Transcriptional sexual dimorphism in elongating bovine embryos: implications for XCI and sex determination genes

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Abstract

Sex chromosome transcripts can lead to a broad transcriptional sexual dimorphism in the absence of concomitant or previous exposure to sex hormones, especially when X-chromosome inactivation (XCI) is not complete. XCI timing has been suggested to differ greatly among species, and in bovine, most of the X-linked transcripts are upregulated in female blastocysts. To determine the timing of XCI, we analyzed in day 14 bovine embryos the sexual dimorphic transcription of seven X-linked genes known to be upregulated in female blastocysts (*X24112*, brain-expressed X-linked 2 (*BEX2*), ubiquitin-conjugating enzyme E2A (*UBE2A*), glucose-6-phosphate dehydrogenase (*G6PD*), brain-expressed X-linked 1 (*BEX1*), calpain 6 (*CAPN6*), and spermidine/spermine N-acetyltransferase 1 (*SAT1*)). The transcription of five genes whose expression differs between sexes at the blastocyst stage (*DNMT3A*, interferon tau (*IFNT2*), glutathione S-transferase mu 3 (*GSTM3*), progesterone receptor membrane component 1 (*PGRMC1*), and laminin alpha 1 (*LAMA1*)) and four genes related with sex determination (Wilms tumor 1 (*WT1*), gata binding protein 4 (*GATA4*), zinc finger protein multitype 2 (*ZFPM2*), and *DMRT1*) was also analyzed to determine the evolution of transcriptional sexual dimorphism. The expression level of five X-linked transcripts was effectively equalized among sexes suggesting that, in cattle, a substantial XCI occurs during the period between blastocyst hatching and initiation of elongation, although *UBE2A* and *SAT1* displayed significant transcriptional differences. Similarly, sexual dimorphism was also reduced for autosomal genes with only *DNMT3A* and *IFNT2* exhibiting sex-related differences. Among the genes potentially involved in sex determination, Wilms tumor 1 (*WT1*) was significantly upregulated in males and *GATA4* in females, whereas no differences were observed for *ZFPM2* and *DMRT1*. In conclusion, a major XCI occurred between the blastocyst and early elongation stages leading to a reduction in the transcriptional sexual dimorphism of autosomal genes, which makes the period the most susceptible to sex-specific embryo loss.

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Introduction

Before sex determination and differentiation occurs, male and female embryos may display phenotypic differences under some environmental conditions (Gutierrez-Adan *et al.* 2006). In the absence of gonads and sex-related hormonal differences, this early sexual dimorphism completely relies on the difference in sex chromosome dosage. Thus, the double X dosage in females and the presence of a Y-chromosome in males not only determine the transcriptional level of sex chromosome-encoded genes but can also exert an effect on the expression of genes present in autosomal chromosomes, thereby determining early sexual dimorphism and the beginning of sex determination (Bermejo-Alvarez *et al.* 2010b).

In eutherian mammals, X-chromosome inactivation (XCI) is required to ensure an equal transcriptional level

for X-linked genes in both sexes. However, both X-chromosomes are active after embryonic genome activation (Patrat *et al.* 2009), and XCI is not fully accomplished during early development, which leads to an upregulation of X-linked genes in female embryos. The developmental stage at which XCI is accomplished is not clear and may differ greatly between species (Okamoto & Heard 2009). In mice, the only species where this phenomenon has been thoroughly studied, the paternal X chromosome undergoes imprinted inactivation from the cleavage stages, and in the cells of the inner cell mass (ICM) of the expanded or hatching blastocyst, a reversal of the inactive state occurs that gives rise to the embryo proper random XCI (Okamoto *et al.* 2004). As a consequence of this dynamic and reversible process, many X-linked genes are upregulated in the female blastocysts (Kobayashi *et al.* 2006). This situation is more pronounced in the bovine

model, where most of the X-linked genes (~90%) are upregulated in the female embryos, suggesting that in the bovine blastocyst, XCI is far from completion (Bermejo-Alvarez *et al.* 2010b). Furthermore, the unequal transcriptional level among sexes of X-linked genes may impose a transcriptional regulation upon autosomal genes, broadening the transcriptional effect. In this sense, the transcription level of several candidate genes depends on the embryo sex (Larson *et al.* 2001, Morton *et al.* 2007, Bermejo-Alvarez *et al.* 2008b, 2010a), and microarray studies have shown a large transcriptional dimorphism in mouse (Kobayashi *et al.* 2006) and, particularly, in bovine embryos (Bermejo-Alvarez *et al.* 2010b).

