

MicroRNA technology in livestock: expression profiling of bovine oocyte and developmental stages of porcine skeletal muscle

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MicroRNA (miR) are a class of small RNAs that regulate gene expression by inhibiting translation of protein encoding transcripts through activation of a specific cellular pathway (Hutvagner et al., 2005). A single miR can target numerous distinct mRNA for decreased translation, and as a result miR appear to be intimately involved in developmental decisions including cell fate, cell cycle progression, apoptosis, adipocyte differentiation, and processes that alter muscle development and growth (Carthew 2006; Garzon et al., 2006; Brennecke et al., 2005; Kajimoto et al., 2006; Anderson et al., 2006; Nakajima et al., 2006; Callis et al., 2007; McCarthy and Esser, 2007). Implication of miR in such a wide array of cellular processes has increased interest in evaluating miR in production livestock; however, published research is limited. Therefore, this review will focus on technologies available for evaluation of miR and the role of miR in economically important traits that are of interest in production livestock.

Functionally important small RNAs were first described in nematodes in 1993 (Lee et al., 1993; Wightman et al., 1993). However, it was not until 2001 that researchers began to understand the function of this family of RNAs that includes miR, and to recognize that their significance was not confined to lower order organisms (Lau et al., 2001; Lee et al., 2001). The small RNA classified as miR are short sequences of 18-26 nucleotide long, encoded by nuclear genes with distinctive properties that comprise 1-5% of known genes. During processing from the primary transcript, the mature miR sequence is loaded into an RNA:protein complex known as the "RNA induced silencing complex" (RISC: Hutvagner et al., 2005; Lai et al., 2005). The sequence of the miR loaded in the complex targets the RISC to specific binding sites in the 3'UTR of mRNA transcripts, resulting in either degradation of the miR:mRNA complex or translocation to P-bodies. In either case, association of RISC with mRNA causes decreased translation of the targeted gene product (Lai, 2005; Chan and Slack, 2006).

Approximately 40% of genes have transcripts that are potential targets for miR, suggesting that miR play an important role in multiple cellular processes. In addition, expression of miR genes is tightly regulated spatially among tissues and temporally during development within tissues in all species studied, indicating the importance of determining miR expression profiles to fully understand livestock biology (Wienholds et al., 2005; Aboobaker et al., 2005). The sequences of miR are extremely highly conserved among species as diverse as nematodes and mammals, supporting the hypothesis that they are of central importance to biology. However, in addition to miR genes widely distributed across order or family, there appear to be genes specific at the genus and perhaps species level. Therefore, developing technologies to detect and evaluate miR within the target species is of importance to determine their functional importance in livestock production.

Currently, there are multiple technologies available that measure individual and global miR abundance including Northern blots, ribonuclease protection assays (RPA), TaqMan assays, microarrays and small RNA clone libraries. TaqMan assays and

microarrays are commercially available, however they are currently limited to representations of human and a few biomedical model species based on the miR sequence and therefore not applicable for all production species. Additionally, while there is opportunity to develop Northern blot and RPA assays specific for production livestock species, one is limited to known sequences in the miR database (miRBase). Our approach was to use clone libraries for evaluation of miR abundance, as this technology is not limited to known sequences in the data base and creates an avenue for discovery of novel miR.

The number of miR sequences in the public database of miR sequence called miRBase has been swiftly increasing (estimates range as high as 800 miR genes in humans; Bentwich et al., 2005). Each newly identified miR is given a unique identifier, generally named with the format miR-X, where X is an integer for example miR-1 or miR-206. Some miR have less intuitive names taken from the genetic locus identified prior to knowledge that the underlying mutation was a miR, e.g. the original gene in nematodes and its mammalian orthologs are named *let-7*, the name of the genetic segregating locus associated to the phenotype originally mapped in worms. With many miR sequences in hand, the focus in the human and biomedical model species has turned to determination of gene targets to evaluate potential miR functions. Initially, gene targets were predicted computationally by utilizing commercially available software such as miRANDA, RNA hybrid and TargetScan (Vatolin et al., 2006; Arora et al., 2007; Yan et al., 2007). Recently, these predictions have been supplemented by an increase in direct experimental identification of gene targets. Technologies available for identification of gene targets and function include but are not limited to evaluation of cDNA libraries with miR probes, exogenous expression of specific miR *in vitro* and creation of miR knock out cell lines and animals (Murchison et al; Tang et al., 2007; Naguibneva et al., 2006; Flynt et al., 2007). Currently, these technologies have been utilized in rodent and human models, but, will also have application in livestock as predictions based on complete genome sequence of livestock species and direct identification of miR through clone libraries of small RNA increase the number of livestock miR sequences in the data base.

The advance and availability of technologies for identification and evaluation of miR has increased the interest in the role of miR in livestock. For the purpose of this short review, we will focus on current literature evaluating the role of miR in skeletal muscle development and reproduction, since the data we have generated profiled miR abundance in skeletal muscle of developing swine and bovine oocytes and therefore provide a connection from current literature to livestock species. Additionally, this review will address other areas of growing interest including the role of miR in adipose tissue and feed efficiency.

