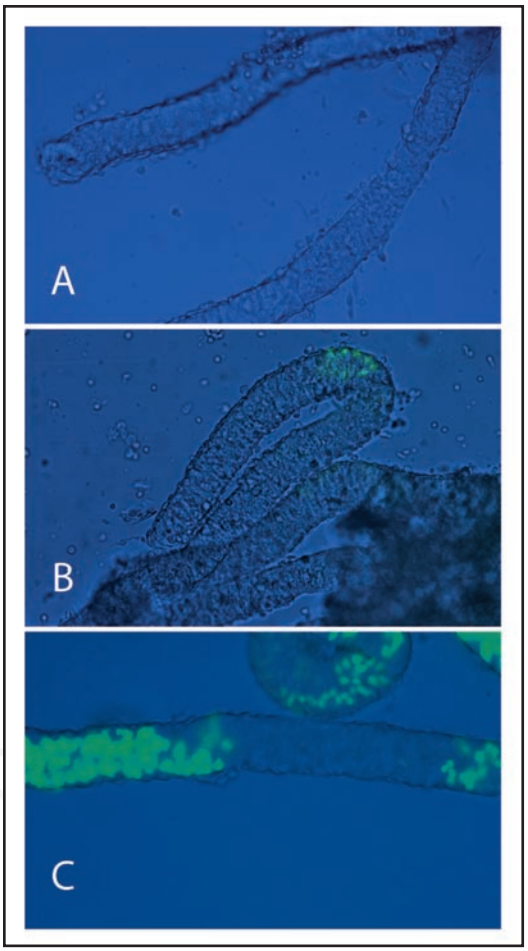

Figure 1. Kit-EGFP expression in neonatal and prepuberal testis tubules. Brightfield and fluorescent merged images of testicular tubules isolated from p18 transgenic mice expressing EGFP under the control of c-kit promoter and 3.5 kb of the first kit intron. The image shows testicular tubules at 1 dpn (A), at 4 dpn (B) and at 7 dpn (C).

We isolated spermatogonia from 7 dpn animals and cultured for 24 or 48 h in the presence of increasing concentrations of ATRA. We found that at 0.3 μM concentration, ATRA has a slight negative effect on cell viability compared to the control (75 ± 3% vs 85.5 ± 6% at 24 h, and 68.8 ± 5% vs 78 ± 5% at 48 h) and no significant mitogenic effect on 7 dpn spermatogonia (data not shown). We also stimulated spermatogonia with 100 ng/ml of KL, a growth factor which stimulates proliferation and survival of spermatogonia.[21,31,32] Nuclear spreads were prepared from cells at the beginning and at the end of the culture time and probed with antibodies against the synaptonemal complex protein 3 (SCP3) to detect meiotic nuclei and to evaluate the degree of meiotic progression (Figs. 2 and 3B). At the beginning of the culture we found that 4.6% showed preleptotene morphology (Fig. 2C, in which typical SCP3 spots are found) while 1.8% of the germ cell population showed early leptotene morphology (Fig. 2D). As shown in Figure 3A and B; and Table 1, after 24 h of culture more meiotic nuclei were found in control cells and their number further increased after 48 h of culture (2.5 ± 0.12 and 7 ± 0.11 fold increase vs T_0, respectively). Treatment with 0.3 μM ATRA induced a further significant increase of the percentage of meiotic nuclei after 24 and 48 h of culture (4.4 ± 0.12 and 12.8 ± 0.2 folds of increase vs T_0, respectively) (Fig. 3A). We determined that this increase was essentially due to cells accumulating at leptotene stage over 48 h of culture (Fig. 3B). Similarly to ATRA, addition of KL also increases the percentage of meiotic nuclei in cultured spermatogonia as measured by the number of nuclei in leptotene (4.5 ± 0.11 and 12.3 ± 0.6 fold of increase vs T_0 after 24 and 48 h, respectively) (Fig. 3A and B). Simultaneous addition of both ATRA and KL did not produce any further increase of meiotic nuclei (4 ± 0.1 and 11 ± 0.8 fold of increase vs T_0) either after 24 and 48 h of culture (Fig. 3A). The increased percentage of meiotic nuclei induced by ATRA or KL treatments was not associated to the increase of SCP3 protein levels, as evaluated by western blot analysis (data not shown), but rather to the typical pattern of SCP3 organization on the meiotic chromosomes (see Fig. 2). To confirm that ATRA and KL increased the number of meiotic cells in vitro, we also performed a quantitative RT-PCR using primers for Dmc1, which encodes a meiosis specific recombinase expressed during the first meiotic prophase,[33] and for the premeiotic marker Stra8 as positive control (Fig. 3C). Treatment with ATRA or KL for 24 h significantly increased Dmc1 levels (about 2 and 3.5 folds compared to the control, respectively), and as expected, ATRA caused a marked increase of Stra8 mRNA levels

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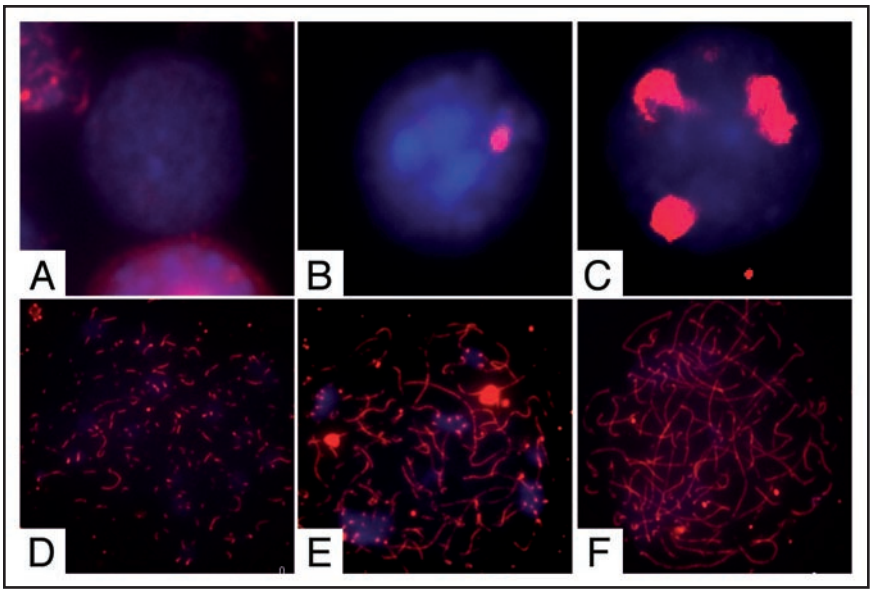

Figure 2. Pattern of SCP3 organization in male germ cells from 7 dpn mice. Representative merged immunofluorescence images showing SCP3 (red) organization on nuclear spreads (blue) in (A) undifferentiated spermatogonia, (B) differentiating spermatogonia, (C) preleptotene spermatocytes, (D) early-leptotene spermatocytes (E) leptotene spermatocytes and (F) zygotene spermatocytes, obtained from freshly isolated or cultured spermatogonia.

(3.7 folds vs control). Interestingly, we found that Stra8 mRNA levels were upregulated also by KL treatment (1.7 fold vs control), and such increase was more evident at the protein level (3.5 fold of increase with respect to control, Fig. 3D).

To verify whether meiotic entry could be stimulated by the two agents also in undifferentiated Kit negative spermatogonia, we treated 4 dpn spermatogonia for 48, 72 and 96 h with ATRA, KL and a combination of both. As previously described,[8,30] Kit and Stra8 expression were strongly upregulated in all the culture conditions in which ATRA was present (Fig. 4A and Suppl. Fig. 1C and D), even though cell viability decreased to about 50% after 4 days of culture in all the treatments (data not shown).We did not find meiotic nuclei up to 72 h of culture in all of the conditions tested, however after 96 h of culture a significant number of cells (Fig. 4B) showed a peculiar (early leptotene-like) SCP3 staining (Fig. 4C). Such staining was different from that found in 7 dpn spermatogonia which entered meiosis in vitro (see Figs. 2D and 3B). The SCP3 pattern exhibited nuclear foci as well as short filamentous structures which were less abundant when compared to leptotene nuclei and was reminiscent of that found in ES cells differentiating into germ-cells.[34] This pattern of staining was present in control cultures (15% ± 1), its percentage increased significantly in cultures treated with ATRA (21.7% ± 1.3) but not with KL (14% ± 0.8) and it almost doubled in the presence of both ATRA and KL (30.2 % ± 0.9).

ATRA increases Kit/KL levels in isolated testicular cells. It has been reported that ATRA stimulates Kit expression in isolated spermatogonia.[7,8] We confirmed these results in 4 and 7 dpn spermatogonia showing that Kit is induced at doses as low as 0.03 μM and that ATRA effect is not mediated by Sertoli cells (Suppl. Fig. 1). We show that Kit induction is evident as early as after 2 h of ATRA stimulation and increased linearly up to 24 h of culture (Fig. 5A) which corresponded to a significant increase at the RNA level, as shown by Northern blot analysis (Fig. 5B). ATRA-dependent increase of Kit expression was exerted at the level of mRNA synthesis rather than of mRNA stabilization. Indeed, we found by western blot analysis that pre-incubation with actinomycin D, a strong inhibitor of all RNA polymerases, completely abolished the increase of *Kit* protein levels induced by ATRA (Fig. 5C). ATRA does not affect the levels of GDNF receptor Ret, a marker for undifferentiated spermatogonia,[35] neither the levels of RAR_{γ}, the RA receptor specifically expressed in spermatogonia[10] (Fig. 5D). As in the case of postnatal spermatogonia, we found that ATRA increased Kit expression also in primordial germ cells (PGCs), harvested at 11.5 dpc, during their proliferative period,[27] but not in haematopoietic stem cells, a cell type which also express Kit[19] (Fig. 5E).

Since Sertoli cells are a known target of retinoic acid,[11] we investigated whether ATRA might regulate in these cells the production of growth factors essential for germ cells proliferation and/or differentiation. Figure 6A shows that, in Sertoli cell cultures obtained from 4 dpn and 7 dpn mice, ATRA strongly upregulated the levels of KL and BPM4 mRNAs after 24 h of culture both at 0.3 and 3 μM concentration. As a positive control we included dbcAMP stimulation which we have previously shown is able to upregulate KL levels in Sertoli cells.[21] In contrast to dbcAMP stimulation, the levels of the stem cell growth factor GDNF were downregulated in 7 dpn cultures by ATRA treatment (Fig. 6B).

Kit-activated signal transduction pathways are required for both ATRA- and KL-induced of meiotic entry. Since Kit is induced by ATRA and is the target of KL in 7 dpn spermatogonia, we hypothesized that the two factors might converge on Kit signalling pathways to increase the number of meiotic cells in culture. In order to test this hypothesis, we isolated and cultured spermatogonia for 48 h in the presence or absence of 5 μM STI571, a selective inhibitor of Kit tyrosine kinase activity,[36] and treated these cells with ATRA or KL. In cells solely treated with STI571, we found the same percentage of meiotic cells as in the control cultures without the inhibitor. The drug, however, was able to completely revert the increase of meiotic nuclei induced by ATRA and KL (0.9 fold of increase compared to STI571 control sample) (Fig. 7A). Since STI571 inhibited both ATRA- and KL-mediated increase of meiotic figures, we analyzed the levels of phospho-Kit and its downstream targets phospho-Akt and phospho-Erk1/2 in spermatogonia after 15 min stimulation with ATRA and KL pretreated or not for 30 min with STI571. Kit protein levels did not change after this pulse of stimulation while, as previous reported[31] we found that KL induced a significant increase of Kit, Akt and Erk1/2 phosphorylations, completely inhibited by the presence of 5 μM STI571 (Fig. 7B). Interestingly, we observed a positive effect on Kit autophosphorylation, Akt and Erk1/2 phosphorylations after only 15 min stimulation with ATRA, which were all prevented by the presence of the tyrosine kinase inhibitor. To determine which signalling pathway was preferentially activated by ATRA and KL, we cultured spermatogonia in the presence or absence of LY29406 (an inhibitor of PI3K pathways) or of U0126 (an inhibitor of the MAPK pathway). Both these inhibitors completely blocked the increase in the number of meiotic figures induced by ATRA or by KL

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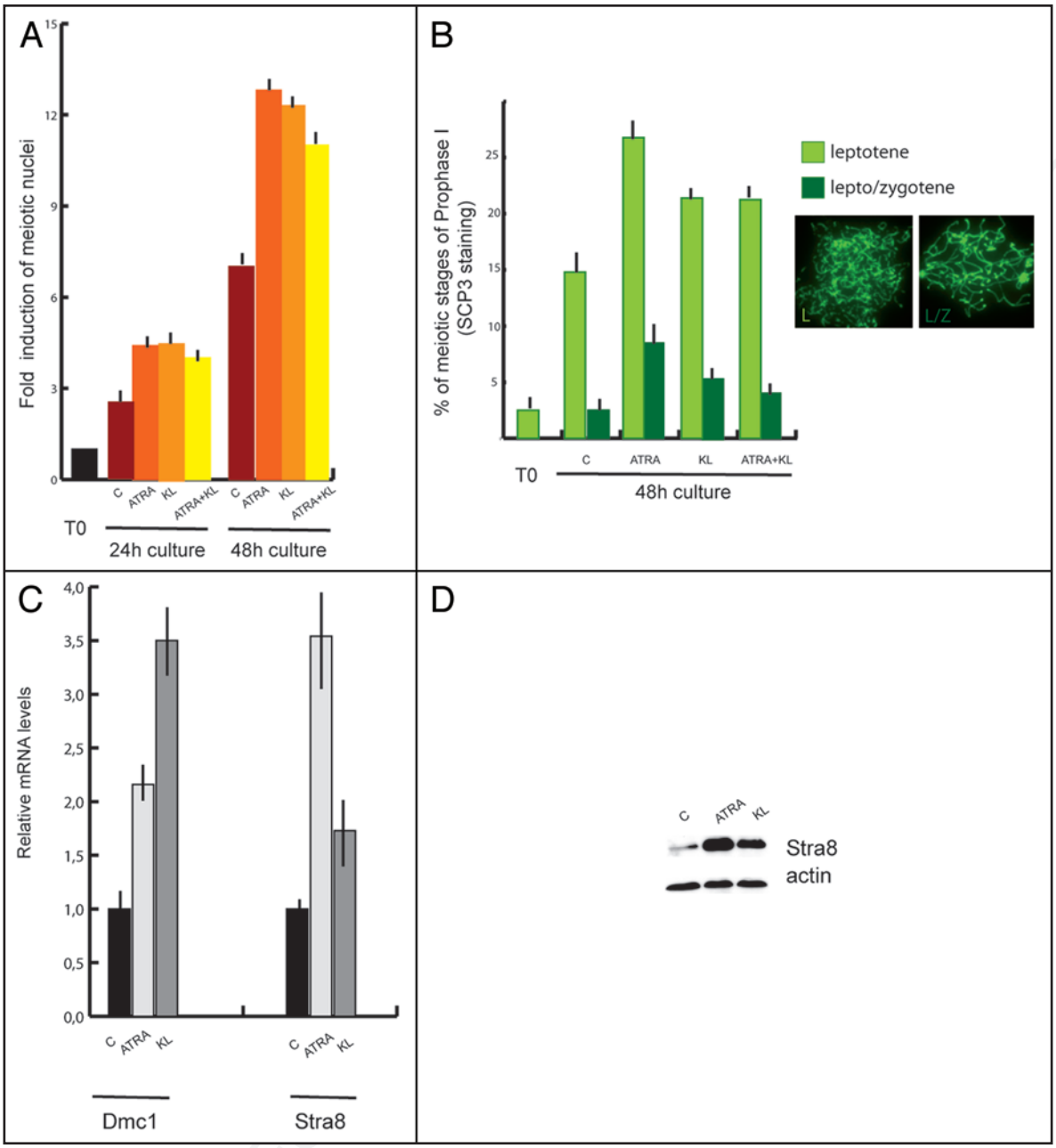

Figure 3. ATRA and KL promote meiotic in 7 dpn differentiating spermatogonia. (A) Histogram representing the fold of increase of meiotic nuclei in control spermatogonia or in cells stimulated with ATRA, KL or ATRA and KL for 24 h or 48 h. The values were obtained as a ratio of the percentage of nuclei with meiotic SCP3 staining at the different time points with the percentage of nuclei with meiotic SCP3 staining at the beginning of the culture (see Table 1 for absolute percentage values). Bars represent SD. (B) Percentage of leptotene (light green and IF left) and lepto/zygotene (dark green and IF right) nuclei in control cultures or cells treated for 48 h with ATRA, KL or ATRA and KL. Bars represent SD. (C) qRT-PCR for Dmc1 and Stra8 in spermatogonia incubated 24 h with ATRA or KL. (D) Immunoblot analysis of Stra8 and actin expression in spermatogonia cultured for 24 h in the absence or presence of ATRA or KL.

(Fig. 8A) and inhibited the increase in phosphorylation levels of Akt and Erk1/2 (Fig. 8B). Since ATRA was activating both PI3K and MAPK pathways, we investigated if the effect was specifically mediated by a RAR receptor. We incubated spermatogonia in the presence of 20 μM Ro-41-5253, an antagonist of RARα receptor, which at this concentration is also able to block the signalling of RARγ.[37] As shown in Figure 8C, we found that pre-incubation with Ro-41-5253 prevented ATRA-mediated phosphorylation of Akt and Erk1/2.

Table 1 **Percentage of leptotene and lepto/zygotene nuclei in culture**

	Cont	ATRA	KL	ATRA + KL
T_0	1.8% ± 0.5 (leptotene)	-	-	-
24 h	4.5% ± 0.22	7.9% ± 0.25	8.1% ± 0.21	7.2% ± 0.7
48 h	12.6% ± 0.2	23% ± 0.4	22.1% ± 0.9	19.6% ± 1.8

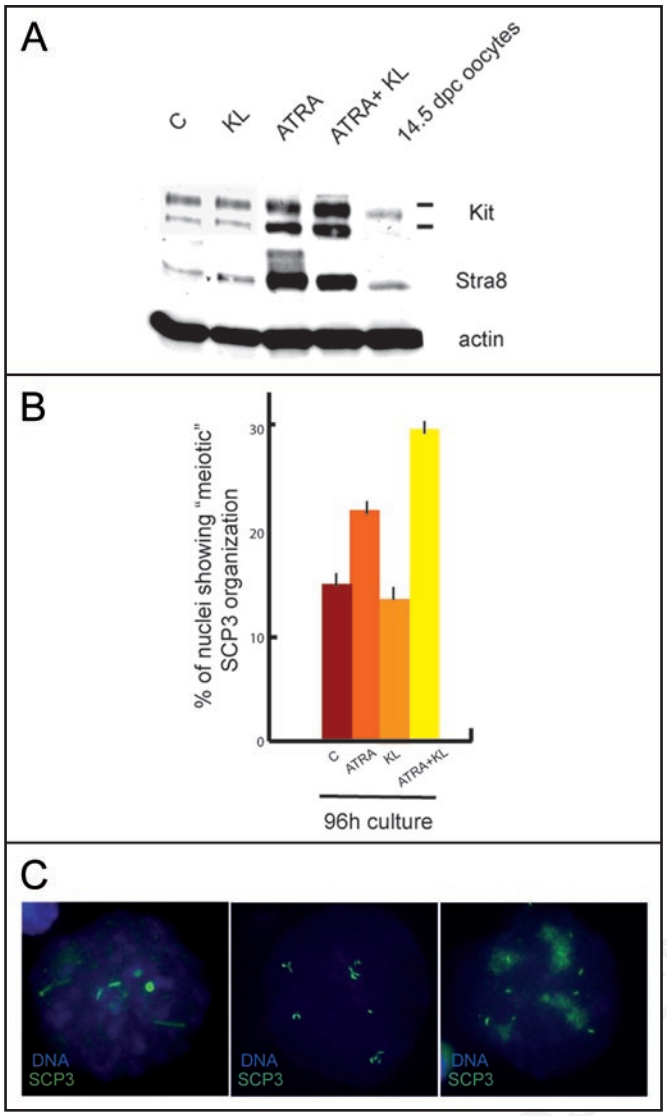

Figure 4. ATRA induces an abnormal SCP3 organization in cultured undifferentiated spermatogonia. (A) Immunoblot analysis of Kit, Stra8 and actin expression in 4 dpn spermatogonia cultured for 96 h in the absence or presence of ATRA or KL. (B) Histogram representing the percentage of meiotic SCP3 organization in control spermatogonia or in cells stimulated with ATRA, KL or ATRA and KL for 96 h. (C) Representative pictures showing an abortive meiotic organization of SCP3.