In the mouse model, XCI is accomplished at the blastocyst stage, slightly before implantation, but unlike in humans and mice, ungulate preimplantation embryo development is characterized by a post-hatching period of conceptus elongation prior to initiation of implantation, which in the cow occurs around day 20 (King *et al.* 1980). Thus, between hatching and implantation, a rapid trophoblast development changes the morphology of the conceptus extraembryonic tissues from a sphere (day 7) to ovoid to tubule (day 14) to filament, concomitant with gastrulation (reviewed in Blomberg *et al.* (2008)). Embryo elongation is orchestrated by epigenetic and transcriptional events, but although microarray analysis revealed transcriptional differences between different stages (Ushizawa *et al.* 2004, Blomberg *et al.* 2008), little is known about XCI and sexual dimorphism at this stage. Indeed, only interferon tau (IFNT2), the factor responsible for maternal recognition of pregnancy in ruminants, has been suggested to be expressed in a sexually dimorphic manner in elongating embryos based on protein activity assays (Flint *et al.* 1997, Kimura *et al.* 2004). Furthermore, transcriptional sexual dimorphism provides a mechanism to explain sex ratio skews caused by selective embryo loss (Bermejo-Alvarez *et al.* 2011).

Transcriptional differences during early embryo development are responsible for sex determination. Although the precise molecular events regulating these processes remain unclear, several gene interactions have been described (Ferguson-Smith 2007, Sekido & Lovell-Badge 2009, Biason-Lauber 2010). To initiate sex determination, these genes must exhibit a sex-specific regulation, which in the absence of gonads can only depend on the differences in gene interactions occurring between XX and XY genomes. For this reason, genes related to sex determination are suitable candidates to exhibit transcriptional sexual dimorphism in developmental stages preceding sex determination, when they may exert other regulatory functions.

The aims of this study were to determine in day 14 elongating bovine conceptuses 1) the timing of XCI by comparing the expression of X-linked transcripts between males and female embryos, 2) sex-related

differences in the expression of autosomal genes known to be expressed in a sex-specific manner at the blastocyst stage, and 3) sex-related transcriptional differences in genes related to sex determination.

Results

To determine XCI evolution during early elongation, the relative abundance of seven X-linked transcripts upregulated in female blastocysts compared with males was analyzed on day 14 embryos. As shown in Fig. 1, the transcription level of five transcripts (X24112, brain-expressed X-linked 2 (*BEX2*), glucose-6-phosphate dehydrogenase (*G6PD*), brain-expressed X-linked 1 (*BEX1*), and calpain 6 (*CAPN6*)) was essentially similar between male and female embryos at day 14, suggesting

Figure 1 Relative poly(A) mRNA abundance of seven X-linked genes (*X24112*, *BEX2*, *UBE2A*, *G6PD*, *BEX1*, *CAPN6*, and *SAT1*) between male and female bovine embryos at the blastocyst stage (A) or at day 14 (B). Lower bar represents the position of the different genes along the X-chromosome in a megabase scale. Data for graph A were obtained from previous observations in groups of blastocysts instead of individual embryos, all of them by qPCR but for *G6PD*, which was obtained by semiquantitative (band intensity) PCR (Gutierrez-Adan *et al.* 2000, Bermejo-Alvarez *et al.* 2010b). Different letters indicate significant differences between sexes based on one-way ANOVA ($P \leq 0.05$). Error bars, S.E.M.

that a substantial XCI occurs during the peri-elongation period in cattle. Nevertheless, two genes (ubiquitin-conjugating enzyme E2A (*UBE2A*) and spermidine/spermine *N*-acetyltransferase 1 (*SAT1*)) displayed significant transcriptional differences between the sexes on both days 7 and 14, although the magnitude of the difference was lower on day 14 (2.8 vs 2 for *SAT1*). There was no apparent relationship between the distribution of the two genes, which partially escaped from XCI and their linear chromosome position with respect to *XIST* (Fig. 1).

