Gene 933 (2025) 149006

Contents lists available at ScienceDirect

Gene

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

Research paper

# Expression of *FET-1* related transcripts during chicken embryogenesis suggests a role in muscle development

Chiron Loubser, Natalya V. Nikitina *

*School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag 3, WITS 2050 Johannesburg, South Africa*

ARTICLE INFO

Edited by: Vitor Silva Pereira

*Keywords:*
Avian
Chicken
Sex determination
FET1
Endogenous retrovirus
Muscle fusion
Gonad development
Sex-specific expression
Cell autonomous sex identity (CASI)

ABSTRACT

The genetic basis of somatic cell identity in avian sex determination is still unknown. *FET1*, an endogenous retrovirus, has previously been demonstrated to be expressed during gonad development. Here, we report expression of *FET1* related transcripts in non-gonadal tissue during chicken development. Both the forward and reverse *FET1* related transcripts were seen in various developing muscle tissues. Both the "full-length" and partial *FET1* transcripts were expressed; the latter however showed a more ubiquitous expression pattern. Female-specific gonadal expression of both sense and antisense transcripts was also confirmed. An anti-FET1 antibody, however, failed to distinguish between the predicted FET1 protein and other endogenous retroviral proteins expressed at E6.5. Our data suggest a possible role for *FET1* related transcripts in sex-specific differences in muscle size and growth rate in the chickens.

## 1. Background

Sex determination is the process whereby sexually reproducing organisms differentiate into a functional male or female. Despite it being one of the most fundamental processes, a great deal of variability exists in sex determination mechanisms. There are two broad mechanisms: genetic sex determination (GSD), where the sex chromosomal composition determines male or female identity; and environmental sex determination (ESD), where environmental factors during development are the main determinants (Bull, 1985). The mammalian XY/XX system in one of the most studied GSD systems. In mammals, males are the heterogametic sex (XY), females the homogametic sex (XX) and SRY, present on the Y chromosome, acts as the primary sex determinant of male gonad identity (Koopman et al., 1991; Sinclair et al., 1990). Both GSD and ESD mechanisms, as well as both the XX/XY and ZZ/ZW GSD systems, have been shown to operate in amphibians, reptiles, and fish. (Devlin & Nagahama, 2002; Gamble et al., 2017; Nakamura, 2009; Sarre et al., 2004; Vicoso et al., 2013). The ZW/ZZ system, where males are the homogametic sex (ZZ) and the females are the heterogametic sex (ZW), is used by birds and snakes. (Smith & Sinclair, 2004; Mullon et al., 2015; Wolf and Bryk, 2011).

Unlike in mammals, gonadal sex in birds is determined by a combination of genetic and hormonal factors. Testis determinants include a double dose of *DMRTt1* (Ioannidis et al., 2021; Lambeth et al., 2014; Smith et al., 2009), which is located on Z chromosome and appears to be the key initiating signal for testis development, while the expression of *FOXL2* (Major et al., 2019) and the presence of high levels of estrogen (Lambeth et al., 2013) drives the formation of the ovaries. Another key distinction between mammalian and avian sex determination is the fact that secondary sex determination in birds is not dependent entirely on estrogen or testosterone produced by the gonads but requires a genetic input. This phenomenon, known as cell autonomous sex identity (CASI) has been demonstrated by the existence of gynandromorphs, male–female chimeras that present as mosaics of male and female secondary sex phenotypes despite having the same levels of circulating sex hormones (Morris et al., 2018; Zhao et al., 2010). At present, there is no

*Abbreviations:* FET-1, female expressed transcript 1; E, embryonic day; HH, Hamburger-Hamilton stage; GSD, genetic sex determination; ESD, environmental sex determination; CASI, cell autonomous sex identity; ERV, endogenous retrovirus; env, envelope gene; ISH, in situ hubridization; min, minute; sec, second; S, Segment; DIG, digoxigenin; AP, alkaline phosphatase; mg, milligram; ml, millilitre; MABT, maleic acid buffer with Tween-20; NBT, nitro blue tetrazolium; BCIP, 5-Bromo-4-chloro-3-indolyl phosphate; SDS, sodium dodecyl sulphate; DTT, dithiothreitol; PBS, phosphate buffered saline; DAB, 3,3'-diaminobenzidine; bp, base pair; nt, nucleotide; lincRNA, Long noncoding RNA; kDa, kilodaltons.

* Corresponding author.
*E-mail address:* natalya.nikitina@wits.ac.za (N.V. Nikitina).

https://doi.org/10.1016/j.gene.2024.149006
Received 14 June 2024; Received in revised form 11 October 2024; Accepted 14 October 2024
Available online 18 October 2024

agreement as to the relative contributions of sex hormones and genetic factors to the different avian secondary sex characteristics (Clinton & Zhao, 2023). For example, male- vs female-specific feathering in chickens appears to be determined by the estrogen level in the skin, while the appearance of the head ornaments (comb and wattles) seems to be controlled by both genetic factors and hormonal levels (Major & Smith, 2016). On the other hand, body weight, muscle and bone size appear to be determined entirely by genes, at least in chickens. For instance, ZW chickens exhibiting female-to-male conversion due to *in ovo* injection of the aromatase inhibitor, Fadrozole, display female-specific body mass (Zhang et al., 2023). ZZ *DMRT1*+/- gene-edited chickens display female gonads, however, their body mass and muscle development remain in the range typical of males (Ioannidis et al., 2021). Currently, candidate genes that control the development of the male-vs-female differences in body mass and muscle size in chickens have not yet been identified.

What characteristics would be expected of a candidate gene (or genes) responsible for cell autonomous sex identity (CASI) in chicken? Since the chromosomal composition distinguishes male from female somatic cells in the gynandromorphs, we would expect the candidate gene(s) to be found either on the W chromosome (and have no Z gametolog) or on Z chromosome (since dosage compensation does not occur in chicken somatic cells (Mullon et al., 2015; Wolf and Bryk, 2011; Zhang et al., 2010) and a double dose of this gene may cause masculinization of somatic tissues in the same way as *DMRT1* acts as a male gonadal determinant). We would also expect it to show expression in some or all of the tissues that exhibit CASI, e.g. muscle cells. One such possible candidate gene is *FET1* (Female Expressed Transcript 1). *FET1* was identified as one of the 26 genes located on the W chromosome and was reported to be expressed in the chicken ovarian cortex at E 4.5-E9.5 (Hirst et al., 2017; Reed and Sinclair, 2002), and at much lower levels in the cortex of male gonads (Hirst et al., 2017). It was initially considered as a candidate W-linked avian female gonadal determinant. However, further experimental work demonstrated that *FET1* gonadal expression is not altered during Fadrozole-induced female-to-male sex reversal in chicken gonads, which does not support a role as a female gonadal determinant (Hirst et al., 2017).

*FET1* was identified as an avian endogenous retrovirus belonging to avi-gamma group (Bolisetty et al., 2012). Endogenous retroviruses (ERVs) are found in all vertebrate genomes, and some of them have been shown to perform important physiological and developmental roles in the host. For example, syncytins, proteins encoded by *env* genes of mammalian ERVs, are critical for the formation and function of placenta due to their ability to promote cell–cell fusion (Dupressoir et al., 2011; Sha et al., 2000). Multiple ERVs are expressed during early embryogenesis and are involved in the establishment of the pluripotency in mammalian stem cells (Wang et al., 2020; Zhang et al., 2022).

Bird genomes contain fewer endogenous retroviruses compared to mammalian genomes, e.g. ERVs comprise 8 % of human and 11 % of mouse genome, but only around 0.2–3.5 % of avian genomes (Zheng et al., 2024). Several chicken ERVs are expressed in various tissues during embryonic development, including ovarian cortex (Carré-Eusèbe et al., 2009) and embryonic epiblast cells (Blanc et al., 2014); however there has not yet been any functional studies of avian ERVs.

We hypothesise that *FET1* may function in somatic sex determination. The aim of this study was to investigate the expression of *FET1* related transcripts and protein in chicken embryonic gonads and somatic tissues. We found that sense and antisense *FET1* related transcripts are present in developing muscle tissues in both males and females, suggesting a possible role in muscle development in chickens.

