REPRODUCTION

REVIEW

The role of non-coding RNAs in male sex determination and differentiation

Raphael H Rastetter, Craig A Smith and Dagmar Wilhelm

Department of Anatomy and Developmental Biology, Monash University, Clayton, Victoria 3800, Australia

Correspondence should be addressed to D Wilhelm; Email: dagmar.wilhelm@monash.edu or to C A Smith; Email: craig.smith@monash.edu

Abstract

A complex network of gene regulation and interaction drives male sex determination and differentiation. While many important protein-coding genes that are necessary for proper male development have been identified, many disorders in human sex development are still unexplained at the molecular level. This suggests that key factors and regulatory mechanisms are still unknown. In recent years, extensive data have shown that different classes of non-coding RNAs (ncRNAs) play a role in almost all developmental and physiological pathways. Here we review what is known about their role in male sex determination and differentiation not only in mammals, but also other species. While for some processes a key role for ncRNA has been identified, we are still far from having a complete picture.

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Introduction

Non-coding RNAs (ncRNAs), in a broad sense, are RNA molecules that, in contrast to messenger RNAs (mRNAs), do not code for proteins. However, more recently, 'ncRNAs' has been more specifically used for RNAs that not only have no protein-coding potential but also have some kind of regulatory function. These regulatory ncRNAs can be further divided, rather arbitrarily, into small RNAs, which are shorter than 200 nucleotides (nt), and long ncRNAs (lncRNAs) with a length above 200 nt. Small ncRNAs are not only defined by their size but also their association with members of the Argonaute (AGO) protein family, for which two clades can be distinguished, the AGO and the PIWI subfamily. Small regulatory ncRNAs can be further sub-divided into microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs), and PIWI-associated RNAs (piRNAs) based on their size, function, mode of action and the AGO protein they bind to (Farazi *et al*. 2008, Kim *et al*. 2009).

At the forefront of investigations have been miRNAs, which regulate gene expression primarily post-transcriptionally through mRNA destabilization and/or inhibition of translation (Pillai 2005, Filipowicz *et al*. 2008, Carthew & Sontheimer 2009). Most miRNAs are transcribed by RNA polymerase II and are processed by Drosha in association with DGCR8 in the nucleus, giving rise to a short hairpin that is exported from the nucleus by exportin 5, and further processed in the cytoplasm by Dicer to give rise to a 21–23-nt long miRNA duplex. One strand of the duplex is then incorporated into the AGO-containing RNA-induced silencing complex (RISC) and mediates sequence-specific binding to target mRNAs, which are subsequently degraded and/or translationally silenced (Farazi *et al*. 2008, Kim *et al*. 2009).

Hundreds of miRNAs are expressed in the developing testis in mammals (Yang *et al*. 2013), and their general role has been analysed using mice lacking Dicer, specifically in somatic or germ cells of the developing testis. Mice homozygous for *Dicer1* deletion in either germ or somatic cells during embryogenesis lead to male infertility due to multiple cumulative defects resulting in the absence of functional sperm (Hayashi *et al*. 2008, Maatouk *et al*. 2008, Papaioannou *et al*. 2009, Huang & Yao 2010, Zimmermann *et al*. 2014), demonstrating that miRNAs are important for proper testis development and function. However, from these experiments it remains unclear which specific miRNAs play important roles in these processes. Progress has been made to identify some of these, which we will discuss in more detail below.

In addition to miRNAs, endo-siRNAs also associate with AGO proteins. These small RNAs are derived from long, perfectly complementary double-stranded RNAs that are formed through sense-antisense transcript pairs, long stem-loop structures and transposon transcripts (Golden *et al*. 2008). Their processing is Drosha/DGCR8 independent but requires multiple Dicer cleavages along the precursor RNA (Watanabe *et al*. 2006, Tam *et al*. 2008). For quite some time it was believed that

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endo-siRNAs do not exist in vertebrates, because Dicer was believed to act exclusively in the cytoplasm, and cytoplasmic long dsRNAs trigger a strong immune response though protein kinase R. However, nuclear action of Dicer has been demonstrated recently, and endo-siRNA expression has been detected mainly in mouse oocytes and ES cells, where they contribute to the repression of transposons. Endo-siRNAs have also been detected in germ cells of the adult testis (Song *et al.* 2011), but it is not known if this type of ncRNA is also involved in sex determination and early testis differentiation.

The third main class of small RNAs, piRNAs, are ~24–31 nt long, derived from single-stranded piRNA precursor transcripts and processed in a Dicer-independent way (Vagin *et al.* 2006, Li *et al.* 2013). piRNAs associate with the PIWI subfamily of AGO proteins and are predominantly expressed in germ cells, where they are involved in transposon silencing through heterochromatin formation and RNA destabilization (Klattenhoff & Theurkauf 2008, Weick & Miska 2014). They play a major role in male development and fertility and will therefore be discussed in more detail below.

In addition to small ncRNAs, new techniques such as sequencing of whole transcriptomes have identified a large number of lncRNAs (Okazaki *et al.* 2002, Carninci *et al.* 2005, Consortium *et al.* 2007). LncRNAs vary in size from 200 bp to several kilobase in size and are transcribed by RNA polymerase II. Similar to mRNAs they are often polyadenylated at the 3′ end and have a 5′ end cap structure. LncRNAs are transcribed from different regions within the genome. Many lncRNAs are transcribed from intergenic regions (Guttman *et al.* 2011, Ulitsky & Bartel 2013), so-called long intergenic ncRNAs (lincRNAs), but there are also numerous lncRNAs transcribed from the sense or antisense strand of protein-coding genes (Wu *et al.* 2014). Some ncRNAs seem to be processed from 3′UTR of mRNAs (Mercer *et al.* 2011), whereas intronic lncRNAs are part of an intron from another transcript (Carninci *et al.* 2005, Consortium *et al.* 2007). Many of these lncRNAs were believed to be by-products from transcription, splicing, RNA processing, etc., but more and more data emerge demonstrating specific expression and function of various lncRNAs (for review, see Fatica & Bozzoni (2014)). It appears that, similar to their heterogeneous origin, lncRNAs have a wide range of functions: they regulate chromatin remodelling by recruiting chromatin modifiers, control the transcriptional rate of genes and influence post-transcriptional processes such as inhibition or induction of translation (Mercer *et al.* 2009, Fatica & Bozzoni 2014).

