Developmental Biology 366 (2012) 317–326

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

# The long non-coding RNA, *MHM*, plays a role in chicken embryonic development, including gonadogenesis

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ARTICLE INFO

*Article history:*
Received 10 October 2011
Received in revised form
1 March 2012
Accepted 26 March 2012
Available online 21 April 2012

*Keywords:*
Chicken embryo
MHM
Gonads
Sex determination
Dosage compensation

ABSTRACT

*MHM* is a chicken Z chromosome-linked locus that is methylated and transcriptionally silent in male cells, but is hypomethylated and transcribed into a long non-coding RNA in female cells. *MHM* has been implicated in both localised dosage compensation and sex determination in the chicken embryo, but direct evidence is lacking. We investigated the potential role of *MHM* in chicken embryonic development, using expression analysis and retroviral-mediated mis-expression. At embryonic stages, *MHM* is only expressed in females. Northern blotting showed that both sense and antisense strands of the *MHM* locus are transcribed, with the sense strand being more abundant. Whole mount *in situ* hybridization confirmed that the sense RNA is present in developing female embryos, notably in gonads, limbs, heart, branchial arch and brain. Within these cells, the *MHM* RNA is localized to the nucleus. The antisense transcript is lowly expressed and has a cytoplasmic localization in cells. Mis-expression of *MHM sense* and *antisense* sequences results in overgrowth of tissues in which transcripts are predominantly expressed. This includes altered asymmetric ovarian development in females. In males, *MHM* mis-expression impairs gonadal expression of the testis gene, *DMRT*1. Both *MHM* sense and antisense mis-expression cause brain abnormalities, while *MHM sense* causes an increase in male-biased embryo mortality. These results indicate that *MHM* has a role in chicken normal embryonic development, including gonadal sex differentiation.



## Introduction

The chicken embryo has a ZZ male: ZW female sex chromosome system. The Z-linked gene, *DMRT*1, is required for testis development during embryonic development and is hypothesized to operate via gene dosage, with males (ZZ) having two doses and females (ZW) having one (reviewed in Chue and Smith, 2011). Z-linked *DMRT*1 is more highly expressed in testes *versus* ovaries, and knockdown of *DMRT*1 expression leads to feminization of male gonads (Smith et al., 2009). It is therefore postulated that *DMRT*1 is a key gene underlying sex determination in the avian system. The higher expression of *DMRT*1 in male tissues (ZZ) suggests that the gene escapes a dosage compensation mechanism. Indeed, birds lack a chromosome wide Z-inactivation system analogous to X chromosome inactivation in mammals (Baverstock et al., 1982; Kuroiwa et al., 2002; Ellegren, 2007; Itoh et al., 2007). On average, most Z-linked genes show mRNA expression levels that are greater than 1.5 fold higher in males (ZZ) compared to females (ZW), as assessed by microarray and individual gene expression studies (Kuroda et al., 2001; McQueen et al., 2001; Itoh et al., 2007; Zhang et al., 2010) reviewed in Arnold et al. (2008). However, there are regions across the Z chromosome that are compensated, or partially compensated (McQueen et al., 2001; McQueen and Clinton, 2009). These regions may harbor genes that are developmentally important and for which dosage inequality between the sexes cannot be tolerated.

A specific cluster of compensated genes has been reported on chicken Zp (Melamed and Arnold, 2007). Within this region is a gene called *MHM* (Male HyperMethylated). *MHM* is hypermethylated on the two Z sex chromosomes of male chickens and is transcriptionally silent. Treatment of male cells with the demethylating agent, 5-aza-cytidine, alters chromatin configuration and allows *MHM* expression in male cells (Itoh et al., 2011). In female cells (ZW), the *MHM* locus is hypomethylated on the single Z chromosome and is transcribed into a long non-coding

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http://dx.doi.org/10.1016/j.ydbio.2012.03.025

RNA that coats the Z chromosome at the site of transcription (Teranishi et al., 2001; Itoh et al., 2011). The *MHM* sequence may mediate the partial dosage compensation seen on the chicken Z chromosome, either locally or more widely. In mammals, X inactivation is regulated by the X-linked gene, *Xist* (Leeb et al., 2009). While not structurally related to *Xist*, *MHM* shows similar features, encoding a high molecular weight non-coding RNA which coats one of the sex chromosomes in cis. It has therefore been speculated that *MHM* could specifically mediate dosage compensation of the Zp gene cluster in chicken, dubbed the "*MHM* valley", by modifying local chromatin organization (Arnold et al., 2008; Teranishi et al., 2001). However, whereas *Xist* is associated with X inactivation, *MHM* is associated with an active Z sex chromosome. Within this same region on the female Z sex chromosome, histone 4, lysine 16 is acetylated (Bisoni et al., 2005). In other dosage compensation systems, histone acetylation is associated with up-regulation of neighbouring genes (Bone et al., 1994; Smith et al., 2005). A causal relationship between MHM and $H_4k_{16}$ acetylation is unclear. However, taken together, these observations have led to the hypothesis that *MHM* plays a role in dosage compensation by altering gene expression in females (ZW). It may do so by up-regulating a subset of genes on the female Z chromosome (those in the "MHM valley") to equalise dosage with that of males (ZZ), via a process that involves local female-specific hyperacetylation of histone $H_4k_{16}$. The *DMRT1* locus is adjacent to the MHM valley, and it has also been hypothesized that MHM RNA could function to repress *DMRT1* expression in female embryos, thereby contributing to sex determination in the chicken (Teranishi et al., 2001; Yang et al., 2010). To further explore the role of *MHM* in chicken embryonic development, we carried out detailed expression and mis-expression studies. We find that RNA transcription occurs from both the sense and antisense strands of the *MHM* locus and that these strands have different temporal and spatial expression patterns during embryonic development. Furthermore, mis-expression of these sequences causes embryonic abnormalities of the brain and gonads, consistent with a role in both dosage compensation and sex determination in the chicken embryo.

## Methods

### *Cloning of the chicken 2.2 kb MHM repeat unit*

The Chicken (*Gallus gallus*) *MHM* locus, first described by Teranishi et al. (2001), comprises a core *Bam*HI- 2.2 kb repeat that has at least 200 copies on the short arm of the Z sex chromosome. The transcript itself was estimated at 9 kb. Using this sequence data, we designed primers to amplify the 2.2 MHM sequence as described by Teranishi et al. (2001) (Genbank AB046698). A 2.2 kb PCR product obtained from chicken genomic DNA was cloned into pSLAX shuttle vector and its identity confirmed by automated sequencing. This sequence was then subcloned into the RCASBP(A) avian retroviral vector for mis-expression studies (Logan and Tabin, 1998).

### *Northern blot analysis*

A 668 base pair *MHM* riboprobe, derived from the 2.2 kb sequence, was used for Northern blotting. Total RNA was isolated from several embryonic day 10.5 male and female gonad, limb and brain samples and approximately 10 μg of each were run on a 1.1% agarose/formaldehyde gel, together with RNA size standards (Promega Corp, Madison, WI, USA). RNA was transferred to a Hybond N membrane (Amersham Biosciences, UK) by capillary transfer and baked at 80 °C for two hours. Membranes were prehybridized with Ultrahyb (Ambion, Austin, Texas, USA) for one hour at 68 °C and then probed with 100 ng DIG-labelled probe overnight at 68 °C. *MHM sense* transcripts were detected with the 668 bp *MHM* antisense riboprobe, synthesized with SP6 RNA polymerase, while the *MHM-antisense* strand was detected on a separate membrane, using the sense probe (T7 RNA polymerase). The next day, membranes were washed to a stringency of 0.1 × standard saline citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) at 68 °C. Bound DIG-labelled riboprobe was detected using an anti-DIG antibody (Roche, Mannhein, Germany). Chemiluminescent signal generated by CDP-Star (Roche) substrate was detected by exposure of membranes to Kodak Hyperfilm (Amersham Biosciences, UK). Northern blots were performed twice. Chemiluminescent exposure time for the sense transcript was 1 min, while exposure for the antisense transcript was over 90 min.

