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Analysis of hepatic microRNA expression in postpartum dairy cows in negative energy balance

A thesis submitted to the National University of Ireland on research carried out in the Animal and Bioscience Research Department, Animal & Grassland Research and Innovation Centre, Mellows Campus, Athenry, Co. Galway, Ireland, in partial fulfilment of the requirements for the degree of Ph.D. in Science

by

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Abstract

This thesis sets about to answer three research questions grounded in transcriptional regulation of hepatic genes in the high yielding postpartum dairy cow under negative energy balance (NEB). For this purpose we used liver tissue from a previously developed dairy cow model comprising of cows under mild negative energy balance (MNEB) and severe negative energy balance (SNEB). The first research question relates to what is the overall expression profile distribution of hepatic miRNAs in the early postpartum period under both conditions and whether hepatic miRNA expression is altered during SNEB. The second question is an *in silico* identification of the gene targets of these miRNAs among sets of differentially expressed hepatic genes previously reported in the same animals. The third question is to determine the functional role of miRNAs in regulating putative target mRNAs. Two high-throughput techniques were used to determine hepatic miRNA expression under NEB in a dairy cattle model. First microarray expression profiling was done to determine the differential expression of hepatic miRNAs under SNEB. Five miRNAs were differentially expressed including miR-140, 31, 17-5p, 1281 and 2885. For all differentially expressed miRNAs, *in-slico* target prediction was carried out on a set of 418 differentially expressed liver genes previously reported in the same animals. A custom Perl script from TargetScan was used to identify putative target sites on bovine 3'UTRs retrieved from Ensemble. Overall 32 down-regulated target genes were identified, including those relevant to NEB such as genes associated with lipid and glucose metabolism and homeostasis and the somatotropic axis. miR-2885 has binding sites in the 3'UTR of *FADS2*, a lipogenic gene which is strongly down-regulated under SNEB. In addition, an up-regulated hepatic transcription factor, *HNF3-gamma* involved in *IGF-1* expression is a putative target of miR-31.

In the second study the overall abundance and distribution of miRNAs under both MNEB and SNEB was profiled using next generation sequencing. Overall, liver-specific miR-122 constituted 75% of expressed miRNA. SNEB resulted in the

down-regulation of miR-143, which has been associated with fatty acid metabolism.Overall 16 up-regulated putative target genes of miR-143 were identified in a set of differentially expressed hepatic genes of same animals from micro-array and RNA-seq based previous NEB model studies. Among these *SLC2A12* and *LRP2* have roles in glucose and lipid metabolism.The third study aimed to determine the functional role of miR-2885 in the regulation of *FADS2* expression. The inhibition of miR-2885 was carried out in an MDBK (Madin Darby Kidney) cell line through lipid-based transfection of a miR-2885 inhibitor and its effect on the expression of *FADS2* was determined. *FADS2* expression was up-regulated over 5-fold validating a role for miR-2885 in *FADS2* regulation.

The thesis layout is as follows; Chapter 1 discusses the impact of NEB on liver physiology and liver gene expression in postpartum moderate yielding dairy cows. Effects of NEB on fertility are also discussed. The postpartum NEB model of moderate yielding dairy cattle and alterations in liver genes from this model are summarized. miRNAs and their role in gene expression regulation, in the context of liver function and disorders is also given. Chapter 2 is a review of current methodologies and data analysis protocols involved in miRNA expression profiling, target predictions and functional analysis. Chapter 3 (a selection from first paper) is a review of research work on miRNAs in domestic livestock. Chapters 4 and 5 (papers 2 and 3) present the results of hepatic miRNA expression analysis from the NEB model. Chapters 4 and 5 also report the putative targets of differentially expressed miRNAs, among differentially expressed liver genes reported previously from the same animals. Chapter 6 presents a functional analysis of miRNA inhibition in a MDBK cell line. Finally, Chapter 7 gives a general discussion on the work done in this thesis followed by a scoping of future work.

