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Sex differences on constitutive long non-coding RNA expression: Modulatory effect of estradiol and testosterone in muscle cells

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Abstract

Introduction: Despite the growing awareness of sexual dimorphism between males and females under pathological and physiological conditions, sex bias in biomedical research in animal models and patients is still present nowadays. The main objective of this work was to investigate sex differences in constitutive long non-coding RNA expression in spinal cord and skeletal muscle from wild-type mice.

Materials and Methods: To assess the influence of gender on long non-coding RNAs, we extracted RNA from tissues of male and female mice and analyzed the expression on nine long non-coding RNAs, selected for being among the most commonly studied or exerting an important role in muscle, at 50, 60, and 120 days of age.

Results and Discussion: We observed age- and tissue-dependent significant sex differences, being more prominent in skeletal muscle. We also studied the effect of sex steroid hormones on long non-coding RNA expression in vitro, noticing a modulation of long non-coding RNA levels upon estradiol and dihydrotestosterone treatment in muscle.

Conclusions: Taken together, results obtained evidenced sex differences on constitutive long non-coding RNA expression and suggested an influence of steroid hormones complementary to other possible factors. These findings emphasize the importance of including both sexes in experimental design to minimize any potential sex bias.

KEYWORDS
DHT, estradiol, hormones, lncRNA, sexual dimorphism, skeletal muscle, spinal cord

1 | INTRODUCTION

One of the hot emerging topics in recent years in biomedical research is long non-coding RNAs (lncRNAs), RNA molecules of more than 200 nucleotides in length that are not translated into protein. The main function of lncRNAs lies in gene regulation at different levels (splicing, translational, epigenetic modulation, and competing endogenous RNA networks), influencing a multitude of cellular processes. To date, lncRNAs have been reported in most model organisms and more than 30,000 of these transcripts have been identified both in human and mice.[1–3] Compared with mRNAs, lncRNAs have shown a stronger tissue-specific expression and function.[4] However, gender influence

in lncRNA expression has been barely studied, even in tissues with pronounced sexual dimorphism. This seems particularly striking considering that lncRNAs have been attributed a role in mammalian sex differentiation and the increasing number of research works highlighting the influence of gender on human disorders or major biological processes.[5,6]

In this sense, most of the few available studies that have approached this issue are focus on diseases such as cancer or multiple sclerosis, where gender is known to modify prevalence, pathogeny, age of onset, disease progression, or treatment efficacy.[7–10] In fact, under physiological conditions, only Xist has been widely reported to have a marked sexual dimorphism, which is essential for X chromosome inactivation and is expressed only in XX individuals.[11,12] However, a recent work has described sex differences on lncRNA expression in the liver of healthy mice, finding 375 sex-biased lncRNAs.[13]

In new fields of research such as lncRNAs, few studies address these possible differences between sexes. Understanding these differences can provide information about distinctive mechanisms in growth and development, dimorphism in fundamental biological pathways and could help to explain gender differences in response to treatment or in the display of pathologies.

The aim of this study was to investigate potential gender-associated differences in lncRNA expression at different ages under normal physiological conditions in mice. To address this question, the levels of some of the most widely studied lncRNAs were evaluated in skeletal muscle and spinal cord from wild-type mice.

Results evidenced that expression of lncRNAs differed as a function of sex, especially in muscle tissue. These distinctions were also age- and tissue-specific. Particularly, in muscle, remarkable differential expression of lncRNAs Hotair, Myoparr, and Meg3 was found at stages of sexual maturation (at 50 and 60 days of postnatal life) and of H19 and Malat1 upon transition to adulthood (at 60 and 120 days after birth). Of note, in spinal cord this pattern differed, pointing out relevant expression differences on Malat1 across all three studied ages and Gas5 being altered during sexual maturation. Further in vitro studies demonstrated a lncRNA-specific response upon androgen or estrogen treatment in skeletal muscle cells. This fact highlights the role of lncRNAs such as Hotair and Malat1, which showed a relevant response to hormone treatment, as potential key players in the sex steroid hormone molecular mechanisms regulating the physiology on this tissue.

2 | MATERIALS AND METHODS

2.1 | Animals

Wild-type B6SJL mice were purchased from Janvier Labs and maintained at the animal facilities of the Centro de Investigación Biomédica de Aragón in a pathogen-free environment and under a standard light/dark (12:12) cycle. Food and water were provided ad libitum. For more details on the mice employed in this research, see Table S1.

The care and use of animals were performed according to the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes. All experimental procedures were approved by the Ethic Committee for Animal Experiments from the University of Zaragoza and were registered with code numbers PI21/15 and PI08/19.