Discussion

It has been recently proposed a model in which RA acts as a meiotic inducing substance (MIS)[13] regulating, via its signalling and metabolism, whether or not female and male germ cells initiate meiosis during embryogenesis.[30] In this study we tested in vitro if its mechanism of action might be conserved in postnatal germ cells. When spermatogonia were stimulated by ATRA, we found that the percentage of meiotic nuclei was significantly increased compared to the control. Interestingly, a similar effect was also exerted by KL, a growth factor essential for spermatogonial survival and proliferation both in vivo and in vitro.[21,23,24] Simultaneous addition of ATRA and KL did not produce any further increase in meiotic nuclei, suggesting that they might be acting on the same cell type (possibly the Kit positive Intermediate and/or Type B spermatogonia) and/or that the pathways activated by the two factors might converge at some level of the pro-meiotic signalling cascade. Indeed, several evidences show that Kit positive spermatogonia are sensitive to both KL and ATRA (present results and refs. 8 and 21), which stimulate a significant increase of Stra8 (refs. 7 and 8 and present results), a fundamental regulator of both female and male meiosis.[6,15,16] Importantly, ATRA and KL similarly upregulate Dmc1, an early meiotic marker that is essential for the process of meiotic recombination.[33]

The evidence that ATRA increased the percentage of meiotic nuclei in isolated postnatal male germ cells confirms its role as a meiotic inducing substance. A microarray analysis performed on ATRA-stimulated spermatogonia (the entire set of raw and normalized data are available in the Array Express public repository at http://www.ebi.ac.uk/arrayexpress, with the accession No. E-MEXP-1126) shows changes in the pattern of gene expression compatible with the ongoing spermatogonial differentiation. For instance, we found upregulation of cyclin D2, previously shown to be strongly upregulated in spermatogonia of the VAD testis after administration of retinoic acid,[38] but also of early meiotic genes, such as Dzip1l, Phf7, Bmp6 and Btg4 (Table 2). At the same time, ATRA downregulates the expression of stemness genes, such as Pcgf2, Pcgf6 and Numb, and of spermatogonial markers known to be turned off at the onset of meiosis, such as Zfpm2, Pole, Egr2, Ptn, Tex16 (Table 2).

The finding that KL regulates meiotic entry of spermatogonia is a novel finding and is in line with the previously reported in vivo expression pattern of KL (highly expressed in Sertoli cells when leptotene spermatocytes appear), and with the observation that male germ cells co-cultured with a KL expressing cell line can undergo transmeiotic differentiation in vitro.[39] These results are also consistent with the data gathered by wide genomic analysis performed with Affymetrix gene chips, which indicate that KL, similarly to ATRA, regulates spermatogonial markers and early meiotic genes.[40] By comparing the effect of KL and ATRA on gene expression pattern we found that spermatogonial markers which are normally turned off at the onset of meiosis are downregulated also by KL treatment, whereas genes involved in stemness were not influenced. At the same time inhibitors of the mitotic cell cycle and early meiotic markers were all found to be equally upregulated by ATRA and by KL treatment[40] (Table 2).

When undifferentiated Kit negative spermatogonia were stimulated in vitro with ATRA they underwent a partial differentiation only after 96 h, but they did not enter meiosis correctly. Although ATRA increased Kit and Stra8 levels and the percentage of SCP3-positive nuclei, these cells failed to correctly organize the synaptonemal complex and, as expected, KL did not show any effect. KL synergized with ATRA in the induction of abortive meiosis in 4 dpn spermatogonia, probably because these cells became Kit positive during the concomitant exposure to ATRA, but they did not undergo a sufficient number of mitotic divisions necessary to become competent to enter meiosis.

We also show that the action of ATRA on spermatogonia is paralleled by a strong differentiative effect on Sertoli cells. In these cells ATRA induces expression of KL and BMP4 (known to promote proliferation and differentiation of spermatogonia[29]) and downregulation of GDNF, which is essential for stem cell renewal.[35] Indeed, it is well established that RA in immature Sertoli cells is

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required to promote spermatogonia differentiation during the prepubertal spermatogenic wave.[11]

We found that ATRA and KL share a common molecular mechanism of action in inducing meiosis, since a specific inhibitor of the Kit tyrosine kinase (STI571) was able to revert the meiotic increase induced by the two agents. Both agents activate either the PI3K or the MAPK pathways which are essential to mediate meiotic entry of spermatogonia in vitro. We observed that ATRA not only functions as a genomic inducer of Kit synthesis, but also as a rapid non genomic agent, by inducing Kit phosphorylation and activation. Such non-genomic effect, recently described also in neuronal systems[41,42] is dependent on a RA receptor, since Kit signalling pathways activated by ATRA in cultured spermatogonia were prevented by the incubation with a specific RAR inhibitor.

The pro-meiotic effect of ATRA and KL that we observe in vitro might be explained by at least four different possible mechanisms (i) by an increase of survival of already meiosis committed cells (pre-leptotene spermatocytes), (ii) by an increase of survival of Kit positive spermatogonia, which then might enter meiosis in a cell-autonomous manner; (iii) by an increase of mitotic proliferation of Kit positive spermatogonia, which then might enter meiosis in a cell-autonomous manner; or (iv) by the induction of meiotic competence in Kit positive spermatogonia. We can rule out the first possibility, since we estimated that the germ cell cultures obtained from 7 dpn mice contain only 4.6% of preleptotene spermatocytes, which cannot account for the increase of leptotene nuclei observed after 48 h of ATRA or KL stimulation (23%). Moreover, it has been demonstrated that preleptotene spermatocytes do not express RARs[10] and we found no preleptotene figures in Kit positive immunomagnetic sorted spermatogonia (our unpublished observation). The second possibility can also be ruled out by the observation that, while addition of either LY294002 or U0126 completely abolishes meiotic differentiation, these inhibitors are not able to suppress KL anti-apoptotic effects on cultured spermatogonia.[31] Moreover, we have previously shown that the induction of an early meiotic gene expression pattern is evident after only 4 hr of KL stimulation, a period of time at which spermatogonial survival is not affected.[40] Finally, we observe that ATRA-mediated increase of meiotic nuclei requires the activation of Kit-dependent pathways, in the absence of any pro-survival effect of ATRA. The third possibility also seems to be unlikely, because ATRA does not stimulate DNA synthesis in differentiating spermatogonia (Pellegrini et al., unpublished), and because KL has been shown to stimulate DNA synthesis in type A, but not in type B spermatogonia[21] which are the presumptive target of stimulation of meiotic entry in the present experiments. Thence, we would favour the fourth possibility, i.e., a direct effect of ATRA and KL on the induction of meiotic competence. The fact that, in vivo, Kit positive spermatogonia enter meiosis after a series of controlled *Kit*-dependent mitotic divisions, and the evidence that Kit requires PI3K and MAPK pathways to regulate both mitotic

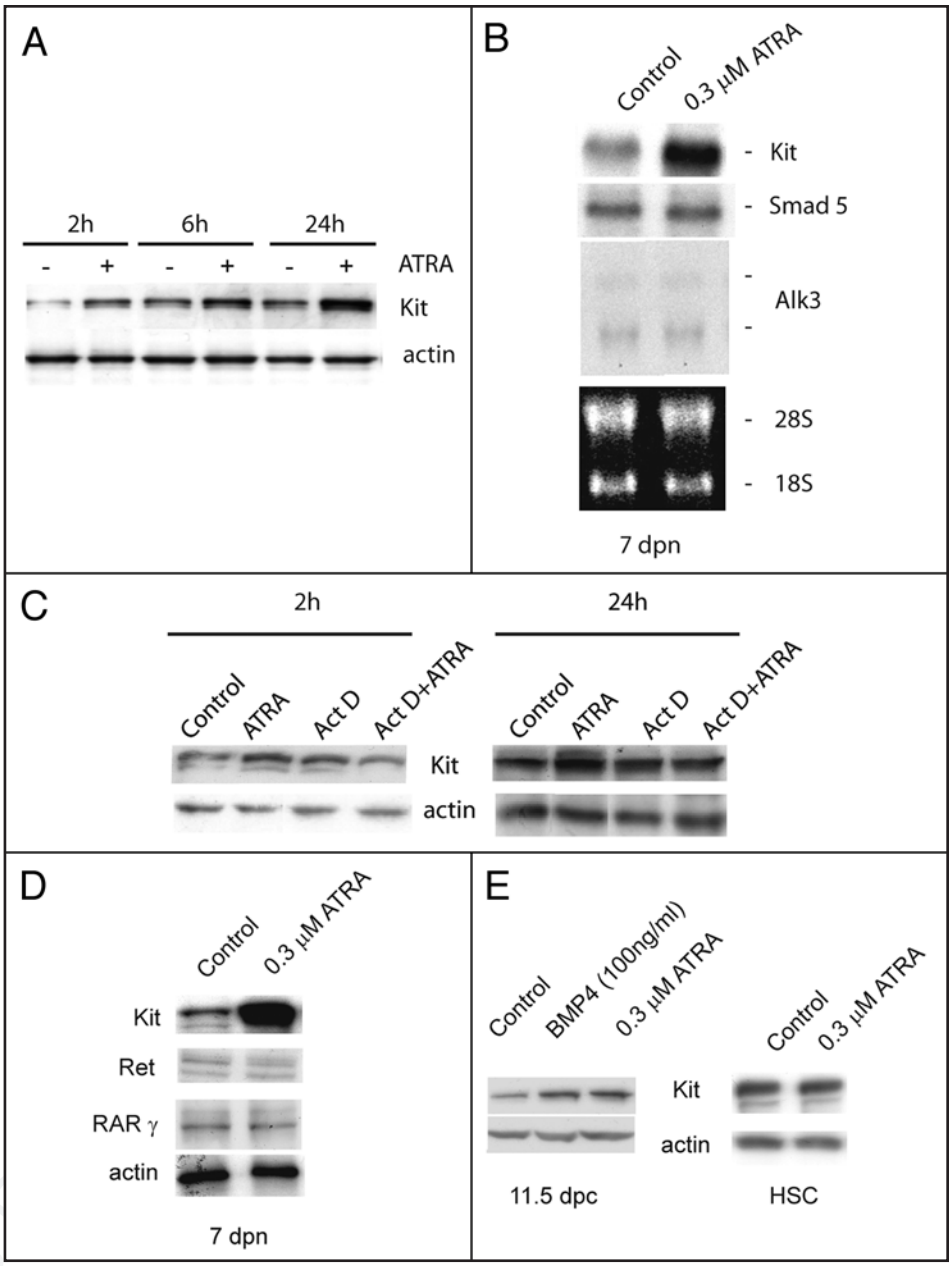

Figure 5. ATRA increases Kit levels in germ cells during development. (A) Time course curve of ATRA stimulated spermatogonia on Kit protein levels after ATRA treatment. Actin is shown as loading control. (B) Northern blot analysis of Kit mRNA levels in isolated germ cells cultured for 24 h with or without ATRA stimulation. Smad5 and Alk3 were included as specific markers for spermatogonial population.[29] 28S and 18S are shown as loading and integrity controls. (C) Kit immunoblotting on spermatogonia treated with Actinomycin D and/or ATRA for 2 h or 24 h. Actin is shown as loading control. (D) Kit, Ret and RAR$_\gamma$ protein levels after 24 h of ATRA stimulation in 7 dpn spermatogonia. (E) Kit levels in PGCs isolated from 11.5 dpc embryos and in hematopoietic stem cells 24 h after ATRA stimulation.

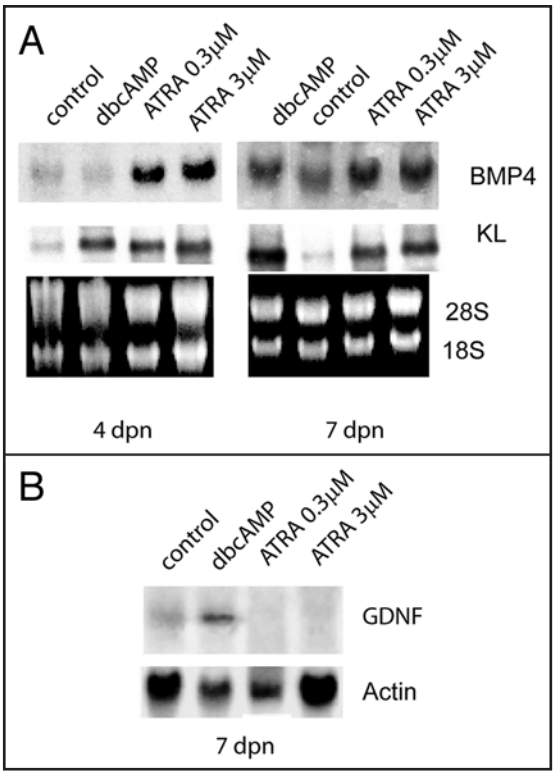

Figure 6. ATRA regulates in Sertoli cells the expression of specific growth factors required for germ cells differentiation. Northern blot analysis (A) for BMP4 and KL, and (B) for GDNF expression in Sertoli cells. SC cultures were obtained from 4 dpn and 7 dpn testes and stimulated o/n with dibutyril-cAMP (dbcAMP, 1 mM), with 0.3 μM or 3 μM ATRA. 28S and 18S or actin were shown as loading and integrity controls.

DNA synthesis[31] and meiotic entry of spermatogonia (this paper), suggest that a link exists between Kit-mediated proliferation and the switch into the meiotic cell cycle. KL induces a gene expression pattern in differentiating spermatogonia[40] that is consistent with a progressive lengthening of the S phase and a progressive shortening of the G_2/M transition, both events known to occur in vivo in male germ cells during the switch from mitosis to meiosis.[43] Presumably, during these Kit-mediated mitotic divisions, accumulation of key factors required for meiosis occur. One of these factors is certainly Stra8, which has been proposed to be essential for the last round of pre-meiotic DNA synthesis in female fetal germ cells,[6] and to regulate the switch from a mitotic pattern of cell division to the meiotic pattern in postnatal male germ cells.[15,16]

In conclusion, we propose a model (Fig. 9) in which RA acts on the somatic cell compartment of the testis, through the induction of KL and on the germ cell compartment, as a direct pro-meiotic factor sharing with KL the same signalling pathway.

Materials and Methods

Cell isolation and culture. Spermatogonia were obtained as we previously reported[21] by differential enzymatic digestion of testes from 4 to 7 days *post natum* (dpn) CD1 albino mice. To precisely define the temporal appearance of Kit expressing spermatogonia, a transgenic line (p18) expressing EGFP under the control of the c-Kit promoter and expanded on a CD1 background,[25,26] was used. Isolation of Kit positive spermatogonia was performed by using magnetic-activated cell sorting (MACS) with CD117 conjugated microbeads (Miltenyi Biotec, Germany). Isolated germ cells were then treated with different factors and cultured for 24 h or 48 h prior to immunofluorescence, western blotting analysis or mRNA preparation. Fresh medium and growth factors were replaced after 24 h when culturing for longer times. PGCs were isolated from 11.5 days *post coitum* (dpc) embryos as we previously reported in ref.[27] Spermatogonia were cultured in modified Earle medium with 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM glutamine without serum supplementation, while PGCs were cultured in spermatogonia medium supplemented with 10% FCS. KL-dependent SV40T-immortalized hematopoietic cells from bone marrow were cultured in RPMI supplemented with 10% FCS and 10 ng/ml KL. Sertoli cell cultures were prepared according to reference 21.

All-trans-retinoic acid (ATRA, Sigma) was dissolved in ethanol and used in a range of 0.03–3 μM. KL and BMP4 were purchased from Società Italiana Chimici (Rome, Italy) and used at 100 ng/ml. STI571, a generous gift of Dr. L. Gnessi (University of Rome "La Sapienza"), was used at 5 μM concentration and Ro 41-5253 (Biomol, DBA Italy) at 7 and 20 μM. DibutyrilcyclicAMP (Sigma) was used at 1 mM concentration. The MAPK inhibitor U0126 (Promega, Italy) and PI3K inhibitor LY294002 (Alexis, Italy) were used at 10 μM concentration.

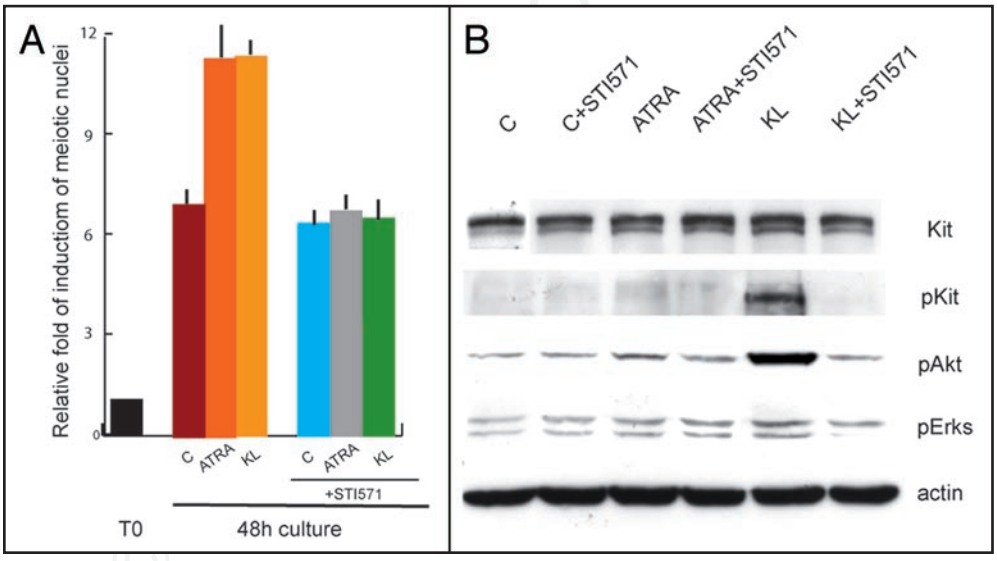

Figure 7. Inhibition of Kit signalling by STI571 completely reverts meiotic progression induced by ATRA and KL. (A) Histogram representing the folds of induction of meiotic nuclei, evaluated as above, in control spermatogonia or in cells stimulated with ATRA, KL or ATRA and KL for 24 h or 48 h in the presence or absence of 5 μM STI571. (B) Immunoblot analysis of Kit activated pathways in control spermatogonia or in cells stimulated for 15 minutes with ATRA or KL, pretreated for 30 minutes with 5 μM STI571.