In this review, we will describe the cellular mechanisms underlying male sex determination and differentiation and the potential functions that ncRNAs have at each step of these processes. We will concentrate on mammalian male development with a specific focus on mice, but also discuss the role of ncRNAs in male sex differentiation in other species. Non-coding RNAs have been identified in all groups of animals, from vertebrates to flies and worms, and among ancient groups, such as cnidarians and sponges (Grimson *et al.* 2008). This indicates that ncRNAs as developmental regulators have a very ancient history and likely played a role very early in metazoan evolution. Both short and lncRNAs have been reported among diverse species. Among the former, small ncRNAs with silencing functions such as miRNAs, endogenous-siRNAs and piRNAs are all present in vertebrate as well as invertebrate cells, and all function to regulate gene expression, including post-transcriptional or translational silencing, and repression of retrotransposon activity (Malone & Hannon 2009). While miRNAs tend to be structurally conserved, piRNAs and lncRNAs generally lack sequence conservation across species. In developing testes, all classes of ncRNAs have been reported in non-mammalian vertebrates, where their analysis is shedding light on their evolution and function. As in mammals, piRNAs are implicated in protecting the male germline from retroviral elements, and indeed are required for proper spermatogenesis in both mammals and non-mammals. In contrast, the exact role of microRNAs in testis determination is less well understood. The role for lncRNAs in the testis is even less clear, but studies in avian and fish cells point to chromatin modification to influence local gene expression (see below).

The development of the bipotential genital ridge in mammals

In mammals, testes and ovaries arise from paired indifferent and bipotential genital ridges, which develop from a thickening of the ventromedial surface of the mesonephros. In mice, the genital ridges are first visible at around 9.5 days *post coitum* (dpc) (Byskov 1986). Mutant and knockout analyses in mouse identified several key protein-coding genes that are responsible for the correct development of the bipotential ridge such as the nuclear receptor subfamily 5, group A, member 1 (*Nr5a1*, also known as steroidogenic factor 1 or *Sf1*), Wilms' tumour suppressor gene 1 (*Wt1*), empty spiracles homeobox 2 (*Emx2*), odd-skipped-related 1 (*Odd1*) and LIM homeobox gene 9 (*Lhx9*) (Luo *et al.* 1994, Sadovsky *et al.* 1995, Miyamoto *et al.* 1997, Schnabel *et al.* 2003, Wang *et al.* 2005). In contrast, ncRNAs have not been implicated in the formation of the indifferent genital ridge to date.

During the formation of the genital ridges, somatic cells coalesce with primordial germ cells (PGCs), the precursor of the gametes, which are specified extragonadally at the base of the allantois at around 6.5 dpc in mouse (Ginsburg *et al.* 1990, Lawson & Hage 1994). PGCs migrate from the allantois through the hindgut and the dorsal mesentery to colonize the developing genital

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ridges between 9.5 and 11.5 dpc (Clark & Eddy 1975, Donovan *et al*. 1986). Master regulators of PGC specification include PR domain zinc finger protein 14, PRDM14 (Yamaji *et al*. 2008), PRDM1, also known as BLIMP1 (Ohinata *et al*. 2005, Vincent *et al*. 2005), and the RNA-binding protein LIN28 (West *et al*. 2009). Several groups have independently discovered that LIN28, and the related protein LIN28B, bind to the primary transcript of the *let-7* miRNA and inhibits its processing into the mature miRNA (Heo *et al*. 2008, Rybak *et al*. 2008, Viswanathan *et al*. 2008). *Let-7* is one of the first characterized miRNAs, which was identified in a study of developmental timing in *C. elegans* (Rougvie 2001). In mammals, the *let-7* family has numerous members and has been shown to bind and repress *Lin28* as well as *Prdm1* (Nie *et al*. 2008, Viswanathan *et al*. 2008), connecting all three players during germ cell specification (Fig. 1).

The differentiation of PGCs in mammals

After entering the gonads, PGCs proliferate until ~13.5 dpc, when they differentiate in a sex-specific manner. In an ovary, PGCs enter the first meiotic prophase, while in a testis they enter mitotic arrest (Bullejos & Koopman 2004, Western *et al*. 2008). XX and XY germ cells were believed to not be sexually dimorphic until this differentiation process is initiated by their surrounding cells, testicular or ovarian somatic cells (Bowles & Koopman 2010). This view has been challenged by two reports demonstrating sexually dimorphic gene expression in PGCs long before initiation of meiosis in an ovary and mitotic arrest in a testis. The first indication came from a study examining miRNAs expressed during early ES cell differentiation (Ciaudo *et al*. 2009). The authors showed that the *miR-302* family of microRNAs was highly expressed in XY, but not XX PGCs at 8.5 and 9.5 dpc, before PGCs arrive at the developing genital ridges (Ciaudo *et al*. 2009). However, it is unclear what regulates this male-enriched expression and what function these microRNAs have at this stage during development. In addition, a protein-coding gene, *Lrrc34*, as well as a lncRNA, *AK015184*, were detected in XX but not XY germ cells from 11.5 dpc, the time of sex determination and 2 days before the onset of sex-specific germ cell differentiation (Chen *et al*. 2012). Similar to the miRNAs, no function has been described for these genes in the PGCs to date.

In addition, several miRNAs have been shown to be expressed during and after PGC differentiation. The microRNA cluster *miR-17-92* is highly expressed in XX and XY PGCs and this expression is reduced in female germ cells following entry into meiosis (Hayashi *et al*. 2008). *miR-17-92* is one of the best-characterized oncogenic miRNA clusters (He *et al*. 2005), suggesting that these miRNAs are important for germ cell survival and proliferation. Similarly, another miRNA cluster known to promote proliferation, *miR-290-295*, is also

