Correlation between estrogen plasma level and miRNAs in muscle of Piedmontese cattle

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Correlation between estrogen plasma level and miRNAs in muscle of Piedmontese cattle

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Abstract

A loss-of-function mutation of the myostatin gene has a very high prevalence in the Piedmontese cattle breed. The effect of such mutation is a double-muscle phenotype due to hypertrophy. However, differences in muscle mass development can still be detected in individuals of this breed. Such differences must be generated by other factors controlling skeletal muscle development. MicroRNAs are short non-coding RNA molecules that modulate gene expression at a post-transcriptional level. MicroRNAs have been demonstrated to be involved in skeletal muscle development and some of them are controlled by steroid hormone signaling. Data on estrogen signaling are lacking, while more studies have been carried out on the effect of androgens. We aimed at identifying putative estrogen responsive miRNAs that might be involved in skeletal muscle development. At a slaughterhouse, we collected both muscle samples from longissimus dorsi and blood samples. Blood 17β-estradiol concentration was assessed and RNA was extracted from muscle samples. The animals we sampled were divided in groups according to estrogen blood concentration and through qPCR expression levels of seven muscle related miRNAs were evaluated. We found that miR-26b ($p<0.01$), miR-27a-5p ($p<0.05$), miR-27b ($p<0.05$) and miR-199a-3p ($p<0.01$) were differentially expressed in experimental groups. Expression levels of miR-26b were approximately 2-fold lower in samples with a low blood estrogen concentration and the other miRNAs showed a tendency to increase their expression levels when blood estrogens were higher. The variations of the circulating concentrations of estrogens in Piedmontese cattle might influence muscle mass development through miRNAs and thus contribute to individual variability in a breed with a high prevalence of a myostatin point mutation.

Keywords: 17β-estradiol, cattle, microRNA, skeletal muscle

1. Introduction

MicroRNAs are a class of small (22 nt), noncoding RNA molecules which can regulate gene expression. MiRNAs can repress translation of mRNA by recognizing and pairing to a seed sequence at the 3'-UTR, but they can also promote complete degradation of target mRNA in case of perfect complementarity (1).

miRNAs modulate gene expression at a post-transcriptional level in many biological processes. They include, but are not limited to, cell cycle progression (2), apoptosis (3), stem cell maintenance and differentiation (4-6).

Many miRNAs play an important role in skeletal myogenesis and muscle regeneration. miR-1, miR-206 and miR-133 have been shown to have opposite effect on myoblasts: while the former two promote differentiation of satellite cells, high expression of miR-133 results in myoblast proliferation rather than differentiation (7,8).

The Piedmontese breed of the north-western part of Italy shows a muscular hypertrophy phenotype which has been attributed to a point mutation in the myostatin gene (9). This mutation has a high incidence in the Piedmontese bovine population and induces both muscular hyperplasia and hypertrophy. However phenotypic variations are still evident. Therefore, other factors must play an important role in determining muscle mass in adult individuals.

When comparing miR-206 expression in Piedmontese and Friesian cattle irrespective of sex, our group found no difference between the two breeds. However, when comparing just female Piedmontese and Friesian cattle, a statistically significant difference was found (10). This evidence points to a possible influence of sex steroids on muscle mass build up, possibly mediated by microRNAs.

The role of gonadal steroids in muscle development has been investigated in depth. Androgens are known to induce muscle hypertrophy (11). A recent study demonstrated that there is a correlation

between androgen stimulation and myostatin expression in satellite cells (12). However, when considering female individuals there is considerably less data on the effect of estrogen and progesterone on skeletal muscle growth. In post-menopausal women hormone replacement therapy (HRT) has a positive effect on skeletal muscle function, as shown by maintenance or increase of muscle size and performance during exercises (13). A partial explanation for this effect is that estrogen might act as a proliferative stimulus for myoblasts through activation of estrogen receptor α (ER-α) (14). Similarly in steers, supplementation of estrogen and trenbolone resulted in increased activation of muscle satellite cells which strengthen the concept that estrogens might directly influence skeletal muscle growth (14,15).

However, the mediators that are involved in this signaling pathways in skeletal muscle cells are not well known. We chose the Piedmontese breed as our model because of the high prevalence of the myostatin mutation to investigate whether estrogens, specifically 17β-estradiol, can modulate miRNAs involved in muscle differentiation and thus explaining phenotypic variations in this breed.