## 2. Methods

### 2.1. Eggs and embryos

All animal experiments were carried out according to the rules and regulations of the University of the Witwatersrand animal ethics committee (WITS Animal Ethics permit number: 2017/01/01/O). All experiments reported in this article were performed on chicken embryos collected at or before embryonic day 10,5 (E10,5), which corresponds to 50 % gestation for chicken embryos. Embryos at such early developmental stages are not subject to ARRIVE guidelines or regulations such as Guidance on the operation of the Animals Act 1986, EU Directive 2010/63 for the protection of animals used for scientific purposes or the NIH (National Research Council) Guide for the Care and Use of Laboratory Animals, all of which pertain only to vertebrate embryos beyond two thirds (66 %) of their normal developmental period (older than E13 for chicken). Male and female embryos were collected and analysed separately, at least 3 embryos were analysed per stage in each experiment.

HH25-26 (E4.5) embryos were used for PCR and RT-PCR. Heads were used for DNA extraction and subsequent sexing PCR and the genital ridges were dissected for RNA extraction. HH22 (E4.0), HH25 (E5.0), HH30 (E6.5–7.0), HH34 (E8.5) and HH36 (E10.5) embryos were used for ISH. PCR reactions and variations thereof were performed on embryos supplied by the Agricultural Research Council (Irene, South Africa) and Avi-Farms (Centurion, South Africa). Four chicken breeds were used for RT-PCR; Venda (local egg layer breed), Potchefstroom Koekoek (dual-purpose local breed), Rhode Island Red (commercial egg layer breed) and White Leghorn (commercial egg layer breed). Specific pathogen-free White Leghorn embryos used for ISH were supplied by Avi-Farms (Centurion, South Africa). Eggs were incubated at 37° C until the required developmental stages. Analysis was performed on pooled tissues from 3 to 9 embryos for each breed, developmental stage and sex.

**Sexing.** Sex of the embryos was determined by PCR according to Clinton et al, 2001 (Clinton et al., 2001). Briefly, the W-linked *XhoI* repeats and the 18S ribosomal gene, an internal control, were amplified. Females are represented by two bands and males by one. The sequences of the primers used are: *XhoI* Forward: CCCAAATATAACACGCTTCACT; Reverse: GAAATGAATTATTTTCTGGCGAC. 18S Forward: AGCTCTTTCTCGATTCCGTG; Reverse GGGTAGACACAAGCTGAGCC. (Table 1). PCR conditions used for sexing: initial denaturation at 94 °C for 2 min, followed by 28 cycles of 94 °C for 5 s, 56 °C for 5 s and 72 °C

**Table 1**
Primers used in this study.

| Targeted gene name (NCBI accession number) | Primer name | Primer sequence (5′-3′) |
|---|---|---|
| Gallus gallus clone pUGD0603 W chromosome-specific XhoI family repeat (M24756.1) | XhoI forward | CCCAAATATAACACGCTTCACT |
| | XhoI reverse | GAAATGAATTATTTTCTGGCGAC |
| Chicken 18S ribosomal RNA (M59389.1) | 18S forward | AGCTCTTTCTCGATTCCGTG |
| | 18S reverse | GGGTAGACACAAGCTGAGCC |
| FET-1 (AY113681.1) | FET-1 GSP forward | TGGCTATGTGGAGACGGCCAGGCACGAA |
| | FET-1 GSP reverse | TCGGCACAGGCAGGCAGAAGCACTTGA |
| | S1 forward | GGACCCTACCAAGTTCTCCT |
| | S1 reverse | TGGTCCCTTTTGTGGTTTGTC |
| | S2 forward | CGAAACTTGACGGGCTTAGG |
| | S2 reverse | GCACTTGATCATCACACAGGT |
| | S3 forward | AAGGCAAAAGGTGGAAAGCG |
| | S3 reverse | CCACCAAACTTACACACCGT |

for 5 s, and final extension at 72 °C for 3 mins.

**RT-PCR.** For the first round of RT-PCR (Fig. 1c), Nucleospin RNA Kit (Macherey-Nagel) was used to extract total RNA. We used whole bodies of HH25 (E4.5) embryos (the heads were collected for sexing). The number of embryos used for RNA extraction was as follows: 8 females and 3 males were used for Venda breed, 8 females 5 males for Potchefstroom Koekoek, and 3 females and 9 males for Rhode Island Red. RNA was reverse transcribed using random hexamer primers and the RevertAid First Strand cDNA synthesis kit (Thermo Fisher). Semi-quantitative PCR conditions were: initial denaturation at 94 °C for 2 min, followed by 28 cycles of 94 °C for 30 s, 69 °C for 30 s and 72 °C for 40 s, and final extension at 72 °C for 3 mins. The same amount of cDNA was used for each PCR reaction. *FET1* primers used: *FET1* GSP Forward– 5′ TGGCTATGTGGAGACGGCCAGGCACGAA3′; *FET1* GSP Reverse – 5′ TCGGCACAGGCAGGCAGAAGCACTTGA 3′ (the location of the primers in the *FET1* sequence is indicated in Fig. 1B). We used the same 18S Forward and 18S Reverse primers as for the sexing PCR to amplify a 256 bp fragment of 18S, as a reference gene. The 660 bp PCR products were gel extracted (Zymoclean Gel DNA Recovery Kit, Zymo Research), and ligated into pGEM-T Easy (Promega) for sequencing.

For the second RT-PCR (Fig. 1D), gonad and breast muscle tissue were collected from HH29-30 (E6.5) White Leghorn male and female embryos and the total RNA was extracted (Quick-RNA Miniprep Kit, Zymo Research). RT-PCR was performed with Protoscript first strand cDNA kit (NEB) using 320 µg of total RNA and the random hexamer mix. PCR, using the same amount of cDNA input, was performed with Q5 Hot start Hi-Fidelity master mix (NEB) using the S1 forward (5′ GGACCCTACCAAGTTCTCCT 3′) and *FET1* GSP reverse (5′ TCGGCACAGGCAGGCAGAAGCACTTGA 3′) primers with the following cycling conditions: initial denaturation at 98℃ for 30 sec, 35 cycles of denaturation at 98℃ for 10 sec, annealing at 66℃ for 10 sec, 72℃ for 40 sec and a final elongation at 72℃ for 2 min. The 1.3 kb PCR products were gel extracted (Zymoclean Gel DNA Recovery Kit, Zymo Research), A-tailed, ligated into pGEM-T Easy and sequenced.

### 2.2. Whole mount in situ hybridization (WISH)

*Embryo collection.* Embryos at HH30 (E6.5), HH34 (E8.5) and HH36/37 (E10.5) were dissected to expose the gonads. We used DNA extracted from the embryonic heads for PCR sexing. Limbs, pectoral muscle and kidneys with attached gonads were dissected from a subset of HH34 embryos in order to enable better probe diffusion. Embryos were fixed overnight in 4 % PFA at 4℃, dehydrated through increasing concentrations of methanol and stored in 100 % methanol at −20 °C.

*Preparation of probe templates.* Three segments of the predicted *FET1* mRNA were PCR amplified and cloned into pGEM-Teasy (Promega).