The relative mRNA abundance of five autosomal genes known to display sex-related transcriptional differences at the blastocyst stage (*DNMT3A*, *IFNT2* – based on probe intensity, GEO GSE17921 – glutathione S-transferase mu 3 (*GSTM3*), *PGMRC1*, and laminin alpha 1 (*LAMA1*)) was analyzed in male or female day 14 embryos. Similar to the X-linked genes, only two autosomal genes (*DNMT3A* and *IFNT2*) maintained the transcriptional sexual dimorphism observed at the blastocyst stage to day 14 (Fig. 2A).

Finally, transcriptional sexual dimorphism was assessed for five transcripts related with sex determination: two transcripts (exons 1–2 and 4–5) of *WT1*, gata binding protein 4 (*GATA4*), zinc finger protein multitype 2

(*ZFPM2*) and *DMRT1* (Fig. 2B). Both *WT1* transcripts were significantly upregulated in male embryos, with no change in their fold changes among exons, excluding a sex-specific alternative splicing affecting these exons at this stage. The opposite situation was observed for *GATA4*, which was upregulated in females, whereas the transcriptional level of *ZFPM2* and *DMRT1* did not differ among sexes. The expression of these genes was analyzed in different adult tissues and days 7 and 14 embryos. Both *WT1* transcripts were expressed in lung, kidney, muscle, testicle, ovary, and day 14 embryos, but the transcription of the exons 4 and 5 was weak and day 7 embryos did not express exons 1 and 2. *GATA4* was expressed in medulla, kidney, gonads, and days 7 and 14 embryos, whereas *ZFPM2* was ubiquitously expressed. In contrast, *DMRT1* expression was restricted to testicle and it was faintly expressed in day 14 embryos.

Discussion

The developmental stages preceding gonad differentiation constitute an ideal model for the study of sexual dimorphism in the absence of concomitant or previous sex hormone exposure. Several recent reports suggest that sex chromosome composition of the genome (XX or XY), irrespective of the endocrine environment, can be responsible for a large proportion of the sexual transcriptional dimorphism observed in adult tissues (Ober *et al.* 2008, Wijchers *et al.* 2010), especially when there is an incomplete XCI leading to an upregulation of X-linked genes in females. XCI has been suggested to greatly differ among species (Okamoto & Heard 2009), and the timing of epigenetic events in ungulate models may greatly differ from the mouse. The balanced expression among sexes of five out of seven X-linked genes in day 14 embryos, in contrast with the situation observed in day 7 blastocysts (Bermejo-Alvarez *et al.* 2010b), indicates that significant XCI occurs after embryo hatching and before maternal recognition of pregnancy, which occurs around day 16 in cattle. This delay in comparison with the mouse model may suggest that, from an epigenetic point of view, the mouse blastocyst is more advanced than the ungulate blastocyst, which, unlike the rodent embryo, is not ready to implant. In agreement with this observation, embryonic stem cell derivation attempts from bovine, sheep, or pig ICM cells have not succeeded, whereas epiblast stem cells have been derived in pigs (Alberio *et al.* 2010). The reason for the incomplete inactivation of two genes (*UBE2A* and *SAT1*) is unclear. The degree of completion of XCI in adult tissues varies among studies depending on the tissue (Talebzadeh *et al.* 2006), from 5.4% of X-linked genes with increased female expression in human lymphoblastoid cell lines (Johnston *et al.* 2008) to 15–25% in fibroblasts and hybrid cells (Carrel & Willard 2005), and the distribution of genes that escape inactivation has been found not to be random along