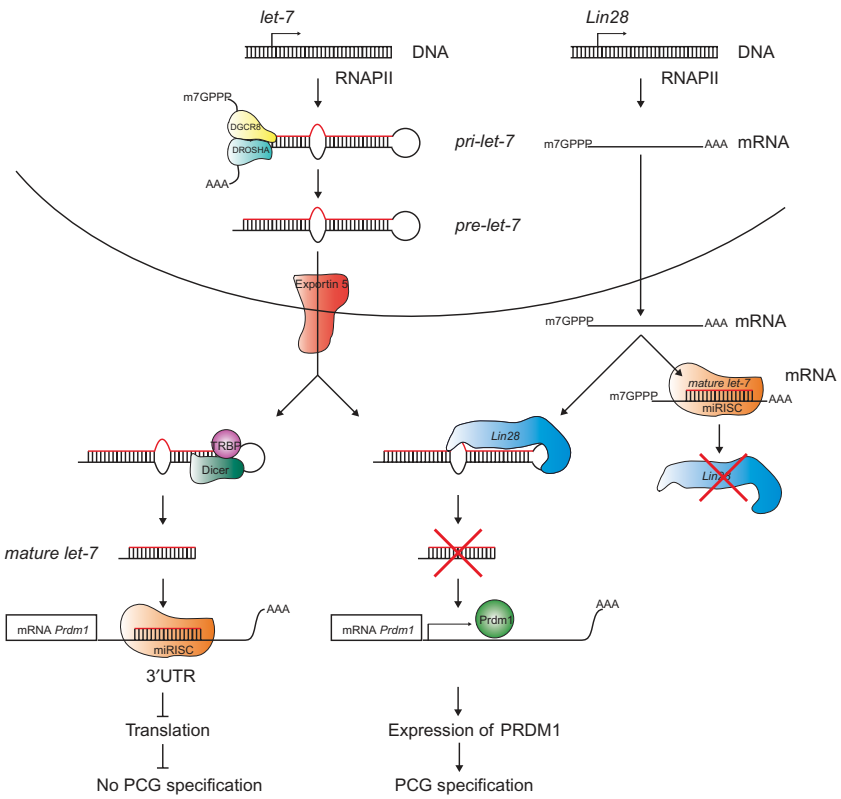

Figure 1 Cross-talk of LIN28, PRDM1 and the miRNA *let-7* during PGC specification. In the absence of LIN28, the mature miRNA *let-7* is generated through the canonical miRNA biogenesis pathway. *Let-7* is transcribed by RNA polymerase II (RNAP II) into *pri-let-7*, which is cleaved by DROSHA and its cofactor DGCR8 to generate *pre-let-7*. *Pre-let-7* is exported by Exportin 5 into the cytoplasm, where it is processed by Dicer and its partner RNA-binding protein TRBP. The guide strand of the mature *let-7* duplex is loaded on the microRISC (miRISC), which then binds to the 3′UTR of *Prdm1* and *Lin28* mRNA leading to an inhibition of *Prdm1* and *Lin28* translation and thereby suppression of PGC specification. In turn, processing of *let-7* is inhibited by the presence of LIN28. LIN28 binds *pre-let-7* and prevents its processing by Dicer and TRBP into mature *let-7*. Reduced levels of mature *let-7* result in the expression of *Prdm1* and *Lin28* and thereby PGC specification.

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highly expressed in PGCs (Hayashi *et al*. 2008). In contrast, the expression of other miRNAs, including *miR-141*, *-200a*, *-200c* and *-323*, decreased during development (Hayashi *et al*. 2008), suggesting that they might inhibit differentiation. However, functional experiments need to be performed to elucidate their function during PGC development.

SRY and sex determination in mammals

In mammals, sex is determined genetically by the presence or absence of the male-determining factor SRY on the Y chromosome. Mouse *Sry* displays a very restricted expression pattern. It is only expressed for ~2 days during embryonic development and only in the developing genital ridge (Hacker *et al*. 1995). Its expression induces a cascade of gene expression and regulation that results in the formation of a testis. Expression of *Sry* is induced at 10.5 dpc in the centre of the gonad, reaches its highest levels at 11.5 dpc and disappears completely after 12.5 dpc (Hacker *et al*. 1995, Bullejos & Koopman 2001, Sekido *et al*. 2004, Wilhelm *et al*. 2005). Mutation analysis of a number of genes revealed insights into the regulation of *Sry* expression (for review see Svingen & Koopman (2013)). Genes that have been implicated in the regulation of *Sry* expression include chromobox homolog 2 (*Cbx2*) (Katoh-Fukui *et al*. 2012), the +KST isoform of WT1 (Hammes *et al*. 2001), steroidogenic factor 1 (SF1), encoded by the gene *Nr5a1* (Pilon *et al*. 2003), as well as the transcriptional co-factor CITED2 in combination with WT1 and SF1 (Buaas *et al*. 2009), GATA-binding protein 4 (*Gata4*) and its cofactor friend of GATA 2 (*Fog2*) (Tevosian *et al*. 2002), combined loss-of-function mutations in the insulin receptor genes, *Ir* and *Igf1r* (Nef *et al*. 2003, Pitetti *et al*. 2013), as well as mutations in the MAPK pathway, including *Map3k4*, its activator *Gadd45γ* and p38MAPKs (Bogani *et al*. 2009, Gierl *et al*. 2012, Warr *et al*. 2012).

Interestingly, in addition to its expression in the fetal testis, where *Sry* is both necessary and sufficient to induce male development, an unusual circular *Sry* transcript has been detected in adult mouse testis (Capel *et al*. 1993). It is likely that the splicing machinery generates this circular transcript due to the long inverted repeat surrounding the *Sry* locus. The function of this circular *Sry* transcript is unknown. Most likely it functions as a ncRNA, as it is not associated with polysomes (Capel *et al*. 1993). Interestingly, in the last couple of years, circular transcripts have been predicted to exist for thousands of genes. They are conserved between species and their expression is highly regulated (Salzman *et al*. 2012, Jeck *et al*. 2013, Memczak *et al*. 2013, Jeck & Sharpless 2014), suggesting that these unusual transcripts indeed have a function. The best characterized function for a circular transcript is the one for the brain-enriched antisense transcript of cerebellar degeneration-related protein 1 (*Cdr1as*) transcript, also known as circular RNA sponge for *miR-7* (*ciRS-7*). Circular *Cdr1as* exhibits 73 target sites for *miR-7*, thereby functioning as a sponge, resulting in a decreased activity of *miR-7* and an upregulation of *miR-7* target genes (Kefas *et al*. 2008, Hansen *et al*. 2013*a*, *b*, Memczak *et al*. 2013). Importantly, the circular *Sry* transcript also possesses several putative target sites for a microRNA, *miR-138*, suggesting that, similar to circular *Cdr1as*, *circSry* could serve as sponge for *miR-138* (Hansen *et al*. 2013*a*). Further investigations are necessary to clarify if circular transcripts play a role in the process of male sex determination and differentiation.

Sex determination and testis development in non-mammalian vertebrates

Interestingly, although the determination of sex is such a fundamental process, the molecular trigger is not widely conserved. *Sry* as the male-determining gene only exists in therian mammals (marsupials and eutherians). The search for alternative sex determination mechanisms has revealed a plethora of different ways depending on the species. Sex can be determined via purely genetic cues, such as in birds and mammals or, for example, through the temperature at which the eggs are incubating, as in many reptiles (Matson & Zarkower 2012, Eggers *et al*. 2014). In contrast to mammals that have a XX female: XY male sex chromosome system, birds have a ZZ male: ZW female system. In addition, the sex chromosomes of birds are not homologous to those of mammals but have evolved from a different autosomal chromosome pair (Marshall Graves 2008). It is therefore not surprising that birds lack the mammalian *Sry* gene. Instead, sex is likely dependent on Z-chromosome dosage, i.e., two doses in males, ZZ, and one dose in females, ZW (Chue & Smith 2011). The best Z-linked candidate gene under this hypothesis is doublesex and mab3 related transcription factor 1 (*DMRT1*), a conserved gene that encodes a zinc finger-like transcription factor. *Dmrt* genes play a prominent role in vertebrate sex determination and gonadal development. In therian mammals, *Dmrt1* is expressed in developing XY gonads after *Sry* and is required only postnatally for proper somatic and germ cell development (Raymond *et al*. 2000). However, in other vertebrates, *Dmrt* genes can have a more central role. In birds, *DMRT1* is expressed in the embryonic gonads, where it is more highly expressed in testes, and knockdown using RNA interference feminises male (ZZ) gonads (Smith *et al*. 1999, 2009). Similarly, a duplicated copy of autosomal *dmrt1*, called *dmrt1bY/dmy*, is the master switch for testis determination in fish species such as medaka (*Oryzias latipes*) (Matsuda *et al*. 2002), and in the amphibian *Xenopus laevis* a divergent *Dmrt* gene on the female W sex chromosome (*dmw*) antagonises autosomal *dmrt1* to have ovary-determining function (Yoshimoto *et al*. 2008). Among reptiles with