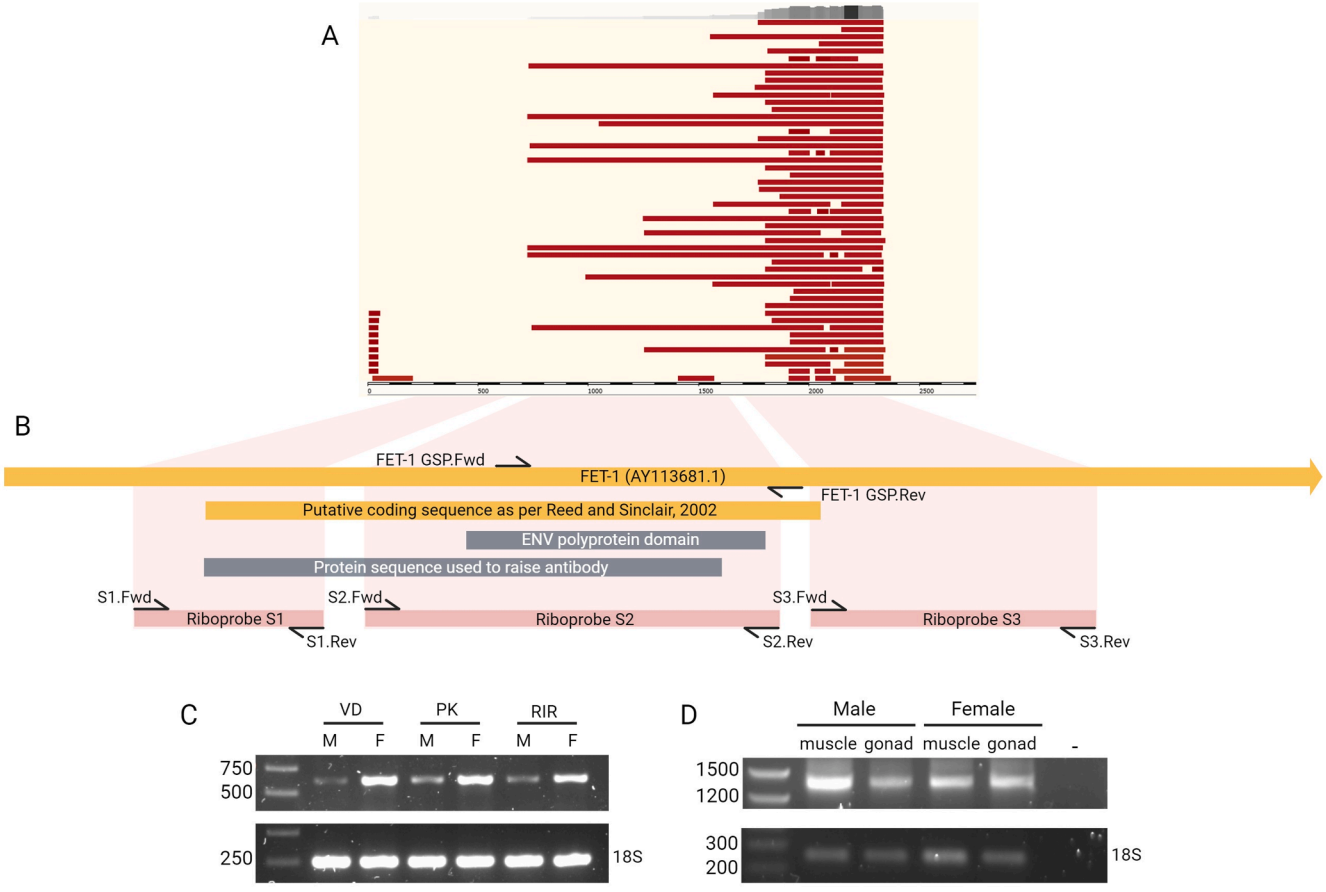


**Fig. 1.** (A) Nucleotide BLAST of *FET1* (GenBank accession number: AY113681) against the ncRNA database (Ensembl), showing alignments of *FET1*-like RNAs to three main regions of the *FET1* mRNA: S1 (corresponding to bases 269–668 of AY113681), S2 (bases 756–1622 bp), and S3 (bases 1670–2279). (B) Diagram indicating the relative positions of the three riboprobes generated in this study with respect to *FET1* mRNA sequence AY113681. Riboprobes S1 and S2 partially overlap the predicted *FET1* coding region. The location of the peptide against which the anti-FET1 antibody was raised is also shown in grey; this sequence includes most of the predicted *env* ORF. (C) RT-PCR on *FET1* mRNA extracted from Rhode Island Red, Venda and Potchefstroom Koekoek chicken breeds. (Top panel). *FET1* expression was observed in both male and female gonads across all three breeds. (Bottom panel) Similar levels of 18S 256 bp band were amplified across all samples. (D) RT-PCR on *FET1* mRNA extracted from the muscle and gonads of E6.5 male and female White Leghorn embryos. Top panel indicates the presence of *FET1* transcripts across all samples examined. For (C) and (D) RNA pooled from 3 to 9 embryos per breed, sex and developmental stage was used. Male and female embryos were collected and analysed separately.

Segment 1 (S1) (corresponding to bases 269–668 of predicted *FET1* mRNA GeneBank accession number AY113681) was amplified with the following primers: forward 5′ GGACCCTACCAAGTTCTCCT 3′, reverse 5′ TGGTCCCTTTTGTGGTTTGTC 3′. Segment 2 (S2) (bases 756–1622) was amplified with forward primer 5′ CGAAACTTGACGGGCTTAGG 3′ and reverse primer – 5′ GCACTTGATCATCACACAGGT 3′. Finally, the most 3′piece, Segment 3 (S3) (bases 1670–2279) was prepared using forward primer 5′ AAGGCAAAAGGTGGAAAGCG 3′, and reverse primer 5′ CCACCAAACTTACACACCGT 3′ (Table 1). Fig. 1B shows the probe template sequence location relative to the sequence of AY113681 (shown as the brown bar at the very top of the panel), the three probe templates are labelled S1, S2 and S3 respectively. The identity of the probe template and the direction in which PCR products were inserted into the plasmid was confirmed by sequencing.

*RNA probes.* DIG-labeled antisense probes were prepared by *Nco*I digestion followed by transcription using SP6 RNA polymerase; sense probes were prepared using *Nde*I and T7 polymerase.

*Whole mount in situ RNA hybridization. In situ* was performed as per our previously published protocol (Nikitina et al., 2009). Briefly, embryos were rehydrated, treated with 10 μg/ml Proteinase K for 30 min, post-fixed and incubated with RNA probes in hybrization mix overnight at 70 °C. Extensive washes with hybridization mix and MABT were performed the next day. Embryos were then incubated with 1:2000 dilution of anti-DIG-AP Fab fragments (Roche) overnight, washed 10x with MABT and stained with a solution of 0.45 mg/ml NBT and 0.175 mg/ml BCIP.

Colour development for embryos hybridized with the S3 sense and anti-sense probes was allowed to proceed for 6 h at room temperature. For embryos hybridized to the remaining probes, colour development was carried out for 48 h at 4 °C. Dissected muscles and gonads were stained for 24hrs at 4 °C. Staining was stopped when the negative control started to develop background staining.

Between 3 and 5 embryos were analysed for each probe, developmental stage and sex.

**Cryosectioning.** Selected embryos were processed through sucrose gradient series, embedded in Cryomatrix (Thermo Scientifc) and snap frozen using liquid nitrogen. 20-μm sections were cut using CM1520 cryostat (Leica), placed on SuperFrost slides (Thermo Scientific), mounted, and viewed and photographed using a Zeiss microscope (Zeiss Axio Zoom V16).

**Antibody against FET1.** Rabbit polyclonal anti-FET1primary antibody was produced by GenScript. The amino acid sequence (UniProt accession number Q8JGM1) against which the antibody was raised (partial ENV domain is in bold): MYRSLIIIGM IGTVVTAWQE NSYLRMTEKI GQTLNRSCWI CTALPRGEGR VPVYGIVVLN WTIPEWVEHG KCKFGIEDKPQKGPKFDLLV INSSRSIFVD RKVNGKDGVG VGWRNLTGLG CSWEYGNFRA ISEVSEAGRW NQGEGCDPRI DKESYWASDKNILGIVGMGA SFTHQENMAR **PCKLPHGYWW LCGDGQARKA LPLNWTGTCT TGYLIPQTTV HEEIPGGLLR TPWIRTKHTYNPLAIYNKGY HSFLRALIPA LGVAQLEKAI LNISATLEIV ENATTDALRA IQEEVSSLSK VVLQNRMALD LLTAKEGGVCTIINQSCCAY INKDLRIETD LRKIWEQTKV LHRTTQDDTN**. The antibody was diluted to 10 μg/ml before use.