2. Materials & Methods

2.1 Animals

24 Piedmontese cows were used for this study. The age of the selected animals ranged from 4 to 13 years and were from farms belonging to Consorzio La Granda (Cuneo, Italy) located in the Piedmont region (north-western part of Italy).

The animals were slaughtered and processed for human consumption according to Italian regulations. At the time of slaughtering, blood samples and muscle samples from *Longissimus Dorsi* were taken. Muscle samples were stored in RNAlater solution (Ambion, ThermoFisher Scientific Inc., Waltham, MA, USA) for transport at the laboratory. Muscle samples were then minced, placed in fresh RNAlater, incubated overnight at 4°C and then stored at -80°C.

2.2 Health assessment of recruited animals

During slaughtering, uterus and ovaries were collected for each animal and they underwent a macroscopic post-mortem examination carried out by a veterinary clinician to assess the presence of developing follicles and their size, the presence of corpora lutea and the presence of macroscopic abnormalities (like cysts).

Only animals with no signs of aberrations and with signs of a regular reproductive activity were included in this study, that is where corpora lutea or developing follicles of normal appearance were detected.

2.3 RNA extraction and RT-qPCR

RNA extraction and reverse transcription was performed as previously described (16). Briefly, 50 to 100 mg of muscle tissue was homogenized with a TissueLyser II (QIAgen, Venlo, Netherlands) in 1 ml TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), total RNA was purified and quantified by

spectrophotometry. Residual DNA was removed with DNAse I Recombinant RNAse-free kit (Roche, Mannheim, Germany) as per manufacturer instruction. For estrogen receoptor (ER) -α and -β analysis 0.5 μg RNA was reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). For miRNA analysis, 100 ng of RNA was reverse transcribed with TaqMan microRNA reverse transcription kit (ThermoFisher Scientific Inc.) using miRNA specific primers from TaqMan microRNA assays (ThermoFisher Scientific Inc.).

Quantitative PCR was subsequently performed using SsoAdvanced Universal SYBR green Supermix (Bio-Rad) for ER-α and β and TaqMan Universal PCR Master Mix for miRNA analysis on a CFX Connect instrument (Bio-Rad).

Primers for ERα were: 5'-AGGGAAGCTCCTATTTGCTCC-3' (forward) and 5'-CGGTGGATGTGGTCCTTCTCT-3' (reverse), based on NCBI sequence NM_001001443.1.

Primers for ERβ were: 5'- GCTTCGTGGAGCTCAGCCTG-3' (forward) and 5'-AGGATCATGGCCTTGACACAGA-3' (reverse), based on NCBI sequence NM_174051.3

Primer efficiency was calculated with CFX Manager (Version 3.0, Bio-Rad) using the linear regression slope of a sample dilution series. For ERα efficiency was 94.5%, for ERβ efficiency was 95.8%.

For mature miRNA detection and quantification, primers and probes were from the respective kits of TaqMan MicroRNA assays (ThermoFisher Scientific Inc.).

Detected mature miRNA sequences were the following: miR-1 – UGGAAUGUAAAGAAGUAUGUAU; miR-26a – UUCAAGUAAUCCAGGAUAGGCU; miR-26b – UUCAAGUAAUUCAGGAUAGGUU; miR-27a-5p – AGGGCUUAGCUGCUUGUGAGCA; miR-27b – UUCACAGUGGCUAAGUUCUGC; miR-206 – UGGAAUGUAAGGAAGUGUGUGG; miR-199a-3p – ACAGUAGUCUGCACAUUGGUUA; miR-16 – UAGCAGCACGUAAAUAUUGGCG. The analyzed sequences are bovine specific as reported in miRCarta v1.1 database but with a high homology with the corresponding human

miRNAs (sequence homology is 100% for analyzed miR-1, miR-26a, miR-27a-5p, miR-27b, miR-206 and miR-199a-3p. Sequence homology is 95.5% for miR-26b and miR-16).

Expression of ER-α and β was normalized using Hypoxantine Phosphoribosyl-transferase (HPRT), while microRNA expression was normalized using miR-16 as housekeeping gene.

Relative quantification was done using the 2-ΔΔCq method (17) and results were expressed as fold changes compared to the low estrogen group.