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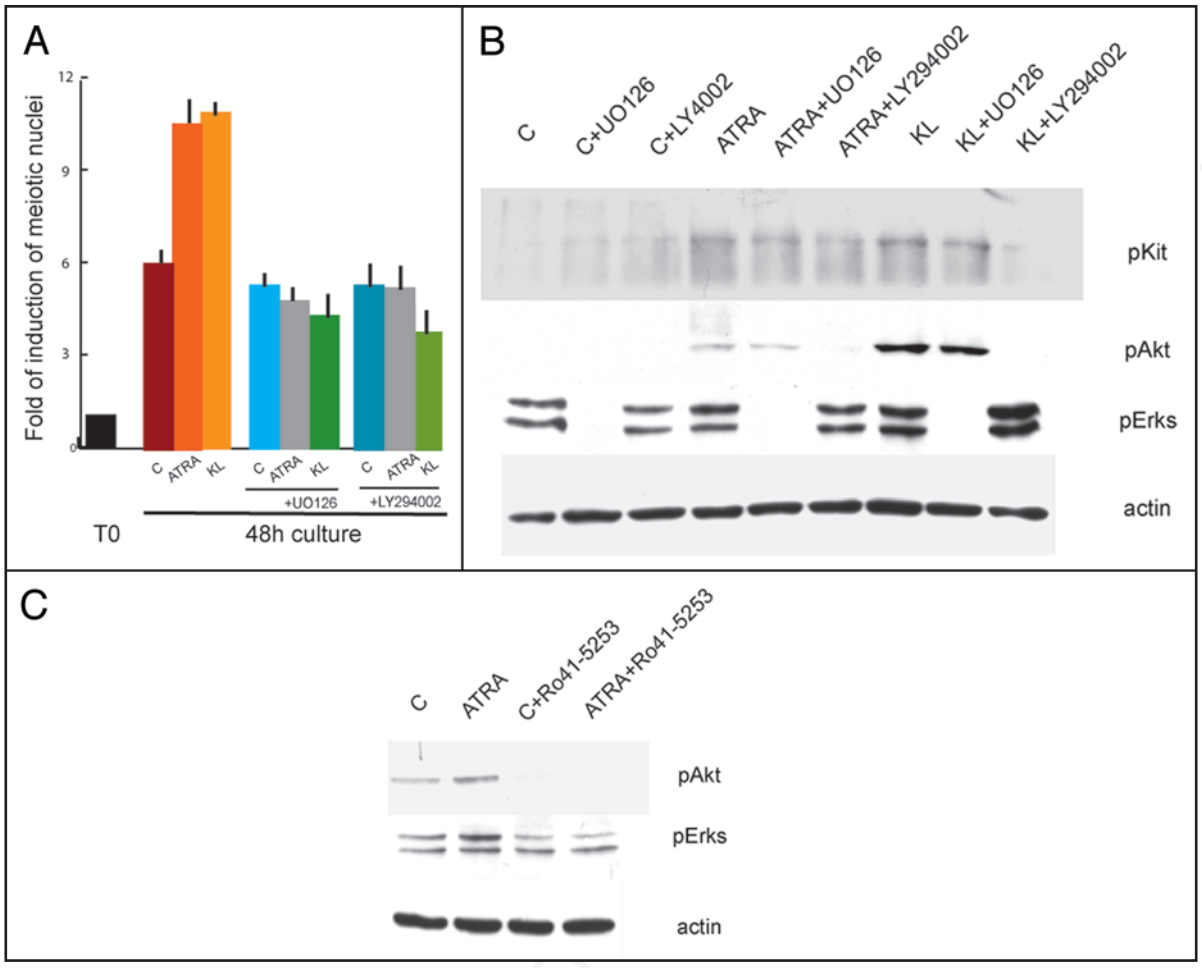

Figure 8. MAPK and PI3K signalling pathways activated by ATRA and KL are required for meiotic entry of spermatogonia. (A) Histogram representing the folds of induction of meiotic nuclei, evaluated as above, in control spermatogonia or in cells stimulated with ATRA and KL for 48 h in the presence or absence of UO126 or LY29406. (B) Western blot analysis of Kit activated pathways in control spermatogonia or in cells stimulated for 15 minutes with ATRA or KL, pretreated with 10 μM UO126 or 10 μM LY29406. (C) Western blot analysis of Kit activated pathways in control spermatogonia or in cells stimulated for 15 minutes with ATRA, pre-treated for 30 min with 20 μM Ro 41-5253.

Viability of 4 and 7 dpn spermatogonia was evaluated by Trypan blue (Sigma) exclusion.

Immunofluorescence and western blotting. For cell spreads, spermatogonia cultured for 24, 48 or 96 h were prepared and stained essentially as described in ref.[28] Slides were washed twice in PBS, and incubated o/n at 4°C with anti-SCP3 rabbit polyclonal antibody (Abcam, Cambridge, UK) diluted in blocking solution (10% goat serum, 3% BSA, 0.5% Triton X-100 in PBS). After washing, secondary antibody was added for 1 h at 37°C. The slides were washed and allowed to dry. Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) was added and the slides were viewed using a Leica microscope. Spreads analysis was performed in five independent experiments. A representative morphological classification of SCP3 organization in various types of germ cells obtained in vitro is reported in Figure 2. For immunofluorescence, spermatogonia cell suspensions were adhered onto poly-L-lysine coated slides and treated as previously described.[29]

For western analysis cells were lysed in 1% Triton X-100, 150 mM NaCl, 15 mM $MgCl_2$, 15 mM EGTA, 10% Glycerol, 50 mM Hepes (pH 7.4) with protease inhibitors. Proteins were separated by SDS-10% polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Amersham). The membrane was blocked in PBS-5% skim milk powder for 1 h. Incubation of the membrane with the primary antibody was carried out at 4°C o/n in PBS-5% BSA and then with the appropriate horseradish peroxidase-conjugated secondary antibody (SantaCruz). Anti-Kit rabbit polyclonal (sc-6283), anti phospho-*Kit* (Tyr 721) (sc-23766) rabbit polyclonal, anti-phospho Erk1/2 mouse monoclonal antibody (sc-7383), anti-actin rabbit polyclonal (sc-7210), anti-*Ret* rabbit polyclonal (sc-167), anti-RAR_γ mouse monoclonal (sc-7387) were from SantaCruz, anti-phospho Akt (Ser-473) was from New England Biolabs, anti Stra-8 antibodies were from Abcam. The horseradish peroxidase conjugate was detected by chemioluminescence with an ECL Kit (Amersham) and autofluorography. For each experiment western blotting analysis were repeated at least four times and densitometry was performed using a Molecular Dynamics Densitometer and ImageQuant software.

Northern blotting and quantitative RT PCR analyses. Spermatogonia were collected into Trizol (Invitrogen) and RNA was purified according to manufacturers suggestions. For Northern blot analysis, total RNA was run in a 1% formaldehyde-agarose gel and blotted onto a Nylon membrane (Hybond-N, Amersham,

Table 2 **List of genes regulated by ATRA treatment of mouse spermatogonia from 7 dpn mice cultured for 24 hr and comparison with the effect of KL treatment on the same cells**

Gene name*	Fold change ATRA vs. control**	Fold change KL vs. control***	Notes****
Zfpm2	0.31	0.24	Downregulated during spermatogonial differentiation.
Polymerase epsilon (Pole)	0.32	0.13	Spermatogonial marker downregulated at the onset of meiosis.
Egr2	0.42	0.39	Spermatogonial marker downregulated at the onset of meiosis.
Pleiotrophin (Ptn)	0.46	0.32	Stimulator of spermatogonial proliferation essential for spermatogenesis.
Polycomb group ring finger 6 (Pcgf6)	0.52	**No Change**	Maintains embryonic and adult stem cells by repressing developmental regulators.
Polycomb group ring finger 2 (Pcgf2)	0.55	**No Change**	Maintains embryonic and adult stem cells by repressing developmental regulators.
Tex16	0.58	0.49	Spermatogonial marker.
Numb	0.62	**No Change**	Upregulated by GDNF in spermatogonia.
Ing1	1.63	1.58	Negative regulator of cell cycle progression.
Phf7	1.73	2.51	PHD finger protein 7, also called Nyd-sp6. A transcription factor highly expressed in the human testis, and absent in spermatocytic arrest. One of the most differentially expressed genes between spermatocytes and spermatogonia in mice.
Dzip1l	1.73	2.18	DAZ interacting protein 1-like An homolog of Dzip1, a RNA binding protein expressed in pre-meiotic spermatogonia.
Cyclin D2	1.96	**No Change**	Proposed to play a role at the crucial differentiation step of undifferentiated spermatogonia into A(1) spermatogonia.
Blimp1 (Prdm1)	2.21	**No Change**	Essential for establishment of primordial germ cells by ensuring their escape from the somatic fate as well as possible reversion to pluripotent stem cells.
Btg4	2.25	4.04	Negative regulator of progression through mitotic cell cycle, expressed at high levels in the testis and in oocytes. Also called PC3B.
Fzd10	2.41	3.12	Wnt receptor, stimulated during atRA-induced neural duifferentiation of the embryonal carcinoma cell line NT2.
Bmp6	3.31	2.09	An oocyte-secreted growth factor.

*Transcripts up or downregulated at least 1.5-fold by ATRA treatment in both of two separate microarray experiments performed with mouse Genome 430 2.0 GeneChip arrays (Affymetrix Inc.,). RNA for microarray analysis was extracted from spermatogonia cultured for 24 hr in the presence or absence of 0.3 μM ATRA. The analysis was performed on duplicate chip arrays, using cRNAs from two different RNA pools, each obtained from two different culture experiments. The entire set of raw and normalized data are available in the Array Express public repository at http://www.ebi.ac.uk/arrayexpress (Accession No.: E-MEXP-1126). **The indicated value of fold change is the average of the two separate microarray experiments. Validity of the data of microarray analysis was confirmed by performing semi-quantitative RT-PCR analysis for randomly selected transcripts on RNA preparations obtained from different cell preparations (not shown). Details about preparation of the samples, bioinformatic and statistical analysis have been described elsewhere.[40] ***KL concentration used was 100 ng/ml. Data from ref. 40. ****References for genes co-regulated by both KL and ATRA treatment are available in ref. 40.

UK) using 10x saline sodium citrate (SSC) buffer. Probes were obtained by RT-PCR from spermatogonia cDNAs (Smad5, Alk3 and Kit) or from Sertoli cell cDNAs (KL, BMP4 and GDNF). PCR products were ^{32}P-labelled with a random prime labelling system (Perkin Elmer). Hybridization was carried out following Quick Hybrid System's instruction (Stratagene). For Quantitative PCRs, contaminating genomic DNA was removed using DNase I Amplification Grade (Invitrogen) and cDNA synthesis was subsequently performed with the SuperScript™ III First-Strand Synthesis System (Invitrogen). Quantitative PCR was performed for Stra8 and Dmc1 genes using SYBR green I fluorescent dye (EPPENDORF) and standard deviations were calculated. Primer sequences for qRT-PCR were: Stra8 for 5'-GTT TCC TGC GTG TTC CAC AAG and rev 5'-CAC CCG AGG CTC AAG CTTC; Dmc1: for 5'-CAT ATC ACT ACT GGG AGC and rev 5'-GTA CTG CTT CAT GGT CTAC. Primer sequences for amplification of probes for Northern blot analysis were: Kit for 5'-TAT GGA CAT GAA GCC TGG CGT and rev 5'-CAT TCC TGA TGT CTC TGG CTA GC; KL for 5'-AAC AGC TAA ACG GAG TCG CC and rev 5'-ACA GTG TTG ATA CAA GCC AC; BMP4 for 5'-TTT GGC CAT GAT GGC CGG GGC CAT ACC TT and rev 5'-TCA GCG GCA TCC ACA CCC CTC TAC CAC CAT; GDNF for 5'GG AGT TAA TGT CCA ACT GGG and rev 5' TAC ATC CAC ACC GTT TAG CG; Alk3 for 5'-ACT TTA GCA CCA GAG GAT ACC and rev 5'-TTT TCA CCA CGC CAT TTA CCC; Smad5 for 5' AAT GAC GTC AAT GGC CAG CTT and rev 5' AGA AGA AAT GGG GTT CAG CG; β-actin for 5'GGT TCC GAT GCC CTG AGG CTC and rev 5'-ACT TGC GGT GCA CGA TGG AGG (also used for normalization in qRT-PCR).

Statistical analysis. The Student t-test and ANOVA have been used to assess the significance, set at $p < 0.05$. All experiments were performed from three to five times and at least in triplicate for each sample. Bars in the histograms represent standard deviations obtained from the indicated independent experiments.

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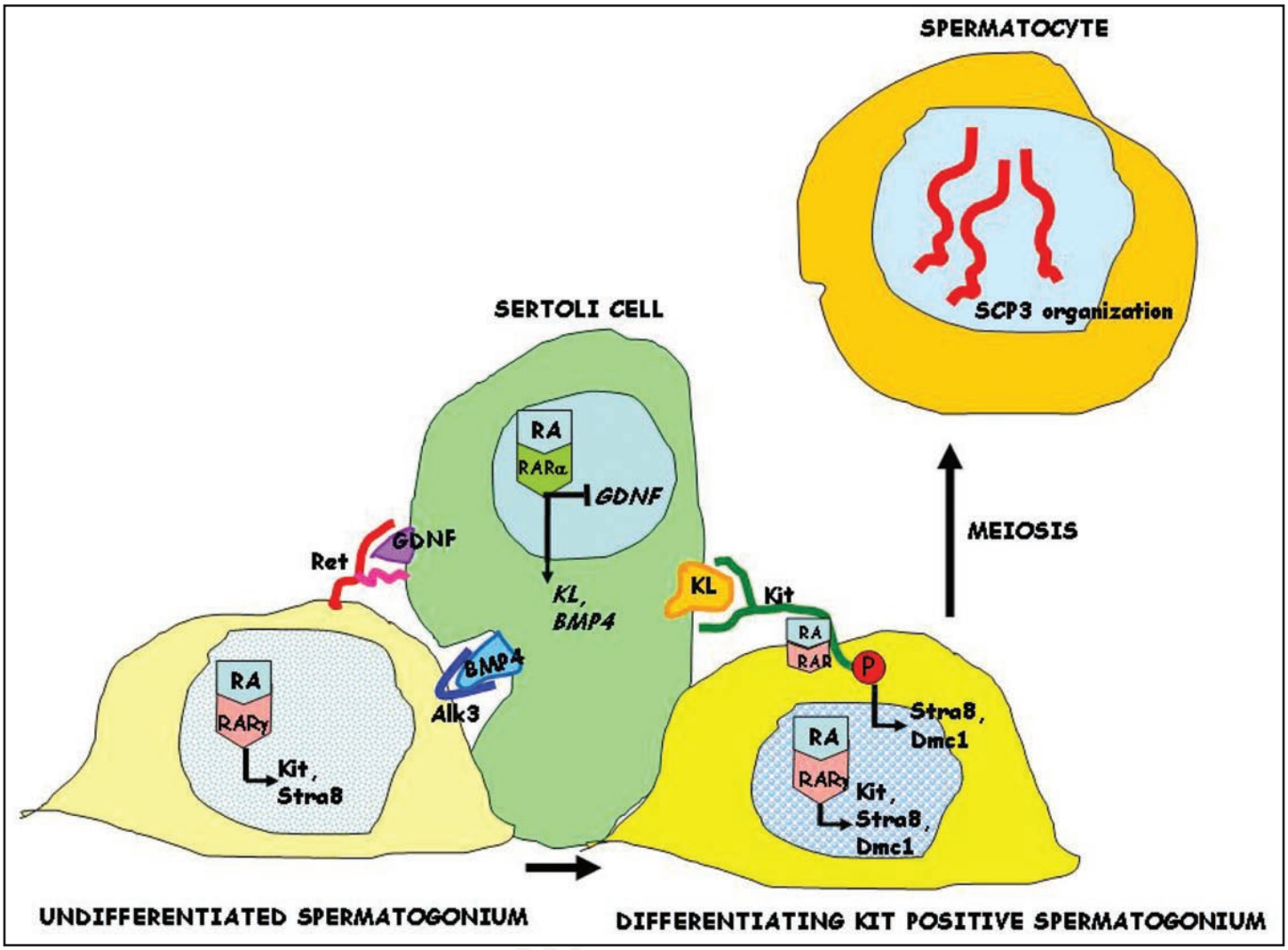

Figure 9. Schematic representation of RA and KL effects on the early steps of spermatogenic differentiation. RA acts in Sertoli cells through the RARα receptor to stimulate the synthesis of growth factors (KL and BMP4), which are essential for germ cell differentiation, and to inhibit the synthesis of the critical growth factor required for spermatogonial stem cell self-renewal (GDNF). RA acts also on undifferentiated and differentiating spermatogonia through the RARγ receptor to stimulate the synthesis of Stra8 and the KL receptor (Kit) but not BMP4 and GDNF receptors (Alk3 and Ret, respectively). Furthermore, similarly to KL, RA directly activates Kit phosphorylation and its signalling cascade in differentiating spermatogonia, through a non genomic mechanism. Activation of Kit dependent signalling is required for differentiation of spermatogonia into spermatocytes , as revealed by the induction of early meiotic markers, such as Dmc1, and by the meiotic organization of nuclear SCP3.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/PelligriniCC7-24-Sup.pdf

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CHAPTER 3

Opposing effects of RA and FGF9 on Nanos2 expression and meiotic entry of mouse germ cells.

Opposing effects of RA and FGF9 on *Nanos2* expression and meiotic entry of mouse germ cells.

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Florencia Barrios, Doria Filipponi, Manuela Pellegrini, Maria Paola Paronetto, Sara Di Siena, Raffaele Geremia, Pellegrino Rossi, Massimo De Felici, Emmanuele A. Jannini[§] and Susanna Dolci*.

Department of Public Health and Cellular Biology, University of Rome 'Tor Vergata', 00133 Rome,

[§] Department of Experimental Medicine, Università dell'Aquila, L'Aquila, Italy

Running title: RA and FGF9 regulate *Nanos2*

Key words: NANOS2, FGF9, RA, *Gata2*, *Taf7l*, meiosis, spermatogenesis

**Author for correspondence*:
Susanna Dolci, MD, PhD
Dept. Public Health and Cell Biology
University of Rome Tor Vergata
Via Montpellier 1, Bldg. E Nord, 00133 Rome, Italy
dolci@uniroma2.it
ph:+390672596252 fax: +390672596268

Abstract

In the mouse, three genes homologous to the *Drosophila Nanos* (*Nos*) gene have been identified. Deletion of one of the *Nanos* genes, *Nanos2*, results in male sterility, due to germ cell loss during the fetal life. Before apoptosis, *Nanos2* null gonocytes enter meiosis, suggesting that Nanos2 functions as a meiotic repressor. Here we show that *Nanos2* is continuously expressed in male germ cells from fetal gonocytes to postnatal spermatogonial stem cells. We observed that the pro-meiotic factor AtRA, an analog of retinoic acid (RA), down-regulates *Nanos2* levels both in fetal and postnatal gonocytes while promoting meiosis. Interestingly, FGF9, a growth factor crucial for sex differentiation and for survival of fetal gonocytes, upregulates *Nanos2* levels in both male and female primordial germ cells (PGCs) and in premeiotic spermatogonia. This effect was paralleled by an impairment of meiotic entry, suggesting that FGF9 acts as an inhibitor of meiosis through the upregulation of NANOS2. We found that NANOS2 interacts with PUMILIO2 and that these two proteins colocalize in the ribonucleoparticle/polysomal fractions on sucrose gradients, supporting the notion that they bind RNA. Finally, we found that recombinant NANOS2 binds two spermatogonial mRNAs, *Gata2* and *Taf7l*, which are involved in germ cell differentiation.