### 2.3. Crude protein extraction

2X Laemmli sample buffer (65.8 mM TRIS pH 6.8, 26.3 % glycerol, 2.1 % SDS, 10 mM DTT and Beta Mercaptoethanol) was used for crude protein extraction. Protein was extracted from three male and three female HH30 (E6.5) embryo gonads, kidneys, pectoral muscle and limbs, through multiple (3–4) freeze thaw cycles. The samples were homogenized, spun down at 17 000 g, and the supernatant collected and used for the SDS-PAGE, Western blot and Dot blot.

### 2.4. Dot blot

Crude protein extracted from male and female embryonic gonads, kidneys, limbs and pectoral muscle were placed onto nitrocellulose membrane and allowed to dry. A spot of FET1 recombinant protein provided by Genscript was included as a positive control. The Dot blot was washed in PBST and blocked in 3 % BSA for 2 h at 37℃ with shaking. The blot was incubated in 1 μg/ml of FET1 polyclonal antibody (Genscript), at 37° C for 2 h with agitation. The following controls were used: FET1 recombinant protein stained with FET1 antibody, limb crude extract treated with pre-immune serum, limb crude extract treated with only secondary antibody (no primary), and limb extract treated with PAX7 antibody. The blot was incubated in 1:2000 dilution of polyclonal Goat anti-rabbit HRP-conjugated antibody (Dako) for 2hrs at 37° C. Colour detection was performed for 10 min in 0.5 mg/ml DAB and 3 % $H_2O_2$.

### 2.5. SDS-PAGE

The protein crude extract was separated a 12 % SDS-PAGE gel, and stained with Coomassie blue (0.1 % Coomassie R250, 10 % glacial acetic acid, 40 % methanol) for 1 h at room temperature, then de-stained with 10 % glacial acetic acid, 20 % methanol.

*Western blot.* The SDS-PAGE was transferred to a nitrocellulose membrane, washed in PBST, blocked with 3 % BSA for 1 hr and incubated in 1ug/ml FET1 antibody overnight at 4℃. The blot was washed and incubated in HRP-conjugated goat anti-rabbit antibody (1:2000 dilution) for 1 h. Staining was performed with 0.5 mg/ml DAB and 3 % $H_2O_2$ in PBS for 15 min.

## 3. Results

Expression of *FET1* was reported in both male and female gonads of egg-layer breeds (e.g. White Leghorn) (Hirst et al., 2017; Reed & Sinclair, 2002). To confirm that this expression profile is not specific to just one breed of chickens, we performed semi-quantitative RT-PCR using total RNA isolated from gonadal tissues of HH26-HH27 (E4.5) embryos from three different chicken breeds. Our results indicated that *FET1* was expressed consistently across all three chicken breeds examined, with the expression appearing higher in females compared to males (Fig. 1C, top panel). 18S RNA was used as a reference gene (Fig. 1C, bottom panel). To confirm the identity of the 658 bp product, we sequenced two male and two female- derived clones. All sequences were virtually identical to nucleotides 984–1641 of AY113681, with only 2 base changes.

*FET1* related integrations were reported to be located on both of the sex chromosomes, as well as in several autosomal locations in the genome assembly of the Red Jungle Fowl (GalGal5, released 12/2015). Given the recent release of genomes for broiler (bGalGal1.mat.broiler.GRCg7b, released 01/2021) and egg-layer (bGalGal1.pat.whiteleghornlayer.GRCg7w, released 09/2021) chicken breeds, we took an opportunity to explore whether some of these *FET1* integrations may be breed-specific. Our TBLASTN and BLASTN search results indicated the presence of 5 full-length integrations on Chromosomes W, 7 on Z, and one on each of Chromosomes 2, 5, 10, 12 and 33 in the most recent assembly of the Red Jungle Fowl genome (GRCg6a, released 04/2018). In the reference broiler genome, full-length integrations were found on Chromosomes Z, W, 2, 5, 10 and 34, while in the White Leghorn genome full-length *FET1* integrations were found on Chromosomes Z, W, 2, 4, 5, 12, 17 and 34. While most of the integrations (especially on Z and W chromosomes) appear to be ancient, a few are different between the broiler and egg-layer genomes, suggesting recent mobilisation and re-integration of the *FET1*-like retroviral element. Of note is the recent integration on Chromosome 4 in the White Leghorn genome, which has 99 % sequence identity to AY113681, and is predicted to encode a 434aa protein with 50 % similarity to syncytin. We also observed multiple

autosomal partial *FET1*-like transcripts of different lengths (Fig. 1a). *FET1*-like transcripts could be broadly divided into three sets based on the length of their homology to the original *FET1* mRNA transcript (AY113681). The longest RNAs, with 92–93 % identity to nt 240–2293, were found in multiple copies on the W and Z chromosomes. The second set of integrations, identical or very similar to nts 728–2293 as well as the third set, nts 1641–2293, did not contain any large ORFs and were found on the W chromosome as well as multiple autosomes. Our RT-PCR primers would have amplified both the first and the second, but not the third transcript set (Fig. 1B); suggesting that male and female gonads express some or all of these two transcript sets at different levels.

Previously reported expression of *FET1* in both male and female gonads (Hirst et al., 2017; Reed & Sinclair, 2002) used a probe that would not have distinguished between the full-length and the shorter *FET1* related transcripts (Fig. 1a). We re-examined the expression pattern of *FET1*-like transcripts in chick embryos by RNA *in situ* hybridization. To distinguish between the different transcript groups, we cloned three sets of probe templates (Fig. 1B). Probe S1 binds to and detects only the predicted *FET1* coding transcripts, and not any of the shorter lincRNAs. Probe S2 detects both the *FET1* coding transcripts and the longer lincRNAs (second transcript set), while probe S3 detects all three sets of the *FET1*-like transcripts present in the tissue.

We performed RNA *in situ* hybridization on HH29/30 (E6.5), HH35 (E8.5) and HH36 (E10.5) embryos, using both sense and antisense RNA probes. Our results are shown in Figs. 2-6, and Supplementary Figs. 1 and 2. Unexpectedly, our results indicate that both the forward and the reverse (antisense) *FET1*-like transcripts are present in embryonic tissues. Full length *FET1* (probe S1) is expressed predominantly in the gonads and various muscle tissues (Figs. 2-5). The earliest expression in the muscle tissues was observed at E6.5, with both the sense and the antisense transcripts seen in male and female tissues (Fig. 2 and Fig. 3, arrowheads; Fig. 5). We occasionally observed asymmetric staining in some embryos (e.g. Fig. 2I, 2 J), which we think is a staining artefact due to poor probe penetration of larger later stage embryos. To confirm that the asymmetrical staining in the pectoral muscle was indeed an artefact,

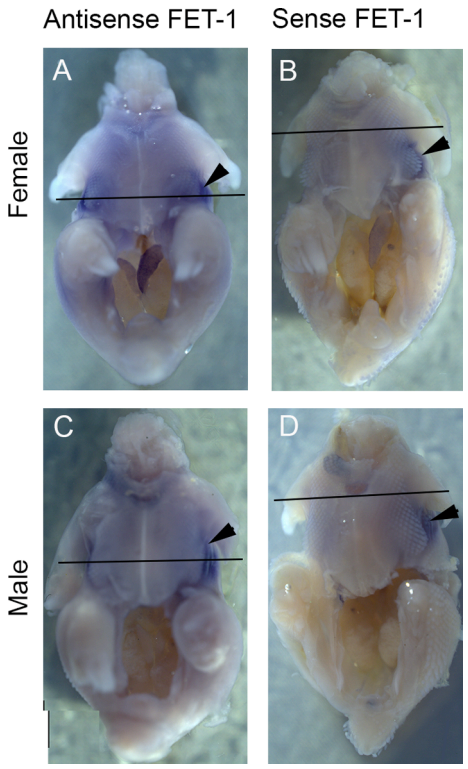


**Fig. 3.** Expression of *FET1* related transcripts in E10.5 chicken embryos. S1 probe was used for *in situ* hybridization, indicating the presence of the longest *FET1* transcripts. "Sense" refers to the *FET1* forward transcript, and "antisense" refers to the opposite direction *FET1* transcript. Black arrowheads mark expression in the pectoral muscles. Black lines indicate the plane of sectioning for Fig. 4. At least three different embryos of each sex and developmental stage were analysed with consistent results.

