THE STUDY OF microRNA IN PLASMA TO DISCOVER BIOMARKERS IMPORTANT FOR MUSCLE GROWTH IN BEEF CATTLE

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

The Study of microRNA in Plasma to Discover Biomarkers
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Producers are constantly looking for ways to improve muscle growth in cattle such that both the quantity and quality of beef can be increased. One approach is to examine expression of microRNAs as a potential selection tool. MicroRNAs are small RNA molecules that have regulatory functions in all metazoans. MiR-133b and miR-27a are known to affect muscle development through the IGF-1 pathway and their effect on the myostatin gene, respectively. Current research in humans has investigated microRNAs as biomarkers for diagnostic purposes. In the current experiment, we quantified the expression of miR-27a and miR-133b in order to evaluate their potential association with muscle growth traits. At approximately 12 month of age, 44 crossbred steers received hormone implants and 44 were not given implants. Plasma was collected from the steers starting from day 0 and in 28 day intervals for 5 months. From the original collection, the plasma samples from 51 steers (28 implanted steers, and 21 control steers) were used for expression analyses. Hemolyzed samples were discarded from the experiment. MicroRNAs were extracted from the plasma, and the expression of the microRNAs was measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) on the

Fluidigm Biomark HD platform. The amount of weight gained from Day 0 until harvest was determined for each steer, and then evenly separated into groups (high-gain and low-gain). The expression of miR-27a and miR-133b was compared between high gain steers and low gain steers. A difference in miR-133b and miR-27a expression was observed in the months of April and August, where an increase in miR-133b and miR-27a expression was observed in the low-gain groups.

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NOMENCLATURE

miRNA	microRNA
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RQ	Relative Quantification

CHAPTER I

INTRODUCTION

In order to meet consumer demands, producers look for strategies to not only increase the amount of muscle found on beef cattle, but also ways to ensure that the production of meat that is tender, juicy, and highly palatable. In order to increase yield, producers will utilize tools that may suppress protein degradation or increase protein synthesis. An extreme example is the production of double muscled cattle. These cattle have a mutation in the myostatin gene, causing a significant increase in muscle mass compared to the average (McPherron and Lee, 1997). It has been found that the suppression of protein degradation causes a decrease in meat tenderness, while increased protein synthesis actually has no effect on meat tenderness (Koohmaraie *et al.*, 2002). Furthermore, it appears that the cattle with the suppressed myostatin gene produce meat that is much more tender than their counterparts (Koohmaraie *et al.*, 2002).

Recently, miRNAs have become of interest for use in identifying the potential of cattle. MicroRNAs are small RNA molecules (around 22-27 nucleotides long) that regulate protein synthesis in the body (Ambros, 2004). Since their first discovery about 20 years ago, it has been discovered that miRNAs provide a variety of functions, such as protection against viral infection, securing genome stability, and repressing protein synthesis (Daneholt, 2006). Recent research has looked into the use of microRNA's found in blood plasma as biomarkers for conditions such as cancer (Tsujiura *et al.*, 2010) and Alzheimer's (Kiko *et al.*, 2014).

Several microRNAs are known to be linked to muscular hyperplasia and muscular hypertrophy. MiR-133 is found in both cardiac and skeletal muscle, and is known to downregulate the insulin-like growth factor, a positive signal pathway that causes muscle growth by increasing protein synthesis and decreasing protein degradation (Wang, 2013). It acts by binding to the 3' UTR region of IGF-1R, leading to a downregulation of the phosphorylation of Akt, which is the central mediator of the insulin-like growth factor (Huang *et al.*, 2011). It has been found that miR-133 increases myoblast proliferation and decreases myoblast differentiation (Huang *et al.*, 2012). Also, both miR-133a and miR-133b have been found to be upregulated during myogenesis (Feng *et al*., 2013). Recent studies have been trying to find miR-133's role in muscle cancers and diseases, and to see if it could be used for therapy (Yu *et al.*, 2014).

Mir-27a has been found to increase muscle cell proliferation by directly inhibiting myostatin (Huang *et al.*, 2012), and that a downregulation of this miRNA leads to muscle atrophy (Wang, 2013).

CHAPTER II

METHODS

Animal Care and Handling

Animal handling and sample collection procedures were approved by the Texas A&M University Animal Care and Use Committee (AUP #2008-234). In this study, a group of 88 F_3 Angus x Nellore steers were used that were approximately 12 months old when the first sample was collected. Implants were applied to 44 of the steers at approximately 12 months of age. The implants used were Component TE-S with Tylan (Elanco, Greenfield, IN, USA). The implants contained 24 mg of estradiol, 120 mg of trenbolone acetate, and 29 mg tylosin tartrate. Weights for the steers were measured every 28 days beginning at the first day the implant was added (Day 0), and continuing until immediately prior to harvest.