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temperature-sensitive sex determination, *Dmrt1* is upregulated at the male- but not the female-inducing temperature at the time of gonadal sex differentiation (Shoemaker *et al*. 2007*a*, *b*). Taken together, these observations point to a key role for *Dmrt* genes in vertebrate sex determination. Nevertheless, the control of sex-determining pathways appears to be remarkably labile especially in lower vertebrates, where different genes have been co-opted to act as master testis determinants (Cutting *et al*. 2013). For example, *amhy*, a duplicated copy of the anti-Müllerian hormone (AMH) is the master testis determinant in one fish species, the Patagonian pejerrey (*Odontesthes hatcheri*) (Hattori *et al*. 2012), whereas in the rainbow trout, an immune-related gene, *sdY*, is the master testis determinant (Yano *et al*. 2012).

Following sex determination, other elements of the pathway tend to be conserved between mammals and non-mammals. These include SOX9, a highly conserved HMG domain transcription factor that is implicated in testis development across all groups from fish to reptiles, birds and mammals (Morais da Silva *et al*. 1996, Western *et al*. 1999). In female embryos of egg-laying species such as birds and reptiles, estrogen is required for proper ovary formation, and testis-enriched DMRT1 can antagonise estrogen production (Elbrecht & Smith 1992, Yao *et al*. 2004, Lambeth *et al*. 2014). In general, studies of non-mammalian species have found that gonadal sex differentiation into ovaries or testes is morphologically conserved among vertebrates, involving the same cell types and many of the same protein coding genes. As noted above, molecular divergence lies primarily at the top of the sex-determining cascades.

Regulation of Sox9 expression by protein-coding and ncRNAs

In mammals, *Sry* is expressed in pre-Sertoli cells, the supporting cells in the testis, which are also the organizers for all other testis-specific cell types. In the short period of *Sry* expression, its key role is the upregulation of *Sox9*, which encodes a transcription factor belonging to the same SRY-like HMG domain family (Bowles *et al*. 2000). Before *Sry* expression is upregulated in the XY genital ridge, *Sox9* is expressed at low levels in both the developing testis and the ovary (Kobayashi *et al*. 2005) due to the binding and activation by SF1 to the testis enhancer sequence (TES) 14 kb upstream of the *Sox9* transcription start site (Sekido & Lovell-Badge 2008). Subsequently, SRY binds together with SF1 to a 1.4 kb core element (TESCO) located within TES resulting in the upregulation of *Sox9* transcription in the testis, whereas *Sox9* expression becomes undetectable in the ovary. Once *Sox9* is expressed at a high enough level, SOX9 itself binds along with SF1 to TESCO to maintain its own expression (Sekido & Lovell-Badge 2008). In addition, two positive feedback loops are described for the maintenance of *Sox9* expression: first, SOX9 upregulates directly or indirectly FGF9, which then activates FGF signalling via FGF receptor 2 (FGFR2), resulting in *Sox9* upregulation (Kim *et al*. 2007, Bagheri-Fam *et al*. 2008). Secondly, SOX9 directly stimulates the expression of the prostaglandin D synthase (*Ptgds*) gene, which in turn produces prostaglandin D_2 (PGD_2), leading to the translocation of SOX9 protein from the cytoplasm into the nucleus and the upregulation of its expression (Malki *et al*. 2005, Wilhelm *et al*. 2005, Wilhelm *et al*. 2007, Moniot *et al*. 2009).

While TESCO also has been located in the human genome ~13 kb upstream of the *SOX9* transcription start site (Sekido & Lovell-Badge 2008), it is not known if TESCO also mediates testis-specific expression of *SOX9* in humans. Mapping of copy number variations in human patients with 46,XX male and 46,XY female development identified a long distance regulatory region upstream of *SOX9*, called *RevSex*, that is likely to harbour an enhancer driving testis-specific expression (Benko *et al*. 2011). Interestingly, this region encodes two lncRNAs, *TCONS_00025195* and *TCONS_00025196* (Smyk *et al*. 2013), which might be involved in the regulation of *SOX9* expression in human testes (Fig. 2).

While the upregulation of *Sox9* expression in the XY genital ridge has been studied in detail, not much is known about what causes its downregulation in the developing ovary. Recently, a microRNA, *miR-124*, has been implicated in the inhibition of *Sox9* expression in the ovary (Fig. 2). This miRNA has been shown to regulate *Sox9* expression in the brain (Cheng *et al*. 2009). *miR-124* was identified in a microarray screen of embryonic mouse gonads as being upregulated in the developing ovary and downregulated in the testis (Real *et al*. 2013), suggesting a role during ovarian differentiation. However, this ovary-enriched expression was not detected by a high-throughput sequencing approach (Rakoczy *et al*. 2013). Nevertheless, inhibition of *miR-124* in XX gonadal cells using antagomirs (small, antisense molecules that bind and inhibit miRNAs) resulted in an upregulation of *Sox9* expression, indicating that *miR-124* is necessary for the repression of *Sox9* in the developing ovary (Real *et al*. 2013). In contrast, overexpression of *miR-124* in XY gonadal cells was not sufficient to repress *Sox9* expression at the mRNA and protein level (Real *et al*. 2013). These knockdown and over-expression analyses were performed in isolated primary cells, and it remains to be tested if this miRNA has an *in vivo* function. Knockout of *miR-124a-1* and *miR-124a-3* in mice does not result in female-to-male sex reversal (Sanuki *et al*. 2011, Park *et al*. 2012), as would be expected if these miRNAs were necessary for the repression of *Sox9* in the ovary. However, all three *miR-124* genes have been reported to be expressed in the developing ovary (Real *et al*. 2013), which could mask any phenotype in single knockout mice, and a triple knockout mouse would be required to draw definite