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Chapter 1

Introduction

1.1 Summary of introduction

The introduction provides a background on the metabolic state of negative energy balance (NEB) postpartum in high yielding dairy cattle and its impact on hepatic gene expression. The role of micro RNAs (miRNAs) in the regulation of gene expression is reviewed followed by the hypothesis and objectives and experimental design of the thesis.

1.2 Dairy cattle under Negative Energy Balance

Genetic selection has helped achieve increased milk production through improving the genetic merit of Holstein Frisian dairy cows in Ireland, mainly by the use of North American Holstein Frisian semen. However this high milk yield trait selection comes at the cost of reproductive performance. This can be related to energy imbalance in these cows arising postpartum when increased milk production takes up a large part of the energy reserves. This alteration leads to a higher energy transfer towards the udder compromising the overall energy needs (Fenwick et al., 2008a). Negative energy balance (NEB) is an altered metabolic state that affects these high yielding dairy cows in the postpartum period. It occurs due to an imbalance between energy requirements for lactation and body maintenance. NEB is known to effect the general health, immunity and fertility of dairy cattle during the postpartum period (McCarthy et al., 2010; Wathes et al., 2009). NEB effects reproductive health through impacts on the biosynthesis of the reproductive hormones (Beam & Butler, 1999). NEB leads to alterations in ovarian cycle, poor conception rates and lower rates of embryo survival (Wathes et al. 2009, 2011) (Figure 1.1). In response to NEB, uncoupling of the somatotropic axis also known as the *GH/IGF-1* (Growth Hormone / Insulin like growth factor-1) axis takes place. The IGF system is comprised of *IGF-I* and *IGF-II* ligands, two receptors, *IGF-1R* (Insulin like growth factor-1 receptor) and *IGF-2 R* (Insulin like growth factor-2 receptor) and six binding proteins called *GFBPs* (Insulin like growth factor binding proteins) (Clemmons et al., 1997).

Postpartum circulating concentrations of *IGF-1* (Fenwick et al,. 2008b & Lucy et al., 2008) and *GHR* are decreased (Butler et al., 2003). Changes in the levels of *IGF-1* are known ot be directly correlated with subsequent conception rate (Wathes et al., 2009 & Taylor et al., 2004).

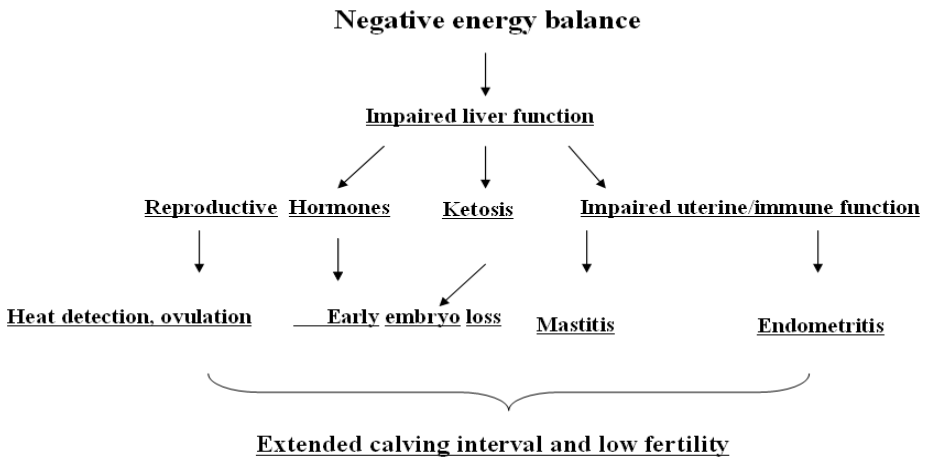


Figure 1.1 Impact of altered liver function under postpartum period NEB on reproductive health and fertility of dairy cattle.