2.2 | Sample collection

Samples were taken from 10 to 12 sex-matched mice at different stages: 50, 60, and 120 days of postnatal life (P50, P60, and P120), as they correspond to the early adolescence, young adulthood, and fully mature adulthood stages, respectively (Figure 1A).[14] Sample collection was carried out upon CO_2 euthanasia. Spinal cord and quadriceps were isolated, frozen in dry ice and stored at −80°C until processed.

2.3 | Cell culture

Mouse C2C12 myoblasts were maintained in culture medium at 37°C and 5% CO_2. To obtain myotubes, cells at >85% confluence were grown with differentiation medium for 4 days.

To investigate the dose-dependent effects of steroid hormones on myoblasts, 50,000 cells per well were seeded in six-well plates and, after settle for 2 h, treated for 48 h with different concentrations of estrogens and androgens in treatment medium (TM) (Figure 2A). A total of 5 nM, 10 nM, 50 nM, and 100 nM concentrations were established for treatment with 17β-estradiol (E2) and 50 nM, 100 nM, 500 nM, and 1 μM concentrations were established for treatment with dihydrotestosterone (DHT). Hormones were added to the TM from a 1000-fold concentrated solution in 50% ethanol, 50% water, so that 0.05% ethanol is present in the medium.

Similarly, for myotubes, C2C12 were seeded at a concentration of 100,000 cells per well in 12-well plates, grown for 48 h and differentiated. Afterward, myotubes were cultured with differentiation treatment medium (DTM) supplemented with E2 or DHT for 48 h (Figure 3A). Hormone concentrations were the same as for myoblasts.

Composition of all mediums used for cell culture is provided in Table S1. Of note, to avoid the estrogen-like activity of phenol red, phenol red-free dulbecco's modified eagle medium (DMEM) (Gibco 31053) was used during hormone treatment; as well as charcoal stripped fetal bovine serum (FBS) to avoid possible hormone interference effect.[15] Moreover, for both myoblasts and myotubes experiments mock-treated controls were included, consisting of myoblasts or myotubes treated with 0.05% ethanol diluted in TM for myoblasts and DTM for myotubes. Besides, the experimental design included the study of *Gpx3* and *Pax7* genes as positive controls to the treatment with E2 and DHT, respectively (see Figure S1).[16,17] Six biological replicates were established per condition.