2.4 Quantification of 17β-estradiol

Serum was recovered from blood samples taken at slaughtering and then frozen at -80°C. Serum samples were then shipped to Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER, Brescia, Italy) where 17β-estradiol concentration was assessed through RIA.

2.5 Statistical analysis

Age of the animals (months) in the three experimental groups was compared using a Kruskal-Wallis non parametric test.

Data from Q-PCR were obtained as quantification cycle values (C_q). Mean C_q values were then normalized using housekeeping genes (HPRT1 for steroid hormone receptors, miR-16 for microRNAs) to calculate a ΔC_q value. Statistic elaboration was performed by using ΔC_q values. Values from the low estrogen group were used as reference to calculate fold changes in miRNA expression among groups by applying the $2^{-\Delta\Delta Cq}$ formula. Differences among groups were assessed using a Kruskal-Wallis non parametric test followed by Dunn's multiple comparison post-hoc tests.

All data comparison were done using IBM SPSS Statistics v24 (International Business Machine Corp., New York, USA). Differences were considered statistically significant when $p \leq 0.05$.

3.Results

3.1 Estrogen signaling in sampled animals

17β-estradiol blood concentration was determined for each animal sampled. Cows were then assigned to one of three groups according to concentrations. Groups were i)less than 10 pg/ml (low estrogen, n=10), ii) between 15 and 30 pg/ml (mid estrogen, n=9) and iii) over 40 pg/ml (high estrogen, n=5).

Age was compared among the three experimental groups and no significant differences were found [Supplementary Fig.1].

The expression levels of ER-α and -β in sampled muscle were compared by Q-PCR among the three groups and no statistically significant difference was found.

3.2 miRNAs expression levels

The expression levels of selected miRNAs were evaluated in all samples. Chosen miRNAs were miR-1, miR-26a, miR-26b, miR-27a-5p, miR-27b, miR-206 and miR-199a-3p [Fig.1].

No statistical difference was found among experimental groups for miR-1, miR-26a and miR-206.

A statistically significant difference was found in miR-26b expression among groups ($p=0.0025$) and more specifically low estrogen group had lower miR-26b levels when compared to mid (2.4 fold decrease) and high estrogen (2.2 fold decrease) groups.

MiR-199a-3p expression was found to be statistically different among groups ($p=0.005$) and had a statistically significant higher expression in the mid estrogen group than in the low estrogen group (3.4 fold increase), but no difference was found to be statistically significant between low and high estrogen groups and the mid and high estrogen groups.

A statistically significant difference in miR-27a-5p and miR-27b was found among groups (p=0.03 for both microRNAs) and post-hoc test showed a statistically significant difference only between low and mid estrogen groups.

4.Discussion

Myostatin mutations have been widely described as responsible for muscle hypertrophy in several bovine breeds. However muscle mass variations can be seen in individuals of the same breed as such a phenotype must be controlled by multiple factors with a variable importance. The role of microRNAs in muscle development has been explored in several species and miRNA families have been identified as capable of mediating differentiation of myoblasts, activation of satellite cells and promote fusion of these cells with mature myofibers (18).

Several miRNA candidates were chosen according to their expressions in muscle cells and to their susceptibility to 17β-estradiol stimulation as found by other research groups.

The candidates we examined included well known and specific regulators of muscle development such as miR-1. This miRNA is involved in myoblast differentiation, in suppression of proliferation of precursor cells and in promoting cell fusion (19). Since in literature no estrogen response elements are reported in the promoter region of miR-1, a direct effect of 17β-estradiol was not expected, but miR-1 expression levels were evaluated nonetheless to explore the possibility that indirect mechanisms of actions could take place. In MCF7 breast cancer cells a modest decrease in miR-1 levels was shown when cells were exposed to estradiol (20).

MiR-26a and miR-26b expression has been previously shown to be down-regulated by estrogen stimulation in breast cancer cells. Such a modulation results in an increased proliferation (21). However, these results were obtained in human cell lines derived from breast ER^+ tumors exposed to 17β-estradiol concentrations that were much higher than what we measured in the blood of the cows

we sampled. The effect of physiological concentration of estrogens on muscle miR-26a and miR-26b might depend on differentially activated pathways. In skeletal muscle, knockdown of miR-26a increases proliferation of Pax7-positive satellite cells and high expression levels of miR-26a are required to promote differentiation of satellite cells (22).