Introduction

NANOS is an evolutionarily conserved protein essential for the survival of primordial germ cells (PGCs). Both in vertebrates (Koprunner et al., 2001; Tsuda et al., 2003) and invertebrates (Fujii et al., 2006; Lehmann and Nusslein-Volhard, 1991; Pilon and Weisblat, 1997; Subramaniam and Seydoux, 1999), NANOS has been shown to act as a putative RNA-binding protein involved in germ cell development. In the mouse, three *Nanos* genes have been identified (Haraguchi *et al.*, 2003). While *Nanos1* is predominantly expressed in the neural system (Haraguchi *et al.*, 2003), *Nanos2* and *Nanos3* are exclusively expressed in fetal and postnatal germ cells (Tsuda et al., 2006; Tsuda et al., 2003). The expression pattern of *Nanos2* is restricted to gonocytes within fetal testis undergoing mitotic arrest, and is then maintained in spermatogonia after birth. Deletion of *Nanos2* results in male sterility due to germ cell loss during the fetal life (Tsuda et al., 2003). *Nanos2* null male fetal germ cells enter meiosis before undergoing apoptosis at around 15.5 dpc (Suzuki and Saga, 2008). Furthermore, *Nanos2* mis-expression in female fetal germ cells prevents meiotic entry, strongly suggesting that *Nanos2* is an anti-meiotic gene (Suzuki and Saga, 2008).

The mechanisms which trigger meiosis have been a subject of debate, but it is now clear that retinoic acid (RA) produced by mesonephroi induces meiosis in female PGCs (Bowles *et al.*, 2006; Koubova *et al.*, 2006), while the same action in the fetal testis is prevented by the presence of the retinoid-degrading enzyme CYP26B1 (Bowles *et al.*, 2006; MacLean *et al.*, 2007). The role of RA as a meiotic inducer is conserved also postnatally, since it is able to induce the meiotic entry of differentiating, KIT positive spermatogonia (Pellegrini et al., 2008). An inverse correlation between RA and NANOS2 levels exists in *Cyp26b1* null fetal testis, a condition in which RA is elevated (MacLean *et al.*, 2007), gonocytes enter meiosis and NANOS2 levels are low (Suzuki and Saga, 2008). The critical role of RA in inducing meiosis in both female and male is played by the Stimulated by Retinoic Acid gene 8, *Stra8* (Anderson et al., 2008; Mark et al., 2008); indeed, *Stra8* null males and females show impairment of gametogenesis due to failure of meiotic commitment (Anderson et al., 2008; Mark et al., 2008). On the other hand, meiotic commitment is aberrantly induced not only in *Nanos2* null mice but also in *Fgf9* knockouts (Colvin *et al.*, 2001).

Fgf9 is known to be expressed by somatic cells in XX and XY gonads at 11.5 dpc, but it becomes XY restricted by 12.5 dpc and is then expressed within the testis cords (Schmahl *et al.*, 2004). *Fgf9* null mice die shortly after birth owing to defects in lung formation, but XY embryos show phenotypic male-to-female sex-reversal and the

surviving gonocytes are found in meiosis (Colvin *et al.*, 2001). The consequences of *Fgf9* ablation on male germ cells are probably independent of the proliferative and differentiative effect on fetal Sertoli cell, since it has been shown that FGF9 is able to promote survival of male germ cells in vitro (DiNapoli *et al.*, 2006).

We report that NANOS2 is continuously expressed in male germ cells from fetal to postnatal development. In particular, we investigated the role of RA and FGF9 on the regulation of NANOS2 in correlation with their ability to influence meiosis. We found that AtRA represses NANOS2 expression, while FGF9 up-regulates *Nanos2* mRNA levels in female and male germ cells and inhibits meiosis in both sexes. Moreover, we show evidences that NANOS2 associates with ribonucleoparticles (RNPs) and polysomes both in fetal and postnatal germ cells. Finally, by GST-RNA pull-down assay we found that NANOS2 shows affinity for two RNA transcripts, *Gata2* and *Taf7l* which are involved in spermatogonial differentiation, strengthening the hypothesis that NANOS2 can act as a meiotic inhibitor through a post-transcriptional regulation mechanism.

Materials and methods.

Cell isolation and culture

Postnatal male germ cells (0 to 7 dpp) were obtained as previously reported by sequential enzymatic digestion of testes from CD1 albino mice (Pellegrini et al., 2008). After preplating of cell suspension, germ cells were cultured in Modified Earle's Medium (MEM, Gibco) with 20 mM glutamine (Gibco), 2 mM Pyruvic acid (Sigma), 1 mM lactic acid (Sigma), non essential aminoacids (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco), without serum supplementation.

To obtain enriched fetal germ cell suspensions, fetal testes and ovaries were collected at different developmental stages and digested with trypsin (Gibco) and DnaseI (Sigma). Cell suspensions were pre-plated in the same medium as postnatal germ cells supplemented with 10% fetal bovine serum, (FBS) and recovered after 3 hr of culture, to allow most of the somatic cells to adhere to the plastic dishes. In the case of organ culture, fetal gonads were cultured for 48h onto 0.8% agarose blocks pre-equilibrated with MEM-10% FBS

All-trans-retinoic acid (AtRA, Sigma) was dissolved at a concentration of 10mM in ethanol and diluted to a final concentration of 0.3 µM (Pellegrini et al., 2008) in culture medium. Control cultures received the corresponding doses of diluted ethanol. FGF9 (from Società Italiana Chimici) was dissolved in PBS 1mg/ml BSA and used to a final concentration of 25 ng/ml. All the factors were replaced in the culture medium after 24 hours.

Separation of KIT positive spermatogonia from KIT negative spermatogonia was performed by magnetic-activated cell sorting (MACS) with CD117 conjugated microbeads (Miltenyi Biotech, Germany) as previously described (Pellegrini et al., 2008).

Definition of the germ cell stages.

Fetal male and female germ cells are defined as PGCs as long as they reach the gonads and proliferate. After gonadal colonization, female PGCs start entering meiosis (at around 13.5 dpc) and are then defined as oocytes. Male PGCs, on the contrary, after gonadal colonization enter mitotic block and are then defined as gonocytes. This definition is retained up to birth, when gonocytes resume mitosis and spermatogonial stem cells can be identified. KIT negative (OCT4 and PLZF positive) spermatogonia

represent the stem cell population of the testis while KIT positive spermatogonia are the differentiating mitotic germ cells which are committed to enter meiosis.

Polysome – RNP fractionation

Prepuberal spermatogonia and fetal gonocytes were subjected to polysome – RNP fractionation on sucrouse gradients as previously described (Lolicato *et al.*, 2008). In brief, 600 µg of cytoplasmic extracts in polysome buffer were loaded onto 15 – 50 % sucrouse gradients. 10 fractions were collected and protein precipitation from each fraction was performed with 72% tricloroacetic acid (TCA) and subsequently resuspended in 2X sample buffer and processed to SDS-PAGE. For the graphic, absorbance at 260nm was continuously recorded while collecting the fractions. EDTA treatment was performed as previously described (Grivna *et al.*, 2006).

Recombinant proteins, transfections, GST protein and RNA pull-down.

Nanos2-myc was produced by RT-PCR using primers using a proof reading polymerase (Pfu, Stratagene) containing BamHI-EcoRI sites at the extremities. The amplified band was cloned in pc*DNA3-myc* (Invitrogen, Milan Italy) or in p*GEX4T1* (GE Healthcare, Milan Italy). For transient transfections, Hek293T were transfected with pc*DNA3myc-Nanos2* by Lipofectamine 2000 (Invitrogen) according to the manufacture's instructions. Pull-down experiments were performed using GST fusion proteins purified from bacterial lysates on glutathione-agarose (Sigma-Aldrich, Milan, Italy). In the case of protein pull down, cell extracts (600 µg of total proteins) of 16.5 dpc gonocytes or 4dpp spermatogonia, precleared for 1 h with glutathione-agarose in lysis buffer, were added to 2 µg of GST protein or GST-NANOS2 fusion protein absorbed on glutathione-agarose in lysis buffer supplemented with 0.05% BSA. After incubation for 90 min at 4°C under constant shaking, beads were washed three times with the same buffer, absorbed proteins were eluted in SDS sample buffer and resolved on a 12% SDS-PAGE for subsequent Western blot analysis. Input represents 1/10 of the initial samples.

For RNA pull-down experiments 1mg of cytoplasmic extracts in polysome buffer was obtained from 7 dpp spermatogonia and processed as described by Keen et al. (2006). RNA and protein containing extracts were incubated with GST or GST-NANOS2 agarose beads for 4 h at 4º. RNA-protein complexes were washed four times in NT2 buffer and RNA was eluted from the beads by incubation at 55º for 30 minutes with 30 µg of Proteinase K. After Trizol extraction (Invitrogen, Milan, Italy) and DNAseI

treatment (Zymo Research, Rome, Italy), RNA was reverse transcribed as described in the following section.

RT-PCR

Total RNA from whole fetal gonads and fetal and postnatal germ cells was extracted with Trizol reagent and treated with DNAse I to avoid potential contamination by genomic DNA. DNA-free RNA was reverse transcribed using SuperScript First Strand Synthesis kit (Invitrogen, Milan, Italy) according to manufacturer's instructions. Primers used for PCR were as follows: *Nanos2 FW* AGTGCCATGGACCTACCGCCCTTT, *Nanos2 RW* TCTCAATTATCGCTTGACTCTGC, *c-Kit FW* GCCACGTCTCAGCCATCTG, *Kit RW* GTCGGGATCAATGCACGTCA, *Stra8 FW* TAGGATCCATGGCCACCCCTGGAGAAG, *Stra8 RW* GAATTCTTACAGATCGTCAAAGGTCTCCA, *Actin FW* GGCTGTATTCCCCTCCATCG, *Actin RW* CCAGTTGGTAACAATGCCATGT. For semiquantitative RT-PCR 25 cycles were performed for the amplification of *Actin* and all other genes, 30 cycles for *Nanos2* amplification from male germ cells or 40 cycles from female germ cells.

RNA from RNA pull-down experiments was reverse transcribed using SuperScript First Strand Synthesis kit (Invitrogen) according to manufacturer's instructions. Primers for putative targets were designed, when possible, to span a region between the last two exons. Primers were the following: *Gata2 FW* CAAGCTGCACAATGTTAACAGGC; *Gata2 RW* ATTCACAGTAATGGCGGCACAAGG; *Taf7l FW* GGAGGAGGAAGAGACAGACAATTC; *Taf7l RW* GTAGAGGGACAGAAGTATGTGGTTCCAC; *Stra8 FW* TGCCGGACCTCATGGAATTT; *Stra8 RW* GAATTCTTACAGATCGTCAAAGGTCTCCA; *Gcnf1 FW* GGATGGAGGTGATTGAACGACT; *Gcnf1 RW* AGTCTCCATCTTGGTCTCTGGCT. PCR conditions were: 95°20s, 58°30s, 72° 30s for 40 cycles.

Immunofluorescence.

Transfected Hek293T cells were adhered onto poly-L-lysine glass slides and fixed for 10 minutes at room temperature in 2% paraformaldhyde. After washing with PBS, cells were permeabilized for 10 minutes with PBS containing 0.1% Triton X-100, and

incubated for 30 minutes at room temperature with PBS containing 0.5% BSA. Samples were then incubated overnight at 4°C in a humidified chamber with anti-MYC antibody from Santa Cruz (sc-2048) at a final concentration of 2 µg/ml and then for 1 hour at room temperature with cyanin 3 (AP 180 C; CHEMICON, CA). Slides were washed and mounted in 50% glycerol in PBS and immediately examined by fluorescence microscopy. Nuclei were counterstained with 1 µg/ml Hoechst (33342; Sigma). Control experiments were performed using non-immune immunoglobulins instead of the specific antibody.

Western blotting.

For western blot analysis germ cells were harvested and washed 3 times with ice cold PBS. Cell lysis was performed with 10mM HEPES pH 7.9, 1% Triton X-100, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol (DTT), 10 mM β-glycerophosphate, 0.1 mM sodium vanadate, and protease inhibitor cocktail (Sigma-Aldich). Forty µg of protein extracts were separated by SDS – PAGE in gradient gels 4 – 20 % (Serva, Milan, Italy) or uniform gels of 15% polyacrylamide and transferred to nitrocellulose membrane (Amersham, Piscataway, NJ). The membrane was blocked in phosphate saline buffer with 0.1% Triton (PBST) and 5% fat-free milk powder for 1 hr at room temperature. Incubation with every primary antibodies was carried out at 4°C overnight O/N in PBST–5% BSA. Appropriate horseradish peroxidase-conjugated secondary antibody (SantaCruz Biotechnology) was used at a 1/5000 dilution in PBST for 1 hour at room temperature. Anti-NANOS2 serum (in this study we used both one obtained from Abnova, Taipei, Taiwan, and a gift from Dr. Saga, SOKENDAI, Japan; in the figure legends we indicated when the commercial antibody was used) was used at a 1/300 dilution in PBST- 5% BSA. Anti-KIT rabbit polyclonal antibody (Albanesi et al., 1996) was diluted at 1/1000. Anti-PUMILIO2 rabbit polyclonal (Abcam) was diluted at 1/3000. Anti-TIAR mouse monoclonal antibody (a kind gift from P. Anderson, Harvard, MA, USA) was diluted at 1/1000. Anti- S6 rabbit polyclonal antibody (Cell Signaling, Beverly, MA) was diluted 1/1000. Anti-ACTIN rabbit polyclonal (A2066, Sigma-Aldrich) was diluted 1/1000. Anti-MYC antibody was from Santa Cruz (sc-2048) and diluted 1/1000. Mouse monoclonal antibody anti human DAZL was from AbD Serotec, Milan Italy and used at a 1/1000 dilution. Anti-STRA8 rabbit polyclonal from Abcam (Ab 49602) was used at a 1/1000 dilution. Anti-SCP3 rabbit polyclonal (1:1000) was from Novus. Anti-OCT4 mouse monoclonal (1:1000) was from SantaCruz (sc-5279). The horseradish peroxidase conjugate was detected by

chemioluminescence with an ECL Kit (Santa Cruz) and autofluorography. Densitometric analysis was performed by ImageQuant™ TL software (GE Healthcare)

Spreads

Cultured spermatogonia or single cell suspensions from cultured fetal gonads were prepared and stained essentially as previously described (Romanienko and Camerini-Otero, 2000). Slides were washed twice in PBS, and incubated o/n at 4°C with anti-SCP3 rabbit polyclonal antibody (Novus) diluted in blocking solution (1:100 in 10% goat serum, 3% BSA, 0.5% Triton X-100 in PBS). After washing, secondary antibody was added for 1 h at 37°C. The slides were washed and allowed to dry. Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) was added and the slides were viewed using a Leica microscope. Spreads analysis was performed in three independent experiments.

Statistical analysis

Continuous variables were summarized as mean and standard deviation (SD). All tests were two-sided and were determined by Monte Carlo's significance. A value threshold of 0.05 was used for the current analysis. T-test was used to test for differences between two independent groups, whereas One-way was used to test for differences among three or more independent groups. For multiple comparisons the Tukey's HSD (Honestly Significant Difference) test was carried out. All statistical tests were carried out using the SPSS statistical analysis software package, version 10.0.

Results

Developmental expression of Nanos2 in the male gonad.

It is known that *Nanos2* mRNA starts to be expressed in male PGCs at around 12.5 dpc (Suzuki et al., 2007). Furthermore, by immunofluorescence analysis it has been very recently shown that undifferentiated spermatogonia type A_s and A_{pr} are the testicular cells which express NANOS2 in the adult testis (Suzuki et al., 2009). However, it is not clear whether NANOS2 levels drop before birth or are maintained elevated in the early postnatal testis. To clearly address this question we performed a western blot analysis on male germ cells isolated at various stages of fetal and postnatal development. As shown in Fig.1A we found that NANOS2 protein levels were high at 15.5 dpc and then decreased up to birth. Since at 15.5 dpc NANOS2 levels were already high, to better define the fetal period when they start to increase, we analyzed protein extracts obtained from germ cells isolated at 12.5, 13.5, 15.5 dpc and 1 dpn. As shown in Fig.1B, we found that at 12.5 dpc Nanos2 levels were low but they consistently increased up to 15.5 dpc. We also found that NANOS2 was expressed after birth and its levels peaked at 5 dpp to decrease at 7 dpp, when differentiating pre-meiotic spermatogonia (KIT positive) and preleptotene spermatocytes are found (Fig.1A and B). Isolated pachytene spermatocytes and spermatids did not express *Nanos2* both at the RNA and protein levels (not shown). Thus, to determine which spermatogonial cell type was expressing *Nanos2* in the prepuberal testis, we isolated pure populations of undifferentiated or differentiating spermatogonia by immunomagnetic cell sorting. KIT negative (undifferentiated) and KIT positive (differentiating) spermatogonia were obtained by using anti-CD117 (Kit) coated beads and analyzed for *Nanos2* expression by semiquantitative RT-PCR. As shown in Fig.1C, *Nanos2* amplification was obtained only in KIT negative spermatogonia while KIT positive cells did not show any signal. Purity of the cell fractions was assessed by western blot analysis probing extracts for KIT and OCT4 (Fig.1C) or RT-PCR amplification of PLZF and OCT4 (not shown), as previously reported (Lolicato et al., 2008).

Retinoic Acid downregulates Nanos2 expression in fetal gonocytes and postnatal spermatogonia

RA has been proposed as a meiotic inducing substance both in fetal female germ cells and in postnatal male germ cells (Bowles et al., 2006; Koubova et al., 2006; Pellegrini et al., 2008). In *Cyp26b* null fetal testis, gonocytes enter meiosis and *Nanos2* mRNA

levels are low (Suzuki and Saga, 2008). This suggests that, apart from *Stra8* induction, the pro-meiotic action of RA might result also from the downregulation of *Nanos2* expression. To test this hypothesis, we cultured testes from 12.5 dpc embryos, the developmental stage in which *Nanos2* starts to be expressed, in the presence of 0.3 μM AtRA for 48h. As shown in Fig.2A AtRA completely abolished the expression of *Nanos2* mRNA, while it up-regulated *Stra8* mRNA levels. The same effect was observed when we cultured disaggregated 13.5 dpc testes for 24 h in the presence of AtRA (Fig.2A, see also Fig.3A). Using chromosomal spreads stained for SCP3, we found that about 33% of male germ cells were in the early meiotic leptotene stage in the presence of AtRA while no meiotic germ cells were present in the control. Using the same culture conditions, in the presence of AtRA about 85% female germ cells were found in leptotene and lepto-zygotene compared to 75% of the control (Fig. 2A, see also Fig. 3B).

We and others have previously demonstrated that G0 arrested gonocytes and undifferentiated, KIT negative, spermatogonia are not able to enter meiosis in vitro upon AtRA stimulation (Pellegrini et al., 2008; Trautmann et al., 2008). Since NANOS2 expression is high in these cells (see Fig.1) we tested if AtRA was able to down-regulate Nanos2 also at these developmental stages. The results showed that 48 hours of AtRA treatment actually down-regulated the levels of *Nanos2* both at mRNA and protein level in 15.5 dpc and 4 dpp isolated male germ cells (Fig. 2B). This effect was accompanied by STRA8 up-regulation (Fig. 2B) but, as expected, did not result in meiotic induction (Fig. 2C).

Fgf9 up-regulates Nanos2 and represses meiosis both in male and female germ cells.