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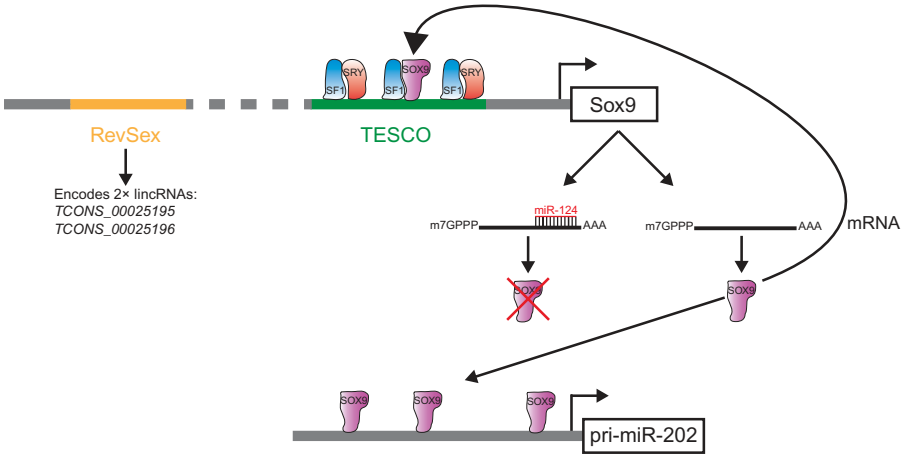

Figure 2 Putative regulation of *Sox9* expression by ncRNAs. In mice, *Sox9* transcription is upregulated by the binding of SF1 and SRY or SF1 and SOX9 to the enhancer region TESCO, which is located 13 kb upstream of the *Sox9* transcription start site. The TESCO sequence is also present in humans, but its relevance for testis-specific *Sox9* expression is not clear. Rather, the analyses of human patients with 46,XX and 46,XY DSD identified a second control region, called RevSex, located upstream of the *SOX9* transcription start site. This region harbours two lincRNAs, *TCON_00025195* and *TCON_00025196*, which might be involved in regulating *SOX9* expression. In addition, *Sox9* expression can be regulated by *miR-124* by binding to the 3′UTR of *Sox9* mRNA, however it is unclear if this regulation occurs during gonad differentiation. Finally, SOX9 likely functions as a transcriptional activator of miRNAs such as *miR-202* and *miR-140*.

conclusions. Also, the ovary-enriched expression of *miR-124* was shown at 13.5 dpc, whereas its expression level at 11.5 dpc, the stage at which *Sox9* expression is downregulated in the ovary (Kobayashi *et al.* 2005), was very low and indistinguishable from the expression level in the XY genital ridge (Real *et al.* 2013), suggesting that *miR-124* might not responsible for the initial down-regulation of *Sox9* in the ovary, but may be involved in the maintenance of *Sox9* repression.

Regulation of miRNA expression by SOX9 during mammalian Sertoli cell differentiation

The expression of SOX9 results in the differentiation of Sertoli cells (Sekido *et al.* 2004, Wilhelm *et al.* 2005), which can be considered as the organizing centres of the developing testis. They drive the differentiation of other cell types by the expression and secretion of essential factors, many of which are directly upregulated by SOX9 or have been at least suggested to be transcriptionally regulated by SOX9 (see below and for review see Svingen & Koopman (2013)). Overall, it induces global cellular and morphological changes, including the migration of endothelial cells from the mesonephros to form the testis-specific vasculature, an increase in proliferation and the formation of testis cords, which comprise clusters of PGCs surrounded by Sertoli cells (Martineau *et al.* 1997, Capel *et al.* 1999, Schmahl *et al.* 2000, Schmahl & Capel 2003, Cool *et al.* 2008, Combes *et al.* 2009), resulting in the formation of a testis.

SOX9 has also been implicated in the transcriptional regulation of miRNAs. We have shown recently that the expression of *pri-miR-202* is downstream of SOX9 in the developing mouse testis (Wainwright *et al.* 2013). Both strands, *miR-202-3p* and *miR-202-5p*, were initially identified in the embryonic gonad by using high-throughput sequencing of small RNAs from differentiating XY and XX gonads (Rakoczy *et al.* 2013). The sequence of *miR-202* is conserved among vertebrates and both strands, *miR-202-5p* and -3p, are upregulated during testis development not only in mouse but also in chicken embryos. In both species, they are expressed in the key Sertoli cell lineage. In chickens, experimental male-to-female sex reversal with oestrogen causes a decline in chicken *miR-202-3p* expression (Bannister *et al.* 2011). Interestingly, *miR-202-3p* also was found to be one of the main miRNAs depleted in the sterile gonads of Xenopus hybrids, suggesting it is important for gametogenesis (Michalak & Malone 2008). To investigate the function of *pri-miR-202* in mouse embryonic gonads *in vivo*, a mouse model was generated in which *pri-miR-202* is over-expressed in somatic cells of the developing testis and ovary using the regulatory region of the *Wt1* (Wainwright *et al.* 2013). Ectopic expression of *pri-miR-202* in XX gonads did not result in any molecular changes to the ovarian differentiation pathway, showing that *miR-202* is not sufficient to switch on testis development (Wainwright *et al.* 2013). However, this does not exclude a possible function during testis differentiation, which would require the generation of a *miR-202*-null mouse model.

Mechanisms involved in the differentiation of Leydig cells and peritubular myoid cells in mammals

One of the factors expressed and secreted by Sertoli cells is desert hedgehog (DHH), which is responsible for the differentiation of fetal Leydig cells and acts through its

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receptor patched 1, PTCH1, expressed on all interstitial cells (Bitgood *et al*. 1996, Clark *et al*. 2000, Yao *et al*. 2002). Interestingly, DHH is not only necessary but also sufficient for Leydig cell differentiation. Ectopic activation of the Hedgehog pathway in an ovary is enough to induce Leydig cells differentiation (Barsoum *et al*. 2009). However, DHH is not the only factor important for Leydig cell differentiation. Activation of Notch signalling reduces and inhibition of Notch signalling increases the number of Leydig cells in the developing testis (Tang *et al*. 2008), demonstrating that also this signalling pathway controls the differentiation of these cells. Similarly, the miRNA *miR-140,* expressed in Sertoli cells and shown to be directly upregulated by SOX9 (Nakamura *et al*. 2011, Yang *et al*. 2011), influences Leydig cell differentiation. Loss of *miR-140* results in an increase in Leydig cell numbers (Rakoczy *et al*. 2013). However, it is not known which genes are regulated by *miR-140* within Sertoli cells that indirectly influence Leydig cell differentiation, although predicted target genes have been shown to play a role in testis development and Leydig cell differentiation and function (Rakoczy *et al*. 2013).

DHH produced by Sertoli cells not only controls the differentiation of Leydig cells, but also another testis-specific cell type, so-called peritubular myoid cells; long, flattened cells that surround Sertoli cells at the testis cords and secret, together with Sertoli cells, extracellular matrix proteins, which form a basal lamina between the two cell types (Tung *et al*. 1984). Peritubular myoid cells become contractile at later stages to pump the sperm into the epididymis. To date, no factors, other than DHH, have been implicated in the differentiation of peritubular myoid cells, including ncRNAs.