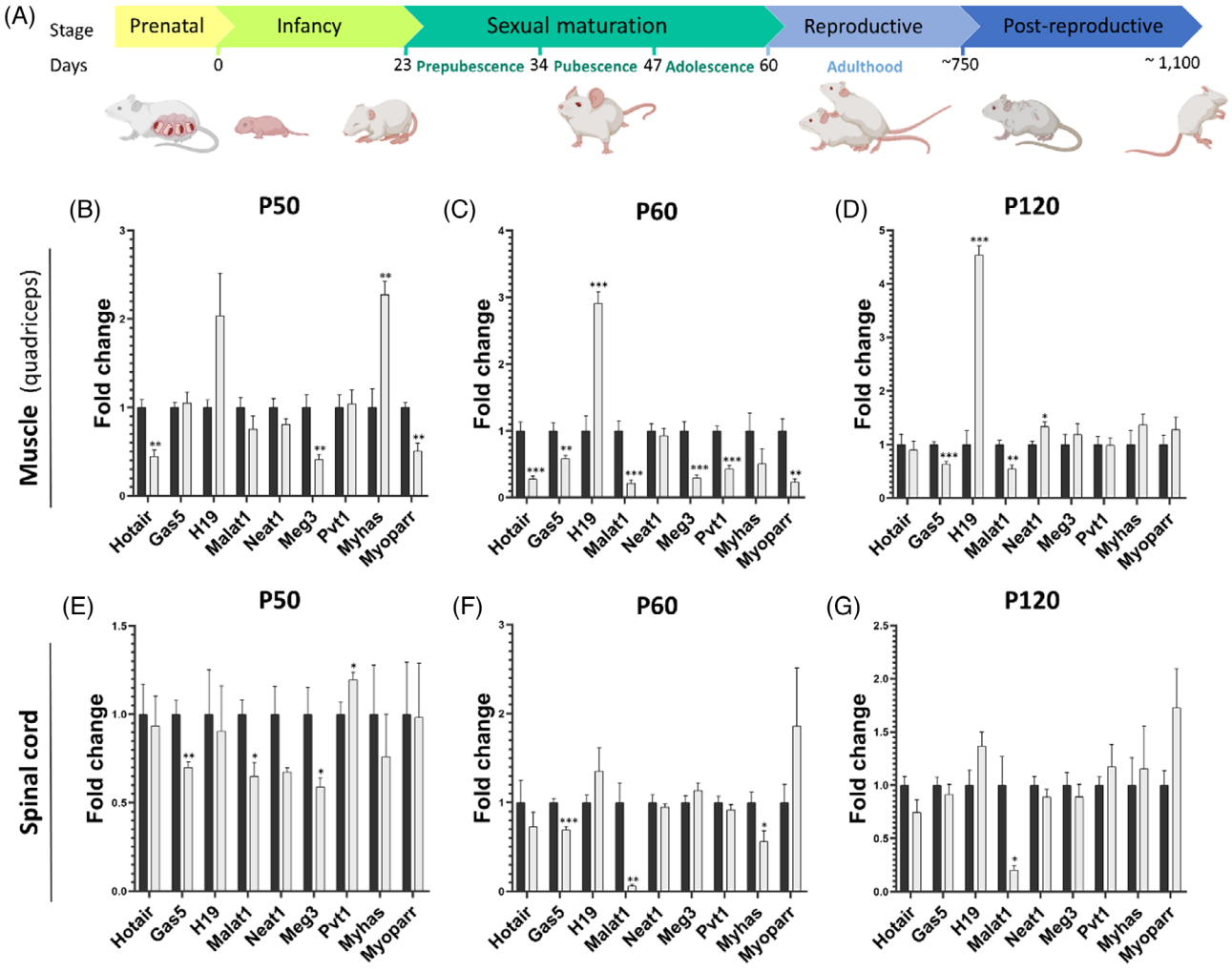

FIGURE 1 Constitutive expression of long non-coding RNAs (lncRNAs) in muscle tissue and spinal cord of male and female mice. (A) Sexual maturation stages in mice lifespan. Figure adapted from Brust et al. (B–D) Fold change expression of selected lncRNAs in quadriceps at postnatal P50 (B), P60 (C), and P120 (D). (E–G) Fold change expression of selected lncRNAs in spinal cord at postnatal P50 (E), P60 (F), and P120 (G). Values for females (in light gray) were referred to those obtained for its age-matched males (dark gray). Data are represented as mean ± standard error of the mean. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

2.4 | RNA extraction

For RNA extraction from tissue, spinal cord and skeletal muscle samples were homogenized in TRIzol Reagent using Tissue Lyser LT (Qiagen). For in vitro experiments, C2C12 cells were directly lysed in TRIzol Reagent. Total RNA was isolated using Direct-zol RNA MiniPrep Kit (Zymo Research), according to manufacturer's protocol.

2.5 | Real-time PCR

For quantification of lncRNA expression, cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Thermo Fisher Scientific). Reverse transcription quantitative PCR (RT-qPCR) was performed from diluted cDNA in triplicates using the Quant Studio 3 Real-Time polymerase chain reaction (PCR) Instrument from Applied Biosystems (Thermo Fisher Scientific). Raw qPCR results are detailed in Tables S2–S5.

Custom self-designed Sybr Green Primers (Thermo Fisher Scientific) that were used in this study are listed in Table S2. Primers were validated in silico with the national center for biotechnology information (NCBI) primer blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and experimentally with an agarose gel (Figure S2) and Sanger sequencing (Supporting Information 2—Sequencing).

The relative gene expression was determined by the 2-ΔΔCT method, using *Gapdh* and *Actb*, and *Gapdh* as housekeeping genes for normalization in in vivo and in vitro experiments.[18]

2.6 | Statistical analysis

Results are shown as the mean value ± the standard error of the mean. To establish significant differences between female and male groups in

in vivo expression studies, a Student's *t*-test was performed for each of the lncRNAs analyzed in the present work. For in vitro studies one-way ANOVA statistical test was used to compare between groups taking as reference mock-treated controls. Outliers for each lncRNA and group were identified by Grubb's test. The software used for the statistical analysis was GraphPad Prism version 8.0.1. Differences were considered as statistically significant if $^{**}p < 0.05$ and highly significant if $^{**}p < 0.01$ and $^{***}p < 0.001$.

3 | RESULTS

3.1 | Sex differences in lncRNA expression in the spinal cord and skeletal muscle in mice

To investigate whether lncRNAs expression is different between sexes, the levels of nine lncRNAs were assessed in nerve and muscle tissue of wild-type mice at different ages (Table 1). Specifically, lncRNAs Hotair, Gas5, H19, Malat1, Neat1, Meg3, Pvt1, Myhas, and Myoparr were evaluated in quadriceps and spinal cord of 50, 60, and 120 days old mice. All these lncRNAs are expressed in both tissues and were selected for being among the most well-studied lncRNAs, with more than 850 entries each in PubMed, and/or playing a role in regulation of skeletal muscle, tissue with a marked sexual dimorphism.[19]

Different age points helped to understand different stages of sexual maturation across mice lifetime: early adolescence (P50), young adulthood (P60), and fully mature adulthood (P120) (Figure 1A).