### *Whole mount in situ hybridization*

One hundred fertile chicken eggs were incubated at 37.8 °C and embryos were harvested throughout development. The whole mount *in situ* hybridization method (WISH) has been described in detail previously (Andrews et al., 1997; Smith et al., 1999). Embryos were sexed by PCR as described previously (Clinton et al., 2001), prior to manipulation of tissues for expression analysis. At least three embryos were used for each analysed time point. Briefly, tissues were dissected under RNase-free conditions and fixed overnight at 4 °C in 4% paraformaldehyde/PBS. Whole embryos (early stages) or isolated tissues were taken. Following fixation, tissues were dehydrated through a methanol series, and stored at −20 °C prior to rehydration and digestion with proteinase-K in PBS+0.1% Triton-X 100 (30 to 90 min, depending upon the size of tissues). Samples were then briefly re-fixed, and incubated overnight at 65 °C in prehybridisation buffer. Two different RNA probes were used in experiments that were repeated at least twice. In the first set of experiments, the same 668 bp fragment used for Northern blotting was used for WISH. The antisense probe (detecting *MHM-sense*) was synthesized using SP6 RNA polymerase and labeled with digoxygenin (DIG). The DIG "sense" probe (detecting MHM-antisense) was synthesized with T7 RNA polymerase. In the second experiment, the entire 2.2 kb repeat unit was used as a riboprobe. Probes were added to tissues overnight in hybridsation solution, washed at 1 × and 0.1 × strigency, and alkaline-phosphatase conjugated DIG antibody was added, following pre-blocking in TBTX buffer (pH 7.0) containing BSA and sheep serum. Tissues were then extensively washed in TBTX+0.1% BSA, and the chromagen was then added (NBT/BCIP in Tris-based buffer, pH 9.0). Color reactions were carried out at room temperature over 2–4 h, prior to imaging. For sectioning, tissues were placed back into chromogen solution and incubated at room temperature over two days, then cryoprotected by placing into 30% sucrose/PBS overnight at 4 °C. Following embedding in OCT compound and snap freezing on dry ice, tissue samples were cut on a cryostat at 10–18 μm and mounted onto slides with Aquamount. In some cases, tissues were treated with DAPI prior to mounting, allowing the visualization of RNA signals relative to the nucleus, followed by false coloring of the RNA signal against DAPI blue immunoflourescence.

### *Mis-expression of 2.2 kb MHM in ovo*

To assess the effects on mis-expressing *MHM*, the RCAS avian viral vector was used (Fekete and Cepko, 1993; Logan and Tabin, 1998) (Smith et al., 2009). The 2.2 kb *Bam*HI MHM unit (Genbank AB046698) was subcloned from pSLAX shuttle plasmid into RCASBP/A proviral DNA and its sequence confirmed. The viral DNA was transfected into chicken fibroblastic DF1 cells and

propagated over several days. RT-PCR analysis confirmed high level of expression of MHM (not shown). Virus was harvested from culture medium, concentrated by ultracentrifugation and titred. High titer virus (at least $10^8$ Infectious Units/mL) was injected into day 0 blastoderms, eggs were sealed with parafilm and development allowed to proceed at 37.8 °C. Control embryos were either not injected, or injected with RCASBP/A virus carrying GFP. These experiments were repeated and involved at least 50 embryos for each experiment. Embryos were generally harvested at embryonic days 10.5 (stage 35). Infection was confirmed by staining tissues with p27 antibody, which detects a viral epitope. Tissues were examined for macroscopic abnormalities, and assessed using either WISH or immunostaining or qRT-PCR for *DMRT*1 and *MHM* gene expression.

### *Immunostaining and histology*

Control and *MHM* mis-expressing tissues were fixed for 15 min in 4% PFA/PBS at room temperature, prior to processing for tissue section immunofluorescence, as described previously (Smith et al., 2003). At least three embryos per time point and/or treatment were examined. Briefly, 10 μm sections were cut of a cryostat, permeablised in PBS+1% Triton X-100 and blocked in PBS+2% BSA. Primary antibodies were raised in-house (rabbit anti-DMRT1 and rabbit Aromatase, both 1:5000) or were obtained commercially (rabbit anti-p27; Charles River Services; 1:1000). Alexa-fluor secondary antibodies were used (donkey or goat anti-rabbit 488 or 594; Molecular Probes). Sections were counterstained with DAPI. For histology of brain samples, tissues were fixed overnight in Bouin's fluid, dehydrated in a tissue processor, embedded in parafim and cut on a microtome at 6 μm. Sections were staining in haematoxylin and eosin prior to mounting in DPX and imaging.

### *Quantitative reverse transcription-PCR (qRT-PCR)*

RNA was extracted from isolated gonads, reverse transcribed as previously described (Smith et al., 2008). Probes were designed UPL Assay Design Center (https://www.roche-applied-science.com) and are as follows. MHM antisense probe 159 together with forward primer 5′-CTC TCA TTC CGG CTT CCA-3′ and reverse primer 5′-TTG TTT TTG TTT GGC CAG TG-3′; MHM sense probe 15 with forward primer 5′-CAA AGC GCT TGA AAG GGT AA 3′ and reverse primer 5′-GCA AAG AAC TAC TCG CCA AAA; DMRT1 probe 59 together with forward primer 5′–AGC CTC CCA GCA ACA TAC AT-3′ and reverse primer 5′-GCG GTT CTC CAT CCC TTT-3′; HPRT probe 38 together with forward primer 5′–CGC CCT CGA CTA CAA TGAA TA-3′ and reverse primer 5′-CAA CTG TGC TTT CAT GCT TTG-3′; Cytokeratin probe 162, together with forward primer 5′-ACT CTG CTC CAG GCA TTG AC-3′ and reverse primer 5′-TCA CGG TTC AGC TCC TCA G-3′; 18S rRNA probe 48, together with forward primer 5-GCA ATT ATT CCC CAT GAA CG-3 and reverse primer 5′-GGG ACT TAA TCA ACG CAA GC-3′. Analysis was performed using LightCycler 480 instrument, LC480 master mix and software (Roche). Relative expression was determined using the comparative CT method (ΔΔCT), with samples normalized against either *18S* rRNA or *HPRT* and expressed as fold change. In addition, the efficiency of each primer/probe combination was determined and only combinations with efficiencies $>80\%$ were used.