The differentiation of the male reproductive tract in mammals

Steroidogenic Leydig cells are localized in the interstitium of XY gonads and induce, through androgen production, the development of secondary male sexual characteristics such as the differentiation of the Wolffian duct into the male reproductive tract including epididymis, vas deferens and seminal vesicles (Eik-Nes 1969, O'Shaughnessy *et al*. 2007). In addition, SOX9 in Sertoli cells upregulates the expression of AMH (Arango *et al*. 2008), which causes the degradation of the Müllerian ducts, which otherwise would differentiate into the female reproductive tract, including oviduct, uterus and upper vagina. While there are good indications that ncRNAs, especially miRNAs, play a role in the female reproductive tract (Hong *et al*. 2008, Nagaraja *et al*. 2008, Gonzalez & Behringer 2009), very little is known about the role of ncRNA in male reproductive tract development. Small ncRNAs, including miRNAs and piRNAs, have been identified in the human epididymis (Zhang *et al*. 2010, Li *et al*. 2012), but their function and whether they are necessary for epididymal differentiation is unknown.

The role of piRNAs in testis development

As mentioned above, piRNAs are predominantly expressed in germ cells and are associated with the PIWI subfamily of AGO proteins. PIWI-interacting RNAs have been identified in both vertebrate and invertebrates, indicating that, as a class, they have a very ancient history within animal cells (Ruby *et al*. 2006, Vagin *et al*. 2006, Houwing *et al*. 2007). PIWI is named for 'P-element induced wimpy testis' and was first identified by an enhancer trap based screen in 1997 in *Drosophila* (Lin & Spradling 1997). Analysis of *Piwi* mutants revealed that PIWI proteins are required for the maintenance and renewal of germline stem cells and the inhibition of retrotransposons mobilization in the male germline (Lin & Spradling 1997, Cox *et al*. 1998, Kalmykova *et al*. 2005). However, increasing evidence also points to a role in the female germline and folliculogenesis (Lim *et al*. 2013).

In mice, 3 *Piwi* genes exist, *Piwil1* (*Miwi*), *Piwil2* (*Mili*), and *Piwil4* (*Miwi2*), which are all essential for spermatogenesis, as null mutation of each resulted in male sterility (Deng & Lin 2002, Kuramochi-Miyagawa *et al*. 2004, Carmell *et al*. 2007). PIWIL2 expression starts at 12.5 dpc and persists into adulthood, whereas PIWIL4 is expressed from 15.5 dpc to 3 days postnatally and PIWIL1 in adult testes from 14 days postnatally (Aravin & Hannon 2008). The different PIWI proteins also recognize and bind different piRNAs, with 26- to 27-nt-long piRNAs binding to PIWIL2, 28- to 29-nt piRNAs to PIWIL4 and the majority of piRNAs binding to PIWIL1 at 30 nt (Aravin *et al*. 2006, Girard *et al*. 2006, Aravin & Hannon 2008). Recent data show that the PIWI/piRNA protein machinery is conserved in various vertebrate lineages, where it plays a conserved role in disabling transposons and protecting germ cells (Lim *et al*. 2013). Table 1 shows the phylogenetic distribution of PIWI homologues and functionally related proteins – Maelstrom, VASA and TDRD1 – amongst vertebrates and *Drosophila*. Similar to mammals, in zebrafish (*Danio rerio*) the *piwi* homologues, *ziwi and zili*, are expressed in embryonic germ cells, where mutagenesis studies show that they are required for germ cell maintenance and transposon silencing (Houwing *et al*. 2007). This is likely to be a feature of piRNAs across the developing gonads of all vertebrate embryos. In adult amniotes, such as mice, platypuses (a monotreme mammal) and chickens, *PIWI* pathway proteins are all expressed in both testis and ovary (Murchison *et al*. 2008, Lim *et al*. 2013), although targeted deletion of PIWIs in mice suggest a requirement for spermatogenesis but not oogenesis (Deng & Lin 2002). In chickens, PIWI homologues have been identified that are called *CIWI* and *CILI* (Kim *et al*. 2012). At the amino acid level, CIWI

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Table 1 piRNA pathway genes amongst vertebrates (reproduced from **Lim SL, Tsend-Ayush E, Kortschak RD, Jacob R, Ricciardelli C, Oehler MK, Grutzner F** 2013 Conservation and expression of PIWI-interacting RNA pathway genes in male and female adult gonad of amniotes. *Biology of Reproduction* **89** 136., with permission).

Species	Piwi/Aub/Ago3	*Piwil1*	*Piwil2*	*Piwil3*	*Piwil4*	*Mael*	*Vasa*	*Tdrd1*
Drosophila	+					+	+	+
Human		+	+	+	+	+	+	+
Mouse		+	+	−	+	+	+	+
Opossum		+	+	−	+	+	+	+
Platypus		+	+	−	+	+	+	+
Chicken		+	+	−	−	+	+	+
Zebrafinch		+	+	−	−	+	+	+
Xenopus		+	+	−	+	+	+	−
Lizard		+	+	−	+	+	+	+
Zebrafish		+	+	−	−	+	+	+

shows 78, 76 and 64% similarity with human, mouse and zebrafish PIWIs respectively, with especially highly conserved PAZ and PIWI domains (80%). RNAi-induced knockdown of *CIWI* and *CILI* results in upregulated expression of the repetitive element *CR1* and an increase in DNA double-strand breakage in isolated chicken PGCs (Rengaraj *et al*. 2014). These data indicate that, similar to zebrafish and mice, the PIWI/piRNA pathway is operational in avian gametes. Thus, the piRNA machinery is conserved in structure and function. In addition to slicing and therefore RNA degradation, PIWI proteins were also involved in transposon silencing by CpG DNA methylation in PGCs (Kuramochi-Miyagawa *et al*. 2004, Carmell *et al*. 2007). Recent data suggest additional functions, including transcriptional regulation, mRNA deadenylation and transgenerational effects (Weick & Miska 2014).

Similar to the differential expression of the PIWI proteins, piRNA expression appears to be highly regulated and occurs in two waves, namely pre-pachytene and pachytene piRNAs (Aravin *et al*. 2007*a*). In mice, the expression of pre-pachytene piRNAs starts at around 12.5 dpc in PGCs (Kuramochi-Miyagawa *et al*. 2004, Aravin *et al*. 2007*a*, Aravin & Hannon 2008). These piRNAs bind to PIWIL2 and PIWIL4 and are involved in transposon defence (Aravin *et al*. 2007*b*, Carmell *et al*. 2007). The second pool of piRNAs is expressed at the pachytene stage of meiosis until the sperm reaches the haploid elongated spermatid stage during spermatogenesis, and are associated with PIWIL1 and PIWIL2 proteins. This burst of piRNA expression in the testis is required for the completion of spermatogenesis (Deng & Lin 2002). Their function appears to be independent of transposon suppression, but is conserved in adult chicken testis. The transcription factor, A-MYB, activates both pachytene piRNA precursors and piRNA biogenesis factors, and is active in adult mouse and chicken testes, pointing to a conserved mechanism of pachytene-enriched piRNAs required for proper spermatogenesis (Li *et al*. 2013). However, in contrast to their function, piRNA sequences themselves have evolved rapidly, with generally poor conservation, even among closely related species.