As a result, significant differences on lncRNA expression between males and females were observed. These differences were tissue and age dependent, being more evident in skeletal muscle (Figure 1B–D) as compared with spinal cord (Figure 1E–G). Particularly, all lncRNAs assessed in quadriceps showed different expression levels in females versus males at the studied ages. On the other hand, five out of the nine lncRNAs (Meg3, Malat1, Gas5, Myhas, and Pvt1) evaluated in spinal cord were differentially expressed in males versus females. Indeed, this result is consistent with the greater sexual dimorphism observed in skeletal muscle as compared with spinal cord.

Regarding age differences in spinal cord, most differences between male and female individuals were observed during adolescence and young adulthood stages (Figure 1E,F). As an exception, Malat1 was differentially expressed between females and males also in fully mature adulthood (Figure 1G). However, no such clear pattern was evident in skeletal muscle, although most differences could be observed at P60 (Figure 1C). Still, some lncRNAs like Hotair, Meg3, and Myoparr were differentially expressed in first stages, while others such as Gas5, Malat1, and Neat1 showed to have more relevance in mature adulthood.

Of note, when gender differences existed, females generally exhibited lower lncRNA expression as compared with males. As exceptions we found lncRNAs Myhas and Pvt1 at P50 in quadriceps and spinal cord, respectively (Figure 1B,E), lncRNA H19 (Figure 1C,D), and lncRNA Neat1 at P120 in quadriceps (Figure 1D).

It has been reported that expression of some lncRNAs can be regulated by estrogens and androgens.[20–27] Given these findings, we wondered whether these differences that we had observed might be due to the effect of these steroid hormones. Especially given that most of the differences in both tissues were found at P60, when steroid hormones begin to peak at higher levels in mice. Thus, we decided to investigate their impact on muscle lncRNA expression.

Marked sex differences in lncRNA expression in skeletal muscle prompted us to study in vitro the effect of treating C2C12 muscle cells with androgen (DHT) and estrogen (E2) steroid hormones.

TABLE 1 Long non-coding RNAs (lncRNAs) selected for the study.

LncRNA	PubMed entries	PubMed entries "name and skeletal muscle"
Hotair	1652	1
Gas5	867	1
H19	3897	74
Malat1	2154	28
Neat1	1228	9
Meg3	1237	38
Pvt1	892	3
Myhas/lnc-mg	6	5
Myoparr	6	6
LncRNA	49,620	483

3.2 | In vitro effect of hormone treatment on lncRNA expression in muscle

3.2.1 | Influence of hormones on lncRNA expression in myoblasts

Based on experimental outcomes, studied lncRNAs could be divided into four different groups according to the response to hormone treatment: lncRNAs up-regulated (1), down-regulated (2), both up- and down-regulated (3), and not significantly modified (viz., Myoparr, Figure 2K) (4) by E2 and DHT hormones.

The first group comprises Hotair and Pvt1 (Figure 2C,D), which increased their expression when myoblasts were treated with E2 hormone, as well as H19 (Figure 2E), which alternatively augmented with DHT treatment.

The second group consists of lncRNAs Neat1, Myhas, Xist, and Meg3 (Figure 2F–I). Again, the lncRNA concentration of some of them decreased upon E2 treatment (Neat1), or DHT treatment (Myhas and Xist). Only Meg3 concentration was reduced in the presence of both E2 and DHT.

Within the third group, Malat1 concentration in myoblasts was reduced in the presence of E2, while increased in the presence of DHT, showing opposite effects for both hormones (Figure 2J).