1.3 Dairy cattle liver under Negative Energy Balance

The liver, as the source of energy supply and center of metabolic activity, is directly affected by NEB. Dairy cow liver is a major contributor to metabolic regulation of the somatotropic axis, essential for growth and reproduction (Gross et al., 2011). Hepatic metabolism is highly sensitive to the imbalance in energy status. In addition, since liver fulfils 90% of the animals glucose requirements,

postpartum rise in glucose demand for milk production results in a dramatic rise in gluconeogenesis in the liver (Nafikov & Beitz, 2007). Under NEB, non-esterified fatty acids (NEFAs) are mobilized from adipose and transported into the liver. Inside the liver, NEFAs are oxidized for energy production. Any incomplete oxidation results in formation of ketone bodies such as beta-hydroxybutyrate (BHB). In addition, NEFA esterification into triacylglycerides (TAGs) occurs. TAGs are either stored or delivered with the

very low density lipoproteins proteins (*VLDL*). Excessive TAGs accumulation in the liver further leads to fatty liver or lipidosis, whereas increased concentrations of ketone bodies in the blood can lead to ketosis in SNEB animals (Adewuyi et al., 2005). An illustration of this process is given in Figure 1.2.

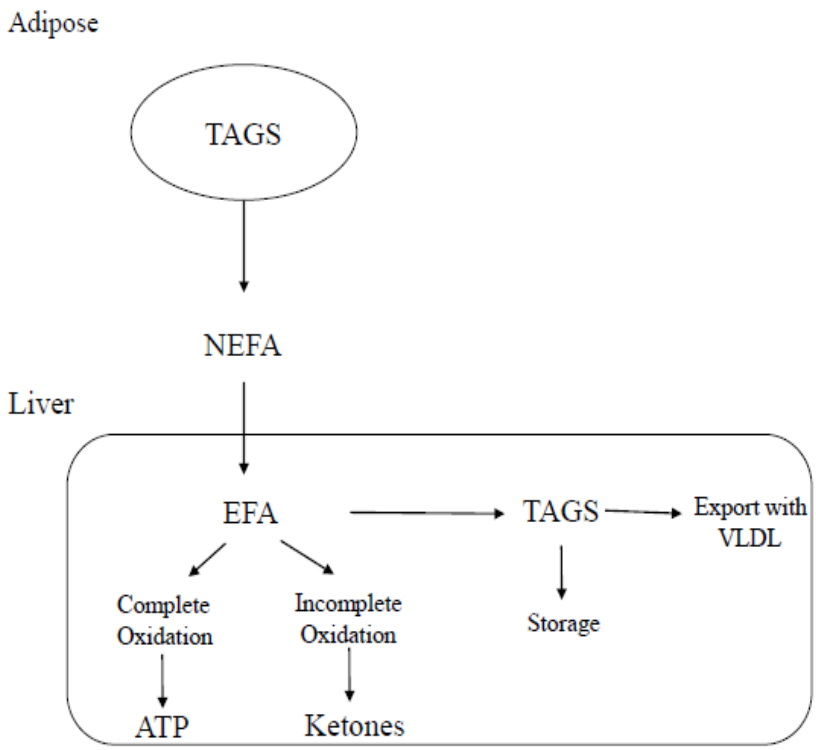


Figure 1.2 An illustration of mobilization of NEFAs from adipose and metabolism of NEFA in dairy cattle liver under NEB.

1.4 Negative Energy Balance model of moderate yielding dairy cows

A dairy cattle model was generated to study postpartum NEB (Figure 1.3). This dairy cattle model was based on controled feeding and altered milking frequency. Previous dairy cattle models generated through feed restriction only have reported the impact of NEB on the expression of hepatic genes and proteins. These models were based on 5-9 days (Kuhla et al., 2009) and 60 hours of the feed restriction

respectively (Loor et al., 2007). The model used in this study reflects the true range of cow EB (energy balance) status recorded on farm in the early postpartum period (Butler et al. 2003b). In this model two groups of cows differing in NEB status were generated; a mild negative energy balance (MNEB) and a severe negative energy balance (SNEB) group (Patton et al., 2006). MNEB cows were maintained as close to positive energy balance as possible by feeding *ad -libitum* a high energy diet together with lower energy output through once a day milking compared to SNEB limited feeding and thrice a day milking.