Figure 2 Relative poly(A) mRNA abundance between male and female day 14 bovine embryos for (A) five autosomal genes that displayed sexual dimorphism at the blastocyst stage (Bermejo-Alvarez *et al.* 2010b) (*DNMT3A*, *IFNT2*, *GSTM3*, *PGMRC1* and *LAMA1*) and (B) five transcripts related with sex determination (*WT1* exons 1–2 and 4–5, *GATA4*, *ZFPM2*, and *DMRT1*). Different letters indicate significant differences between sexes based on one-way ANOVA ($P \leq 0.05$). Error bars, S.E.M.

the chromosome, but rather clustered and mapping primarily to the distal portion of the X chromosome short arm (Xp) (Miller & Willard 1998, Carrel & Willard 2005), far from the *XIST* gene (Huynh & Lee 2003). However, we could not establish a relation between linear distribution and escape from inactivation, which may be caused by the three-dimensional structure of the chromosome or by gene-specific regulation.

Consistent with a major regulatory role of the upregulated X-linked transcripts on the global transcriptional sexual dimorphism, the partial completion of XCI was accompanied by a reversion of the transcriptional sexual dimorphism observed for autosomal genes at previous stages, although two genes still differed between the sexes. *DNMT3A*, a *de novo* DNA methyltransferase, was upregulated in male day 14 embryos, as was observed in day 7 blastocysts (Bermejo-Alvarez *et al.* 2010b). This upregulation has been linked to a lower methylation level for several regions in female embryos (Bermejo-Alvarez *et al.* 2008b, Gebert *et al.* 2009), stem cells (Zvetkova *et al.* 2005), and peripheral leukocytes (Fuks *et al.* 2004), as well as to a higher global transcriptional level in female bovine blastocysts suggested by gene ontology (Bermejo-Alvarez *et al.* 2010b). These molecular differences may have consequences for the developmental origin of adult diseases (Bermejo-Alvarez *et al.* 2011). In this sense, Beckwith–Wiedemann syndrome, a human epigenetic disorder originating during the preimplantation period related with altered methylation patterns, occurs at a relatively high frequency in monozygotic twins, and in almost all cases, the affected twins are female (Lubinsky & Hall 1991, Weksberg *et al.* 2002), whereas in sheep, a methyl-deficient maternal diet during the periconceptional period affects the methylation status and phenotype to a greater extent in males (Sinclair *et al.* 2007). *IFNT2* was upregulated in females as was suggested based on array probe intensity in bovine blastocysts (Bermejo-Alvarez *et al.* 2010b) and on antiviral activity in bovine blastocysts (Kimura *et al.* 2004) and elongated Red Deer embryos (Flint *et al.* 1997), although no significant differences were observed for elongated bovine embryos (Kimura *et al.* 2004). The differences among studies may be based on the different techniques used, as antiviral activity sensitivity and specificity are relatively low compared with qPCR. According to its pregnancy recognition role, and in agreement with previous findings (Ushizawa *et al.* 2004), we observed that *IFNT2* was intensively expressed in day 14 embryos compared with blastocysts, where its detection by qPCR approach was difficult. The higher expression of this pregnancy recognition gene may confer the female embryos a slight implantation advantage over the males, which may partly explain the primary sex ratio in cattle, which has been found to be slightly higher (57%) than 1:1 (Rizos *et al.* 2008, Bermejo-Alvarez *et al.* 2010a). Nevertheless, the intense reduction in the transcriptional sexual

dimorphism occurring between days 7 and 14, suggests that sex-specific embryo loss is likely to occur between hatching and the initiation of elongation, as the wide sex transcriptional differences at the blastocyst stage provides a molecular basis for sex-specific mortality rates. In agreement with this notion, embryo loss before pregnancy recognition results in a similar energy and time loss for the species than a non-fertile mating, and specifically in cattle, survival to day 14 represents a useful measure of developmental competence, as very little embryonic loss occurs after day 14 (Dunne *et al.* 2000).