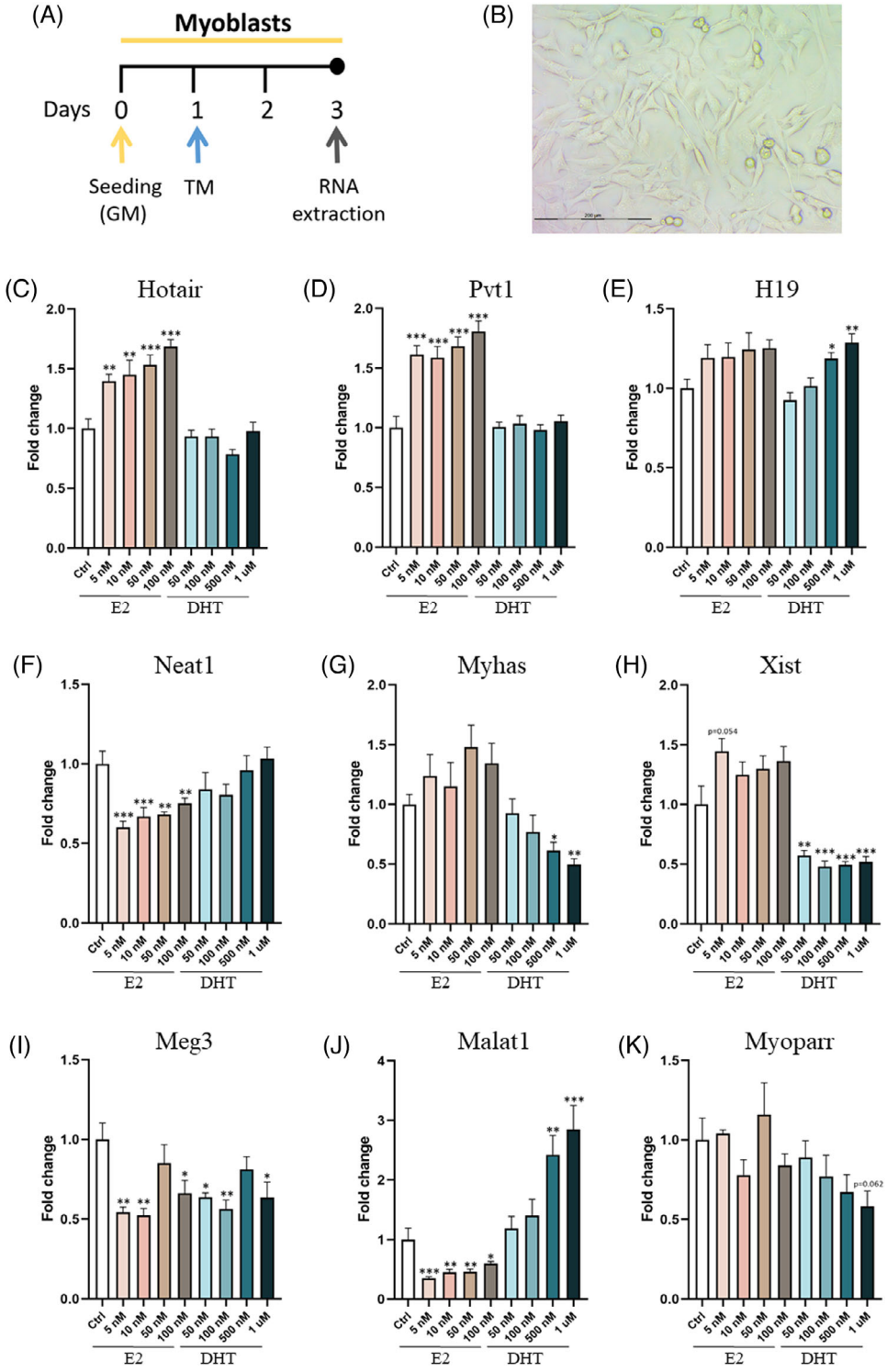

FIGURE 2 In vitro effect of female and male hormones on myoblasts. (A) Experimental design of hormone effect on undifferentiated C2C12 muscle cells. C2C12 cells were seeded and allowed to settle for 2 h. Afterward, cells were washed and cultured with treatment medium (TM) for 48 h. Finally, C2C12 myoblasts were harvested for quantitative PCR (qPCR) analysis. (B) Myoblast morphology. (C–K) Fold change expression of lncRNAs Hotair (C), Pvt1 (D), H19 (E), Neat1 (F), Myhas (G), Xist (H), Malat1 (I), Meg3 (J), and Myoparr (K) after treatment with 17β-estradiol (E2) or dihydrotestosterone (DHT) as compared with untreated controls (Ctrl). Gas5 expression in C2C12 cells was below qPCR detection threshold. Data are represented as mean ± the standard error of the mean. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. GM, growth medium.

As shown, the effect of E2 and DHT treatment on myoblasts can be highly variable in terms of lncRNA expression. However, results obtained suggest a possible modulatory effect of hormones on lncRNA expression levels in these precursor cells of muscle fibers. This modulatory effect would be different depending on the lncRNA and, in general (except for Myhas, H19, and Malat1 in DHT treatment), non-dose dependent.

3.2.2 | Influence of hormones on lncRNA expression in myotubes

Similar to the above, we found lncRNAs whose concentration was up-regulated in the presence of E2 (Hotair, Meg3, Pvt1, Neat1, and Myoparr, Figure 3C–G), DHT (H19, Figure 3H), or both hormones (Malat1 and Myhas, Figure 3I,J). Notably, in myotubes, only Xist concentration was down-regulated when exposed to one of these two steroid hormones (DHT), although it was also positively influenced by E2 (Figure 3K).

In light of results, we can conclude that sex steroid hormones modified lncRNA expression in myotubes. Interestingly, only three of the studied lncRNAs (Hotair, Pvt1, and H19) followed the same pattern in myoblasts and myotubes. This alternative trend involved in some cases a change in the hormones they respond to (such as Myoparr or Xist response to E2), a switch in effect direction (Neat1 or Malat response to E2), or both at the same time (Myhas and Meg3).

Although all lncRNAs showed a response to hormone treatment in myotubes (vs. eight out of nine in myoblast), in terms of dosage, a higher concentration of hormone was required to see a response at the expression level in five of the nine lncRNAs studied. This suggests a greater sensitivity in myoblast responsiveness to sex steroid hormones as compared with myotubes, in which this effect was generally independent of the doses tested.