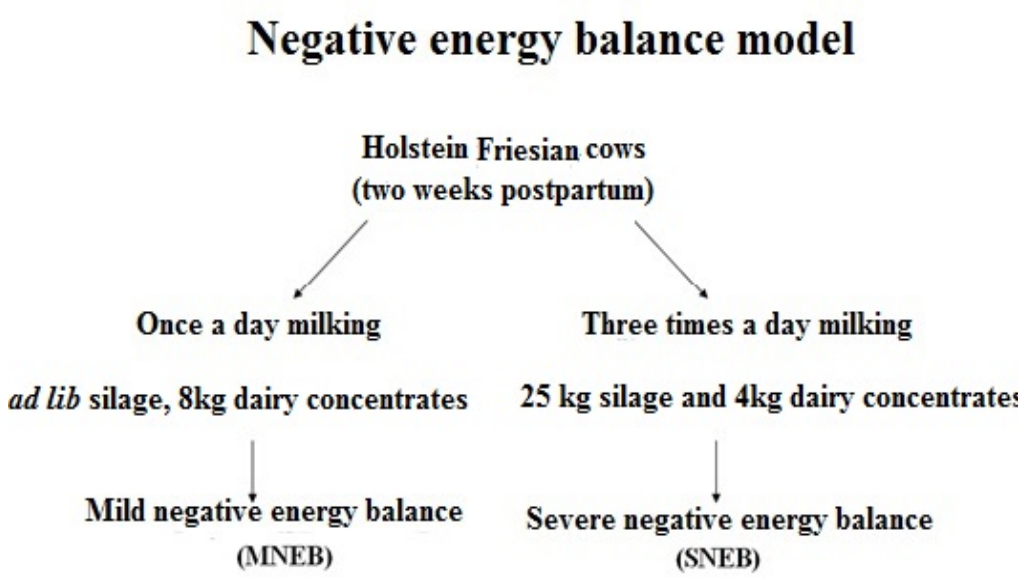


Figure 1.3 Negative energy balance model of medium yielding postpartum dairy cows. The cows were divided in to mild energy balance (MNEB) and severe negative energy balance (SNEB) group based on different feeding and milking regimes.

1.5 Physiological alterations in dairy cattle model of Negative Energy Balance

1.5.1 Energy balance status and measurements in Negative Energy Balance model

Energy Balance (EB) calculation was based on the French NEL system (net energy for lactation). Body weight, dietary energy intake, milk yield and milk composition were used to calculate net EB, in unité fourragère lait (UFL) day.$^{-1}$ One UFL unit is equal to the net energy for lactation equivalent of 1 kg of standard air-dry barley as described previously (Jarrige et al., 1989).

- For the period from day 2 post calving to slaughter until tissue collection EB was recorded. SNEB cows had undergone significant ($P < 0.05$) changes in energy balance (−3.6-fold). MNEB and SNEB groups showed a mean EB of -4.93 ± 0.971 and -8.75 ± 0.987 UFL/day respectively.

- Average DMI (daily matter intake) was 10.5 ± 0.74 and 8.1 ± 0.79 kg day−1, respectively, and average milk yield was 18.7 ± 2.62 and 23.2 ± 1.17 kg day−1, respectively in MNEB and SNEB groups.

1.5.2 Measurement of plasma concentrations of metabolites in Negative Energy Balance model

Blood was sampled weekly from two weeks prior to calving up to and including the day of slaughter; Blood samples were taken each morning approximately 1 hour before feeding. NEFA, cHB, *IGF-1,* cholesterol and urea concentration levels in blood plasma were recorded (Table 1.1 and Figure 1.4).

Table 1.1 Plasma metabolite concentrations under MNEB and SNEB from postpartum dairy cattle model (McCarthy etal.2010).