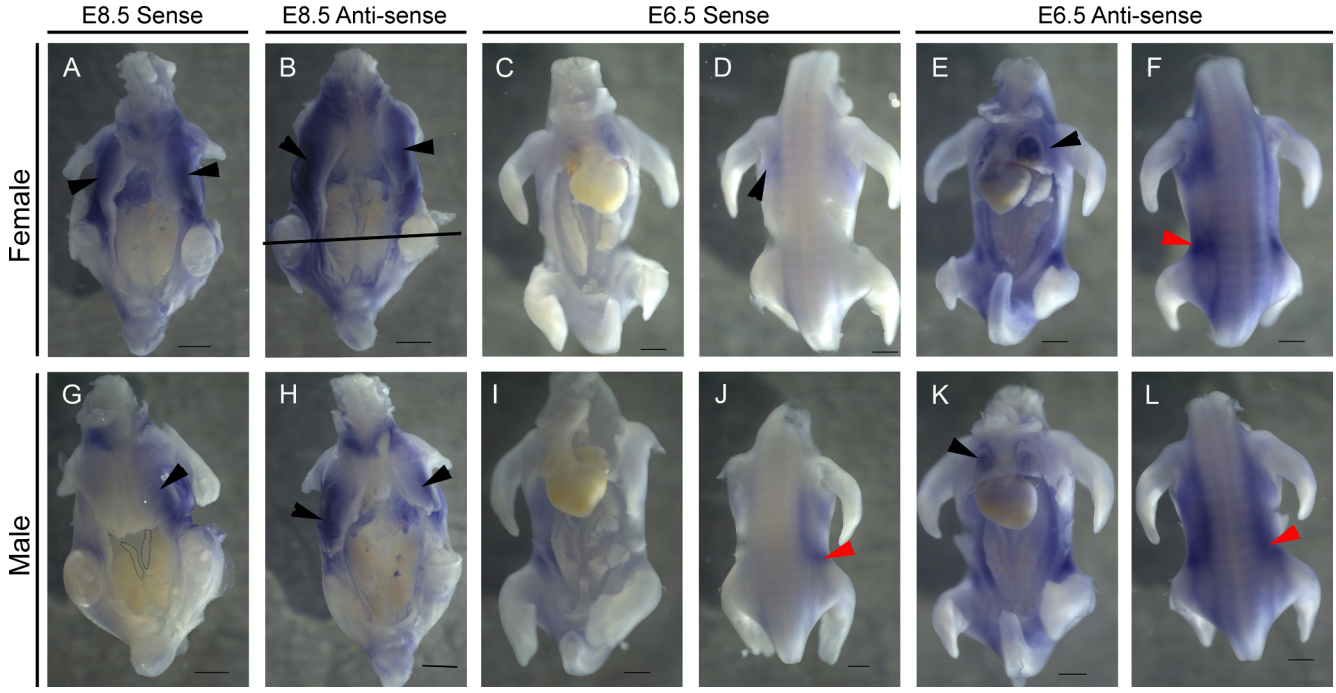


**Fig. 2.** *FET1* related transcripts are expressed in developing muscles in E6.5 and E 8.5 chicken embryos. The S1 probe was used for *in situ* hybridization, indicating the presence of the longest *FET1* transcripts. "Sense" refers to the *FET1* forward transcript, and "antisense" refers to the opposite direction *FET1* transcript. Black arrowheads point to the expression in the pectoral muscles, while the red arrowheads denote expression in the body wall. Black line in (B) indicates the plane of section. Scale bar – 0.5 cm. At least three different embryos of each sex and developmental stage were analysed with consistent results.

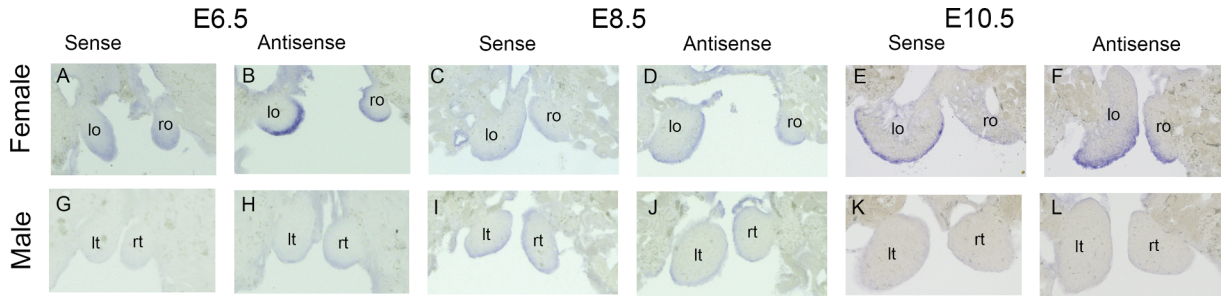


**Fig. 4.** Both sense and antisense *FET1* related transcripts are present in gonads. Sense *FET1* transcripts were observed in ovarian cortex, with higher expression in the left ovary (A, C and E). Antisense *FET1* transcripts were present in the cortex of both ovaries (B, D and F). A much lower level of expression of both the sense and the antisense *FET1* transcripts was observed in the testicular cortex at E6.5 and E 8.5 (H, I nd J). lo-left ovary, ro-right ovary, lt-left testis, rt-right testis. At least two different embryos of each sex and developmental stage were analysed with consistent results.

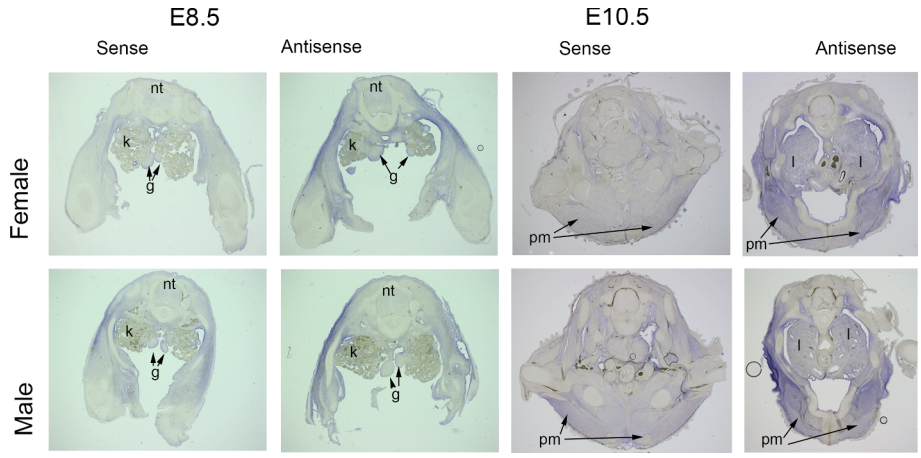


**Fig. 5.** Expression of *FET1* related transcripts in muscle tissues. g-gonads, l-lungs, k-kidneys, nt-neural tube, pm-pectoral muscle. At least two different embryos of each sex and developmental stage were analysed with similar results.