4 | DISCUSSION

Multiple studies have demonstrated that males and females are morphologically, physiologically, and hormonally different, exhibiting distinct biological responses in both physiological and pathological situations.[28] This highlights the relevance of including both genders in experimental studies. Inclusion of female samples/individuals in basic, pre-clinical, and clinical research has largely increased in recent years, being essential, for example, to identify potential different gender responses to drugs.[29] However, many papers still use a single sex, do not specify the gender used in the experiments conducted or do not analyze both sexes separately. This is a misleading approach if we lack the understanding of these possible existing differences across genders that may be biasing our results.

Regarding lncRNAs, as mentioned above, only a few studies related to diseases in which gender had already been shown to modify associated pathology, age of onset, disease progression, or treatment efficacy have taken into account the possible influence of sex-dependence on lncRNA expression.

In this scenario, the present work studied constitutive lncRNA expression levels between sexes in quadriceps and spinal cord of healthy mice at different ages evidencing significant differences. These results are in agreement with previous works in liver conducted by Goldfarb and Waxman demonstrating sex-biased lncRNA expression in liver of mice and those referring Xist selective expression in females.[11–13]

Two very different tissues were included in this work: quadriceps and spinal cord. Muscle provided a tissue model with marked sexual dimorphism, whereas spinal cord served as a counterpoint. And, indeed, gender differences were observed in both tissues, with a greater number of distinctions in muscle, and these being tissue- and, in general, age dependent.

It is well known that molecular functions of lnRNAs underlie gene expression regulation. This leads to hypothesize that lncRNAs could be involved in the mechanisms that cause male and female morphological, physiological, and functional differences. This hypothesis is bolstered by the involvement of lncRNAs in development and cell differentiation, as well as the results shown above.

Thus, lncRNAs that have been found to be differentially expressed between male and female, for example, in muscle, could be involved in the regulation of fiber-type composition, mitochondrial content and energy metabolism, insulin sensitivity, protein synthesis and degradation (proteostasis), or muscle contraction.

In this sense, females have shown to present a slower fiber phenotype as compared with males.[30] Interestingly, H19, which was found up-regulated in female muscle, has been shown to promote slow fiber shift.[31]

As far as mitochondrial content and metabolism regulation concerns, Hotair and H19 have shown to play a role.[31,32] As so, nuclear OXPHOS gene expression is opposed by Hotair while enhanced by H19. According to the literature, skeletal muscle of females could have a higher oxidative phosphorylation capacity than males,[33] which is consistent with the lower and higher expression of Hotair and H19 in females here reported.

Lastly, H19 has shown to promote skeletal muscle sensitivity to insulin,[34] which is interesting taking into account the higher insulin-stimulated glucose uptake of skeletal muscle in females.[35] In addition, Malat1, Hotair, Gas5, and Meg3 have been related to protein turnover and autophagy.[36]

Likewise, lncRNAs could also play a role on the physiological differences existing between males and females in the central nervous system.

Furthermore, we hypothesized that the different features displayed by both sexes in this study could be mainly due to hormonal effect, because selected ages of animals in this study were carefully chosen to follow sexual maturation progress in physiological conditions, in which no influence of any disease took place. This idea was reinforced by the fact that in both tissues most of the differences were found at sexual maturation stages (P60) and by the studies that had previously