## Results

### *MHM-sense and MHM-antisense sequences.*

Teranishi et al. (2001) first described the *MHM* transcript as a 9 kb RNA, containing a 2.2 kb repeat unit. This unit is reiterated on the chicken Z sex chromosome (Genbank accession number AB046698). We amplified a 2.2 kb sequence from chicken gDNA, which corresponded to this sequence, shown in Fig. 1. *MHM* is thought to encode a long non-coding RNA. However, there are several short potential open reading frames derived from both sense and antisense strands, the longest being approximately 420 bp (MacVector software, Accelrys). These could potentially encode small bioactive peptides. In the studies described here, a 668 bp RNA probe was designed against a region within the 2.28 kb *MHM* RNA sequence, as shown in Fig. 1. The UCSC genome browser indicates that there are several splice variants of the *MHM* sequence. Indeed, Northern blot analysis of embryonic tissues revealed a number of female specific transcripts, in both the sense and antisense orientations, (Fig. 2). Several transcripts of varying sizes derived from the sense strand were detectable in female tissues (from 1.7 kb to over 12 kb), with the most abundant species being of higher size (7 kb and above). No transcripts were detected in males (Fig. 2(A)). The *MHM* sense strand studied here is identical to the original *MHM* sequence as published by Teranishi et al. (2001) (clone pCC1-11). However, the *MHM* sequence present on the Genbank database (AB046689.1) is in the opposite orientation with respect to our sequence. The *MHM* antisense strand was also expressed female-specifically, but at much lower levels and with different sized transcripts. (Northern blot chemiluminescent exposure time was 1.5 h for MHM-antisense *versus* an exposure time of 1 min for the MHM sense strand).

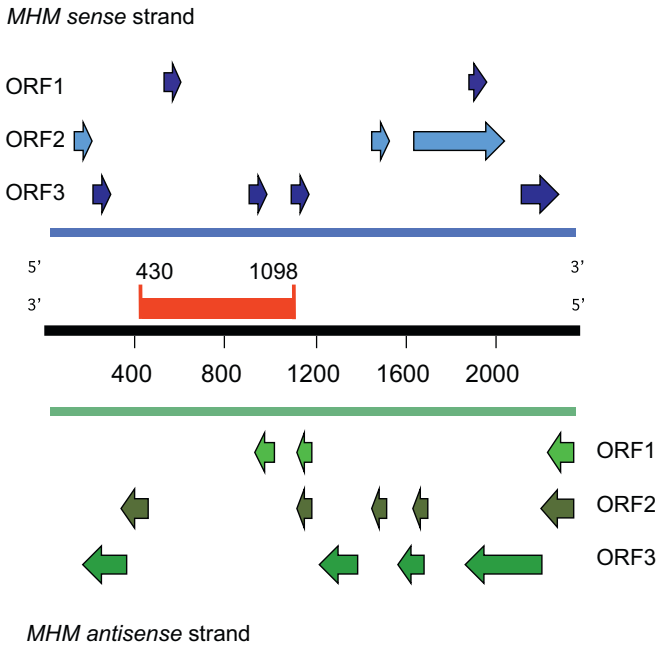


**Fig. 1.** Potential open reading frames (ORFs) in each frame identified from 2376 bp MHM sequence, AB046698. *MHM-Sense* sequences 5′ −3′ (blue). *MHM-antisense* 3′ −5′ (green). The position of the 668bp riboprobe sequence used in this study is shown in red. The longest potential ORFs are approximately 400 bp. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### *Spatial expression of MHM*

Whole mount *in situ* hybridization (WISH) and tissue sectioning was used to examine *MHM* sense and antisense strand expression in whole embryos at embryonic day (E) 1, 2 and 4 and in isolated urogenital systems up to day 8.5 (Figs. 3 and 4). Both the 668 bp and 2.2 kb riboprobes gave the same overall staining patterns. While both sense and antisense strands were expressed in a female-specific manner, the sense strand showed

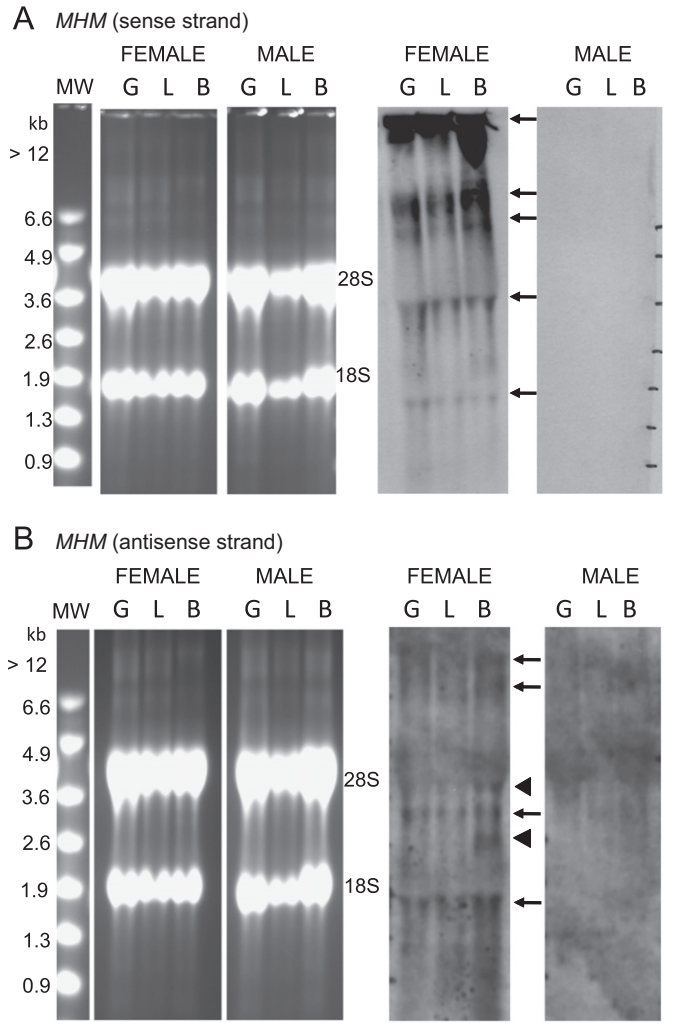


**Fig. 2.** Northern blot analysis of day 10.5 embryonic gonad (G), limb (L) and brain (B) total RNA. (A) *MHM* sense strand expression. Ethidium bromide staining showing equal loading in female and male samples as indicated by the 28S and 18S bands. Five RNAs are detected in female but not male tissues, at 1.8 kb, 3.6 kb, 7 kb, 9 kb and over 12 kb. (B) *MHM* antisense strand expression. 28S and 18S bands stained with ethidium bromide indicate equal loading. Weak bands are detectable in female tissues, notably at 3.4 kb and 1.8 kb. Additional bands are present in brain (arrowheads).

stronger expression, in agreement with the Northern blotting. Strong female-specific expression of *MHM sense* strand was detected throughout embryos (Fig. 3). Expression was first detectable in whole embryos after day 1, throughout the body (Fig. 3(A)). However, specific tissues showed higher *MHM sense* expression than others. These included the brain, branchial arches, heart and the urogenital system. In the urogential system, *MHM sense* was expressed in the (female) gonads, with expression increasing over development, from day 4 to at least day 8 (Fig. 3(B)). Lower level expression was also observed in the female mesonephric kidneys. No *MHM sense* expression was detected in males.

The antisense *MHM* strand was also expressed, but at lower levels and with a more restricted pattern. It was first detected at E1 (stage 10) in the developing neural tube of the female (Fig. 4). By E4.5, *MHM-sense* expression was observed in the developing female brain and limbs (Fig. 4(A)). In the urogenital system, *MHM-antisense* was weakly expressed in the gonads (Fig. 4(B)).