Plasma Collection and Processing

Blood was collected from the group of 88 steers every 28 days over a period of 7 months. The samples were collected using Vacuette® 18 ga x 1.5 inch multiple sample needles (PN 450048, Greiner Bio-One North America, Monroe, NC, USA) by venipuncture and put into 10 ml BD Vacutainer® tubes containing K_2-EDTA (PN 366643, BD Diagnostics, Franklin Lakes, NJ, USA). The tubes were gently inverted 10 times immediately after collection as directed by the manufacturer. The blood was processed as soon as possible, except for the first (April) and second (May) sample collections, where the blood was processed 1 and 2 days following collection, respectively.

The blood was processed by centrifugation at 1300 x *g* for 10 minutes,. Next, the plasma was removed with care in order not to disturb the buffy coat, and transferred to clear, 1.5 mL microcentrifuge tubes, at 500 µL aliquots and stored at -80°C.

Isolation and Quantitative RT-PCR

Except when noted otherwise, all the procedure used during miRNA synthesis and qRT-PCR followed manufacture's protocols (Exiqon, Inc., Woburn, MA, USA). The miRNAs were extracted from 500 µL of plasma with the miRCURY ^{TM} RNA-Isolation Kit-Biofluids (Exiqon). The miRNA's were quantified using the Qubit® microRNA Assay Kit on the Qubit® 2.0 fluorometer. However, the RNA volume input was not adjusted to normalize RNA mass. Universal cDNA Synthesis Kit II (Exiqon) was used to perform reverse transcription. The qPCR reaction cocktail (10 µL) was compromised of 1X ExiLENT SYBR® Green Master Mix (Exiqon), 1X ROX (Life Technologies, Carlsbad, CA), 1 µL of primer mix, and 2 µL of diluted template cDNA. Amplification was performed in the ABI 7900HT real-time thermal cycler in "9600 Emulation" mode (Applied Biosystems, Inc., Foster City, CA).

Data Analysis

The cattle were sorted based on how much weight they gained during the time that data was collected (from Day 0 to Harvest). The median value for weight gain was then determined, and the cattle that gained more weight than the median were considered "high gain", while those that gained less weight than the median were labeled as "low gain". The steer that had the median value was not included in the data analysis. The relative expression quantities (RQ) were calculated using the relative quantification method as described by Livak and Schmittgen (Livak

and Schmittgen, 2001). The calibrator that was used was for this experiment was the sample collected in August from steer 160.

Samples that reached the threshold past 35 cycles or failed to reach the threshold before the end of the analysis were changed to 45 in order to make the RQ value 0. Furthermore, any obvious outliers were removed from the data. The standard error for each group was determined and presented using standard error bars. A two-tailed t-test was used in order to determine statistical significance.

CHAPTER III

RESULTS AND DISCUSSION

Expression of miR-133b

The expression of miR-133b in the plasma samples throughout the experiment is illustrated in **Figure 1**. There was a statistically significant difference (P<0.05) between groups in April and August (denoted by *). In both the month of April and August, the Low Gain group had a significantly higher expression of miR-133b when compared to the High Gain group.