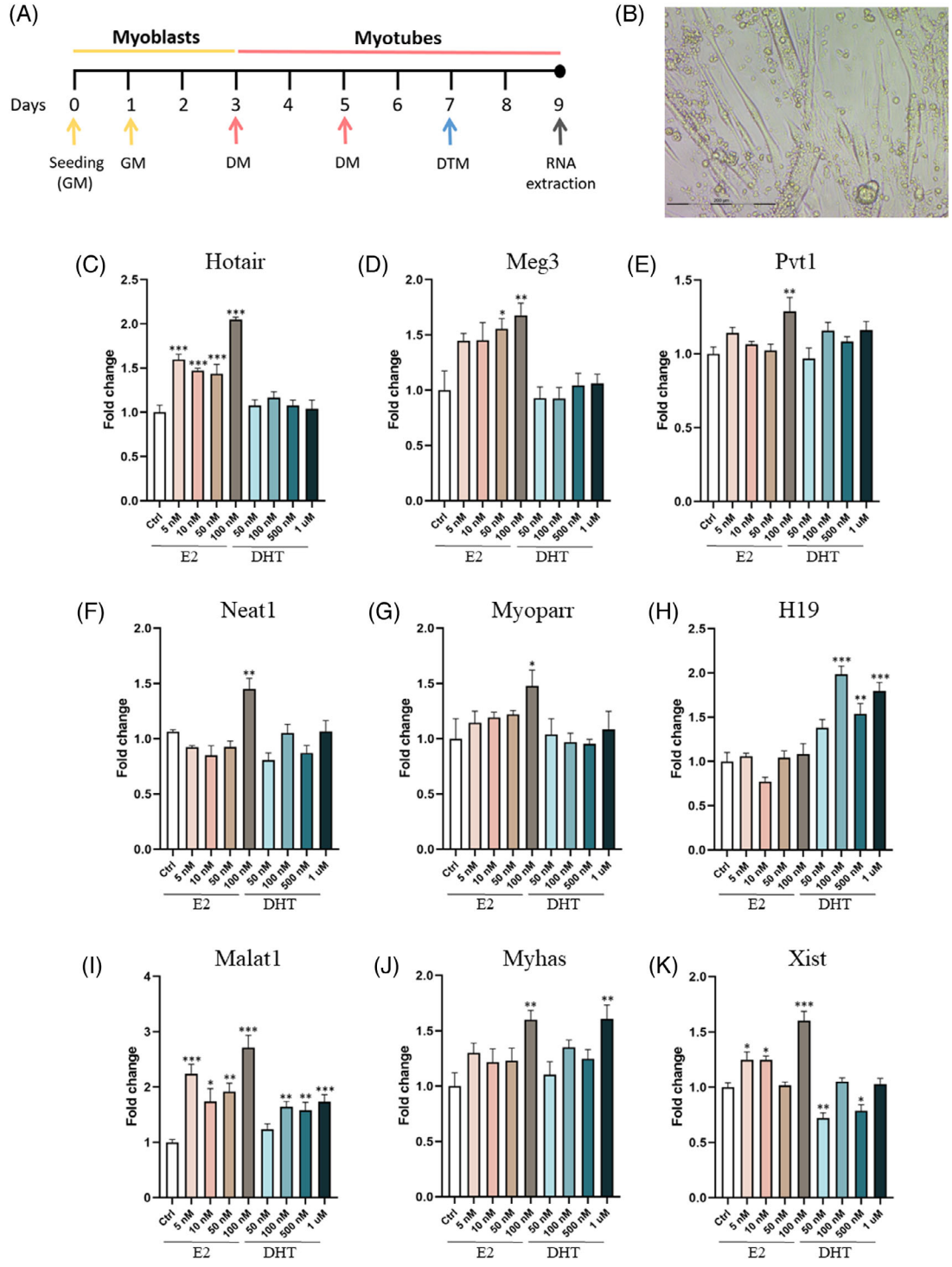

FIGURE 3 In vitro effect of female and male hormones on myotubes. (A) Experimental design of hormone effect on differentiated C2C12 muscle cells. C2C12 cells were seeded, allowed to settle overnight, and maintained in growth medium (GM) for 2 days. Myogenic differentiation was then induced from day 3 to day 7 by switching the cells to differentiation medium (DM). Day 7 myotubes were switched to differentiation treatment medium (DTM) for 48 h. Finally, C2C12 myotubes were harvested for quantitative PCR (qPCR) analysis. (B) Myotubes morphology. (C–K) Fold change expression of lncRNAs Hotair (C), Meg3 (D), Pvt1 (E), Neat1 (F), Myoparr (G), H19 (H), Malat1 (I), Myhas (J), and Xist (K) after treatment with 17β-estradiol (E2) or dihydrotestosterone (DHT) as compared with untreated controls (Ctrl). Gas5 expression in C2C12 cells was below qPCR detection threshold. Data are represented as mean ± standard error of the mean. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

TABLE 2 Comparative summary of outcomes obtained in vivo in quadriceps and in vitro in myotubes.

LncRNA	In vivo outcomes (quadriceps)	In vitro outcomes (myotubes)
Hotair	↓ in females at P50 and P60	↑ E2
Gas5	↓ in females at P60 and P120	Expression below detection threshold
H19	↑ in females at P60 and P120	↑ DHT
Malat1	↓ in females at P60 and P120	↑ E2, ↑ DHT
Neat1	↑ in females at P120	↑ E2
Meg3	↓ in females at P50 and P60	↑ E2
Pvt1	↓ in females at P60	↑ E2
Myhas	↑ in females at P50	↑ E2, ↑ DHT
Myoparr	↓ in females at P50 and P60	↑ E2
Xist	↑ in females, no expression in males[a]	↑ E2, ↓ DHT

Abbreviation: LncRNAs, long non-coding RNAs.
[a]Result widely documented in the literature, not included in Figure 1.

suggested that lncRNAs could be modulated by estrogens' and androgens' action.[20–27]

To study possible hormonal regulation, C2C12 muscle cells were subjected to treatment with E2 or DHT. Moreover, this study intended to differentiate between two different types of muscle cells: myoblasts (undifferentiated) and myotubes (fully differentiated). In light of results above presented, it can be concluded that steroid hormones influenced lncRNA expression, although the effect of E2 and DHT treatment on myoblasts and myotubes can be highly variable.