### *Localization of MHM expression in embryonic gonads*

Given the previous suggestion that *MHM* RNA could influence gonadal sex differentiation, we examined gonadal *MHM* expression in more detail. Tissues stained during whole mount *in situ* hybridization were cryo-protected and sectioned. In gonads examined at embryonic day 6.5 and 10.5, punctate *MHM* sense RNA localisation was detected throughout both left and right gonads and in mesonephric kidneys of female embryos (Fig. 5(A)). A similar pattern was seen in sectioned brains (not shown). High power magnification of the RNA signal suggested nuclear localization, which was confirmed by DAPI nuclear counterstaining (Fig. 5(A)). This nuclear expression pattern was observed with both the 688 bp and the 2.2 kb riboprobes detecting *MHM sense*. Such punctate nuclear RNA staining is quite distinct from the diffuse cytoplasmic staining typical of mRNA ISH. There appeared to be a single focus of localization within each nucleus. In contrast, the *MHM-sense* strand was present in the cytoplasm of the developing ovarian cortex of female gonads (Fig. 5(B)) with weak staining detected in scattered cells of the underlying medulla. (Embryonic day 10.5 tissues are shown.) The cortex is the site of germ cell proliferation and folliculogenesis, and the medulla is the site of oestrogen biosynthesis. Counterstaining of some sectioned whole mount specimens with DAPI to delineate the nucleus, followed by false colouring of the RNA signal, clearly confirmed the *MHM-antisense* staining to be cytoplasmic. This contrasts with the nuclear staining for *MHM-sense*. No specific staining for either *MHM-sense* or *MHM-antisense* could be detected in male

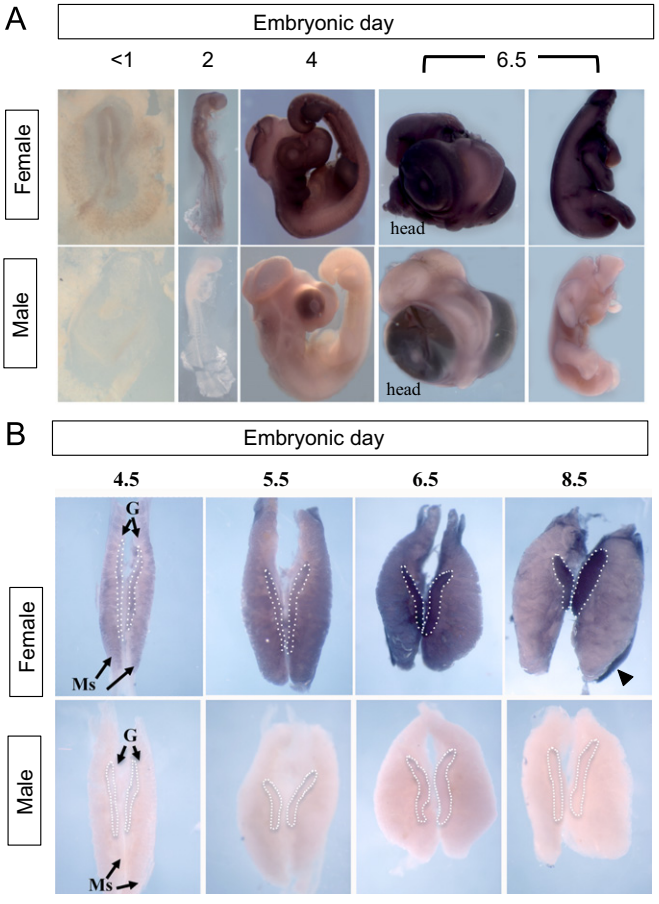


**Fig. 3.** Female-specific expression of *MHM* sense RNA, using either 0.668 kb or 2.2 kb riboprobe. (A) Whole embryo expression, delineated with a 2.2 kb *MHM* riboprobe. Expression in female embryos is detected first throughout from day 1. Strong expression is seen by day 4. Males do not show expression. (B) *MHM* expression in the urogenital system, using the 668 bp riboprobe. Expression is detectable in female gonads (G, outlined), in the Müllerian duct (arrowhead) and at lower levels in the mesonephros (Ms), from day 4.5–8.5. Gonadal expression increases over time. No expression is detectable in male gonads.

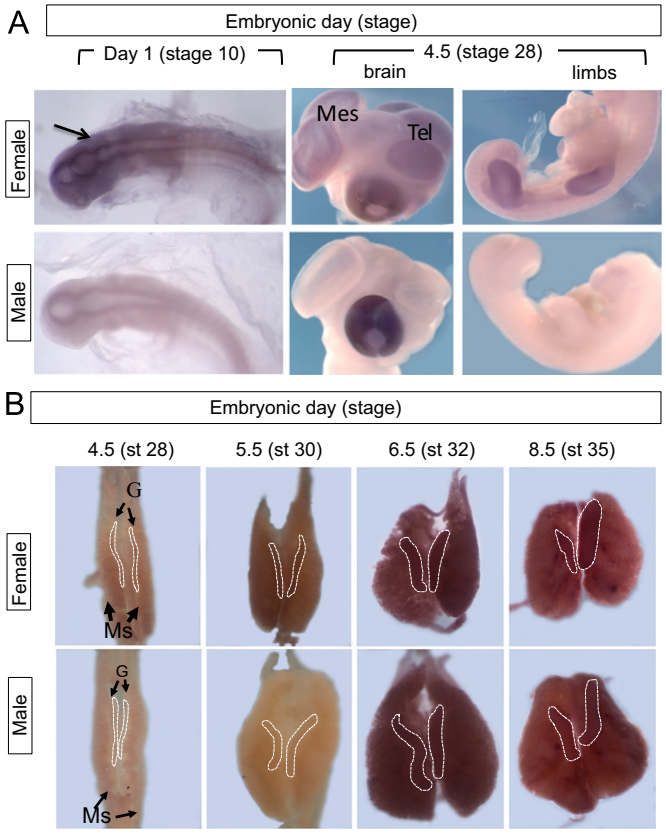


**Fig. 4.** Female-specific expression of *MHM-antisense* RNA. (A) *MHM-antisense* RNA expression in day 1 female embryos. At E4.5, expression is detectable in the telencephalon (Tel) and mesencephalon (Mes) of the brain, and in the fore- and hindlimbs. Males did not show expression. (B) Low level-*MHM-antisense* expression in the gonads (G) outlined in white. Ms=mesonephric kidneys. Weak expression observed from day 4.5 in the females with stronger expression observed at 6.5 and 8.5. Little expression is observed in the males. Scale bar=50 μm.

gonads of any age. Only non-specific background staining was seen in over-stained male samples (so-called "edge effects", Fig. 5(C)).

### *Mis-expression of MHM in female embryos*

*MHM-sense* was mis-expressed *in ovo* by injecting day 0 blastoderms with *RCAS* virus expressing the 2.2 kb *MHM* repeat unit. This procedure leads to widespread expression of the transgene throughout the embryo (Smith et al., 2009). Phenotypic effects were assessed either at E4.5 (stage 28) or E10.5 (stage 35). We originally hypothesised that mis-expression of *MHM* sequences in male embryos (in which the gene is normally silent) would have phenotypic consequences. In fact, mis-expression of *MHM-sense* induced abnormalities in both sexes. The major defects observed were perturbation of normal gonadal differentiation, brain defects and a general overgrowth of tissues. In females, both *MHM-sense* and *antisense* over-expression disrupted the normal asymmetric development of the gonads (Fig. 6). Viral delivery of *MHM sense* and antisense strands in developing urogenital systems was confirmed by *WISH* (Fig. 6). Typically, control female gonads are asymmetrical at E10.5, marked by a large left ovary and regressing right gonad (Fig. 6(A) and (B)). *MHM-sense* mis-expression in embryonic female gonads prevented the normal regression of the right gonad, resulting in the development of bilateral enlarged ovaries. Both of these ovaries were significantly larger than normal asymmetric female gonads (Fig. 6(B)). Both left and right *MHM-sense* infected ovaries expressed aromatase, a medullary marker, and had thickened outer cortex, as shown in Fig. 6(B). Immunohistochemical detection of viral p27 antigen (Fig. 6(B)) confirmed robust expression of virus and the transgene in these tissues. The enlarged ovaries of the *MHM-sense* over-expressing females gonads had an increased proliferation index, according to PH3 staining (not shown). Interestingly, the same ovarian overgrowth was observed in female embryos over-expressing *MHM-antisense* RNA (Fig. 6(B)). As for *MHM sense*, the antisense strand induced bilateral ovary formation in female embryos, with robust aromatase enzyme expression delineating two enlarged ovaries. As for *MHM-sense*, *MHM antisense* delivery into tissues was confirmed by viral p27 staining and WISH for the expressed *MHM*-antisense (Fig. 6).