Whereas the biogenesis of miRNAs and siRNAs requires the catalytic activity of Dicer, the generation of piRNAs is Dicer independent (Aravin *et al*. 2006, Vagin *et al*. 2006, Watanabe *et al*. 2006, Houwing *et al*. 2007). Biogenesis of primary piRNAs begins with the transcription of long, single-stranded piRNA precursor transcripts (Aravin *et al*. 2006, Watanabe *et al*. 2006), which are exported from the nucleus and cleaved into primary antisense piRNAs. In addition, a secondary biosynthesis mechanism exists, the so-called ping-pong mechanism, which results in the amplification of piRNAs (Brennecke *et al*. 2007). Here, the antisense piRNA derived from the primary pathway is loaded onto PIWIL2 in mammals and the protein-RNA complex binds and slices transposon RNA, resulting in the degradation of the transposon RNA and the generation of sense piRNAs. Sense piRNAs in turn associate with PIWIL4 to bind and cleave the antisense transcript derived from piRNA clusters to amplify the number of antisense piRNA (Aravin *et al*. 2007*b*, Brennecke *et al*. 2007). In addition, other piRNAs are derived from the 3′UTRs of protein-coding genes, which has been shown to be conserved from *Xenopus* to mammals (Robine *et al*. 2009, Ha *et al*. 2014). Using shotgun cloning or high-throughput next-generation sequencing, piRNAs have been identified in embryonic and adult chicken testis (Zhang *et al*. 2013, Rengaraj *et al*. 2014). Despite some challenges associated with the low sequence conservation of small ncRNAs, chicken piRNAs have been annotated and mapped in clusters across the chicken genome. They can be derived from either repetitive elements or exons (Yang *et al*. 2012).

The role of piRNAs in sex determination

Is there a role for piRNAs in regulating sex determination during embryogenesis? It is unlikely that this is the case in mammals, as piRNA expression is predominantly germ cell-specific (Girard *et al*. 2006, Grivna *et al*. 2006, Lau *et al*. 2006, Watanabe *et al*. 2006) and PIWI proteins as well as piRNAs are essential for germ cell proliferation, maintenance and/or differentiation and therefore fertility (e.g., Deng & Lin 2002, Carmell *et al*. 2007,

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Kuramochi-Miyagawa *et al*. 2010), but not sex determination.

In contrast, evidence from invertebrates points to a key role at least in one species. Among flies, moths and other insects, sex is determined cell-autonomously throughout the body of the entire embryo. The silkworm *Bombyx mori* uses a ZW female: ZZ male sex chromosome system, with the W sex chromosome known to be female-determining. The female W chromosome is enriched in piRNAs, and one of these, dubbed *Feminiser* (*Fem*), acts as the primary female-sex determining gene (Kawaoka *et al*. 2011, Kiuchi *et al*. 2014). The *Fem* sequence is reiterated on the silkworm W sex chromosomes, and encodes a mature piRNA that is expressed in embryos and acts to regulate the *Doublesex* gene. *Doublesex* is a major sex-determining gene among insects. The primary transcript is spliced in a sexually dimorphic fashion, leading to two different proteins, with female- and male-specific functions respectively. These isoforms regulate downstream targets that coordinate female vs male development throughout the body and not just in the gonads. When *Fem* is experimentally inhibited, *Doublesex* is spliced in the male mode, leading to male development. In normal female gonads, the *Fem* piRNA acts to cleave a Z-chromosome linked transcript, *Masc*, that otherwise produces a zinc finger protein that directs the male-specific splicing of *Doublesex* (reviewed in Whitworth & Oliver (2014)). The vertebrate homologue of *Doublesex* is *DMRT1*, a highly conserved zinc finger-like transcription factor with a pervasive role in testicular morphogenesis (Gamble & Zarkower 2012, Matson & Zarkower 2012). Although differential splicing of *DMRT1* has been reported among vertebrates (Huang *et al*. 2005), there is currently no evidence to suggest that vertebrate *DMRT1* might be regulated by a piRNA. However, this has not been thoroughly investigated.

The role of lncRNAs in dosage compensation

In species with genetic sex determination, such as the XX female: XY male system in mammals and the ZW female: ZZ male system in birds, males and females have a difference in sex chromosome-linked gene dosage, which has resulted in the evolution of dosage compensation mechanisms. In mammals, this is realized by the inactivation of one of the X chromosomes through coating by a lncRNA called *Xist* (*X* inactive specific transcript). The 19-kilobases-long transcript *Xist* is only transcribed from the inactive X chromosome and coats the X chromosome in *cis*, repressing the expression of hundreds of genes. Prior to inactivation, a lncRNA that is antisense to *Xist*, called *Tsix*, is expressed. Upon differentiation, the expression *Tsix* is downregulated from one of the X chromosomes, resulting in the *Xist* and inactivation of this X chromosome. On the active X, the maintained expression of *Tsix* prevents full-length *Xist* expression and X-linked gene expression is unaffected (reviewed in Moran *et al*. (2012)) This phenomenon of dosage compensation in mammals is clearly regulated by lncRNAs. In other groups, such as birds, there is no chromosome-wide inactivation of one sex chromosome in the homogametic sex. However, there is potential involvement of lncRNAs in dosage compensation in chickens as well. Chickens and other birds have a ZZ male: ZW female sex chromosome system. As noted above, the Z-linked transcription factor gene, *DMRT1*, is thought to play a central role in avian sex determination by directing testis development in ZZ embryos. Overexpression of DMRT1 induces the male-specific genes, *HEMGN*, *SOX9* and *AMH* (Lambeth *et al*. 2014) (Fig. 3). Meanwhile, knockdown of *DMRT1* expression with RNAi results in feminization of the gonads, and an upregulation of both FOXL2 and Aromatase, which are female marker genes (Smith *et al*. 2009). *MHM* is a 2.2 kb reiterated sequence located on the chicken Z sex chromosome, but apparently absent in most other birds. It is only transcribed in female cells, where it produces a 9-kb-lncRNA that accumulates in the nucleus near its site of transcription, quite close to the *DMRT1* locus (Teranishi *et al*. 2001). Although there is no chromosome wide Z inactivation mechanism in birds to equalise Z dosage, *MHM* is located within a region of the Z shows a higher degree of equalized Z expression than elsewhere, and corresponds with hyperacetylation of histone H4, which is associated with increased gene expression (Bisoni *et al*. 2005, Melamed & Arnold 2007). It has therefore been suggested that it may regulate local dosage compensation, perhaps by upregulating nearby genes and hence hyperacetylation of histone 4 in ZW female cells.