Metabolite	Plasma concentrations
NEFA	Concentration of NEFA increased * (+ 4.7-fold) in SNEB cows.
BHB	Concentrations of BHB increased in SNEB with increasing days postpartum *(+7.4-fold) ($P < 0.01$).
TAG	No significant difference in plasma TAG level in both groups. SNEB liver TAG were increased *(+3.9-fold)($P > 0.01$).
Cholesterol	Plasma concentrations rose with increasing days postpartum in the case of MNEB ($P < 0.0001$).
Urea	Mean concentrations of urea tended to be greater in SNEB ($P=0.062$).
Glucose	Glucose concentrations decreased in SNEB group * (−1.5-fold), ($P < 0.001$).
IGF	IGF-1 concentrations decreased in SNEB (*−4.8-fold) ($P < 0.02$).
Glycogen	Liver glycogen decreased in SNEB *(−10.4-fold) ($P < 0.001$).

Abbreviations: IGF = insulin like growth factor, BHB = beta hydroxyl butyrate, NEFA = non-esterified fatty acid and TAGs = triacyal glycerides.

*The fold changes in concentration levels under SNEB (severe negative energy balance) compared to MNEB (Mild negative energy balance) are given.

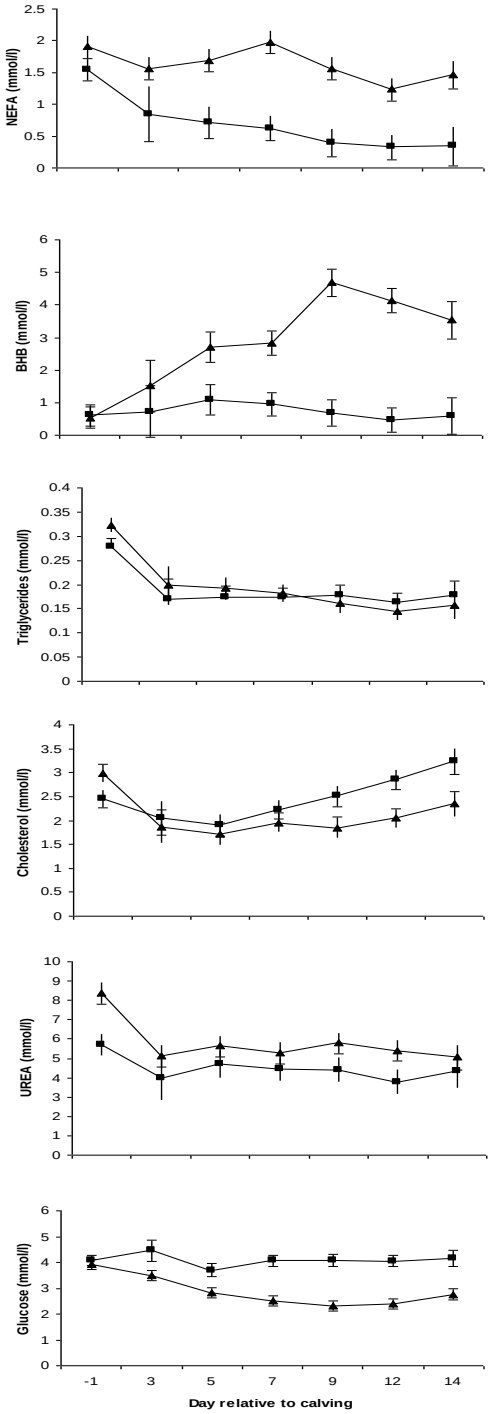


Figure 1.4 Plasma metabolites and effect of days relative to parturition in animal model of NEB in medium yielding postpartum dairy cows. Plasma metabolites are represented on the Y-axis, with day relative to parturition on the X-axis.