### *Mis-expression of MHM sequences in male embryos*

*MHM* is not normally expressed in male embryos, due to epigenetic silencing (refer to Fig. 2). To assess the effects of *MHM* mis-expression on male embryos, qRT-PCR, whole mount staining and immunofluorescence were used on embryonic day 10.5 samples. For both *MHM* sense and antisense sequences, mis-expression perturbed testis development but did not induce

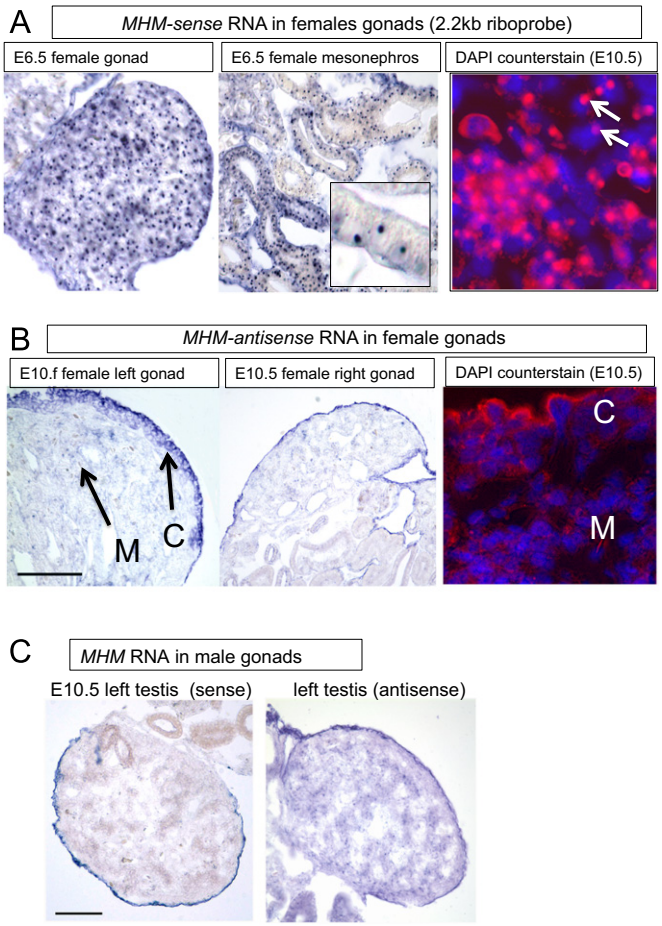


**Fig. 5.** Localization of *MHM sense* and *antisense* transcripts in E10.5 gonads, using 0.668 kb or 2.2 kb riboprobe. (A) Punctate expression of *MHM-sense* in a E6.5 female gonad and in the neighboring mesonephric kidney (insert shows nuclear localization). DAPI counter-staining confirms a nuclear localization in E10.5 ovary (arrows). (B) *MHM-antisense* expression in the cytoplasm of the left ovarian cortex. (C ) and in scattered medullary cells (M). The right gonad lacks a cortex and shows medullary staining only. False DAPI counterstaining for nuclei (blue) shows that MHM-Forward is cytoplasmic (red). Bar=100 um. (C) No specific staining for either RNA in E10.5 testis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

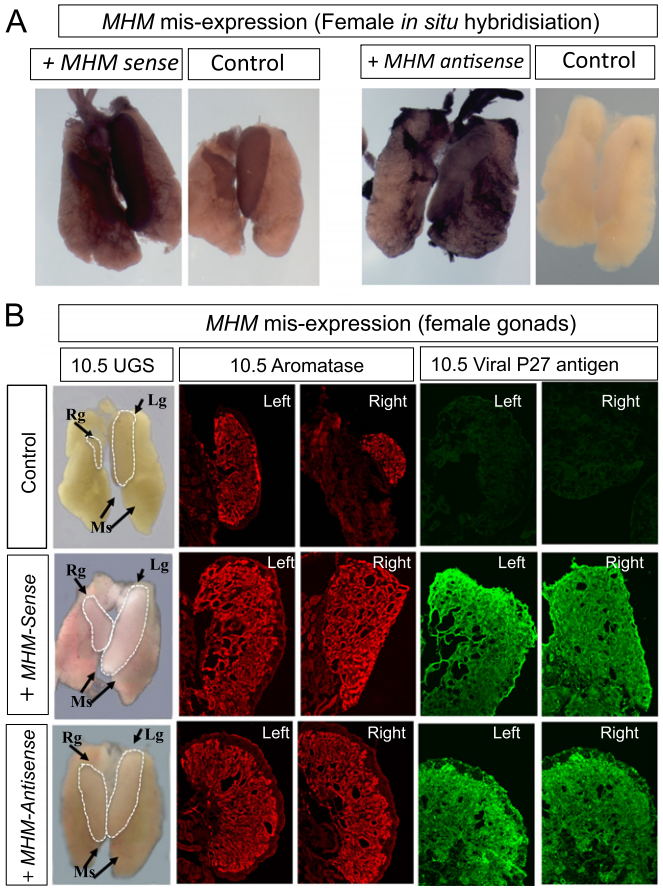


**Fig. 6.** Effects of *MHM-sense and antisense* mis-expression in day 10.5 female chicken embryos. (A) MHM *in situ* hybridization following MHM sense and MHM antisense mis-expression. (B) Lack of gonadal asymmetry and production of bilateral ovaries in MHM sense and antisense infected female embryos. Aromatase enzyme immunofluorescence delineated bilateral enlarged ovaries. Viral P27 staining confirmed robust virus infection in these gonads. UGS=urogenital system; lg=left gonad; Rg=right goand; Ms=mesonephric kidneys.

feminisation. *MHM* also caused an increase in male-biased embryo mortality. *MHM* whole mount *in situ* hybridization showed modest but detectable mis-expression of *MHM sense* strand RNA in male gonads, compared to controls, which lacked *MHM-sense* expression (Fig. 7(A)). This established that virally-delivered exogenous *MHM-sense* sequence in males was not silenced, unlike the endogenous copies of this gene. However, mortality was high among these males infected with virus carrying *MHM* sense, and it is postulated that those males expressing high levels of exogenous *MHM* were those died (see Table 1). Among the surviving males, which had a relatively low level of *MHM* sense mis-expression, *DMRT*1 mRNA levels were not significantly different to those of controls, showing a normal pattern of male enrichment compared to females at E10.5 (Fig. 7(A) and (B)). However, lower levels of DMRT1 protein were variably detected in developing testes, compared to controls. Aromatase, a female marker, was not expressed in these male gonads mis-expressing *MHM sense* (as assessed by immunofluorescence; not shown). MHM sense therefore perturbed gonadal DMRT1 protein expression but did not induce male-to-female sex reversal.