Myoblasts and myotubes are physiological, morphological, and functionally different. In this sense, myoblasts are the precursor cells of myotubes or, in other words, muscle fibers. They are present in muscle embryonic development and, in low proportion, during adult muscle regeneration. After muscle damage caused by trauma, disease, denervation or intense physical exercise, muscle satellite cells are activated and differentiate into myoblasts. These myoblasts will subsequently give rise to muscle fibers. For this reason, the response to this type of cell could be of interest especially when studying diseases or conditions that affect muscle as well as stages of embryonic development. Myotubes, on the other hand, would be the predominant cells in post-embryonic muscle and, therefore, are those that could be best compared with a healthy adult muscle in physiological conditions.

Notably, for most of the lncRNAs studied (Neat1, Meg3 Pvt1, Myhas and Myoparr), higher concentrations of hormone were required to see an effect in myotubes. Further sensitive response of lncRNA regulation to E2 and DHT hormones in myoblast could suggest a stronger involvement of this regulation pathway in muscle development and regeneration. This would be in line with the role of lncRNAs in embryonic development and myogenesis previously reported.[17,37]

As for results on myotubes, it was striking that in most cases the in vitro results were not in agreement with what would be expected based on in vivo findings (Table 2). For instance, Hotair levels increased with E2 treatment in C2C12 myoblasts and myotubes, while they were decreased in female mouse muscle when compared with male mice at P50 or P60. In this sense, only the effect of hormones on Xist and Neat1 seemed to be consistent with in vivo evidence. Xist response to hormones would also be consistent with its previously reported role as chromosome X-inactivator.[11,12] Hence, results obtained in this study suggest that the DHT hormone could contribute to Xist silencing in males to prevent repression of the single chromosome X (effect observed in myoblasts and myotubes, Figures 2H and 3K). Likewise, the results in myotubes give rise to a possible role of estradiol as an enhancer of Xist.

Importantly, an augmented response to E2 treatment has generally been noted. However, C2C12 is a female mouse-derived cell line, which could also limit responsiveness to DHT treatment.[38]

Alternatively, the observed influence of hormones on lncRNA expression might be related to the effect of hormones on muscle, rather than to the basal sex differences existing in the muscle. In other words, lncRNAs might also be acting as mediators of muscle response to DHT and E2, which includes protein synthesis, cell proliferation and adenosine triphosphate (ATP) production promotion (DHT), muscle mass and strength improvement (DHT and E2), and collagen content of connective tissue increase (E2).[39–41] This would explain, for example, that Malat1 increased in the presence of both DHT and E2, if it were related to the enhancement of muscle mass and strength in response to hormones.

Overall, as treatment of C2C12 with DHT and E2 alone did not explain the differences observed in quadriceps, it seems that other regulatory factors rather than steroid hormones would be playing a role in the different features observed in vivo between genders.

In this regard, lncRNA could be regulated by other mechanisms such as myokines, transcription factors (such as myogenic regulatory factors in muscle), and other hormones (i.e., the growth hormone), which also present a sex-biased behavior and may contribute to differences observed between in vitro and in vivo results. Additionally, other factors, including epigenetics, may also play a role. In fact, previous published works suggest that epigenetic modulation, such as DNA methylation or histone modifications, may contribute to the dimorphism observed in certain regions of the brain, heart, and innate immunity.[42–44]

5 | CONCLUSIONS

We can conclude that there are significant physiological differences in mouse lncRNA profile expression between sexes in quadriceps and spinal cord. These differences are in general tissue and age dependent. In addition, further studies will be needed to explore the molecular network leading to lncRNA differences between genders and the biological significance of these differences.

In particular, a better knowledge of lncRNA profile in mice should be taken into consideration especially when performing in vivo or clinical studies involving lncRNAs, so that sex-independent analysis or, at least, work with balanced sexes are included in the experimental design. Furthermore, this study opens the door to the study of lncRNAs in the

context of diseases in which gender differences, so far unexplained, are found.

AUTHOR CONTRIBUTIONS

Tresa López-Royo, Raquel Manzano, and Rosario Osta were implied in conceptualization of the article and devised experimental design. Laura Moreno-Martínez was in charge of maintenance and cross-breeding of mice. Tresa López-Royo, Laura Moreno-Martínez, Leticia Moreno-García, Ana Cristina Calvo, and Raquel Manzano collected animal samples and performed the experiments. Tresa López-Royo interpreted the data, performed the statistical analysis, wrote the main text, prepared tables, and produced figures. Raquel Manzano and Rosario Osta discussed and reviewed the manuscript. All the authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available within the article. Further information could be asked to the corresponding author upon reasonable request.

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