Two autosomal potential activators of *SRY* (Sekido & Lovell-Badge 2009) displayed opposite transcriptional sexual dimorphism. *WT1*, an essential factor for the development of kidneys and gonads (Barbaux *et al.* 1997), was upregulated in males, whereas *GATA4* mRNA, a regulatory factor not essential for sex determination (Biaison-Laubier 2010), was more abundant in females. A combination of *GATA4* and *WT1* strongly activate *SRY* promoter in mouse, pigs, and humans, particularly the *WT1* (+KTS) isoform (Miyamoto *et al.* 2008). Unfortunately, *WT1* (+KTS) isoforms have not been described in cattle, so we decided to analyze the mRNA abundance of the two proximal (1 and 2) and distal exons (4 and 5), observing a similar sexual dimorphism. The synergic effect of both genes on sex determination may explain their contrasting regulation according to sex at this early stage, as if both were upregulated in the male, they may trigger sex determination mechanisms. *GATA4* is essential for cell differentiation to extra-embryonic endoderm (Soudais *et al.* 1995), and thus, there may be sex-related differences in differentiation processes. On the other hand, both genes are regulators of transcription and therefore they may act by regulating the expression of other sexually dimorphic genes. In this sense, GATA transcription factors have been reported to increase *IFNT2* transcription (Bai *et al.* 2009). The other two genes analyzed did not show significant differences. *ZFPM2* is an essential factor for *GATA4* to interact with *SRY* (Tevosian *et al.* 2002), and *DMRT1* is the only regulator of male development conserved in the whole vertebrate phylum, whose deletions cause sex reversal in humans and male infertility in mice (reviewed in Waters *et al.* 2007). Interestingly, in contrast with the three other genes, the expression of *DMRT1* was limited to the testicle and day 14 embryos (Fig. 3), and there are no functions known for this gene other than sex determination or differentiation, which may suggest a partial transcriptional activation of sex determination pathways before a major regulation finally triggers sex determination.

In conclusion, a major XCI occurs between the day 7 blastocyst and day 14 early elongation stages in bovine, suggesting that XCI timing may differ greatly among species. Consistent with a major role of X-linked gene upregulation in global transcriptional sexual dimorphism, transcriptional sexual dimorphism of autosomal

Figure 3 PCR products of the transcripts related with sex determination and the housekeeping gene *H2AFZ* from cDNA obtained from different adult tissues (Br, brain; Me, medulla; Lu, lung; Kd, kidney; Ms, muscle; Te, testicle and Ov, ovary), day 7 blastocysts (D7), day 14 embryos (D14) or negative control prepared in the absence of reverse transcriptase (C-).

genes was reduced in more advanced stages of embryo development, which makes the period between hatching and maternal recognition of pregnancy as the most suitable for a sex-specific embryo loss. However, two genes, related with DNA methylation (*DNMT3A*) and pregnancy recognition (*IFNT2*), maintained the sex-specific differences observed at the blastocyst stage. Furthermore, the expression of four sex determination-related genes was observed in day 14 bovine conceptuses and two of the genes (*WT1* and *GATA4*) showed a sex-specific transcriptional regulation.

Materials and Methods

In vitro embryo production, embryo transfer, and recovery

Immature cumulus-oocyte complexes obtained from ovaries collected at slaughter were matured for 24 h in TCM-199 supplemented with 10% (v/v) FCS and 10 ng/ml epidermal growth factor at 39 °C under an atmosphere of 5% CO₂ in air with maximum humidity. IVF was performed with frozen-thawed semen at a final concentration of 1×10⁶ spermatozoa/ml during 20 h gamete co-incubation under the same conditions. Presumptive zygotes were denuded and transferred to 25 µl culture droplets of synthetic oviduct fluid medium supplemented with 5% FCS under mineral oil under a humidity-saturated atmosphere of 5% CO₂, 5% O₂, and 90% N₂. Day 7 blastocysts were transferred to the ipsilateral horn of synchronized recipients (20 blastocysts per recipient) using an established multiple embryo transfer model (Clemente *et al.* 2009, Forde *et al.* 2010). In total, four crossbred heifers (18–24 months old) were used as recipients. Animals were housed indoors on slats for the duration of the experiment and were fed a diet consisting of grass and maize silage supplemented with a standard beef ration. Estrous cycles were synchronized by administration of two i.m. injections of the prostaglandin F_{2α} analog, cloprostenol (PG; Estrumate; Chanelle, Loughrea, Galway, Ireland), 11 days apart. Standing estrus was defined as day 0. The recipients were slaughtered on day 14 and the reproductive tract was removed, sealed in a plastic bag and placed in a sealed polystyrene box for transportation to the