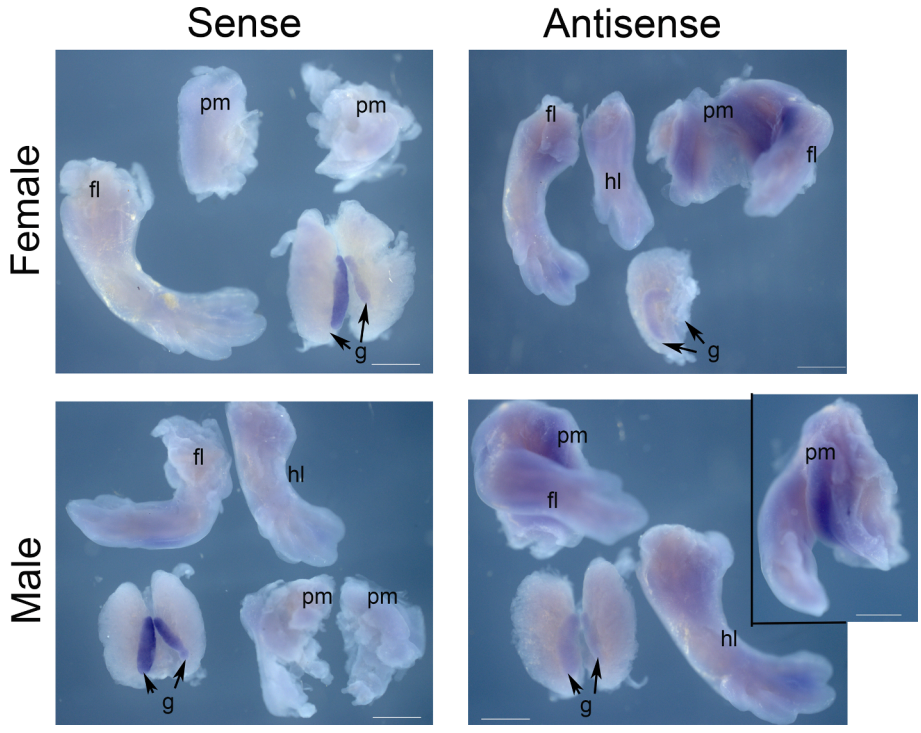


**Fig. 6.** *In situ* hybridization detecting *FET1* antisense and sense transcripts in dissected male and female gonads, pectoral muscles and limbs of E8.0 embryos. g-gonads, fl-forelimb, hl-hindlimb, pm-pectoral muscle. At least three different embryos of each sex and developmental stage were analysed with consistent results.

we repeated *in situ* hybridisation using dissected muscles, limbs and gonads of E8.0 embryos (Fig. 6). We found that both sides of the pectoral muscle stained to the same extent, confirming that inadequate probe penetration is most likely the cause of lower levels of staining on one side of the pectoral muscle seen in, for example Fig. 2G, 2I, 3B and 3D. Additionally, a novel area of *FET1* expression was seen in the muscles of both front and hindlimbs for both the sense and the antisense *FET1* related transcripts (Fig. 6).

To further confirm the presence of *FET1* related transcripts in muscle tissues, we performed RT-PCR on total RNA isolated from E6.5 male and female pectoral muscle (Fig. 1D), we also included RNA from gonads. An expected band of 1.3 kb was obtained in all cases. We cloned these bands, and sequenced 6 separated clones from female gonads, 5 from female muscle, 3 from male gonads and 6 from male muscle. All these clones had 95 % identity to *FET1* AY113681 and 99 % identity to genomic regions on the W chromosome (which appear to contain other full length *FET1* integrations). Our sequencing data has been submitted to Mendeley Data (https://doi.org/10.17632/sw497k9d6p.1).

Both the sense and the antisense *FET1* transcripts are expressed in the cortex of female gonads (Fig. 4A-F), while low levels of *FET1* transcripts are seen in the cortex of male gonads (Fig. 4G-L). Interestingly, the sense transcript is seen mostly in the left ovary, but the antisense transcript is present in both ovaries (compare Fig. 4E and Fig. 4F). Weak staining was seen in the male gonads (Fig. 4G-L). Taken together, these findings suggest possible roles for full-length *FET1* in gonad and muscle development.

As expected, probes S2 and S3 were seen in the same tissues as probe S1. For S2, novel regions of expression included staining of the surface ectoderm in females at E4.5 and 6.5 (Supplementary Fig. 1). For probe S3, both the sense and the antisense transcripts show ubiquitous expression in both male and female embryos from E4.5 onwards (Supplementary Fig. 2).

Our results shown in Figs. 2-5 appear to demonstrate a very similar, almost identical expression patterns for of *FEt1* related sense and antisense transcripts. These results were obtained after 48-hour staining with NBT/BCIP, to ensure all areas of *FET1* presence in the tissue were visualized. When we repeated *in situ* experiments and allowed for a limited staining time of 24hrs, it became apparent that colour development proceeds at different rates in different tissues. For sense *FET1* transcripts, blue staining appeared first in the gonads, and then within the muscle tissue, while the opposite was seen for antisense *FET1* transcripts. This may be related to different levels of *FET1* sense and antisense transcripts present in these tissues, though this observation will need to be confirmed with a more quantitative method, such as qRT-PCR.

*FET1* was predicted to encode a 434-amino acid protein (Reed & Sinclair, 2002), however different *FET1* integrations on both Z and W chromosomes have stop codons that will likely result in shorter peptides being produced, if indeed these integrations are expressed. We wanted to find out whether the predicted *FET1*-encoded protein is produced in embryonic tissues. To this end, a polyclonal anti-FET1 antibody was raised in rabbits. We used this antibody to perform Dot blots on total protein extracted from male and female E4.5 gonads and male and female E6.5 muscle tissue (Fig. 7A). We found positive FET1 immunoreactivity in all the tissues examined, which was unexpected because it was not consistent with the highly tissue-specific expression of the *FET1* S1 probe. The “secondary antibody only” control did not produce any staining (not shown), suggesting that non-specific binding of the secondary antibody was not the issue. To confirm the specificity of the antibody, we performed a Western blot using crude protein extract from male and female gonads, pectoral muscle, and limbs. We found that multiple bands were recognized by the antibody, including a band corresponding to the predicted full-length FET1 protein (Fig. 7B; bottom panel, red arrows). This result may suggest that peptides of different lengths are produced from different genomic integrations of FET1, however it is also possible that our antibody exhibits cross-reactivity to other related endogenous viral proteins, which are present and expressed in the chicken embryos. NCBI conserved domain database search reveals similarities to two protein domains within FET1: 1) ENVV1-like HR1-HR2 domain forming part of a transmembrane subunit found in various endogenous retroviruses and 2) ENV polyprotein (coat polyprotein) (Marchler-bauer et al., 2015). Both of these conserved regions were included in the peptide that was used to produce the antibody, supporting possible cross-reactivity of the antibody. It would be desirable to generate a more specific antibody for future studies. We believe this could be achieved by using short (20aa) peptides from regions of FET1 that lie outside the conserved domains, i.e. aa1-185 or aa 210–434 of predicted FET1 protein sequence (AAM52407.1). It may be necessary to generate and test several antibodies against different regions of the protein.

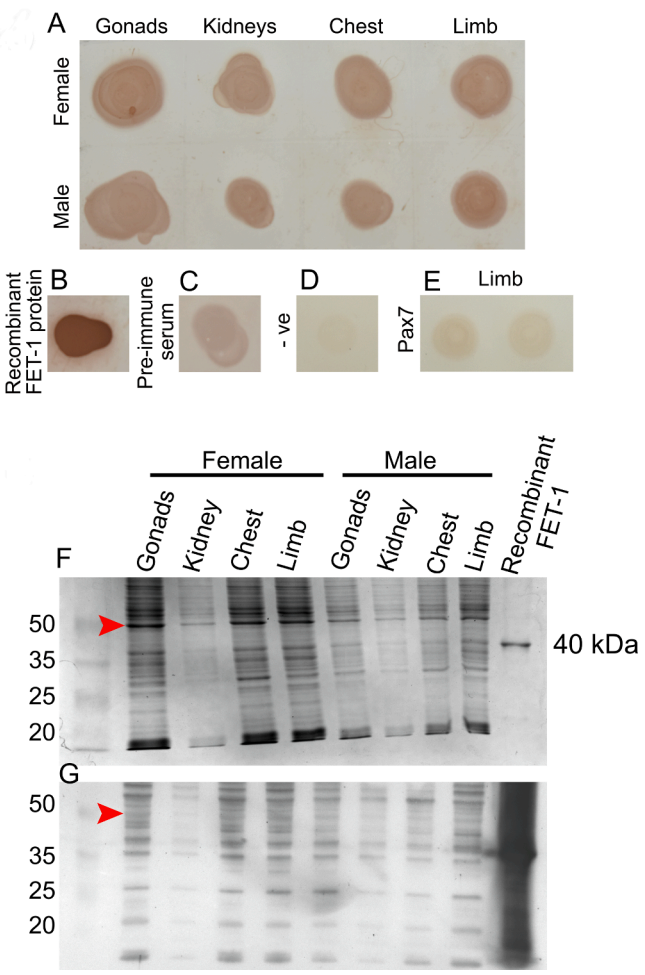


**Fig. 7.** FET1 antibody shows cross-reactivity to other proteins. (A) Dot blot of total protein extract from male and female gonads, kidneys, pectoral muscle and limb, stained with FET1 polyclonal antibody. Both male and female samples show equal staining. (B) Positive control: recombinant FET1 protein detected with FET1 antibody. (C) Crude protein extract from limbs incubated with pre-immune serum, showing weak staining. (D) Negative control: limb protein extract incubated with secondary antibody only. (E) PAX7 antibody was used on limb protein extract as an additional control. (F) Coomassie blue staining of total protein extract from male and female gonads, kidneys, pectoral muscle and limbs. Red arrow indicates the expected size and location of FET1 at 50 kDa. (G) Western blot of total protein extract using anti-FET1 antibody. Cross reactivity to multiple other bands, most likely other endoogenous retroviral proteins, was observed. Red arrow indicates staining at the expected band size for FET1 at 50 kDa. 40 kDa FET1 recombinant protein is used as a control.