1.6 Alteration in liver gene expression in Negative Energy Balance model

Complex metabolic adjustments evident from hepatic gene expression profiles under SNEB based on data from microarrays and RNA-seq technologies were reported in two studies from this NEB model previously (McCabe et al., 2012; McCarthy et al., 2010). The genes differentially expressed under SNEB were significantly associated with processes involved in lipid metabolism, molecular transport, small molecule biochemistry, gene expression regulation, cellular compromise and DNA replication and recombination. Increased TAGs accumulation in the liver was reflected by overrepresentation of the *PPAR* (peroxisome proliferator-activated receptors) signalling pathway. Somatotrophic axis members; *IGF-1*, *GHR*, *IGFBP2* (*IGF* binding protein 2) and *IGFBP3* (*IGF* binding protein 3) were also differentially expressed under SNEB. These genes also contribute to the overrepresented p53 signalling pathway, associated with stress (Table 1.2 and Figure 1.5).

These studies speculate that the biosynthesis of *IGF-1* in liver is nutritional dependent and limited feed intake can influence its hepatic expression under SNEB. Polyunsaturated fatty acids (PUFA) are essential for cell viability and their synthesis is also impaired during SNEB. Feeding cows a diet enriched in unsaturated fatty acids during the late prepartum and early postpartum period is known to improve postpartum general and reproductive health (Thatcher et al. 2006). Members of the *FADS* (fatty acid desaturase) family, *FADS1* (fatty acid desaturase 1) and *FADS2* ((fatty acid desaturase2) critical for PUFA metabolism were down-regulated in SNEB animals. *FADS2* which desaturates linoleic and linolenic acid and EFAs (Essential fatty acids) to ω3- and ω6-polyunsaturated fatty acids (PUFAs) was the most down-regulated gene under SNEB. *FADS1* also mediates desaturation of the long chain polyunsaturated fatty acids *(LC-PUFAs)* arachidonic acid (C20:4) and eicosapentaenoic acid (C20:5). These PUFAs are also precursor of embryotrophic hormone synthesis.