High miR-27a and miR-27b expression levels are necessary for satellite cells differentiation: down-regulations of these miRNAs by Pitx2 overexpression results in a blocked differentiation and in increased proliferation of precursor cells (23). This effect is achieved through a repression of Pax3 by miR-27b (24). We also have previously shown that miR-27b is able to target and downregulate myostatin in Piedmontese cattle (16).

MiR-206 belongs to the same family as miR-1 and due to a similar, but not identical sequence, they share some targets. MiR-206 has been shown to promote myoblasts differentiation and to prevent their proliferation in an in vitro model using C2C12 cells (25). We also recently demonstrated that bovine satellite cells strongly up-regulates miR-206 during in vitro differentiation into myotubes (26). Moreover in MCF7 breast cancer cells, miR-206 and ER-α are part of a regulatory feedback mechanism, in which miR-206 can decrease ER-α and in turn ER-α can decrease miR-206 expression levels (27). The effect of miR-206 overexpression in ER-α-positive ovarian cancer cells is a proliferation inhibition.

MiR-199a-3p is part of the mir-199a/214 cluster. The expression levels of these miRNAs have been reported to be reduced by administration of 17β-estradiol in ovariectomized mice and to be increased by progesterone (28). In a dystrophic zebrafish model, increased expression of miR-199a-5p was associated with disruption of the normal myogenic differentiation during development by affecting WNT signaling, pointing to an important role for this miRNA in a normal differentiation pathway (29).

Using these data as starting point, we decided to assess in skeletal muscle of Piedmontese cattle whether physiological variations in circulating 17β-estradiol levels could modulate miRNAs known

to participate in muscle homeostasis. Bovines are a species in which estrogen plasma levels are low (in the range of pg/ml), so we expected small variations in blood concentrations to have a detectable effect on several miRNAs.

Estrogens have a variable plasma concentration according to the stage of the estral cycle the bovines are in. More specifically their concentration increases during the proestral phase up to the estrus and then abruptly declines.

No differences were found in the expression of both estrogen receptors (α and β) among the experimental groups, suggesting that no changes in hormone sensitivity were taking place. When miRNAs expression was examined, we found that increasing estrogen levels were able to slightly increase the expression of miR-26b and induced a tendency to increase miR-199a-3p, miR-27a-5p and miR-27b. Given the role these miRNAs play in skeletal muscle development, estrogen seems to act to promote differentiation and to inhibit cell proliferation in vivo in skeletal muscle of beef cattle and more specifically in the Piedmontese breed. Since the reproductive activity is cyclical the skeletal muscle is exposed to fluctuating concentration of 17β-estradiol. MiRNAs might then mediate a similar cyclical activity in skeletal muscle cells, by either promoting proliferation or differentiation of satellite cells during the estral cycle.

We detected small variations in response to fluctuations in estrogen serum concentrations, which could have a limited biological significance in determining muscle mass development. However, in breeds such as the Piedmontese one where mutated myostatin has a prevalence above 98%, such small changes might play an important role to determine differences among individuals.

Acknowledgements

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Figure Caption

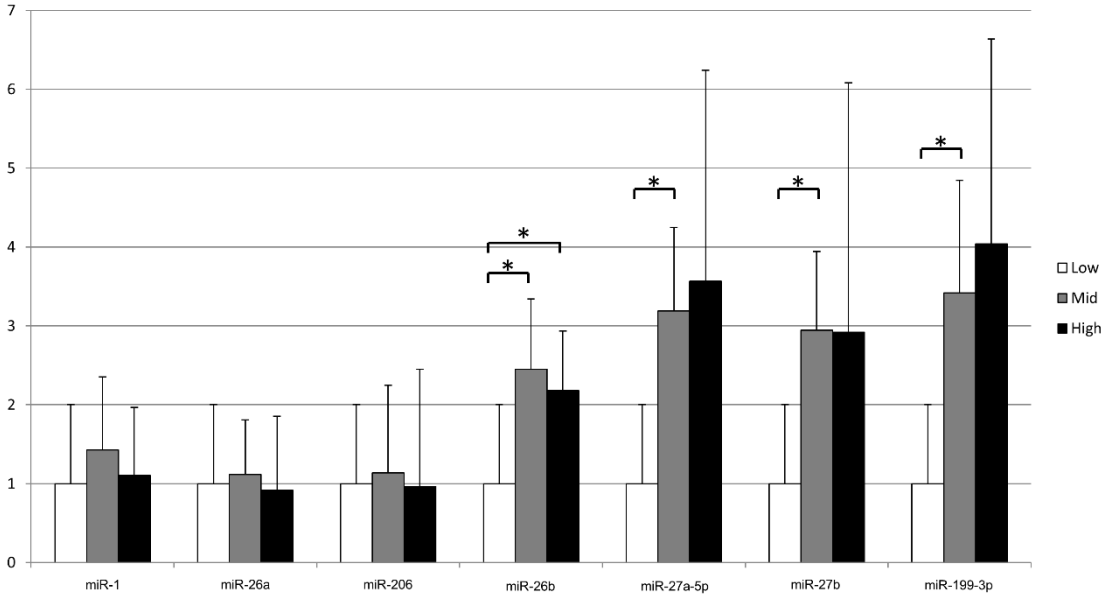
Fig.1 miRNAs expression levels in bovines with different estrogen serum concentration