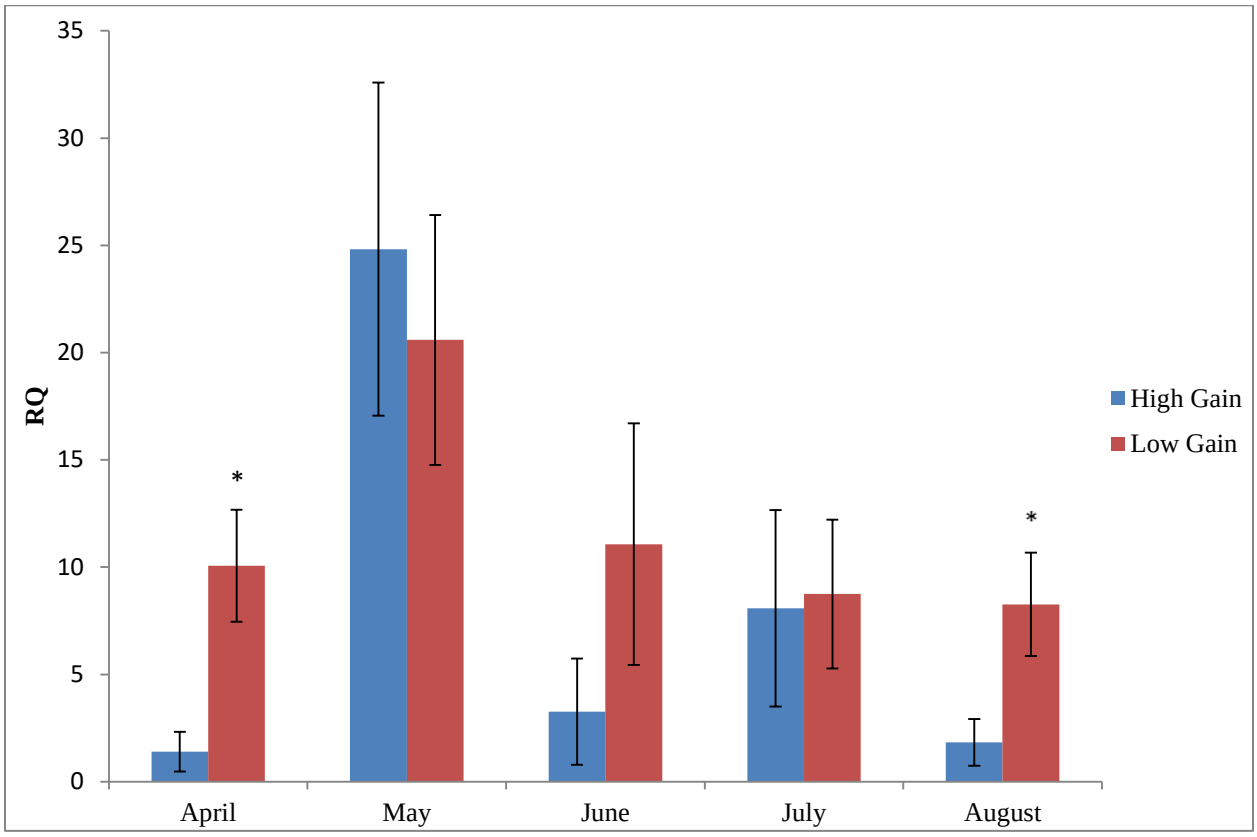

Figure 1. *Plasma miR-133b Expression in Steers Based on Day 0 to Slaughter Weight Gain.(* denotes P<0.05).*

Expression of miR-27a

The expression of miR-27a in the plasma samples throughout the experiment is illustrated in **Figure 2**. There was a statistically significant difference (P<0.05) between groups in April and August (denoted by *). The Low Gain group in both the months of April and August experience a higher expression of miR-27a when compared to the corresponding High Gain group.