Evaluation of miR in the area of reproduction is of interest as reproductive performance of the dam impacts stayability in the herd and subsequent production costs. The first report of a role for miR processing in reproductive function was reported for the female germline during meiosis of the mouse (Murchison et al., 2007). In this experiment, mice had been created lacking the ribonuclease named Dicer, that is responsible for a critical step in processing the primary transcript of miR genes into the mature, RISC-loaded form. Therefore, animals lacking Dicer would be expected to have a global deficiency of miR activity. Oocytes of mice lacking Dicer activity exhibited arrested meiosis, suggesting that miR function is important to the very earliest stages of

development. A separate study also reported that maternal miR contribute to zygotic development in the mouse as deletion of Dicer from the oocyte blocked cell division (Tang et al., 2007). Together these data suggest that miRNA are critical for progression of oogenesis. However, while miR have been evaluated in the mouse ovary and oocyte (Murchison et al., 2007; Tang et al., 2007; Ro et al., 2007), there is no current literature quantifying miR abundance in production livestock, leading us to evaluate miR abundance in bovine oocytes. A profile of miR that are highly expressed in oocytes, compared to profiles of other tissues in cattle, may identify oocyte-specific miR that have a role in oocyte development, oogenesis and reproductive performance of the animal. Initial results suggest the presence of miR in bovine oocytes not previously documented in miRBase (unpublished data), highlighting the need for research in livestock to compliment that being performed in model species.

In addition to reproductive performance, we are interested in evaluating skeletal muscle and adipose tissue, as profit margin in production livestock is influenced by nutrient partitioning between these tissues. While an increase in the amount of intramuscular fat increases meat quality grade, associated excess subcutaneous adipose tissue is undesirable and decreases total profit. Similarly, an increase in muscle mass can alter hot carcass weight and increase end profit, but not if that increase is associated with diminished intramuscular fat and decreased quality grade. Therefore, understanding the mechanisms that regulate cellular processes of adipose and skeletal muscle development and growth is of interest.

MicroRNA have been reported to have a role in skeletal muscle development including proliferation and myogenesis (Yang and Wu, 2007; Nguyen and Frasch, 2006; Brennecke et al. 2005). Two miR (miR-181 and miR-214) affect skeletal muscle development through regulation of differentiation and organization of muscle precursor cells, respectively, indicating muscle-specific function despite the fact that their expression has been detected in all tissues examined (Naguibneva et al., 2006; Flynt et al., 2007). In contrast, at least three muscle-specific miR (miR-1, miR-133 and miR-206) display abundance increases with differentiation *in vitro*, but despite the similarity of the expression profiles these three miR regulate different stages of myogenesis (Anderson et al., 2006; Nakajima et al., 2006; McCarthy and Esser, 2007; Chen et al., 2006). MiR-133 increases proliferation of C₂C₁₂ myoblasts by repressing the serum response factor, while miR-1 and miR-206 promote differentiation by targeting histone deacetylase 4 and p180 subunit, respectively (Chen et al., 2006; Kim et al., 2006).

Muscle-specific miR have also been reported to regulate a gene that directly impacts economic traits in livestock. Texel sheep have a phenotype of increased muscle mass that was initially thought to result from a mutation in the protein myostatin. However, research determined that mRNA level of myostatin was not altered and mutations were not present in the coding region of the protein in Texels (Clou et al., 2005). Subsequently it was determined that Belgian Texel sheep carry a mutation in the 3'UTR of the myostatin-encoding transcript that creates a target site for miR-1 and miR-206 containing RISC complexes, resulting in decreased myostatin protein and consequent increase in muscle mass.

The increased interest in identifying miR and determining their role in skeletal muscle led us to evaluate miR expression profiles at specific stages of muscle development in the fetal pig and adult animal, and initiate similar studies in cattle. We

have identified an initial group of miR that change in expression during specific developmental stages and therefore may target genes that regulate this process. Expression profiling of polyadenylated transcripts in the same tissue will provide complimentary data to permit identification of putative targets of miR regulation and complete the picture of gene regulation during development of the single most important tissue in livestock production.

In addition to skeletal muscle, adipose tissue impacts carcass value including meat quality grade and yield. Adipose tissue was once thought to only be a site of lipid storage with little function in regulation of cellular processes. Today, adipose tissue is identified as an endocrine organ with a role in diseases associated with obesity, immunology, and hormone production. Adipose tissue differentiation and metabolism have also been reported to be regulated by miR through evaluation of adipocytes and adipose tissue of drosophila and humans (Xu et al., 2003; Esau et al., 2004). Mir-14 is a cell death suppressor that regulates the cell cycle and subsequent fat metabolism by altering diacylglycerol and triacylglycerol levels in drosophila (Xu et al., 2003), while Esau et al. (2004) identified a role for a second miR, miR-143, in adipocyte differentiation of human preadipocytes. Mir-143 abundance increased during differentiation of adipocytes and inhibition of miR-143 decreased differentiation. Together these data suggest that miR have a functional role in the biology of adipose tissue and are of interest to animal scientists.

Feed costs represent greater than 50% of the total cost in most livestock production systems. Therefore, identifying factors that regulate feed efficiency could significantly alter feed cost. A recent study evaluated genetic factors that contribute to feed efficiency utilizing whole-genome association (Barendse et al., 2007). In addition to identifying DNA variation near proteins associated with energy utilization and gene regulation, distribution of miR motifs were significantly different for single nucleotide polymorphisms (SNP) associated with residual feed intake versus the total number of SNP. While function validation of these miR motifs will need to be determined, these data suggest that these miR motifs are associated with feed efficiency.

In summary, miR are a class of small RNAs that regulate gene translation and developmental processes that may impact livestock production including reproduction, skeletal muscle, adipose tissue, and feed efficiency. Utilizing the clone library technology, we have profiled miRNA abundance in the bovine oocyte and porcine muscle at different stages of skeletal muscle development. These miR will provide us with an initial group of expressed miR that change in expression in individual tissues and during specific developmental stages and thereby identify miR that regulate cellular processes and impact economically important traits.

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