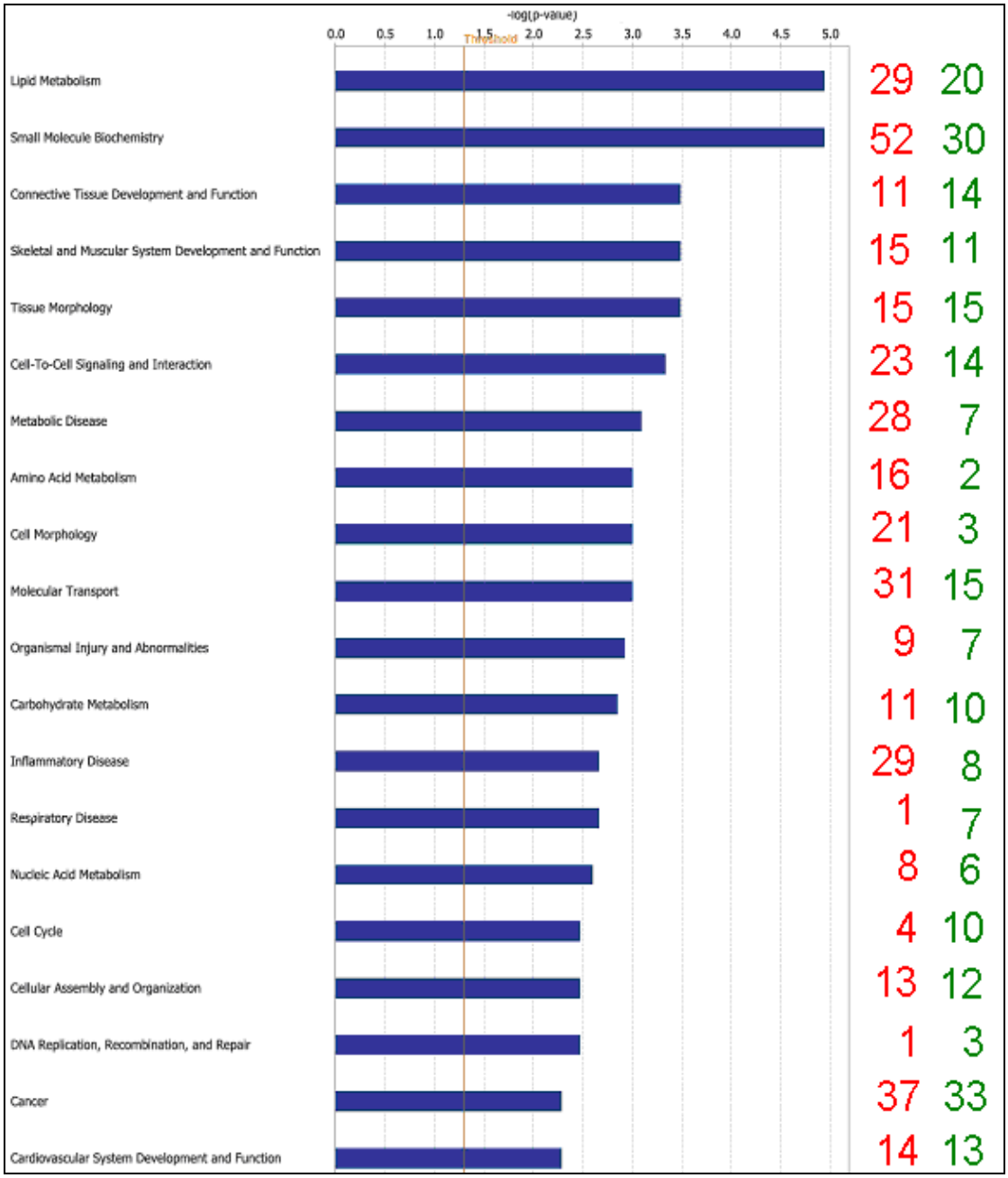


Figure 1.5 Representation of differentially expressed genes according to top 20 molecular and cellular functions most significantly affected by SNEB (McCarthy et al., 2010). The blue bars indicate the likelihood [-log (P-value)] that the specific molecular and cellular function category was affected by negative energy balance compared with others represented in the list of differentially expressed genes. The number of up- and down-regulated genes in each group is represented on the right hand side by red and green numbers respectively. The cut-off ( ) is shown at $P < 0.05$ (1.301 log scale).

Table 1.2 KEGG pathways associated with all DE hepatic genes under SNEB from RNA-seq study (McCabe *et al.*, 2012)

Pathway code	Pathway name	Overrepresented
04976	Bile secretion	0.001483
00140	Steroid hormone biosynthesis	0.002046
04950	Maturity onset diabetes of the young	0.004947
00910	Nitrogen metabolism	0.007765
00061	Fatty acid biosynthesis	0.011248
05219	PPAR signalling pathway	0.018112
03320	Bladder cancer	0.018941
04910	p53 signalling pathway	0.020756
04115	Insulin signalling pathway	0.021653
04977	Vitamin digestion and absorption	0.026059
00592	α-linolenic acid metabolism	0.030405
04975	Fat digestion and absorption	0.034363
04080	Neuroactive ligand-receptor interaction	0.040178

Decrease in PUFA level associated with down-regulated *FADS* expression in SNEB cows can be a potential contributor to the poor fertility of dairy cow (Wathes et al. 2007b). Overall SNEB was found to alter important biological pathways in postpartum high yielding dairy cattle liver (McCabe et al., 2012; McCarthy et al., 2010).

1.7 Role of miRNAs in post-transcriptional gene regulation

Complex multicellular organisms require precise spatial and temporal regulation of gene expression (Phillips, 2008). Gene expression is regulated on multiple levels before, during and after transcription. The gene expression regulation capability of non-coding RNA was reported more than 15 years ago (Clemens et al., 2000; Fire et al., 1998; Lee, Feinbaum & Ambros, 1993), but their importance has only been recently appreciated. According to recent reports 98% of the human genome is non-coding but it is not without function (Ium, 2012). The two main classes of non-