Successful mis-expression of *MHM-antisense* in male embryos was also confirmed by qRT-PCR and whole mount *in situ* hybridization. Higher levels of *MHM antisense* mis-expression were achieved compared to *MHM-sense* (Fig. 7(C)). Mis-expression of *MHM antisense* caused a significant drop in *DMRT1* mRNA levels compared to controls, with correspondingly reduced protein levels, as assessed by immunofluorescence (Fig. 7(D)). In females, a modest but insignificant fall in DMRT1 was also noted. Other autosomal genes were also tested for changes in expression level following *MHM antisense* mis-expression (*Cytokeratin, HPRT*) but these RNAs were not affected (not shown). This indicates that the effect of *MHM* antisense was specific to *DMRT*1and not a global effect on all genes.

### *MHM mis-expression and brain development*

Both *MHM-sense* and *MHM-antisense* mis-expression induced brain abnormalities, in both sexes (Fig. 8). For example, *MHM-sense* and antisense expression were not detected in male brains infected with control RCASBP-GFP virus at E4.5 or 10.5 (Fig. 8), and brain structure appeared normal. *MHM-sense* or antisense mis-expression was readily visualised in males by WISH staining (Fig. 8), with brain deformities observed in these samples. The brains were characterised by enlarged telencephalon, diencephalon and mesencephalon. At E10.5 eye defects and exencephaly were also observed (Fig. 8). A similar result was obtained for female brains (not shown). H & E staining showed grossly abnormal brains in *MHM*-infected embryos compared to controls, with impaired ventricle development, by day 10.5 (Fig. 8). Other tissues, such as the heart, showed overgrowth. This is shown in Fig. 8, where WISH confirmed *MHM-antisense* mis-expression in a E10.5 male heart that was larger relative to the control (RCASBP-GFP) male heart.

### *Mis-expression of MHM-antisense increases embryo lethality*

Mis-expression of the *MHM sense* strand induced increased male-biased embryonic mortality. Typically, injection of RCAS viral vectors carrying a non-toxic transgene, such as GFP, causes approximately 30–35% at embryonic day 10.5. This is unrelated to the gene being expressed (Table 1). The mis-expression of *MHM antisense* strand specifically caused 53% mortality among male embryos, significantly higher than the percentage mortality seen in RCAS.GFP or RCAS.MHM antisense infected males. (Fishers exact test). Female embryos did not show this increased mortality (Table 1).

## Discussion

*MHM* is a chicken Z-linked gene that is hypermethylated and silent in male cells but hypomethylated and expressed in female cells, although its role in development is unclear. The studies reported here reveal a potential role for *MHM* in regulating embryonic growth and gonadal development. In embryonic chicken tissues, the *MHM* locus is transcribed in both sense and antisense directions. Our expression analysis confirms previous reports that *MHM* is expressed exclusively or predominantly in female cells (Teranishi et al., 2001;Itoh et al., 2011). However, the *MHM-sense* and *MHM-antisense* sequences have different and dynamic expression profiles in female tissues. In their original study reporting the presence of *MHM* on the chicken Z chromosome, Teranishi et al. (2001) described the expression pattern of the sense sequence. In that study, the *MHM* sequence was found to be transcribed in a strand specific fashion, with only the sense strand being synthesized and localizing to the nucleus of embryonic fibroblasts. We have expanded this expression analysis, confirming nuclear localization of the RNA, and showing that it is expressed throughout the developing embryo, but most strongly in brain and urogenital system. This expression pattern was first delineated with a 668 bp riboprobe. When the *in situ* hybridization studies were repeated with the entire 2.2 kb repeat

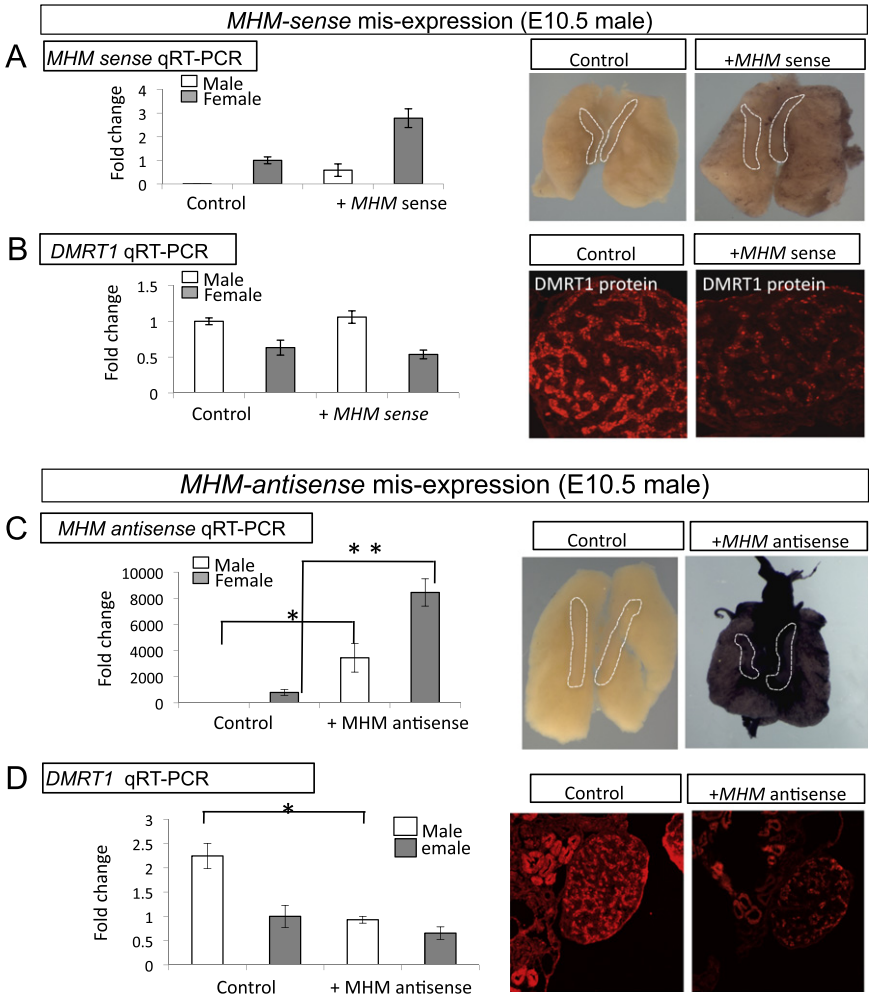


**Fig. 7.** Effects of *MHM mis-expression on male (testis) development* and *DMRT*1 expression. (A) Quantification of *MHM sense* mis-expression by qRT-PCR and *in situ* hybridization. Immunostaining for DMRT1 showed lower protein levels in MHM sense *vs* control testes. (B) No Effect of *MHM antisense* mis-expression on DMRT1 mRNA, but DMRT1 protein staining (red) is lower in gonads mis expressing MHM-sense. Immunostaining showed lower levels of *DMRT*1 protein in MHM-antisense *vs* control testes. (C) Quantification of *MHM-antisense* expression by qRT-PCR and *in situ* hybridization. Mis-expression is detected in both sexes. (* $<0.01$; ** $<0.001$; unpaired *t*-test). (D) Mis-expression of *MHM-antisense* reduces DMRT1 mRNA (* $<0.01$) and protein (red staining). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 1**
Effects of *MHM* mis-expression on embryo mortality among embryonic day 10.5 chicken embryos. Number of dead embryos as a percentage of total embryos infected with virus expressing *MHM sense* or *MHM-antisense* at day 0 (blastoderm stage). Total survival rate is also shown. Male embryos mis-expressing MHM sense showed greater mortality compared to GFP control males (bold value=statistically significant between the two ratios; Fisher's Exact test, two tailed $p=0.0497$).