## 4. Discussion

Differences in body mass between males and females are seen in most vertebrate species, with the males generally being larger and developing greater body mass than females in both birds and mammals. Sex-specific muscle development is entirely under hormonal control in mammals, with gonadal sex-reversal resulting in the secondary sex characteristics that are typical of the hormonal level, not the chromosomal sex (Acién & Acién, 2020). In birds, sex-specific body mass and muscle development is entirely under genetic control (Clinton & Zhao, 2023; Ioannidis et al., 2021; Major & Smith, 2016; Zhao et al., 2010). Our results demonstrate that *FET1* full length forward and reverse transcripts are expressed in the developing muscles in both males and females. Interestingly, the predicted FET1 –encoded protein appears to be a syncytin, with 32 % and 35 % amino acid identity to mouse syncytin B and human syncytin 2 respectively. Syncytins were reported to be responsible for the sex-specific differences in muscle growth rate in several mammals. Syncytin B knockout male, but not female mice display > 20 % reduction in muscle mass, which makes the knockout male mice resemble wild-type females (Redelsperger et al., 2016). This and the presence of *FET1* transcripts during muscle tissue development makes *FET1* a possible genetic determinant for somatic muscle cell identity in chickens. Functional analysis is required to establish this role with certainty.

*FET*1-related sense and antisense transcripts are expressed predominantly in the gonadal cortex of females. This expression pattern suggests a possible role in ovarian follicle maturation, but not in the determination of gonadal identity.

Vertebrate skeletal muscles originate from the dorsal portion of somites. The dorsal part of a somite forms dermomyotome, the region that will give rise to back dermis and striated muscles of the limbs and body wall. Muscle precursors undergo epithelial-to-mesenchymal transition and migrate to their final destinations, where they aggregate to form nascent muscles (Chal & Pourquié, 2017). Two types of trunk muscles are recognised: epaxial, which comprise mostly the muscles of the back, and hypaxial, which include the shoulder girdle and muscles of the limb and body wall. We observed expression of *FET1* in both types of muscle groups, suggesting that its function is not limited to a particular muscle group.

Muscle fibre formation is a two-stage process. Primary myogenesis, involving fusion of myoblasts to produce myotubes, occurs between E3 and E7 during chicken embryogenesis. This is followed by secondary myogenesis (occurring from E8 onwards), which entails fusion of myoblasts and the myotubes produced during primary myogenesis, resulting in muscle fibres (Chal & Pourquié, 2017). Our *in situ* hybridisation results support the notion that *FET1* is involved both in later stages of primary myogenesis and in secondary myogenesis, since we observed expression in muscle tissues from E 6.5 until E10.5 (the latest stage examined), but not in the myotomes at E4.5.

Mammalian syncytin proteins have critical functions as fusogens during placenta, muscle and bone development and remodelling. Our results demonstrate the presence of *FET1* transcripts but due to the non-specific nature of our antibody, we were unable to convincingly demonstrate that these *FET1* transcripts are translated. The functional significance of the presence of the antisense *FET1* RNA is not clear; however, it is tempting to speculate that it is involved in regulation of the sense *FET1* transcription and activity in selected tissues. Antisense transcription appears to be a common feature in multiple mammalian endogenous retroviruses. Even though the antisense transcripts do not usually encode a protein, they can inhibit the expression of their sense counterpart in multiple different ways via formation of double-stranded RNA duplexes; alternatively, increased expression of the sense strand may occur due to protection from endonuclease degradation (Romerio, 2023).

The sex dimorphic expression pattern of *FET1* is of interest given the fact that, in chicken, selective breeding for more efficient feed utilization and larger muscle mass have resulted in simultaneous reduction in fecundity due to polyovulaion (as seen in broiler breeds), while breeding for higher egg production has resulted in smaller bird size and lower growth rates (as seen in egg-layer breeds)(Hocking, 2014; Buzala & Janicki, 2016). We propose that this phenomenon may be explained by the existence of genes such as *FET1*, which may play roles in both gonadal and muscle development.

*FET1* has been identified as an ancient endogenous avian retrovirus (ERV) belonging to the avigamma 1 class of avian ERVs (Bolisetty et al., 2012). Vertebrate ERVs were demonstrated to play significant roles in the development and physiology of their hosts (Reviewed in Frank and Feschotte, 2017, Current Opinion in Virology). ERV-encoded proteins syncytins are thought to be responsible for the evolution of the placenta in eutherian mammals; these proteins are derived from the envelope proteins of captured retroviruses and the original fusogenic properties of these proteins co-opted to promote cell–cell fusion and syncytium formation (Dupressoir et al., 2011). It is possible that its function in the cells of the developing chicken pectoral muscle involves mediating the fusion of myoblasts to form muscle fibres. Given that greater muscle development in mammalian males compared to females is thought to be entirely dependent on the levels of steroid hormones, it would be of interest to examine and compare the FET1 and syncytin B regulation in birds and mammals, respectively.

## Ethics approval

All experiments involving chicken embryos were performed in accordance with the rules and regulations of the Animal Ethics Committee of the University of the Witwatersrand, Johannesburg. Ethics approval number 2017/01/01/O.

## Authors' contributions

CL and NN developed the idea. CL and NN designed the experiments. CL performed all experiments. CL and NN analysed the data and wrote the manuscript. All authors read and approved the final manuscript.

## CRediT authorship contribution statement

**Chiron Loubser:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Natalya V. Nikitina:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Data curation, Conceptualization.

## Funding

This work is based on the research supported wholly by the National Research Foundation of South Africa (Grant Numbers: 105821) to NN. CL is a recipient of the NRF Scarce Skills Master's Scholarship.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2024.149006.

## Data availability

The sequencing data has been deposited to Mendeley Data Repository and is freely available under doi: 10.17632/sw497k9d6p.1

## References


Acién, P., Acién, M., 2020. Disorders of sex development: classification, review, and impact on fertility. J. Clin. Med. 9. https://doi.org/10.3390/jcm9113555.

Bolisetty, M., Blomberg, J., Benachenhou, F., Sperber, G., Beemon, K., 2012. Unexpected diversity and expression of avian endogenous retroviruses. mBio 3, 1–10. Doi: 10.1128/mBio.00344-12.

Bull, J.J., 1985. Sex determining mechanisms: An evolutionary perspective. Experientia 41, 1285–1296. https://doi.org/10.1007/BF01952071.

Buzala, M., Janicki, B., 2016. Review Review : Effects of different growth rates in broiler breeder and layer hens on some productive traits. Poult. Sci. 95, 2151–2159. https://doi.org/10.3382/ps/pew173.

Clinton, M., Haines, L., Belloir, B., Mcbride, D., 2001. Sexing chick embryos : A rapid and simple protocol. Br. Poult. Sci. 42, 134–138. https://doi.org/10.1080/713655025.

Clinton, M., Zhao, D., 2023. Avian sex determination: a chicken egg conundrum. Sex. Dev. https://doi.org/10.1159/000529754.