XY *Fgf9* null gonocytes enter meiosis before undergoing apoptosis, suggesting that FGF9 prevents meiosis in XY germ cells (DiNapoli et al., 2006). Since this phenotype resembles that of *Nanos2* null gonocytes, we hypothesized that FGF9 could directly or indirectly up-regulate *Nanos2* expression in germ cells. To test this possibility, we chose a developmental age in which *Nanos2* mRNA levels are low in the fetal testis and are obviously absent in the fetal ovary. Thus, 12.5 dpc male or female gonads were cultured for 48 h in the presence or absence of 25 ng/ml FGF9. By semiquantitative RT-PCR we found that the levels of *Nanos2* mRNA were up-regulated by FGF9 treatment in fetal testes and, interestingly, *Nanos2* mRNA expression was stimulated also in fetal ovaries (Fig.3A and B). The same effect was observed when disaggegated 13.5 dpc gonads were cultured for only 24h in the presence of FGF9 (Fig. 3A). A control PCR

minus RT was run for *Nanos2* mRNA in order to exclude genomic contamination of the cDNA samples (Fig.3A) Since the expression of NANOS2 in female germ cells was found to be detrimental for meiotic entry (Suzuki and Saga, 2008), we prepared SCP3 stained nuclear spreads from fetal ovaries after 48h of culture with FGF9. The results showed that while in the control most of the female germ cells were in leptotene and lepto-zygotene (75%), less than one third of them entered meiosis in the presence of FGF9 (22%; Fig. 3B). The nuclear morphology of the non meiotic nuclei was similar to that of G0 arrested gonocytes (Fig 3 B, compare also with Fig. 2C) showing faintly stained chromatin by DAPI.

Finally, we investigated if FGF9 was able to affect meiosis also in 7 dpp spermatogonia. We found that FGF9 reduced the number of meiotic cells after 48h of culture, as judged by chromosomal spread counts (40% in the control versus 20% in the FGF9 treated cells, Fig. 4A) and by the decrease of SCP3 levels (Fig. 4B). The role of FGF9 in meiotic inhibition was not mediated by the decrease of STRA8 levels, since they did not change after FGF9 stimulation. To understand which cell population was responsive to FGF9, we then isolated KIT positive and KIT negative spermatogonia by immunomagnetic cell sorting and stimulated with the factor for 24 h. FGF9 sensitivity was present in both cell types, since it induced up-regulation of NANOS2 levels either in KIT negative and KIT positive spermatogonia, as shown by RT-PCR and by western blot analyses (Fig. 4C).

Nanos2 associates with RNPs and polysomes and interacts with Pumilio2

In the *Drosophila* germline progenitor cells, the pole cells, NANOS has been shown to interact with PUMILIO (PUM) to repress *Cyclin B* mRNA translation (Kadyrova *et al.*, 2007). To date, none of NANOS mammalian homologs has been demonstrated to act as translational repressors, but we have recently shown that NANOS3 is able to interact with PUM2 and associates with the RNPs, in an heterologous system (Lolicato *et al.*, 2008). To test whether NANOS2 function might be involved in the control of translation, a *Myc*-tagged *Nanos2* expressing vector was transfected in Hek293T. By immunofluorescence analysis using anti-MYC antibodies, we found that NANOS2-MYC was distributed both in the cytoplasmic and the nuclear compartments of transfected cells (Fig. 5A). Cytoplasmic extracts were then fractionated on a sucrose gradient and analyzed by Western blot. Figure 5B shows that NANOS2-MYC was found mostly in the RNP fractions but also in the ribosomal and light polysome fractions. We then followed the distribution pattern of endogenous NANOS2 on sucrose

gradients using 16.5 dpc or 7 dpp spermatogonia extracts (Fig. 6A). Similarly to what observed with the transfected cells, NANOS2 co-localized with the RNP fractions as well as with those containing the ribosomal subunits and light polysomes. The sedimentation pattern of PUM2 on the sucrose gradients mirrored that of NANOS2. On the contrary, TIAR, another RNA binding protein known to be a translational repressor (Mazan-Mamczarz *et al.*, 2006) essential for germ cell survival (Beck *et al.*, 1998), was found to be exclusively present in the RNP fractions (Fig. 6A). The finding that NANOS2 also associates with polysomes was further verified by fractionating 7 dpp spermatogonial extracts on sucrose gradients in the presence of EDTA, to disrupt polysomes. As shown in Fig.6, NANOS2 association with the light polysomal fractions was completely reverted toward the ribosomal subunits and the RNPs in the presence of EDTA, as occurs for DAZL, an RNA binding protein which has been shown to be present both in the RNP and in the polysomal fractions (Tsui *et al.*, 2000).

NANOS2 interacts with PUM2 and binds to target mRNAs.

We have previously shown that PUM2 is able to bind not only NANOS3 but also NANOS2 when used as a bait in pull-down experiments (Lolicato et al., 2008). Since the sedimentation profile of NANOS2 and PUM2 suggested that they may associate in vivo (Fig. 6), we tested the ability of NANOS2 to interact with the endogenous PUM2 from germ cell extracts in a pull-down assay. We prepared protein extracts from fetal gonocytes (16.5 dpc) or (4 dpp) postnatal spermatogonia and incubated them with a GST- or a GST-NANOS2-agarose coupled resin (Fig. 7A). We found that PUM2 from both the fetal and postnatal cell extracts was able to interact with GST-NANOS2, while a faint background band was observed in the presence of GST (Fig. 7B).

Preliminary data obtained by microarray analysis reported that significant alterations occur in the transcriptome of the developing male gonad of *Nanos2*-null mice (Saba R, 2009). To verify if murine NANOS2 works as an RNA-binding protein, we undertook a GST-RNA pull-down approach, by probing GST or GST-NANOS2 agarose beads with ribonucleoproteic extracts from postnatal spermatogonia. RNA molecules bound to GST-NANOS2 or to GST were then extracted and reverse transcribed to obtain cDNAs. Using primers that spanned the 3'UTR of the putative target genes, we looked for some mRNAs whose expression was altered in the *Nanos2* knockouts and/or for transcripts which displayed a sexual dimorphic expression pattern during fetal gonad development. Specifically, we screened the cDNAs obtained from the RNA pull-down for *Stra8*, *Taf7l*, *Kit*, *Gcnf1* and *Gata2*, by PCR analysis. We found

that *Gata2* and *Taf7l* mRNAs were retained specifically by GST-NANOS2 beads (Fig. 7C), while we did not observe any specific signal for *Kit*, *Stra8* or *Gcnf1* (Fig. 7C). We actually found that perfect consensus binding sites for PUM2 [UGUANAUA(A/G)NNNN(C/G/U)(C/G/U)(C/G/U)(C/G/U)(C/G)CC] (White et al., 2001) are located within the 3' UTR of both *Gata2* and *Taf7l* mRNAs, supporting the notion that a complex of NANOS/PUMILIO is essential for mRNA binding. When looking in the Geo Profile database for *Taf7l* and *Gata2* expression in germ cells, we found that these two genes were not only differentially regulated in the developing male and female gonads, but their levels were higher in the differentiating type B compared to type A spermatogonia (Supplementary Fig. 1A and B). Interestingly, *Gata2* mRNA levels were found significantly increased in the postnatal testis in a period in which high levels of *Kit* are also found (Suppl. Fig.1 C).

Discussion.

NANOS2 is a potential RNA-binding protein which functions cell autonomously to control germ cell sexual differentiation during fetal development. It has been proposed to act as an anti-meiotic gene since its deletion, in mice, is associated with abortive meiosis in fetal gonocytes, before they undergo apoptosis. Furthermore, its misexpression in female PGCs prevents meiosis and makes the nuclear morphology of these cells similar to that of G0 arrested gonocytes (Suzuki and Saga, 2008).
In the present paper we used fetal germ cells and postnatal spermatogonia as models to understand the function of NANOS2 and its relationship with the beginning of meiosis.

Nanos2 expression and meiotic commitment

By analyzing the developmental profile of NANOS2 expression in the testis during the perinatal period, we found that NANOS2 is continuously expressed from the fetal to the postnatal period and it shows two peaks of expression: one at around 15.5 dpc and another one at around 5 dpp. The first peak temporally corresponds to the mitotic block period, which male germ cell undergo prenatally. In this period gonocytes choose not to enter meiosis and retain stem cell features, as demonstrated by the ability to complete spermatogenesis when transplanted into adult testes (Ohta *et al.*, 2004). The second peak corresponds to a period in which NEUROGENIN 3 (NGN3) expressing cells, the undifferentiated spermatogonia (Nakagawa et al., 2007; Yoshida et al., 2004) start differentiation into KIT-positive cells (Pellegrini et al., 2008). During this period

the spermatogonial stem cell factor PLZF (Promyelocytic Leukemia Zinc Finger) tightly controls *Kit* mRNA expression in stem cells (Filipponi *et al.*, 2007), and they are completely lost when *Kit* is not repressed (Buaas *et al.*, 2004; Costoya *et al.*, 2004). By purifying KIT positive and KIT-negative spermatogonia we found that NANOS2 expressing cells were present only within the KIT-negative spermatogonia population, which corresponds to the PLZF expressing spermatogonia at 4-5 dpn, indicating that *Nanos2* is a spermatogonial stem cell expressed gene. In agreement with our results, during the revision of this paper, two studies have shown that NANOS2 is mostly expressed in A_s ands A_{pr} spermatogonia from the adult testis and that its conditional deletion in adulthood leads to spermatogonia stem cell loss (Sada et al., 2009; Suzuki et al., 2009)

Evidences from the genetic studies have suggested NANOS2 acting as a suppressor of meiosis in male fetal germ cells (Suzuki and Saga, 2008). We found that AtRA, a derivative of RA and a well-known pro-meiotic factor (Bowles et al., 2006; Pellegrini et al., 2008), was able to down-regulate *Nanos2* both at mRNA and protein levels in male PGCs (12.5 dpc) and in undifferentiated spermatogonia. In mitotically arrested gonocytes (15.5 dpc), on the contrary, we observed that the strong decrease of *Nanos2* mRNA levels induced by AtRA was paralleled only by a less intense decrease of the protein levels. Since this different behaviour was consistently reproduced, we can hypothesize that NANOS2 protein stability is higher in mitotically arrested germ cells compared to proliferating germ cells. We found that AtRA increased the percentage of proliferating male PGCs entering meiosis but was uneffective on mitotically arrested gonocytes or on undifferentiated spermatogonia. For arrested gonocytes, this discrepancy can be explained by the existence of a narrow window for meiotic competence, which corresponds to the period in which Nanos2 starts to be expressed (12.5 dpc) and is needed to repress the meiotic fate (Saga, 2008; Suzuki and Saga, 2008). After this period, high levels of NANOS2, due to increased protein stability and/or to enhanced synthesis stimulated by factors such as FGF9 (see below), “masculinise” gonocytes. The evidence that AtRA does not trigger meiosis in undifferentiated spermatogonia, notwithstanding the modulation of NANOS2 and STRA8, suggests that these cells lack other molecular factors required for meiotic entry. One of these factors could be the KIT tyrosine kinase receptor, whose downstream signalling is essential for meiotic entry of differentiating spermatogonia in vitro (Pellegrini et al., 2008). In line with this hypothesis is the observation that *Kit* is expressed in proliferating fetal germ cells of both sexes up to 12.5-13.5 dpc (Manova

and Bachvarova, 1991), when the "meiotic window" is active, and is then re-expressed in the male only postnatally, in differentiating spermatogonia, the meiotic competent cells of the testis.

In search for factors that could up-regulate NANOS2 levels and negatively influence meiosis, we found that FGF9 was a good candidate. Fibroblast growth factors (FGFs) play a role in the proliferation and survival of many cell types (Ornitz and Itoh, 2001). *Fgf9* is expressed in the undifferentiated gonad of both sexes at 11.5 dpc, but it becomes XY restricted by 12.5 dpc and is maintained in the testis cords (Schmahl *et al.*, 2004). Sertoli cell precursors fail to proliferate in *Fgf9* mutants and testis differentiation is disrupted (Schmahl *et al.*, 2004). The majority of germ cells are lost at 12.5 dpc, however the surviving gonocytes within the *Fgf9* null gonads are found in meiosis (DiNapoli *et al.*, 2006), suggesting that FGF9 normally promotes survival and prevents meiosis of fetal male germ cells. Indeed, we found that FGF9 up-regulated Nanos2 and strongly inhibited meiosis in female PGCs in vitro. This observation is in line with the evidence that *Nanos2* misexpression in female germ cells inhibits meiosis and masculinises germ cell nuclei (Suzuki and Saga, 2008). FGF9 up-regulated NANOS2 also in postnatal spermatogonia, and such increase was not observed after a short incubation time (not shown), suggesting that a transcriptional mechanism likely mediates this effect. Sensitivity to FGF9 was present in the undifferentiated and in the differentiating spermatogonia, since both these cell types express an FGF9 receptor, as confirmed by microarray analysis (Rossi *et al.*, 2008). We observed a negative effect of FGF9 on meiotic entry also in differentiating spermatogonia, suggesting a common mechanism shared between female and male germ cells in the control of the mitotic/meiotic switch. It has been hypothesized that a possible mechanism for NANOS2 in the prevention of meiosis is to repress STRA8 expression during the period in which CYP26B1 levels decrease in the male gonad and RA is increased in the mesonephroi. In our studies we did not observe STRA8 down-regulation after FGF9 stimulation in vitro of postnatal spermatogonia, suggesting that NANOS2 might act independently or downstream of STRA8, even if we cannot exclude that other mechanisms are active in vivo.

Nanos2 subcellular localization and molecular interactions.

In contrast to what we observed for NANOS3 (Lolicato *et al.*, 2008), NANOS2 was found associated not only with RNPs, but also with the ribosomal and polysomal fractions. This localization is uncommon for translational repressors, but it has been

recently shown that translational repression can occur also at the polysomal level, during protein synthesis, through a miRNA repressive mechanism (Cannell et al., 2008; Gebauer and Hentze, 2004; Petersen et al., 2006). Furthermore, it cannot be ruled out the possibility that NANOS2, and eventually PUM2, might function both as translational repressors and activators, as it has been shown for FMRP (Zalfa *et al.*, 2006), or at other levels of post-transcriptional regulation of mRNA expression, such as the control of mRNA stability.

When transfected in Hek293T cells, NANOS3 associates with PUM2 and is found within the RNPs (Lolicato *et al.*, 2008). Since it has been shown that NANOS2 can complement the NANOS3 defect in *Nanos3* null mice, we reasoned that it could also recognize the same partners in male germ cells. Indeed, we found that NANOS2 was able to bind PUM2 expressed either in fetal gonocytes or in postnatal spermatogonia, suggesting that PUM2 can interact in vivo with NANOS2, probably both acting in posttranscriptional mRNA regulation. In the attempt to discover potential Nanos2 targets, we looked for genes showing a sexual dimorphic expression pattern during gonadal differentiation and/or more abundantly expressed in differentiating spermatogonia. Among several genes tested (*Kit, Taf7l, Gata2, Stra8 and Gcnf1*), *Gata2* and *Taf7l* mRNAs were found to interact with GST-NANOS2. GATA2 is a zinc finger transcription factor expressed in fetal oocytes up to 15.5 dpc but not in fetal gonocytes (Siggers et al., 2002). There are no studies on *Gata2* expression in postnatal gonads, however its mRNA levels are found strongly increased in differentiating spermatogonia, according to the microarray analysis database (Geo Profiles) at NCBI. While the role of GATA2 in germ cells is unknown, it is well established that in hematopoietic stem cells this factor controls *Kit* expression at the transcriptional level (Orkin, 1992). *Taf7l* is a component of the transcriptional machinery specific of preleptotene spermatocytes and haploid spermatids (Pointud et al., 2003) and its deletion leads to reduced male fertility (Cheng et al., 2007). Thus, the identification of two transcription factors involved in the differentiation of spermatogonia as targets of post-transcriptional control by NANOS2 is in line with the evidence that NANOS2 negatively regulates differentiation of spermatogonia stem cells (Sada et al., 2009). Studies to identify other NANOS2 mRNA targets, whose post-transcriptional regulation is determinant for spermatogonia differentiation, will be important to understand how commitment of stem cell occurs in the initial phase of spermatogenesis.

In conclusion, our results can be interpreted as schematically depicted in Fig. 8. RA and FGF9 produced locally by the somatic cells of the gonads act in opposite ways to regulate *Nanos2* expression and meiotic entry both in fetal and postnatal germ cells. In the fetal ovary, FGF9 levels sharply decrease at 11.5 dpc in coincidence with the raise of circulating levels of RA which stimulate *Stra8* expression in PGCs and their commitment to meiosis. In the fetal testis, FGF9 levels are kept high and circulating RA is degraded by CYP26B1 (not depicted) in somatic cells. High levels of FGF9 without RA signalling in male PGCs upregulate *Nanos2* determining their transition into mitotically arrested gonocytes. In the postnatal testis, *Nanos2* is expressed exclusively in SSCs and Sertoli cells safeguard the delicate balance between stem cell self renewal and meiotic commitment of spermatogonia by modulating the levels of FGF9 and RA.

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FIGURE LEGENDS

Figure 1. **Developmental expression of NANOS2.**
A) Western blot analysis of NANOS2 from fetal (15.5 dpc) throughout postnatal life (7 dpp). Male germ cells express NANOS2 at 15.5 dpc and the protein levels decrease around birth, and peak again at 5 dpp to decrease later at 7 dpp. On the right panel it is reported the densitometric analysis of western blots from three separate experiments. Bars represent mean ±standard deviations (SD), $p<0.001$. B) Western blot analysis of NANOS2 expression starting at earlier developmental stages. On the right side, the densitometric analysis of the western blots from three independent experiments is shown. Bars represent mean ±SD, $p<0.001$. C) On the left, schematic representation of spermatogonia development in the prepuberal testis (7 dpp). Two populations can be distinguished: the undifferentiated spermatogonia, that do not express KIT, and the differentiated KIT positive spermatogonia. Adapted from (de Rooij and Mizrak, 2008). Middle panel shows that *Nanos2* expression is restricted to the undifferentiated spermatogonia population by semiquantitative RT-PCR or by western blot analysis (right panel).

Figure 2. **AtRA downregulates *Nanos2* both in fetal and postnatal male germ cells.**
A) Right side panel, upper part, male fetal gonads were isolated at 12.5 dpc and cultured in vitro for 48 hours in the presence of AtRA. RT-PCR analysis shows that *Stra8* and *Nanos2* mRNA expression are oppositely modulated by AtRA treatment. Lower panel, testes from 13.5 dpc embryo were collected, disagregated, and kept in culture for 24 hours in the presence of AtRA. RT-PCR analysis shows that AtRA strongly reduces *Nanos2* expression also at this developmental stage. The histogram represents the percentage of meiotic nuclei scored in control or in AtRA treated cultures of 12.5 dpc gonads. Three independent experiments performed in triplicate are represented. Bars show mean ±SD, $p<0.001$. B) Isolated male germ cells of 15.5 dpc and 4 dpp were stimulated in vitro with AtRA for 48 hours. Western blot (middle panels) and semiquantitative RT-PCR (external panels) analyses show that, concomitantly with STRA8 upregulation, NANOS2 is down regulated in AtRA treated cells. The right panel represents the densitometric analysis of the western blots from three independent experiments performed in triplicate. Bars represent mean ±SD, $p<0.001$. C) Nuclear spreads from 15.5 dpc (left panels) or 4 dpp (right panels) spermatogonia were prepared after 48 hours of culture in the presence or absence of AtRA and probed for SCP3.