| Embryos | GFP control | | +*MHM* sense | | +*MHM* antisense | |
|---|---|---|---|---|---|---|
| PCR Sex | Male $n=29$ | Female $n=20$ | Male $n=78$ | Female $n=95$ | Male $n=55$ | Female $n=44$ |
| *Embryo lethality (%)* | | | | | | |
| Lethality | **9 (31.0)** | 6 (30.0) | **42(52.5)** | 37(39.0) | 17(30.9) | 15(34.1) |
| Survival rate | 34 (69.4) | | 94 (54.3) | | 67 (67.7) | |

region as a probe, the same expression pattern was recorded, with some additional staining in the branchial arches of female embryos. We describe here that the *MHM antisense* strand was also transcribed, albeit at much lower levels, with restricted expression in brain, limbs and urogential system. The nuclear localization of *MHM sense* strongly supports the idea that it is a non-coding RNA, and that it has a function directly associated with chromosomal DNA. In contrast, the antisense strand is cytoplasmic and may therefore be translated into a protein.

Northern blotting with a 668 bp probe derived from the core 2.2 kb repeat unit of *MHM* detected several differently sized transcripts. One of these transcripts corresponds to the 9 kb high molecular weight species described by Teranishi et al. (2001). In that study, a single band was detected in Northern blots of fibroblasts derived from female chicken embryos and in male embryos when treated with the demethylating agent, 5-azacytidine. In our study, several other larger and smaller transcripts were detected in various tissues. These may represent splice

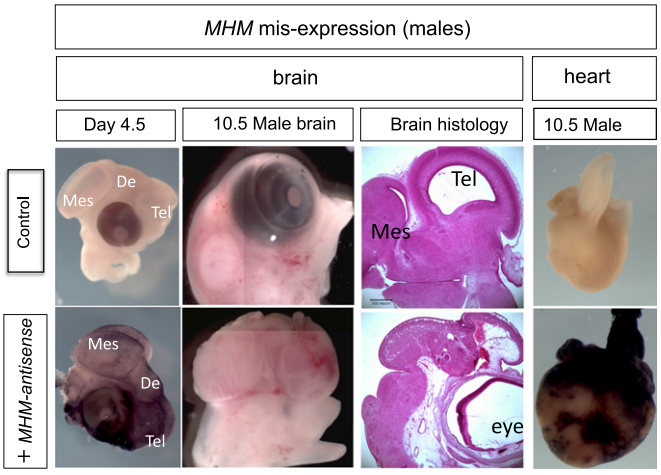


**Fig. 8.** Brain abnormalities following *MHM* mis-expression in day 4.5 and 10.5 chicken embryos. Male control and *MHM-antisense* mis-expressing embryos are shown. (Comparable results were obtained for MHM-sense strand). The control male brain at day 4.5 shows normal morphology and no *MHM* expression. In embryos infected with MHM-antisense, mis-expression is detcted in the telencephalon (Tel), diencephalon (De) and in the mesencephalon (Mes). By day 10.5, abnormal exencephaly is seen in MHM-antisense infected embryos. H and E staining of MHM-misexpressing embryos shows failure of normal ventricle development. Embryos misexpressing *MHM-antisense* also have enlarged hearts.

variants of *MHM*, or *MHM*-related sequences, as recently described by Itoh et al. (2011). The 668 bp riboprobe used here for Northern blotting has sufficient homology to the *MHM*-related sequences (64B7H1 and 28F4D2) to have detected these transcripts in addition to MHM. However, *in situ* hybridization analysis using the entire 2.2 kb sequence as a probe showed the same nuclear signal as the 688 bp probe, strongly suggesting that all of these related sequences are non-coding RNAs.

We observed widespread *MHM-sense* expression in developing female embryos, with particularly strong expression in brain and gonads, while *MHM-antisense* had a lower and more restricted expression pattern (compare Figs. 2 and 3). The more widespread expression of *MHM sense* is consistent with a role in dosage compensation in most or all cells. Mis-expression of this sequence causes general enlargement of tissues and brain defects. These effects may reflect a perturbation of a subset of genes that are normally required to be dosage compensated, especially those expressed in the brain. A number of *MHM-sense* transcripts were detected in the embryonic brain, as assessed by Northern blotting (Fig. 2). This is consistent with previous reports of *MHM* expression in embryonic chicken brains (prior to gonadal sex differentiation) (Scholz et al., 2006). Mis-expression of *MHM-sense* and *antisense* sequences caused exencephaly of the brain in many embryos of both sexes. The exencephaly was characterized by greatly expanded cerebral hemispheres, and abnormal folding, and thickened neuroepithelium. The exact role of *MHM* in development is unknown, but evidence is now strong that it encodes a long-non coding RNA which correlates with both open chromatin and histone acetylation in female cells (Itoh et al., 2011), all of which point to a role in dosage compensation of Z-linked genes. Any perturbation of this system would lead to aberrant gene expression. Hence, the effects of *MHM* mis-expression in the brain could be due to abnormal elevation of gene expression, namely, in those genes that are normally subject to dosage compensation via *MHM*-induced up-regulation (those in the so-called *MHM* valley of the Z sex chromosome). Under this hypothesis, elevated gene expression would cause defects in both sexes. This idea could be tested by examining the expression level of genes within the MHM-valley of embryos over-expressing *MHM-sense*. It is interesting to note that the MHM valley is reported to be enriched for genes associated with cellular physiology, reproduction and development and developmental disorder (Melamed and Arnold, 2007).

However, a key point that has arisen from our studies is the mechanism of action of exogenous *MHM*. In our studies, *MHM* was mis-expressed using the RCAS avian retroviral vector. This vector integrates randomly into the genome at the blastoderm stage. It therefore presumably does not directly target exogenous *MHM* sequence to its native location on the Z and hence would only affect those genes in the neighborhood of the viral genomic integration site (anywhere in the genome, and varying from embryo to embryo). Hence, random integration of the viral vector into the genome could produce variable effects, depending upon which group of neighbouring genes was affected by the exogenous *MHM*. This assumes that *MHM* RNA only coats DNA at the site of transcription, *in cis*, but it could potentially act *in trans*. The tissue-specific effects of mis-expression that we report here are in fact consistent with a *trans* function for MHM, that is, an effect upon specific developmental pathways irrespective of the site of exogenously delivered *MHM* integration. One approach to test this assumption is to infect chicken cells *in vitro* with RCAS-viral delivered *MHM*, generate monoclonal lines of cells with the same genomic integration site, determine the site of integration by inverse PCR, and assess the expression levels of adjacent genes. The alternative possibility is that *MHM* acts *in trans*, on genes distant to its site of transcription. It is interesting that mis-expression of *MHM-antisense* produced the same brain overgrowth abnormalities. *MHM-antisense* was found it localize in the cytoplasm, implying a different function to the sense strand. How both strands can induce qualitatively similar abnormalities in the brain is unclear.