Devlin, R.H., Nagahama, Y., 2002. Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. Aquaculture 208, 191–364. https://doi.org/10.1016/S0044-8486(02)00057-1.

Dupressoir, A., Vernochet, C., Harper, F., Guegan, J., Dessen, P., Pierron, G., Heidmann, T., 2011. A pair of co-opted retroviral envelope syncytin genes is required for formation of the two-layered murine placental syncytiotrophoblast. Proc. Natl. Acad. Sci. 108, E1164–E1173. https://doi.org/10.1073/pnas.1112304108.

Frank, J.A., Feschotte, C., 2017. Co-option of endogenous viral sequences for host cell function. Curr. Opin. Virol. 25, 81–89. https://doi.org/10.1016/j.coviro.2017.07.021.

Gamble, T., Castoe, T.A., Nielsen, S.V., Banks, J.L., Card, D.C., Schield, D.R., Schuett, G. W., Booth, W., 2017. The Discovery of XY Sex Chromosomes in a Boa and Python. Curr. Biol. 27, 2148–2153.e4. https://doi.org/10.1016/j.cub.2017.06.010.

Hirst, C.E., Major, A.T., Ayers, K.L., Brown, R.J., Mariette, M., Sackton, T.B., Smith, C.A., 2017. Sex reversal and comparative data undermine the W chromosome and support Z-linked DMRT1 as the regulator of gonadal sex differentiation in birds. Endocrinology 158, 2970–2987. https://doi.org/10.1210/en.2017-00316.

Hocking, P.M., 2014. Unexpected consequences of genetic selection in broilers and turkeys: problems and solutions. Br. Poult. Sci. 55, 1–12. https://doi.org/10.1080/00071668.2014.877692.

Ioannidis, J., Taylor, G., Zhao, D., Liu, L., Idoko-Akoh, A., Gong, D., Lovell-Badge, R., Guioli, S., McGrew, M.J., Clinton, M., 2021. Primary sex determination in birds depends on DMRT1 dosage, but gonadal sex does not determine adult secondary sex characteristics. PNAS 118. https://doi.org/10.1073/pnas.2020909118.

Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., Loverll-Badge, R., 1991. Male development of chromosomally female mice transgenic for SRY. Nature 351, 117–121.

Lambeth, L.S., Cummins, D.M., Doran, T.J., Sinclair, A.H., Smith, C.A., 2013. Overexpression of Aromatase Alone is Sufficient for Ovarian Development in Genetically Male Chicken Embryos. PLoS One 8, 68362. https://doi.org/10.1371/journal.pone.0068362.

Lambeth, L.S., Raymond, C.S., Roeszler, K.N., Kuroiwa, A., Nakata, T., Zarkower, D., Smith, C.A., 2014. Over-expression of DMRT1 induces the male pathway in embryonic chicken gonads. Dev. Biol. 389, 160–172. https://doi.org/10.1016/j.ydbio.2014.02.012.

Major, A.T., Ayers, K., Chue, J., Roeszler, K., Smith, C., 2019. FOXL2 antagonises the male developmental pathway in embryonic chicken gonads. J. Endocrinol. https://doi.org/10.1530/JOE-19-0277.

Major, A.T., Smith, C.A., 2016. Sex Reversal in Birds. Sex. Dev. https://doi.org/10.1159/000448365.

Marchler-bauer, A., Derbyshire, M.K., Gonzales, N.R., Lu, S., Chitsaz, F., Geer, L.Y., Geer, R.C., He, J., Gwadz, M., Hurwitz, D.I., Lanczycki, C.J., Lu, F., Marchler, G.H., Song, J.S., Thanki, N., Wang, Z., Yamashita, R.A., Zhang, D., Zheng, C., Bryant, H., 2015. CDD : NCBI ' s conserved domain database. Nucleic Acids Res. 43, D222–D226. https://doi.org/10.1093/nar/gku1221.

Morris, K.R., Hirst, C.E., Major, A.T., Ezaz, T., Ford, M., Bibby, S., Doran, T.J., Smith, C. A., 2018. Gonadal and Endocrine Analysis of a Gynandromorphic Chicken. Endocrinology 159, 3492–3502. https://doi.org/10.1210/en.2018-00553.

Mullon, C., Wright, A.E., Reuter, M., Pomiankowski, A., Mank, J.E., 2015. Evolution of dosage compensation under sexual selection differs between X and Z chromosomes. Nat. Commun. 6, 7720. https://doi.org/10.1038/ncomms8720.

Nakamura, M., 2009. Sex determination in amphibians. Semin. Cell Dev. Biol. 20, 271–282. https://doi.org/10.1016/j.semcdb.2008.10.003.

Nikitina, N., Bronner-Fraser, M., Sauka-Spengler, T., 2009. Whole-mount in situ hybridization on lamprey embryos. Cold Spring Harb Protoc 2009, pdb.prot5125. Doi: 10.1101/pdb.prot5125.

Redelsperger, F., Raddi, N., Bacquin, A., Vernochet, C., Mariot, V., Gache, V., Blanchard-Gutton, N., Charrin, S., Tiret, L., Dumonceaux, J., Dupressoir, A., Heidmann, T., 2016. Genetic evidence that captured retroviral envelope syncytins contribute to myoblast fusion and muscle sexual dimorphism in mice. PLoS Genet. 12, 1–18. https://doi.org/10.1371/journal.pgen.1006289.

Reed, K.J., Sinclair, A.H., 2002. FET-1: A novel W-linked, female specific gene up-regulated in the embryonic chicken ovary. Mech. Dev. 119, 87–90. https://doi.org/10.1016/S0925-4773(03)00097-2.

Sarre, S.D., Georges, A., Quinn, A., 2004. The ends of a continuum: Genetic and temperature-dependent sex determination in reptiles. Bioessays 26, 639–645. https://doi.org/10.1002/bies.20050.

Sinclair, A.H., Berta, P., Palmer, M.S., Hawkins, J.R., Griffiths, B.L., Smith, M.J., Foster, J.W., Frischauf, A.-M., Lovell-Badge, R., Goodfellow, P.N., 1990. A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature 346, 240–244. https://doi.org/10.1038/346240a0.

Smith, C.A., Roeszler, K.N., Ohnesorg, T., Cummins, D.M., Farlie, P.G., Doran, T.J., Sinclair, A.H., 2009. The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. Nature 461, 267–271. https://doi.org/10.1038/nature08298.

Smith, C.A., Sinclair, A.H., 2004. Sex determination: Insights from the chicken. Bioessays 26, 120–132. https://doi.org/10.1002/bies.10400.

Vicoso, B., Emerson, J.J., Zektser, Y., Mahajan, S., Bachtrog, D., 2013. Comparative Sex Chromosome Genomics in Snakes: Differentiation, Evolutionary Strata, and Lack of Global Dosage Compensation. PLoS Biol. 11, e1001643.

Wolf, J.B., Bryk, J., 2011. General lack of global dosage compensation in ZZ/ZW systems? Broadening the perspective with RNA-seq. BMC Genomics 12, 91. https://doi.org/10.1186/1471-2164-12-91.

Zhang, X., Li, J., Wang, X., Jie, Y., Sun, C., Zheng, J., Li, J., Yang, N., Chen, S., 2023. ATAC-seq and RNA-seq analysis unravel the mechanism of sex differentiation and infertility in sex reversal chicken. Epigenetics Chromatin 16. https://doi.org/10.1186/s13072-022-00476-1.

Zhang, S.O., Mathur, S., Hattem, G., Tassy, O., Pourquié, O., 2010. Sex-dimorphic gene expression and ineffective dosage compensation of Z-linked genes in gastrulating chicken embryos. BMC Genomics 11, 13. https://doi.org/10.1186/1471-2164-11-13.

Zhao, D., McBride, D., Nandi, S., McQueen, H.A., McGrew, M.J., Hocking, P.M., Lewis, P. D., Sang, H.M., Clinton, M., 2010. Somatic sex identity is cell autonomous in the chicken. Nature 464, 237–242. https://doi.org/10.1038/nature08852.