Merged images of SCP3 staining, which does not correspond to correctly assembled synaptonemal complexes, and DAPI.

Figure 3. **FGF9 stimulates *Nanos2* expression and inhibits meiosis in fetal gonads.**
A) Semiquantitative RT- PCR from control and FGF9 treated cultures of 12.5 dpc whole gonads (48h) or disaggregated 13.5 dpc (24h) gonads. The expression of *Nanos2* is upregulated upon FGF9 stimulation in both sexes, at both stages and in both culture conditions. On the right, a control PCR analysis without reverse transcriptase was run with cDNAs from 13.5 dpc male disaggregated gonads cultured with and without FGF9 or AtRA for 24 h Densitometric analysis of three independent experiments. Bars represent mean $\pm$SD, $p<0.001$. B) Nuclear spreads of the corresponding cultures, immunostained for SCP3 to detect the presence of synaptonemal complexes. Graphical representation of meiotic chromosomes counts both in fetal male and female gonads, from three independent experiments, is shown on the right. Bars represent mean $\pm$SD, $p<0.001$.

Figure 4. **FGF9 stimulates *Nanos2* expression and inhibits meiosis in postnatal spermatogonia.**
Spermatogonia from prepuberal testes (7dpp) were cultured in the presence or absence of FGF9 for 48h. A) Nuclear spreads of the corresponding cultures stained for SCP3 and DAPI. The histogram represents the percentage of meiotic nuclei scored in control or in FGF9 treated cultures from three independent experiments. Bars shows mean $\pm$SD, $p<0.001$. B) Western blot analysis for NANOS2 (Abnova antibody), STRA8, SCP3 and TUBULIN. NANOS2 levels are increased by FGF9 while SCP3 levels are decreased. STRA8 is not affected. The bar indicates the specific NANOS2 band, used for densitometry, while asterisk denotes a non specific one. Densitometric analysis of the western blots from three separate experiments is shown on the right. Bars represent mean $\pm$SD, $p<0.001$. C) Immunomagnetic sorted KIT positive and KIT negative spermatogonia were analyzed for the sensitivity to FGF9. Semiquantitative RT-PCR on mRNAs (left panel) or western blot on protein extracts (right panel) from KIT- and KIT+ spermatogonia were probed for NANOS2, ACTIN and KIT.

Figure 5. **NANOS2 co-sediments with RNPs and polysomes in Hek293T.**

A) Transfection of *Myc*-tagged *Nanos2* in Hek293T cells. Immunofluorescence analysis with anti-MYC antibodies shows that NANOS2 is distributed mainly in the cytoplasm but also in the nucleus. B) RNP-Polysome fractionation on sucrose gradients of Hek293T cells transfected with *myc-Nanos2*. Western blot analysis of the collected fractions shows that NANOS2 sediments mainly in the light fraction of the gradient (fraction 10, i.e. RNPs) but it is also present through the ribosomal subunits (fractions 7, 8, and 9) and to a less extent in the polysomes (fractions 3, 4, 5, 6). S6 pattern and UV absorbance profile demonstrate the correct fractionation assay.

Figure 6. **NANOS2 associates with RNPs and polysomes of fetal and postnatal male germ cells.**

A) Fetal (16.5 dpc) and postnatal (7 dpp) male germ cells were collected and cytoplasmic extracts were fractionated on sucrose gradients. Western blot analysis of the collected fractions shows that NANOS2 sediments with the RNPs fraction, with the ribosomal subunits and the polysomal fractions. PUM2 showed a sedimentation pattern similar to NANOS2, suggesting that both proteins can interact in vivo. In contrast, the translational repressor TIAR is found exclusively in the RNPs fraction in both fetal and postnatal stages. S6 and UV absorbance are presented as controls of correct fractionation. B) Untreated (Mg^{++}) or EDTA-treated (EDTA) 7 dpp spermatogonial extracts were fractionated on sucrose gradients and analyzed by UV spectrometry (not shown) and western blotting for NANOS2 and DAZL.

Figure 7. **NANOS2 interacts with PUMILIO2 to regulate target mRNAs.**

Protein and RNA pull-down were performed to investigate the interaction between Nanos2 and Pumilio2. A) Coomassie staining of GST and GST-coupled NANOS2 used for pull-down experiments. B) Western blot of cell extracts from fetal (16.5 dpc) and postnatal (4dpn) male germ cells loaded onto GST or GST-NANOS2-agarose beads and probed for PUM2. A specific band for PUM2 is observed in the presence of GST-NANOS2 in both fetal and postnatal cell extracts. C) RT-PCR for potential target mRNAs of NANOS2.

Figure 8. **Opposing effects of RA and FGF9 on *Nanos2* expression and meiotic entry of mouse germ cells.**

The niche between Sertoli cell precursors and fetal gonocytes may be equivalent to the stem cell niche of the postnatal testis. Fetal gonocytes and spermatogonia stem cells

could have an equivalent cell fate signed by the expression of *Nanos2*. Such fate would be lost when spermatogonia are committed to meiosis. In the fetal testis, Sertoli cell precursors secrete FGF9 to induce *Nanos2* expression and masculinisation of PGCs (that become gonocytes). FGF9 upregulates *Nanos2* and prevents meiosis. Postnatally, the balance between FGF9 and RA secreted by Sertoli cells regulates the levels of *Nanos2* expression in undifferentiated spermatogonia (NANOS2 positive cells) and in differentiating spermatogonia (STRA8 and KIT positive cells). In the fetal ovary FGF9 secretion from somatic cells drops early during gonadogenesis and as a consequence PGCs are not masculinised. High RA levels ensure meiotic entry through *Stra8* and possibly *Kit* induction

Supplementary Figure 1. **Developmental expression of Taf7l and Gata2.**
Microarray data were obtain from GeoProfiles Database (NCBI). A) Sexual dimorphic expression of *Taf7l* (on the left) and *Gata2* (on the right) in the fetal ovary (pale green) and the fetal testis (dark green) at 13 dpc. Both transcripts are found exclusively in the ovary at this developmental stage. B) Expression of *Taf7l* (on the left) and *Gata2* (on the right) in type A spermatogonia (palest green), type B spermatogonia (paler green), pachytene spermatocytes (pale green), and spermatids (dark green). Both transcripts are found in type A and type B spermatogonia. C) *Gata2* developmental expression in the postnatal testis shows a critical expression window at 8 dpp, that corresponds to the developmental period in which KIT positive spermatogonia are particularly abundant.

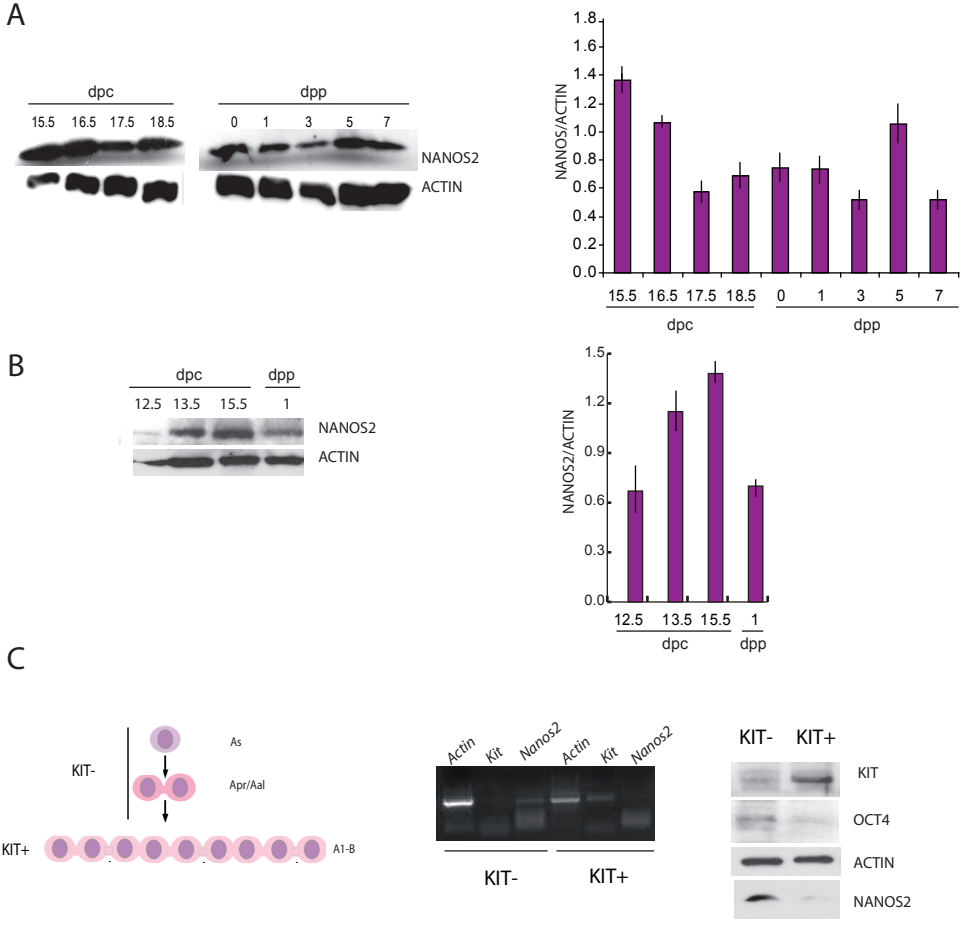

A
dpc
15.5 16.5 17.5 18.5
dpp
0 1 3 5 7
NANOS2
ACTIN
NANOS/ACTIN
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
15.5 16.5 17.5 18.5 0 1 3 5 7
dpc
dpp
B
dpc
dpp
12.5 13.5 15.5 1
NANOS2
ACTIN
NANOS2/ACTIN
1.5
1.2
0.9
0.6
0.3
0.0
12.5 13.5 15.5 1
dpc
dpp
C
KIT-
As
Apr/Aal
KIT+
A1-B
Actin Kit Nanos2 Actin Kit Nanos2
KIT-
KIT+
KIT- KIT+
KIT
OCT4
ACTIN
NANOS2

FIGURE 1

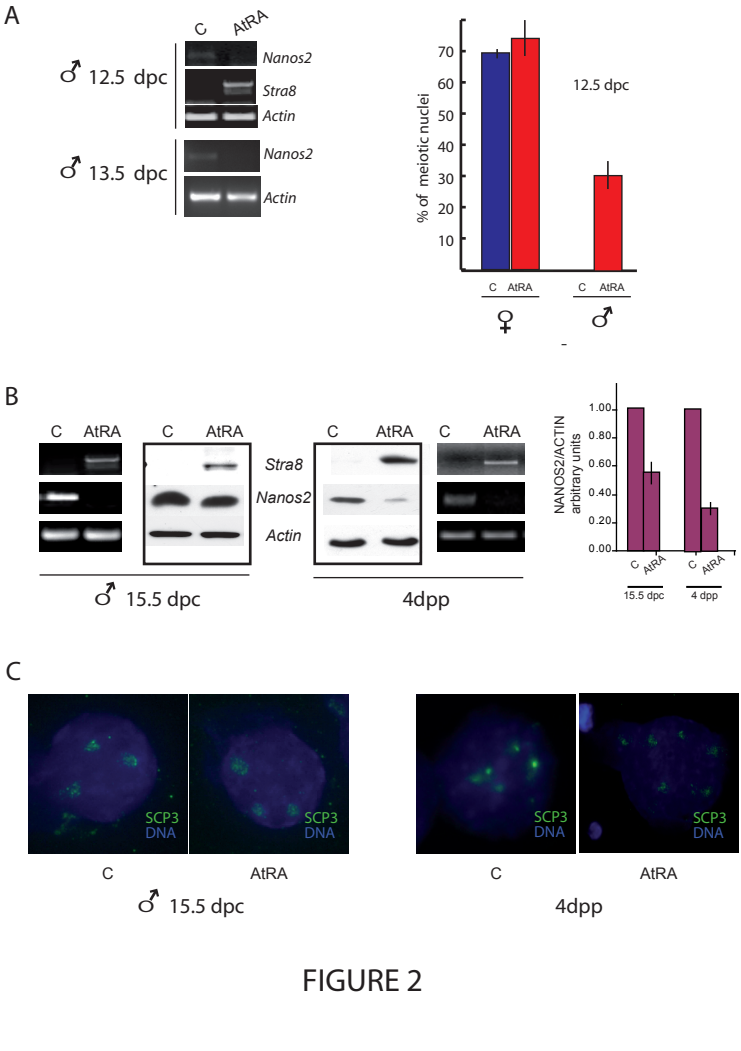
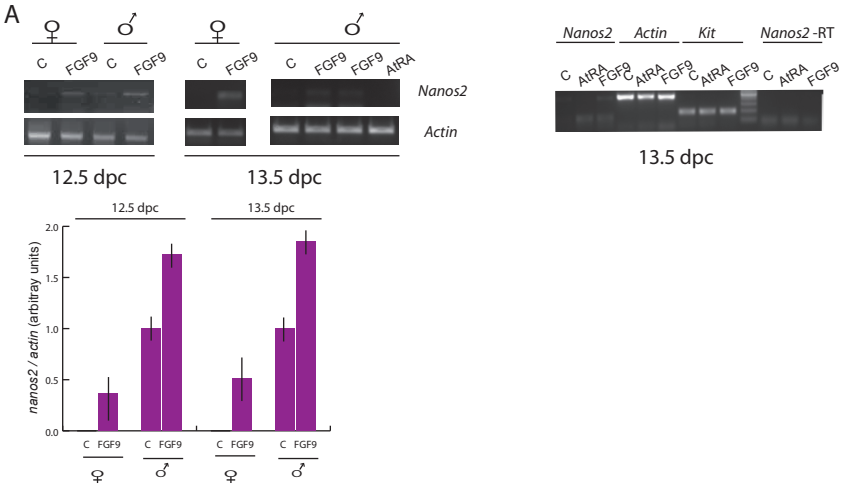

A
C
AtRA
♂ 12.5 dpc
Nanos2
Stra8
Actin
♂ 13.5 dpc
Nanos2
Actin
% of meiotic nuclei
70
60
50
40
30
20
10
12.5 dpc
C AtRA
C AtRA
♀
♂
B
C AtRA C AtRA
C AtRA C AtRA
Stra8
Nanos2
Actin
♂ 15.5 dpc
4dpp
NANOS2/ACTIN arbitrary units
1.00
0.80
0.60
0.40
0.20
0.00
C AtRA C AtRA
15.5 dpc
4 dpp
C
SCP3
DNA
C
AtRA
♂ 15.5 dpc
C
SCP3
DNA
C
AtRA
4dpp

FIGURE 2


A
♀
♂
C
FGF9
AtRA
Nanos2
Actin
12.5 dpc
13.5 dpc
Nanos2 -RT
Kit
nanos2 / actin (arbitray units)
2.0
1.5
1.0
0.5
0.0

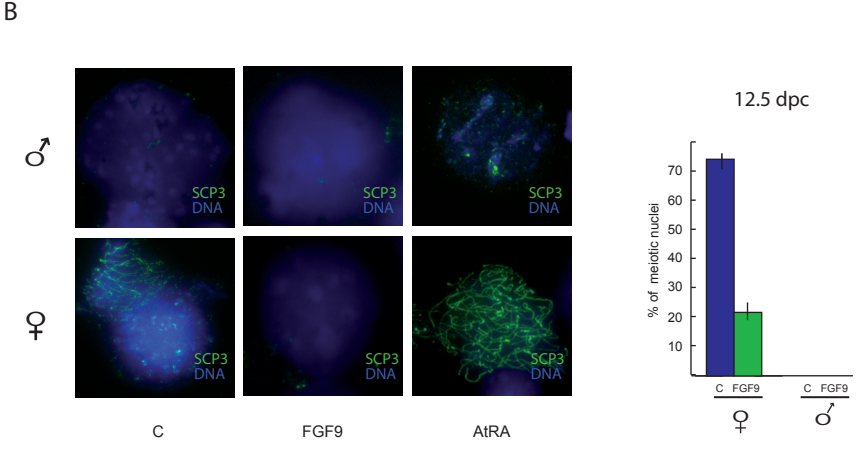
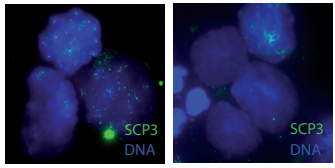
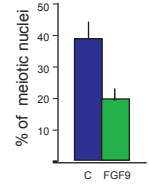

B
SCP3
DNA
C
FGF9
AtRA
12.5 dpc
% of meiotic nuclei
70
60
50
40
30
20
10

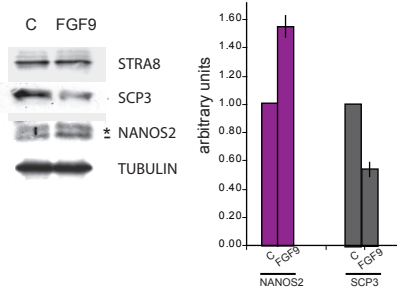
FIGURE 3

A


SCP3
DNA
SCP3
DNA
C
FGF9


% of meiotic nuclei
50
40
30
20
10
C FGF9

B


C FGF9
STRA8
SCP3
NANOS2
TUBULIN
arbitrary units
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
C FGF9
NANOS2
C FGF9
SCP3

C

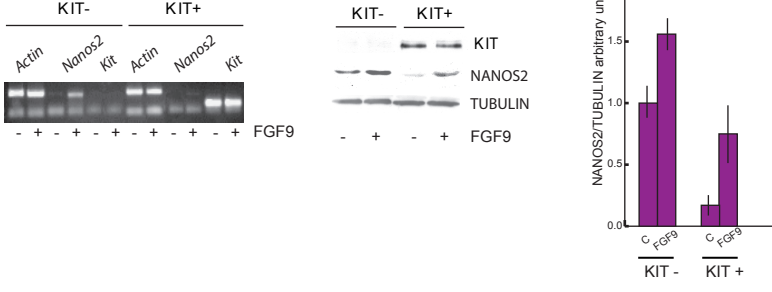

KIT-
KIT+
Actin
Nanos2
Kit
Actin
Nanos2
Kit
- + - + - + - + - + - + FGF9
KIT- KIT+
KIT
NANOS2
TUBULIN
- + - + FGF9
NANOS2/TUBULIN arbitrary units
2.0
1.5
1.0
0.5
0.0
C FGF9
C FGF9
KIT -
KIT +