Graph showing the expression levels of selected miRNAs as fold changes among bovine grouped according to their 17β-estradiol serum concentration. Low, less than 10 pg/ml (n=10); mid, between 15 and 30 pg/ml (n=9); high, more than 40 pg/ml (n=5). Brackets indicate where the difference is statistically significant ($p<0.05$).

Supplementary Fig.1 Age comparison among experimental groups

The graph shows the distribution of the age of the animals grouped by 17β-estradiol serum concentration.

Fig.1



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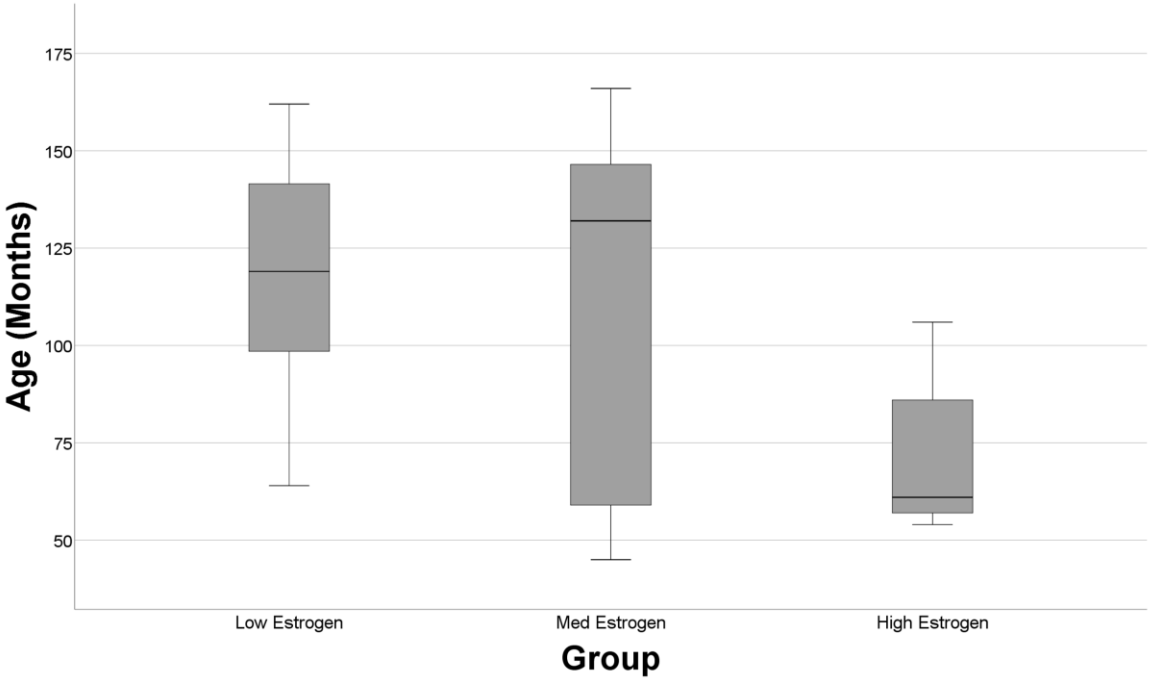
Supplementary Fig.1



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