laboratory (within 60 min). After removal of the ovaries and the oviducts, the uterine horns were trimmed free of excess tissue before being flushed with 40 ml PBS. Embryos were located under a stereomicroscope and then snap frozen individually in liquid nitrogen and stored at –80 °C until analysis.

RNA extraction, RT, and quantification of mRNA transcript abundance

Molecular biology procedures were carried out as described previously (Bermejo-Alvarez *et al.* 2010b). Poly(A) RNA was extracted individually from 33 day 14 embryos of a similar size following the manufacturer's instructions using the Dynabeads mRNA Direct Extraction KIT (DynaL Biotech, Oslo, Norway) with minor modifications: lysis was achieved in 100 µl lysis buffer for 10 min with occasional gently pipetting and then the fluid lysate was hybridize with 20 µl prewashed beads for 5 min with gently shaking. After hybridization, two washes in 100 µl washing buffer A and two more in washing buffer B were performed. Finally, beads were eluted in 30 µl Tris–HCl. Immediately after extraction, the RT reaction was carried out following the manufacturer's instructions (Bioline, Ecogen, Madrid, Spain) using poly(T) primer, random primers, and MMLV reverse transcriptase enzyme in a total volume of 40 µl to prime the RT reaction to produce cDNA. Tubes were heated to 70 °C for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 100 units of reverse transcriptase. They were then incubated at 42 °C for 60 min to allow the RT of RNA, followed by 70 °C for 10 min to denature the RT enzyme.

Embryo sexing was performed on both DNA and cDNA. The DNA present in the lysis buffer after poly(A) RNA recovery was extracted with phenol/chloroform treatment and finally suspended in 50 µl milliQ water (Bermejo-Alvarez *et al.* 2010a). We used 8 µl each sample were used to perform embryo sexing by PCR by both the BRY/Sat and the amelogenin gene methods (Bermejo-Alvarez *et al.* 2008a). The result was confirmed on cDNA by the presence of the Y-specific transcripts *YZRSR2* and *DBY* (Bermejo-Alvarez *et al.* 2010b).

The quantification of all mRNA transcripts was carried out in the 33 samples by real-time quantitative RT-PCR in two independent experiments involving ten males and seven females (X-linked genes and *DNMT3A*) and nine males and seven females (other genes) with two repetitions per sample for all genes of interest. Experiments were conducted to contrast relative levels of each transcript and histone *H2AFZ* (*H2AFZ*) in every sample. PCR was performed by adding a 2 µl aliquot of each sample to the PCR mix containing the specific primers to amplify *H2AFZ*, the non-annotated transcript *X24112* (*BEX2*), *UBE2A*, *G6PD*, *BEX1*, *CAPN6*, *SAT1*, DNA methyltransferase 3 A (*DNMT3A*), *IFNT2*, *GSTM3*, progesterone receptor membrane component 1 (*PGRMC1*), *LAMA1*, (*WT1*, exons 1 and 2 or exons 4 and 5), *GATA4*, *ZFP281*, also known as *FOG2*), and doublesex and mab-3-related transcription factor 1 (*DMRT1*). Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 1. For quantification, qRT-PCR was performed as described previously (Bermejo-Alvarez *et al.* 2010b). PCR conditions were tested to achieve efficiencies close to 1 and then the

Table 1 Details of primers used for qRT-PCR.