FIGURE 4

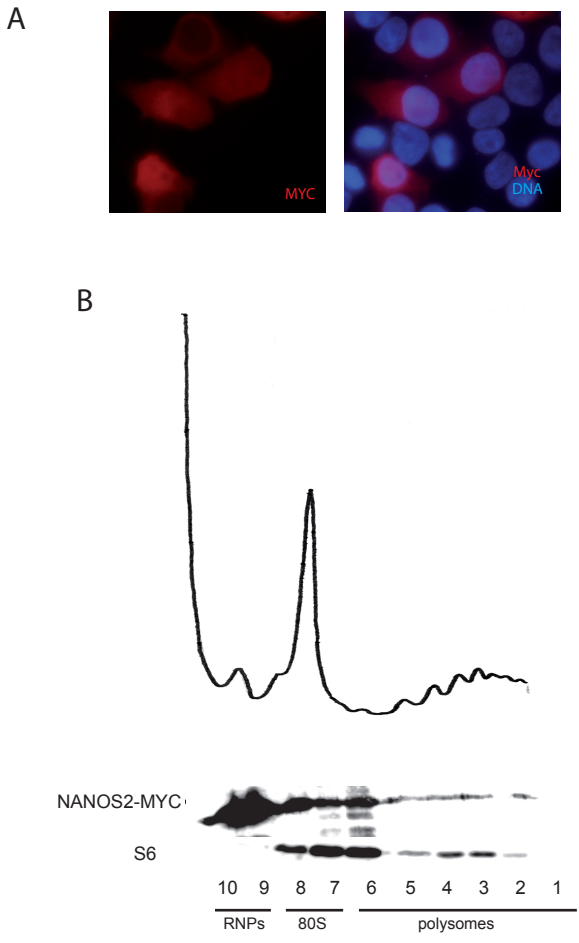


FIGURE 5

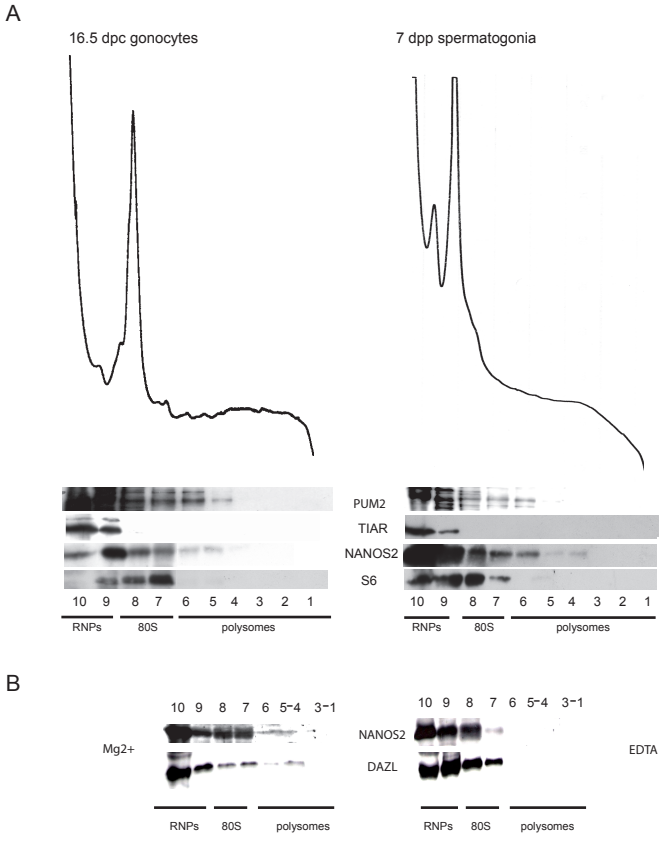

A
16.5 dpc gonocytes
7 dpp spermatogonia
PUM2
TIAR
NANOS2
S6
10 9 8 7 6 5 4 3 2 1
RNPs
80S
polysomes
B
Mg2+
EDTA
NANOS2
DAZL
10 9 8 7 6 5-4 3-1
RNPs
80S
polysomes

FIGURE 6

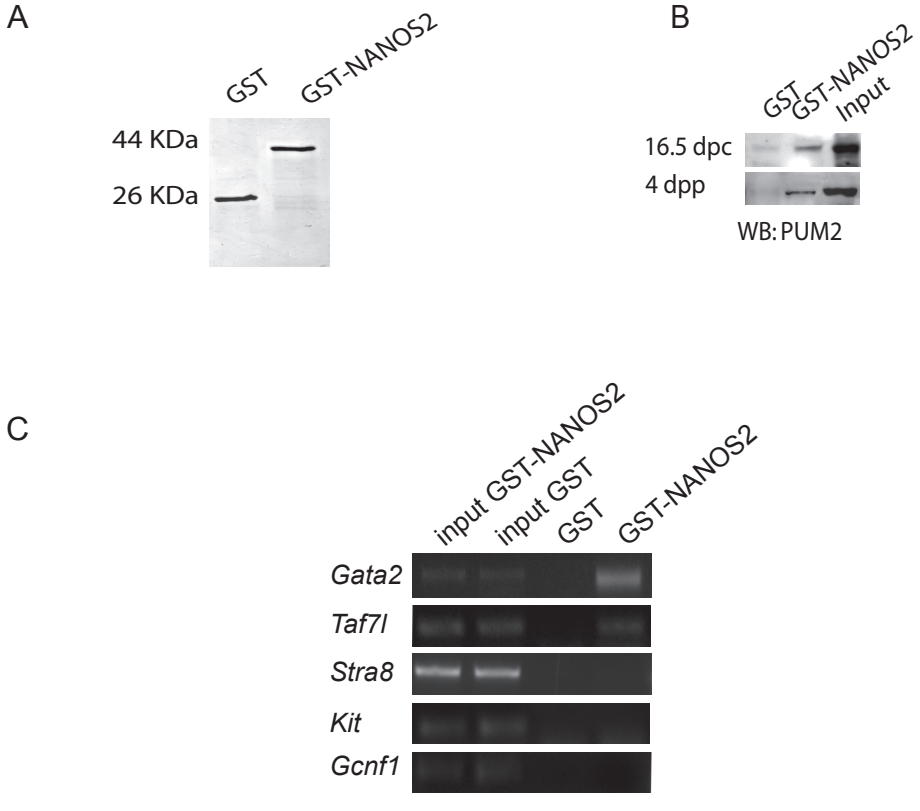
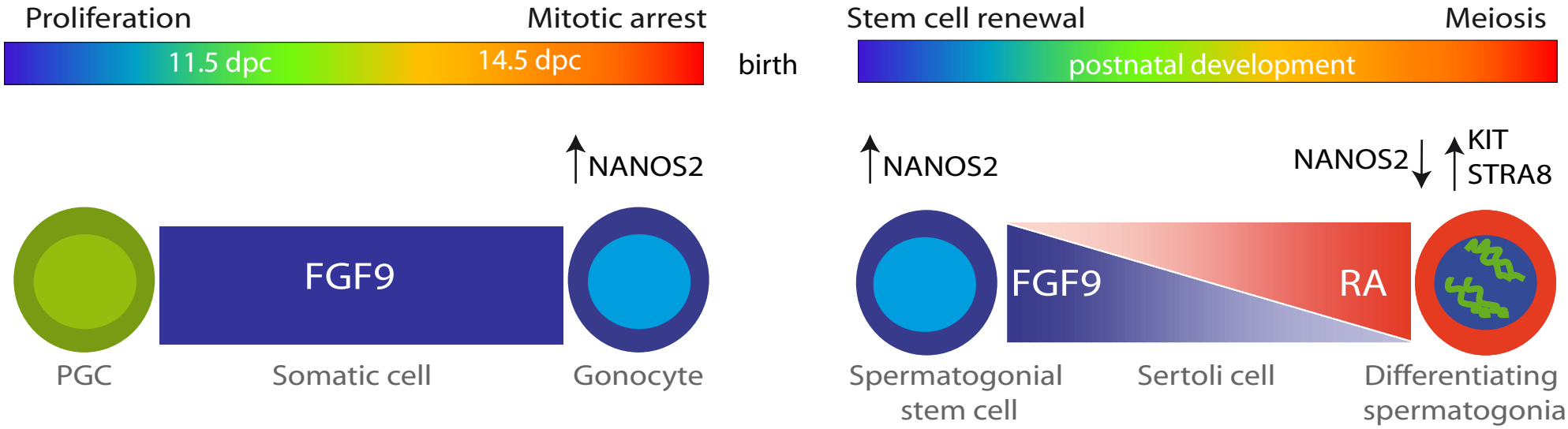

A
GST
GST-NANOS2
44 KDa
26 KDa
B
GST
GST-NANOS2
Input
16.5 dpc
4 dpp
WB: PUM2
C
input GST-NANOS2
input GST
GST
GST-NANOS2
Gata2
Taf7l
Stra8
Kit
Gcnf1

FIGURE 7


Proliferation
Mitotic arrest
11.5 dpc
14.5 dpc
birth
Stem cell renewal
Meiosis
postnatal development
NANOS2
FGF9
NANOS2
NANOS2
KIT
STRA8
FGF9
RA
PGC
Somatic cell
Gonocyte
Spermatogonial stem cell
Sertoli cell
Differentiating spermatogonia

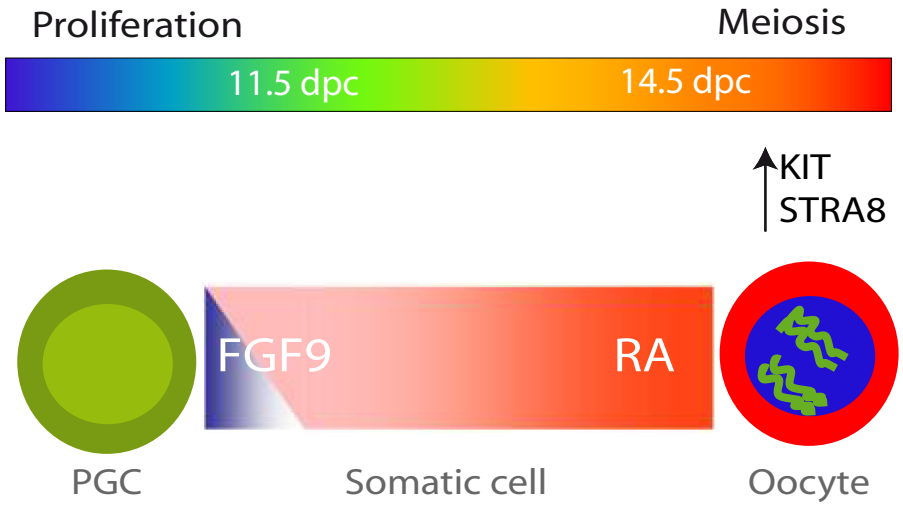
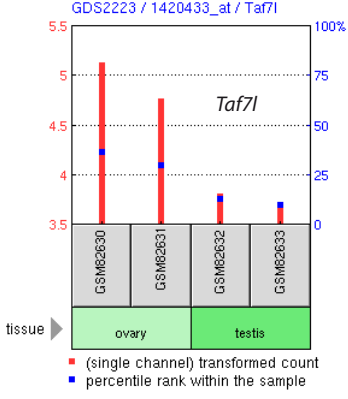

Proliferation
Meiosis
11.5 dpc
14.5 dpc
KIT
STRA8
FGF9
RA
PGC
Somatic cell
Oocyte

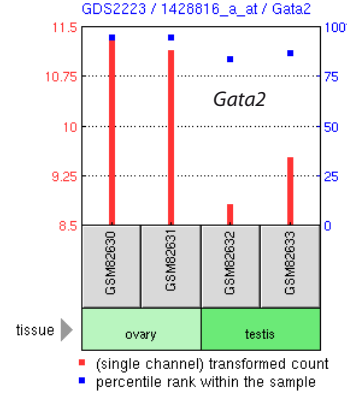
FIGURE 8

SUPPLEMENTARY FIGURE 1

A

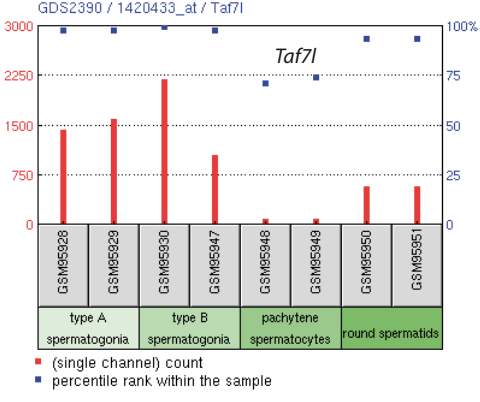
Fetal ovary versus fetal testis

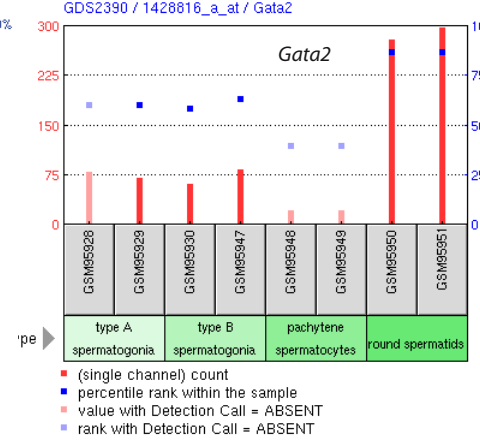




B

Spermatogonia (type A and type B), spermatocytes, spermatids





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CHAPTER 4

The role of Sohlh transcription factors on spermatogonia differentiation.

The role of *Sohlh* transcription factors on spermatogonia differentiation.

Manuscript in preparation.

Florencia Barrios[§1], Doria Filipponi[§2], Federica Bramucci[1], Manuela Pellegrini[1], Aleksandar Rajkovic[3], Sergio Ottolenghi[4], Pellegrino Rossi[1], Emmanuele A. Jannini[5] and Susanna Dolci*[1].

[§] These two authors contributed equally to this work.

[1] Department of Public Health and Cellular Biology, University of Rome 'Tor Vergata', Rome, Italy.

[2] Present address: Institute of Molecular and Cell Biology, IMCB, Singapore.

[3] Baylor College of Medicine, Texas, USA

[4] Department of Biotechnology and Biosciences, University of Milan 'Bicocca', Milan, Italy

[5] Department of Experimental Medicine, University of L'Aquila, L'Aquila, Italy

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**Author for correspondence*:
Susanna Dolci, MD, PhD
Dept. Public Health and Cell Biology
University of Rome Tor Vergata
Via Montpellier 1, Bldg. E Nord, 00133, Rome, Italy
dolci@uniroma2.it
ph:+390672596252 fax: +390672596268

ABSTRACT

How *Kit* expression is regulated in the germline is still an open question. Two transcription factors specifically expressed in germ cells, namely SOHLH1 and SOHLH2, have been recently involved in the process of spermatogonia differentiation. In the male, deletion of each transcription factor leads to the disappearance of *Kit*-expressing spermatogonia in the prepuberal testis. In the female, ablation of either *Sohlh1* or *Sohlh2* is responsible of oocyte loss in the neonatal ovary. To investigate if *Kit* expression might be regulated by these two transcription factors, we first performed a time course expression study of SOHLH1 and SOHLH2 during fetal and postnatal development. A temporal correlation among these genes was found only in postnatal spermatogonia. Transfection experiments of *Kit* regulatory regions driving EGFP expression in Hek293T showed that E-box sequences within the promoter are essential for *Kit* transactivation when either *Sohlh1* or *Sohlh2* were co-expressed. The concomitant presence of both factors in the transfection showed a cooperative effect on Kit trnsactivation. EMSA experiments further showed that SOHLH1 and SOHLH2 can independently and cooperatively bind a probe containing two spaced E-boxes. Finally, by co-immunoprecipitation experiments we found that the two proteins can interact in vivo. Altogether, our results suggest that SOHLH1 and SOHLH2 directly activate Kit transcription in postnatal spermatogonia.

INTRODUCTION

Germ cells are the ethernal link between generations. Meiosis is the process that turns diploid germ cells into haploid gametes. To study germ cell development is critical to unravel secrets of pluripotency.

The precursors of gametes, the primordial germ cells (PGCs), are first induced in the epiblast as a consequence of BMP4 signalling from the extrembryonic ectoderm (Ohinata et al., 2009). After its specification, PGCs proliferate and migrate towards the sexually undifferentiated gonadal ridges. Proliferation and survival of PGCs in the embryonic gonad is critically dependent on the signalling pathway guided by the *Stem Cell Factor* (SCF), produced by somatic cells, and its receptor in germ cells, the tyrosine kinase *Kit* (Dolci et al., 1991; McLaren, 1998). Since this early KIT signaling will be essential for survival and differentiation of male and female germ cells throughout all the life span.

How *Kit* expression is regulated in germ cells is still an open question. During germ cell development, *Kit* expression pattern is bimodal in both sexes. In fetal oocytes, it is downregulated after meiotic entry, and is then upregulated perinatally to control oocyte growth (Manova et al., 1993). In fetal male germ cells, *Kit* is downregulated in concomitance with the mitotic block of gonocytes and then its expression is resumed after birth when undifferentiated spermatogonia differentiate into *Kit* positive spermatogonia to acquire meiotic competence (Pellegrini et al., 2008). Before meiotic commitment, PLZF, a transcriptional repressor that prevents the depletion of the spermatogonia stem cell (SSC) pool (Buaas et al., 2004; Costoya et al., 2004) is essential for the repression of *Kit* expression in these cells (Filipponi et al., 2007).

Basic Helix Loop Helix (bHLH) transcription factors play important roles in cellular differentiation during organogenesis (Massari and Murre, 2000). Studies on the haemopoietic and melanoblast lineages shed light on two bHLH proteins that regulate

Kit transcription: *Tal1* and *Mitf*, are two members of the MYC superfamily of bHLH transcription factors that have been demosntrated to regulate *Kit* expression in haemopoietic stem cells and melanoblasts, respectively (Levy et al., 2006; Robb and Begley, 1997; Robb et al., 1996; Widlund and Fisher, 2003). Recently, two testis and ovary-specific bHLH transcription factors genes, *Sohlh1* and *Sohlh2*, have been reported to be essential for both spermatogenesis (Ballow et al., 2006a) and oogenesis (Pangas et al., 2006). They share 50% of aminoacid sequence identity, as well as a similar developmental expression pattern in both fetal and postnatal gonads (Ballow et al., 2006a; Ballow et al., 2006b; Pangas et al., 2006). Moreover, *Sohlh1* knockout and *Sohlh2* knockout mice have common phenotypes that in homozygosity lead to sterility in either testis or ovaries (Ballow et al., 2006a; Ballow et al., 2006b; Hao et al., 2008; Pangas et al., 2006; Toyoda et al., 2009). In the female germline, through binding to canonical E-box sequences, SOHLH1 has been demonstrated to transcriptionally stimulate the promoter activity of two genes critical for the progression of primordial follicles into primary follicles, *Lhx8* and *Zp1*(Pangas et al., 2006). This step of oogenesis, that strongly depends on KIT signalling, is similarly affected in *Sohlh1* knockout (Pangas et al., 2006; Toyoda et al., 2009) and *Sohlh2* knockout females (Toyoda et al., 2009). Interestingly, also the differentiation of spermatogonia is critically dependent on KIT signalling, and is strongly disrupted in either *Sohlh1* knockout (Ballow et al., 2006a) or *Sohlh2* knockout mice (Hao et al., 2008; Toyoda et al., 2009). All these evidences suggest that SOHLH1 and SOHLH2 could be key regulators of *Kit* expression in the germline.

In this paper, we address the specific sequences that are bound by SOHLH1 and SOHLH2 within the *Kit* regulatory regions that are critical for its expression in male germ cells. We accurately define the developmental expression of *Sohlh1* and *Sohlh2*

within fetal gonads and postnatal spermatogonia. Finally we show that these two bHLH interact *in vivo*.

MATERIALS AND METHODS

Cell culture and transient transfection.

Transient transfections using Hek 293 cells, maintained in Dulbecco's modified Eagle's medium with100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM glutamine, were performed by phosphate cloride.

Plasmids concentration was as follows: 1.5 μg *pcDNA-Sohlh1*, 1.5 μg *pcDNA-Sohlh2*, or 0.75 μg of each in case of co-trasfection, 0.9 μg for constructs containing the *Kit* regulatory regions (p13, p18, p316, p178, and p149, see text and Filipponi et al., 2007).1μg *pCMV-Tir-myc* plasmid (Loiarro et al., 2005), was included in the mixture as a control for transfection efficiency. One μg of *pcDNA 3.1* was used as a carrier to equalize the total amount of transfected DNA, when *pcDNA-Sohlh1* or *pcDNA-Sohlh2* was omitted. In all co-transfection experiments, the total amount of transfected mammalian expression vectors was kept constant. All transfections were performed at least three times.