However, another hypothesis is that *MHM* may play a role in chicken gonadal sex differentiation (Fig. 3). In male cells (ZZ), *MHM* is hypermethylated and transcriptionally silent, whereas in female cells (ZW), it is hypomethylated and transcribed. Given its close proximity to *DMRT1*, it has been suggested that *MHM* may influence or dampen expression of *DMRT1* in female cells, as these DMRT1 levels are always lower than in males. This could occur through *MHM* lncRNA coating the chromosome adjacent to the *DMRT*1 locus, inducing local chromatin conformational changes that may interfere with transcription factor binding. Indeed, mis-expression of *MHM* in early ZZ chicken embryos appears to disrupt *DMRT1* expression (Roeszler *et al*. 2012), while injection of *MHM* expression plasmids into adult chicken testes quenches *DMRT1* expression. Further analysis of *MHM* will involve knockdown or knockout *in vivo*. In addition, *MHM* likely has other roles beyond gonadal sex differentiation, as it is widely expressed in female embryos. The absence of *MHM* in most other birds may reflect the poor conservation of lncRNAs in general. Since lncRNAs are implicated mainly in chromatin confirmation, they may have

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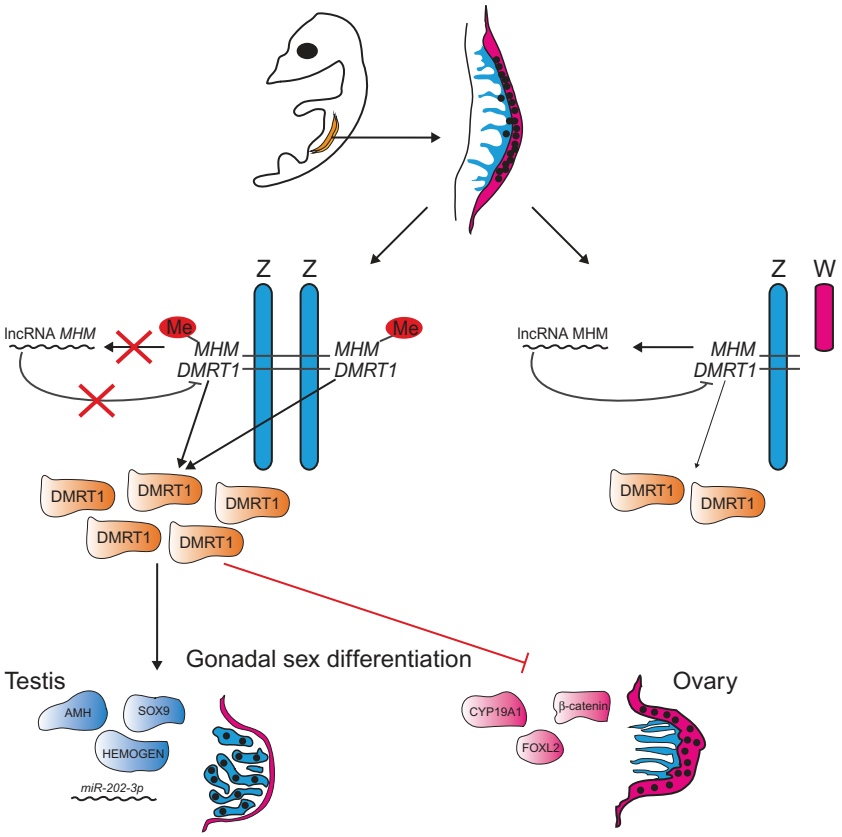

Figure 3 Potential roles of the long non-coding RNAs MHM in chicken. In ZZ male gonadal cells, Z-linked *MHM*, a long non-coding RNA, is methylated and transcriptionally silent. The neighbouring *DMRT1* gene is transcribed, and is required for testis development, activating genes such as *HEMOGEN* and *SOX9*. *MiRNA-202-3p* is also expressed in testis and may play a role is cord organisation. In ZW female cells, *MHM* is hypomethylated and transcribed into long-non-coding RNA that coast the Z adjacent to the *DMRT1* locus. It may quench *DMRT1* expression, leading to less DMRT1 protein and allowing ovarian pathway genes to become active (e.g., *FOXL2*, *CYP19A1* and β-catenin).

fewer sequence constraints than, for example, microRNAs. Other sequences could perform analogous roles, even if not structurally homologous.

Conclusions and future directions

In recent years, substantial progress has been made in understanding the role of some ncRNAs, such as piRNAs, in male sex determination and differentiation, while the investigation of other ncRNAs has just begun. The expression of miRNAs in embryonic gonads at the time of sex determination and also at later stages during testis differentiation and in the postnatal testis has been studied extensively in different species. Surprisingly, depending on the experimental setup, there is little overlap between the different studies with respect to the miRNAs that were detected (e.g., Papaioannou *et al*. 2009, Aguilar *et al*. 2010, Rakoczy *et al*. 2013). In addition, deletion of Dicer or Drosha demonstrated only a relatively late role for miRNAs in the postnatal testis (Hayashi *et al*. 2008, Maatouk *et al*. 2008, Papaioannou *et al*. 2009, Huang & Yao 2010), suggesting that miRNAs might not play a role during the early stages of sex differentiation. However, miRNAs appear to be very stable, at least in the testis, resulting in a loss of all miRNAs only after several days following Dicer deletion (Papaioannou *et al*. 2009). Hence, the deletion of Dicer or Drosha in the developing testis is not a useful strategy to investigate the function of miRNAs at these early steps of sex determination and differentiation. In contrast, specific miRNAs that have been shown to be expressed at these stages need to be examined individually. From the few examples tested to date, it appears that, similar to other systems, miRNAs might only play a modulatory role during sex determination, and loss-of-function or gain-of-function analysis causes only a relatively mild phenotype.

The class of ncRNAs about which we possibly know the least as far as its role during male sex differentiation are lncRNAs. Similar to miRNAs, these RNAs have been shown to be expressed (Chen *et al*. 2012), but functional analysis is lacking. However, recent technical advances, especially genome editing tools such as the TALEN and CRISPR/CAS9 systems, offer the exciting opportunity to perform *in vivo* loss-of-function analysis for specific ncRNAs, including miRNAs and lncRNAs, that is feasible considering both the cost and time involved and is not restricted to species such as mice, fish and *Drosophila*. These functional analyses will not only provide a more complete picture of the evolution and the molecular mechanisms driving male sex differentiation, but will also provide new candidate genes likely to be causative for disorders of sex development and male infertility in humans.

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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