Gene	Primer sequence (5'–3')	Fragment size (bp)	GenBank accession no.
<i>H2AFZ</i>	AGGACGACTAGCCATGGACGTGTG CCACCACCAGCAATTGTAGCCTTG	209	NM_174809.2
<i>X24112</i>	TACAGCTGACCAAACTAAAGGC TGCTTAGCTTCTGAGTTTGGAC	204	CK846762
<i>BEX2</i>	AGAACCTATGGCCCTCCTTTCG AAGCTGGTAATAGGAAACGCG	409	NM_001077087.1
<i>UBE2A</i>	GGGCTCCGTCTGAGAACAACATC CATACTCCCGCTTGTTCCTCGG	336	XM_864331
<i>G6PD</i>	CGCTGGGACGGGGTGCCCTTCATC CGCCAGGCCTCCCGCAGTTCATCA	347	XM_583628.4
<i>BEX1</i>	ACCTAGAGGAAATCGCAGACGG AAGCATCTTGCCATGAAACAGG	434	NM_001077034
<i>CAPN6</i>	TTTTAAATGATTGCGTGCTGAAC AAAGCCACATCCTCCATGAAGC	135	AU278104.1
<i>SAT1</i>	GCCAGGTTGCAATGAAGTGTCG AAACATGCAACAACGCCACTGG	320	NM_001034333.1
<i>DNMT3A</i>	CTGGTGCTGAAGGACTTGGGC CAGAAGAAGGGGCGGTCATC	317	AY271299
<i>IFNT2</i>	GCTATCTCTGTGCTCCATGAGATG AGTGAGTTCAGATCTCCACCCATC	359	NM_001015511.3
<i>GSTM3</i>	ATCGCCCGCAAGCACAATATG TCCAGGCACTTGGGCTCAAAC	290	NM_001046560.1
<i>PGRMC1</i>	TGTGTGCACAAATCCAGAAAG AATCATGCAGTTAGGTCAATCG	265	NM_001075133.1
<i>LAMA1</i>	CCCTGCCAGCAATGCACACATC TCGGATGCCGTTCTGTTGAAGG	341	AF010231
<i>WT1</i> (1–2)	ATGGCACGGGGTACGAGAGTG GCAGCCTGGGTAAGCACACATG	191	XM_866138.2
<i>WT1</i> (4–5)	TTCCGACCACCTGAAGACCCAC CAGAGCGCCAGCTGCAGCTTGC	168	XM_866138.2
<i>GATA4</i>	CCCGCCACCACTGCTTCCAG CGGGTGGATGGAGGACCCGT	171	NM_001192877.1
<i>ZFPM2</i>	TCACCGCGCATCAGCGGAAC CGCGATGAGCTGAGCGGCTT	224	XR_084585.1
<i>DMRT1</i>	AGCAGAGGGCATGTGGAGAAC TTCATCTGCCACTGGTTTCTG	251	NM_001078060.1

comparative cycle threshold (C_T) method was used to quantify expression levels. Quantification was normalized to the endogenous control, *H2AFZ*. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative C_T method, the ΔC_T value was determined by subtracting the *H2AFZ* C_T value for each sample from each gene C_T value of the sample. Calculation of $\Delta\Delta C_T$ involved using the highest sample ΔC_T value (i.e. the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔC_T sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta C_T}$.

To analyze the expression of the genes related with sex determination in adult tissues, male and female adult tissues (brain, medulla, lung, kidney, muscle, testicle, and ovary) were collected from a local slaughterhouse and immediately snap frozen in liquid nitrogen. RNA extraction was performed with ULTRASPEC (Ecogen, Madrid, Spain), avoiding DNA contamination by DNase treatment following the protocol described by Schmittgen & Livak (2008) with minor modifications. PCR

was performed and products were visualized in 2% ethidium bromide-stained gels under u.v. light.

Statistical analysis

Data were analyzed using the SigmaStat (Jandel Scientific, San Rafael, CA, USA) software package. Relative mRNA abundance differences among sexes were analyzed by one-way ANOVA. Error bars in Figs 1 and 2 represent the s.e.m. for the average $2^{-\Delta\Delta C_T}$ values.

Declaration of interest

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

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