Mis-expression of *MHM* sequences also resulted in gender-specific effects in the gonads. The female urogenital system is normally asymmetrical, characterized by a large left ovary and a smaller regressed right gonad (Fig. 6(B)). *MHM-sense* mis-expression induced variable hypertrophy of the gonads, resulting in enlarged bilateral ovarian development (Fig. 6(B)), demonstrating that ectopic *MHM-sense* prevented the normal regression of the right female gonad, instead stimulating ovarian differentiation. The right ovary appeared morphologically identical to a normal left ovary, with a well-developed, aromatase-positive medulla and thickened outer cortex. Both the left and right ovaries of these embryos were considerably larger than control left ovaries. This suggests that *MHM-sense* may be involved in regulating the normal growth and asymmetry of the female urogenital system. The exact mechanism involved is unknown, but warrants further examination. As for brain development, *MHM-antisense* had the same effect as the sense strand on ovarian development. Gonads were not masculinised but developed symmetrically.

In male control embryos, *MHM* is not expressed in the gonads, according to *in situ* hybridization, while the viral vector delivered modest ectopic expression (Fig. 7(A)). The overall structure of the developing testis was not greatly affected, and male gonads were not feminised (aromatase was not expressed), as would be predicted if *MHM* had a repressive effect upon *DMRT1* mRNA expression (Teranishi et al., 2001; Yang et al., 2010). However, DMRT1 protein staining was reduced in *MHM* sense–infected male embryos compared to controls. This suggests that *MHM* is influencing DMRT1 synthesis as the post-transcriptional level, or that MHM is having an effect upon the morphogenesis of testis cords, or the actual number of Sertoli cells expressing DMRT1. Similarly, mis-expression of *MHM-antisense* in male embryos caused a decline in both gonadal *DMRT*1 mRNA and protein. This effect was variable among embryos, which could reflect the inherent variability of the viral vector at delivering robust transgene mis-expression. However, as for *MHM-sense* mis-expression,

overall testis structure was maintained and the female marker, aromatase, was not activated. Hence, gonads were not feminized. The 2.2 kb *MHM sense* and *antisense* strands may therefore have the ability to perturb *DMRT*1, but not completely repress the gene. It is possible that the 9 kb MHM transcript may show more marked effects upon the developing testis, but due to technical limitations of the RCAS viral vector, we were unable to express this larger transcript. Another possibility is that more extensive inhibition of *DMRT*1 may have occurred in those male embryos in which increased lethality occurred, presumably due to very high *MHM-sense* levels.

An association between MHM and chicken sex determination has been proposed by previous studies. It has previously been hypothesized that *MHM* RNA could contribute to the repression of *DMRT*1 expression in female embryos by inducing local chromatin modification, reinforcing the sexually dimorphic profile of this key sex gene. *MHM* RNA accumulates on the female Z chromosome very close to the *DMRT*1 locus (Teranishi et al., 2001). A recent study of adult chickens reported the down-regulation of *DMRT*1 gene expression following transient transfection of 13-week old chicken testes with a plasmid vector expressing the 2.2 kb MHM sequence (Yang et al., 2010). Hence, although most studies infer that the *MHM* transcript could lead to up-regulation of neighbouring genes as a dosage compensation mechanism, this putative role for *MHM-sense* invokes down-regulation of the *DMRT1* gene. In addition, Yang and colleagues recently reported the effects of experimental sex reversal on the methylation status of *MHM* in adult chickens (Yang et al., 2011). In that study, female-to-male sex reversal was achieved by treating embryos at day 3 with an aromatase enzyme inhibitor, which blocks oestrogen synthesis. As adults, these ZW male birds showed enhanced methylation of the *MHM* locus in the gonads, but not in the liver. This indicates that the regulation of MHM may be different between tissues, pointing to potential link between gonadal development and the activity of the *MHM* locus. Presumably, male factors activated by the inhibition of oestrogen, or a loss of female factors, can leads to enhanced epigenetic modulation of *MHM* in the gonad, compatible with testis development.

The method of gene mis-expression used here delivers the transgene to all tissues of the embryo. Our aim was to closely mimic endogenous *MHM* expression, which is detectable in several tissues from early stages of development. Despite robust delivery of the virus to all tissues, as assessed by viral p27 epitope staining, abnormalities were seen in specific tissues (primarily, the brains and gonads). This suggests that the endogenous role of *MHM* (forward and reverse sequences) relates to brain and gonad development. The different subcellular localization patterns of *MHM-sense* and *MHM-antisense* suggest that the two sequences have different functions. The locus is potentially complex. A number of different transcripts have now been identified for the sense strand, including some with intron-exon structure, some with little homology outside a core region, and some with poly A tails (Itoh et al., 2011). Nevertheless, our 2.2 kb probe showed RNA localization within the nucleus, suggesting a common mode of action for MHM-related sequences that involves non-coding RNA-chromatin interaction. Mis-expression of both sense and antisense strands induced similar phenotypes, namely, brain and gonadal abnormalities. It is possible that the two strands may interact with each other, for example, by inducing expression of the opposite strand, hence inducing the similar mis-expression phenotypes. This would be reminiscent of the interaction between mammalian *Xist* and its antisense transcript, *Tsix* (Lee et al., 1999, Luikenhuis et al., 2001). If *MHM* functions to elevate local expression of Z-linked genes that are developmentally important, as part of a dosage compensation mechanism (Arnold et al., 2008, McQueen and Clinton, 2009), mis-expression may elevate such gene expression to the point of lethality, which would be more detrimental in males (ZZ), as was found here. This could be tested *in vitro*, by examining Z-linked gene expression cells over-expressing *MHM-antisense versus MHM-sense*.

The data presented here provide the first report of a bidirectionally expressed long non-coding RNA in an avian system, and the functional analysis of these sequences. Our results indicate that the *MHM* locus is transcribed from both sense and antisense strands in chicken embryos, with several different transcripts expressed in female tissues. The sense RNA strand is located exclusively in the nuclei of female cells in all tissues that were examined. In contrast, the antisense strand is more lowly expressed, and has an intriguing cytoplasmic localization. This establishes the *MHM* locus as a complex region, with both strands having potential roles in development. Furthermore, Itoh et al. (2011) recently reported differences between the two *MHM* loci on the Z sex chromosomes of male chicken fibroblastic cells, in terms of methylation sensitivity and potential chromatin status.

Current investigations to further analyse *MHM* will focus on RNA interference, with the aim of knocking down endogenous expression in cultured cells and *in ovo*, and then assaying global gene expression by microarray. If *MHM* plays a role in local dosage compensation, changes in expression of MHM-valley genes would be predicted. The exact mode of action of MHM, and whether it participates in dosage compensation or sex determination, or both, requires further analysis. There is a growing body of evidence linking long non-coding RNAs in local gene silencing (Mercer et al., 2009) (Ponting et al., 2009) This would be the proposed mode of action of *MHM* upon the neighboring *DMRT*1 gene. Yet *MHM* is also implicated in dosage compensation, in a way that would involve up-regulation, not down-regulation, of neighboring genes. The possible role of long non-coding RNAs in gene activation is less well understood (Dean, 2006). Further analysis of MHM in a tractable model such as the avian embryo will enhance our understanding of long non-coding RNA function.

## Acknowledgements

This research was supported an Australian Research Council Discovery Grant awarded to CAS and AHS, and the Victorian Government''s Operational Infrastructure Support Program. We thank Daniela Bodemer for carrying out the histology, Dr. Katie Ayers for help with early blastoderm expression analysis, and Dr. Peter Farlie for the analysis of brain and neural abnormalities.

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