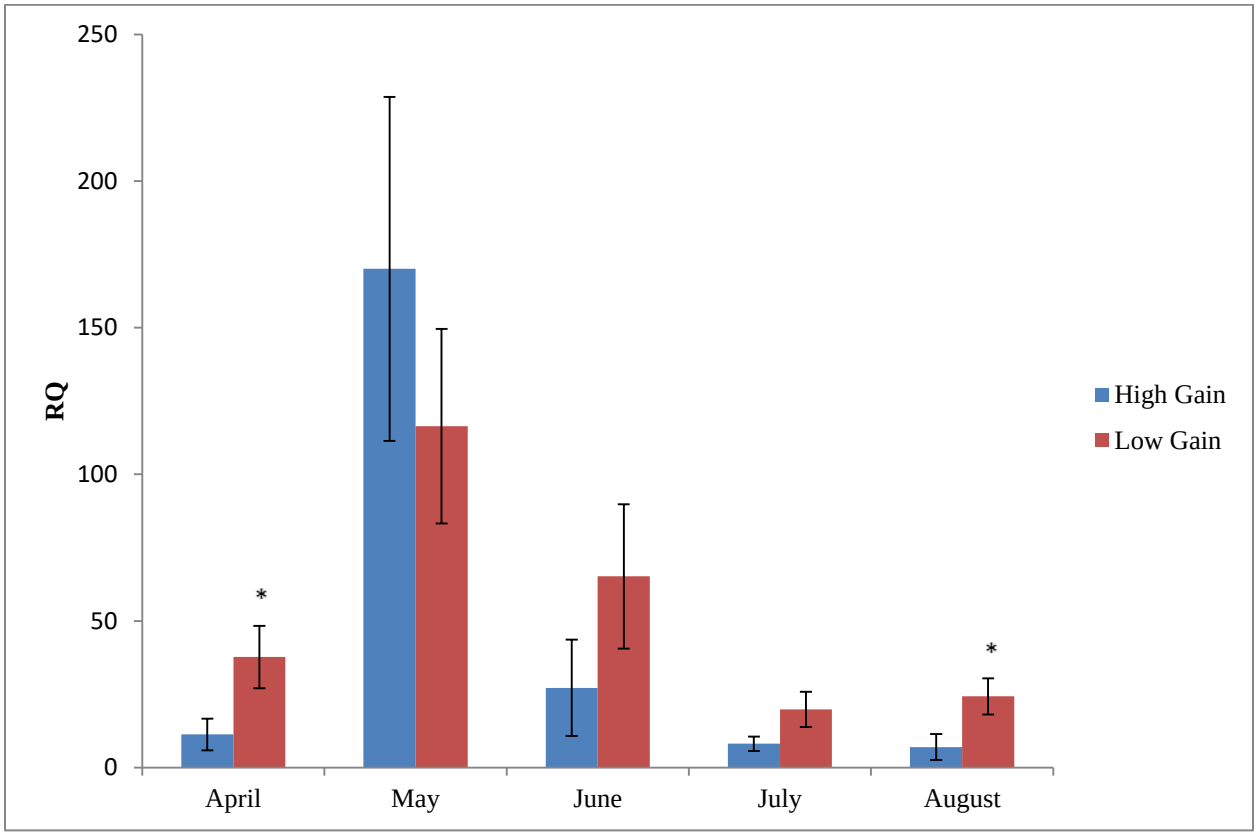

Figure 2. *Plasma miR-27a Expression in steers based on Day 0 to Slaughter Weight Gain (* denotes P>0.05).*

Discussion

It was noted that there was a significant increase in expression levels for both miR-27a and miR-133b in both the High Gain group and the Low Gain group during the month of May. This increase can may be attributed all of the steers entering a growth phase as cattle were placed on feed.

During the months of April and August, the expression level of miR-133b in the Low Gain group was significantly higher than that of the High Gain group. One of the functions for miR-133b is its role in downregulating IGF-1 expression by regulating IGF- 1 receptors (IGF-1R) (Huang *et al.*, 2011; Wang, 2013). The receptors are regulated by miR-133b due to it's ability to bind to the 3'UTR of IGF-1R (Huang *et al.*, 2011). This decrease in IGF-1R causes a decrease in the phosphorylation of Akt, which ultimately affects the PIK3/Akt signaling pathway, which is an integral to skeletal muscle development and growth. (Huang *et al.*, 2011). The IGF-1 protein is very important for skeletal muscle growth and development. IGF-1 has been identified as an important component for anabolic pathways that are involved in skeletal muscle cells (Musarò *et al.*, 2001). Administering IGF-1 to cultured muscle cells has been shown to stimulate cell proliferation and then myogenic differentiation (Engert *et al.*, 1996). The addition of a locally expressed isoform of IGF-1 has been shown to decrease muscle degeneration in mice (Musarò *et al.*, 2001). Therefore, regulating the IGF-1 receptor has effects on skeletal muscle growth and development. The results from this experiment are consistent with previous results that indicate miR-133b ultimately has a negative effect on muscle growth. The higher expression of miR-133b in the Low Gain group when compared to the High Gain group during April and August could be an indication of its increased expression and role in the animal during these months. Furthermore, the differing levels of expression throughout the months could indicate differing amounts of growth that is occurring at the time. Further experiments could include measuring the IGF-1 and IGF-1R levels and activity in plasma, in order to observe how they relate to the levels of miR-133b. Also, the animals' growth during times of differing miR-133b expression could be examined in order to observe how specific times of high growth and low growth alter the plasma miR-133b expression.

Research has shown that miR-27a negatively regulates myostatin production by binding the 3'UTR of myostatin (Huang *et al.*, 2012). Myostatin, a member of the transforming group factor-β superfamily, has been established in its role in skeletal myogenesis and myoblast proliferation (Huang *et al.*, 2012). Myostatin negatively regulates skeletal muscle growth by regulating proliferation. It has been found that myostatin ultimately results in an accumulation of hypophosphorylated Rb protein, which causes myoblasts to remain in the G_1-phase of the cell cycle (Thomas *et al.*, 2000). In this experiment, the Low Gain group experienced elevated expression of miR-27a in the months of April and August when compared to the respective High Gain groups. The increased expression of miR-27a in the Low Gain groups could be an indication that since miR-27a is not being used in the muscle, it was excreted into the plasma. In future trials, muscle biopsies could be obtained in order to observe the concentration of miR-27a in the muscle when compared to miR-27a concentration in the plasma. One interesting thing to consider is that both miR-133b and miR-27a experienced statistically significant data in April and in August. This could be an indicator of different periods of growth that are occurring during these times.

CHAPTER IV

CONCLUSION

The expression levels of miR-27a and miR-133b in plasma was observed in Angus-Nellore steers over a period of 5 months. A significant difference in the expression of miR-133b and miR-27a was observed between High Gain and Low Gain groups in the months of April and August. In both cases, the Low Gain group had greater expression of microRNA in plasma than the High Gain group. The difference in plasma miR-133b expression could be an indication of this miRNA acting in order to upregulate skeletal muscle growth and development, while the increased expression in miR-27a could be a result from this miRNA not being used in the muscle, and thus ending up in plasma. Further research in miR-133b and IGF-1 expression in plasma and a comparison in miR-27a in plasma and muscle could be performed in order to test this hypothesis.

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