Postnatal male germ cells (0 to 7 dpp) were obtained as previously reported by sequential enzymatic digestion of testes from CD1 albino mice (Pellegrini *et al.*, 2008). After preplating of cell suspension, germ cells were cultured in Modified Earle's Medium (MEM, Gibco) with 20 mM glutamine (Gibco), 2 mM Pyruvic acid (Sigma), 1 mM lactic acid (Sigma), non essential aminoacids (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco), without serum supplementation.

For fetal time course studies, one gonad of each stage (12.5, 13.5, 15.5, dpc) was isolated and cleaned from surrounding tissues. Proteins were extracted by additon of 10 μl of sample buffer 4X, boilled for 5 minutes, and frozen for subsequent analysis in 10 or 12 % SDS-PAGE.

All-trans-retinoic acid (ATRA, Sigma) was dissolved in ethanol and used to a final concentration of 0.3 μM (Pellegrini et al., 2009).

Separation of KIT positive spermatogonia from KIT negative spermatogonia was performed by magnetic-activated cell sorting (MACS) with CD117 conjugated microbeads (Miltenyi Biotech, Germany) as previously described (Lolicato et al., 2008; Pellegrini et al., 2008).

Electrophoretic Mobility Shift Assay

Nuclear extracts were obtained from 7 dpp spermatogonia, from Hek293 mock or Sohlh1 or/and Sohlh2 transfected as previously reported (Filipponi et al., 2007). The probe containing one putative E-box (CACGTG) within kit first intron was amplified from CD1 genomic DNA using the following primers: FW-TCTAGATTCTGGGAATCAGGGACT; RW-TCTAGACCTGAGTATGGCTATCA. The band was purified using purification kit from Roche, digested with XbaI and cloned into pPCR-Script. The fragment was subsequently excised and labeled with ^{32}P. Binding reactions were carried out in a volume of 10 μl. Ten μg of nuclear extracts in binding buffer (20 mM HEPES, pH 7.5, 1 mM $MgCl_2$, 10 μM $ZnCl_2$, 4% glycerol, 100 mg/ml BSA) were incubated on ice for 20 min, in the presence or absence of 100X molar excess of unlabeled wt or mutated double-stranded oligonucleotides. The mixture was incubated for a further 20 min in the presence of 20000cpm of labelled probe at room temperature. DNA loading dye was added, and samples were electrophoretically separated through a 0.5X Tris-borate-EDTA–nondenaturing polyacrylamide gel before autoradiography.

Immunoprecipitation of Sohlh1.

For immunoprecipitation, 1mg of total protein extracts from 7 dpp spermatogonia were incubated in the presence of 1 μg anti-SOHLH1 antibody (Abcam) overnight at 4°C. The immunocomplexes were recovered with protein A-Sepharose presaturated with PBS containing 0.05% BSA (Sigma). After three washes in PBS at 4°C, under constant shaking, immunocomplexes were eluted from beads with SDS-sample buffer. Proteins were separated by SDS-PAGE, blotted and probed with anti-SOHLH2 antibody or with anti-SOHLH1 antibody.

Western blotting.

For western blot analysis cells were harvested and washed 3 times with ice cold PBS. Cell lysis was performed with 10mM HEPES pH 7.9, 1% Triton X-100, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol (DTT), 10 mM β-glycerophosphate, 0.1 mM sodium vanadate, and protease inhibitor cocktail (Sigma-Aldich). Twenty to forty μg of protein extracts were separated by SDS – PAGE in uniform gels of 10 – 12% polyacrylamide and transferred to PVDF membrane (Amersham, Piscataway, NJ). The membrane was blocked in phosphate saline buffer with 0.1% Triton (PBST) and 5% fat-free milk powder for 1 hr at room temperature. Incubation with every primary antibodies was carried out at 4°C overnight O/N in PBST–5% BSA. Appropriate horseradish peroxidase-conjugated secondary antibody (SantaCruz Biotechnology) was used at a 1/5000 dilution in PBST for 1 hour at room temperature. The horseradish peroxidase conjugate was detected by chemiluminescence with an ECL kit (Amersham, Milan, Italy) and autofluorography. Primary antibody used were as follows: Anti-SOHLH1 rabbit policlonal antibody was from Abcam (Ab 49272, Cambridge, UK) and was used at 1/2000 dilution; Anti-SOHLH2 guinea pig polyclonal antibody (Dr. A. Rajkovic, to be described elsewhere) was used at a 1/500 dilution; Anti-KIT rabbit polyclonal antibody (Albanesi et al., 1996) diluted at 1/1000. Anti-actin rabbit polyclonal (A2066, Sigma-Aldrich) was diluted 1/1000. Anti-EGFP

rabbit polyclonal antibody was from Società Italiana di Chemici and used at a 1/1000 dilution. Anti-myc epitope monoclonal antibody was from Santa Cruz (sc-2048) and diluted 1/1000.

Statistical analysis

Continuous variables were summarized as mean and standard deviation (SD). All tests were two-sided and were determined by Monte Carlo's significance. A value threshold of 0.05 was used for the current analysis. T-test was used to test for differences between two independent groups, whereas One-way was used to test for differences among three or more independent groups. For multiple comparisons the Tukey's HSD (Honestly Significant Difference) test was carried out. All statistical tests were carried out using the SPSS statistical analysis software package, version 10.0.

RESULTS

Expression pattern of Sohlh1 and Sohlh2 in germ cells.

It has been proposed that the ablation of either *Sohlh1* or *Sohlh2* affects postnatal development of germ cells in steps in which *Kit* expression is critical (Ballow et al., 2006a; Ballow et al., 2006b; Hao et al., 2008; Pangas et al., 2006; Toyoda et al., 2009). To better understand the expression pattern of *Sohlh1* and *Sohlh2* during development, we performed a western blot analysis on male and female fetal gonads and on postnatal spermatogonia. Interestingly we found that SOHLH1 was not expressed in female germ cells, while it was turned on in male germ cells at around 15.5 dpc, when gonocytes are found in mitotic block. SOHLH2 was found to be expressed as early as 12.5 dpc both in female and male germ cells,and it was then kept expressed in both sexes up to the perinatal period (Fig1 A and data not shown). In both sexes, the expression pattern of SOHLH2 was mirrored by that of PLZF (Fig. 1A), a transcriptional repressor which we previously showed to block Kit expression in spermatogonia (Filipponi et al., 2007). To evaluate SOHLH1 and SOHLH2 expression in postnatal spermatogonia, we isolated purified populations of KIT negative (undifferentiated) and KIT positive (differentiating) cells, by using anti-KIT coupled immunomagnetic beads. We found that SOHLH1 was present in both undifferentiated and differentiating spermatogonia, although it was enriched in KIT positive cells (Fig.1B). On the contrary SOHLH2 was found to be restricted to undifferentiated, KIT negative, spermatogonia (Fig. 1B). This sharp contrast in the expression pattern of SOHLHs within these two cell populations might suggest distinct roles for these two transcription factors.

To investigate if, as KIT, SOHLH1 and SOHLH2 might be regulated by retinoic acid, we estimulated spermatogonial cell cultures with the retinoic acid analogue ATRA (all-trans retinoic acid). 0.3 μM ATRA was added to cultures of undifferentiated

spermatogonia (obtained from 4 dpp testes) or of a mixed population of undifferentiated and differentiating spermatogonia (7 dpp testes) for 24 h of culture. Cell extracts were then obtained and by western blot analysis we observed that SOHLH1 and SOHLH2 were upregulated upon ATRA treatment at both the developmental stages. As positive controls we also probed the membrane for KIT and STRA8, two demonstrated targets of ATRA (Oulad-Abdelghani et al., 1996; Pellegrini et al., 2008)(Fig 1C).

SOHLH1 and SOHLH2 regulate *Kit* promoter and intronic activities.

Since it has been shown that differentiating Kit positive spermatogonia are the only affected cell type affected in both *Sohlh1* or *Sohlh2* knock out males (Ballow et al., 2006a; Ballow et al., 2006b; Hao et al., 2008; Pangas et al., 2006; Toyoda et al., 2009), and, in particular in *Sohlh1* null mouse testes the *Kit* mRNA levels are lower than in the wildtype counterpart, we reasoned that they could directly regulate *Kit* expression in these cells. To test this hypothesis, we searched for the existence of canonical E-box sequences (namely CAGCTG and CACGTG) within constructs containing the Kit regulatory region necessary to modulate its expression in the germline (Cairns et al., 2003; Filipponi et al., 2007).The p18 construct is made of approximately 6 Kb of *Kit* promoter, within which we found two E-box CACGTG sequences and four E-box CAGCTG sequences (Fig. 2A). and 3.5 Kb of the first *Kit* intron driving the expression of an EGFP cassette (Cairns et al., 2003; Filipponi et al., 2007). This last segment of *Kit* first intron contains two more E-box sequences: one is the CACGTG type and the other is the CAGCTG type (Fig.2A).

To test if SOHLH1 and / or SOHLH2 were able to modulate the promoter activity of *Kit*, we co-trasfected p18 with *Sohh1* or with *Sohlh2* expressing plasmids, or with both, in Hek293T cells, and then we analyzed the levels of EGFP expression by western blot analysis. To normalize the transfection efficiencies, we introduced in all the samples a

normalizing vector expressing an epitope of *Myd88*, a construct that we previously validated to be constantly expressed and inert (Filipponi et al., 2007). Interestingly, we observed that either SOHLH1 or SOHLH2 were able to significantly enhance EGFP expression when compared to transfected cells in which the *Sohlhs* expressing plasmids were omitted (Fig. 2C). The upregulation of EGFP was further stimulated when both transcription factors were simultaneously introduced in the cells (Fig. 2C). These results suggested that not only SOHLH1 and SOHLH2 can independently stimulate *Kit* promoter activity but also that their cooperation results in a stronger transactivation.

To investigate if the E-boxes present in the Kit intron were dispensable for SOHLHs responsiveness, we cotransfected *Sohlh1* or *Sohlh2* expressing plasmids with the p13 construct, which contains only the 6Kb of the *Kit* promoter driving EGFP expression (Fig. 2A). Similarly to what obtained by transfecting the p18 construct, we found that both *Sohlh1* or *Sohlh2*, independently or together, can significantly upregulate *Egfp* expression (Fig. 2C). However, due to the 3.5 kb differences between p13 and p18 constructs, it was not possible to make an interassay comparison of the EGFP expression levels.

We then sequentially deleted the p13 construct to establish which of the six E-boxes present within the promoter were critical for SOHLH1 and/or SOHLH2 responsiveness. By digestion of p13 with appropriate restriction enzymes, the following constructs were generated (Fig. 2B): p316, which contains the two E-boxes of *Kit* promoter proximal to the *Egfp* transcriptional start site; p178, which contains only the most proximal E-box and p149, the basal promoter, which does not contain any E-box and does not support EGFP expression when transfected in Hek293T cells. When using p316 in co-trasfection experiments, we found that both SOHLH1 and SOHLH2 were still able to to upregulate EGFP expression however the additive effect between the two transcription factiors was almost lost. The same results were obtained when transfecting p178, which

contains only one E-box. By transfecting p149, we confirmed that the basal promoter did not support EGFP expression in Hek293T cells and it was not responsive to Sohlh1 nor Sohlh2. This latter was expected since no E-box sequences are contained in the this construct. Together, these results support the hypothesis that SOHLH1 and SOHLH2 can both independently and cooperatively transactivate *Kit* promoter in an heterologous system.

Kit intronic sequences are bound in vitro by Sohlh1 and Sohlh2.

Since we observed that E-box containing regions were essential to enhance *Egfp* expression by SOHLH1 and SOHLH2, we aimed to evaluate if these transcription factors were able to bind *in vitro* the E-box sequences. We designed primers to amplify by PCR a specific region of 220 bp containing the two E-box sequences present in the 3.5 kb of the first Kit intron. The fragment was ^{32}P labeled in order to perform an electromobility gel shift assay (EMSA) with nuclear extracts obtained from Hek293T transfected with *Sohlh1*, *Sohlh2*, or with both vectors (Fig. 3). Nuclear extracts from *Sohlh1* transfected cells (Fig. 3, lane 2) showed three retarded bands, the slower migrating one being the specific one(Fig.3, lanes 2 and 3) as shown by the competition with an excess of the cold probe (Fig.3, lane 3). Nuclear extracts from *Sohlh2* transfected cells showed a specific retarded prominent band (Fig. 3, lane 4) which was not observed in the presence of an excess of the cold probe (Fig. 3, lane 5). This retarded band showed a slower electrophoretic mobility compared to the one obtained with SOHLH1 containg extracts. When both transcription factors were transfected, a retarded band was observed (Fig. 3, lane 6) which showed an intermediate electrophoretic mobility, which was faster than the one generated by SOHLH2 and slower than the one generated by SOHLH1 (Fig. 3). As a proof of its specificity, this band was not observed when the cotransfected extracts were primed in the presence of

an excess of cold probe (Fig. 3, lanes 3, 5, and 7). When nuclear extracts of mock-transfected (*pCMV-Egfp*) Hek293T cells were primed with the labelled probe, a pattern of two bands was observed (Fig. 3, lane 8which were competed by the cold probe (Fig. 3, lane 9). The electrophoretic mobility of the two bands was different from those generated by SOHLH1 and SOHLH2 transfected nuclear extracts, suggesting that other bHLH transcription factors (possibly MYC) are present in Hek293T. All together, these results suggest that SOHLH1 and SOHLH2 can bind the E-box sequences as homo-dimers or multimers and also as heterodimers or multimers.

Sohlh1 and Sohlh2 interact in vivo.

The modulation of *Kit* promoter by both SOHLH1 and SOHLH2 and the peculiar retarded band obtained in EMSA experiments when both the transcription factors were present, suggested that SOHLH1 and SOHLH2 might interact in vivo. We tested this hypothesis directly in postnatal spermatogonia. Cell extracts obtained from 7dpp male germ cells were immunoprecipitated with an anti-SOHLH1 antibody and then the immunocomplexes were probed for SOHLH2. By western blot analysis we found that anti-SOHLH1 antibodies, but not preimmune IgGs, were able to specifically immunoprecipitate Sohlh2 (Fig. 4), suggesting that the two proteins interact in vivo.

DISCUSSION

Unravelling the transcriptional network that controls the tissue and time divergent expession pattern of *Kit* is of central importance in physiological and pathological situations.,In the haemopoietic and melanoblast lineages, bHLH transcription factors such as TAL1 and MITF,respectively, have been shown to be essential for the control of *Kit* expression. In the search of bHLH transcription factors with analog role in the germline, we found that SOHLH1 and SOHLH2, two recently identified bHLH transcription factors specifically expressed in germ cells, can regulate Kit transcription.

Expression pattern of SOHLH1 and SOHLH2 in germ cells.

It has been previously shown that *Sohlh1* transcripts are initially found at around 12.5 dpc in male gonads and by 14.5 dpc in the female gonads. Its expression is then maintained from fetal through the postnatal development in spermatogonia and primordial oocytes, respectively (Ballow et al., 2006a; Pangas et al., 2006). *Sohlh2* transcripts have been shown to be detectable in the gonads as early as 13.5 dpc (Ballow et al., 2006b)., After birth, the localization of the SOHLH2 protein is restricted to cells in the primordial to primary oocyte stages (Ballow et al., 2006b), similar to the SOHLH1 protein (Pangas et al., 2006). The expression pattern of SOHLH2 in the testis is also similar to that of SOHLH1, being both expressed in spermatogonia (Ballow et al., 2006a; Ballow et al., 2006b). Since *Sohlh2* has been shown to be upregulated in *Sohlh1* knockout animals (Ballow et al., 2006a), but the reciprocal upregulation does not occur in *Sohlh2* knockouts (Hao et al., 2008; Toyoda et al., 2009), it has been hypothesized that SOHLH2 acts upstream of SOHLH1, potentially regulating its expression (Toyoda et al., 2009).

In line with these observations, we found that SOHLH2 expression preceeded SOHLH1 as it was found to be already turned on by 12.5 dpc in both in male and female fetal germ cells. SOHLH1 was first detected by 13.5 dpc in the male gonads, while in the fetal ovaries it first appeared by 17.5 dpc (not shown). Interestingly, we found that expression of SOHLH2 preceded that of SOHLH1 also in postnatal spermatogonia: SOHLH2 is restricted to the undifferentiated spermatogonia population, while SOHLH1 is found enriched in the KIT-positive, differentiating, cell population. The discrepancy between the expression of these bHLH transcription factors and *Kit* during the fetal development suggests that their putative role in *Kit* expression might be restricted to the postnatal development of spermatogonia. Interestingly, both SOHLH1 and SOHLH2 are strongly up-regulated by ATRA, as in the case of *Kit*, either in undifferentiated or differentiating spermatogonia, further confirming that both proteins are involved in spermatogonia differentiation.

SOHLH1 and SOHLH2 bind *Kit* regulatory regions and heterodimerize.

It is well known that bHLHs function by forming homo- or heterodimers and binding to a consensus sequence “CACGTG” or “CAGCTG” called the E-box. By computer assisted analysis we found that six canonical E-box sequences are contained within the Kit promoter region (p13 plasmid) and two are found within 3.5 kb of the first *Kit* intron (p18 plasmid). When we used these regions in transfection experiments, we found that both SOHLH1 and SOHLH2 can independently increase the expression of the reporter gene, EGFP. Furthermore we observed that co-transfection of both the transcription factors strongly increased EGFP expression. After sequentially deleting the E-box containing regions within the intron and the promoter, responsiveness to SOHLH1 and SOHLH2 was still maintained, but it was completely lost when all the E-box containing regions were deleted. Interestingly, cooperativity between SOHLH1 and SOHLH2 was

abolished when only one or two of the first E-boxes within the promoter were present in the construct driving EGFP expression (p316 and p178, respectively), suggesting that homo or heterodimers of the two proteins are required for *Kit* transcription. By EMSA and co-immunoprecipitation we tested this hypothesis. By gel shift analysis, we found that both SOHLH1 and SOHLH2 produced two different retarded bands, one faster and one slower respectively, when using a probe containing two E-boxes. When co-expressed, both proteins produced a third retarded band with an electrophoretic mobility consistent with the possibility of an heterocomplex formation. Indeed, immunoprecipitation of SOHLH1 from spermatogonia extracts showed that a fraction of SOHLH2 was associated to the protein, indicating that in this cell type SOHLH1 and SOHLH2 interact. However, the presence of residual SOHLH2 in the supernatant of the immunoprecipitation revealed that not all the amount of the protein is associated to SOHLH1.

All together our results suggest a model for a transcriptional network that developmentally regulates *Kit* expression in postnatal spermatogonia (Fig 5). Undifferentiated spermatogonia contain SOHLH1 and SOHLH2 heterocomplexes that bind the *Kit* promoter to potentially activate its transcription. However, the presence of PLZF, also tightly bound to Kit promoter in the same cells, prevents *Kit* transcription. This hypothesis is supported by the observation that, within the 6.9 kb fragment that we analysed, the two PLZF responsive elements of the *Kit* promoter closely flank the first and the last E-box. Thus, the down regulation of PLZF in early differentiating spermatogonia could then release cells from repression allowing *Kit* expression to be primed by SOHLH1 and SOHLH2. Once *Kit* transcription is initiated by the two transcription factors, the presence of SOHLH1 is sufficient to maintain elevated levels of *Kit* in differentiating spermatogonia.

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CHAPTER 5

Future directions.

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GRAZIE, INFINITE GRAZIEEEEEEEEE

A Manuela
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