coding RNAs are large non-coding RNAs and small non-coding RNAs including piwi-interacting (piRNA), repeat associated small interfering RNA (rasiRNA), small temporal RNA (stRNA), small interfering RNA (siRNA) and micro RNA (miRNA). miRNAs are single-stranded 19-24nt in length and silence gene expression post-transcriptionally through complimentary base pairing to target mRNA (Bartel, 2009a; Mattick, 2003). The first miRNA *lin-4* was discovered in the round worm Caenorhabditis elegans *(C. elegans*) about two decades ago (Lee et al., 1993) and was found to have complimentary sequences to multiple target sequences on the 3'UTR of the *lin-14* gene involved *C. elegans* larval development (Lee et al., 1993). miRNA research has progressed rapidly in the last decade and miRNAs have been implicated in the regulation of genes involved in various biological processes ranging from cell cycle and cell proliferation to development, immunity and metabolism pathways as well as various diseases and (Farazi et al., 2013; Hulsmans & Holvoet, 2013) disorders in including those of the liver like non-alcoholic fatty liver disorder (NAFLD) (Bala et al., 2009). Recently miRNAs have been found to have roles in many biological processes and miRNA mediated regulation of genes in energy metabolism, hepatic functions and disorders have been reported (Anders et al., 2013; Bala, Marcos & Szabo, 2009; Davalos et al., 2011; Poy, Spranger & Stoffel, 2007). miRNAs contribute to glucose and lipid metabolism and insulin signalling and the role of miRNAs in the regulation of *IGF* has become well established (Davalos et al., 2011; Hu et al., 2013a; Hu et al., 2013b; Poy et al., 2007; Yang et al., 2013; Zampetaki & Mayr, 2012a) (Figure 1.6).

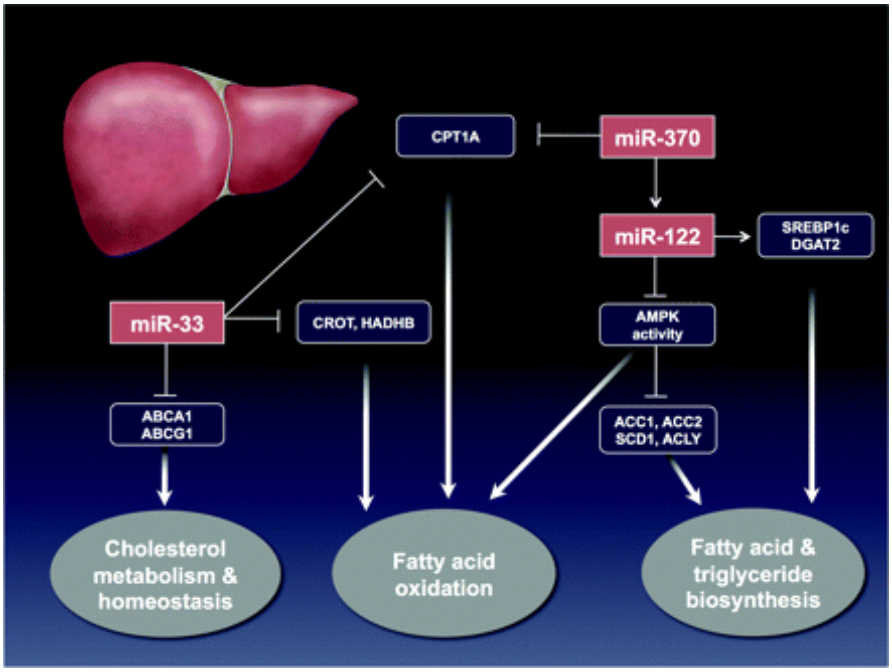


Figure 1.6 Liver miRNAs with their direct targets genes with roles in liver metabolic functions. Sourced from (Zampetaki & Mayr, 2012a).

1.8 Hypothesis

miRNA expression in the liver of moderate yielding postpartum dairy cows is altered due to SNEB and these altered miRNAs have potential roles in the regulation of hepatic genes differentially expressed under SNEB.

1.9 Objectives

- The first objective relates to the overall expression profile distribution of dairy cattle hepatic miRNAs in the early postpartum period in both MNEB and SNEB conditions and whether hepatic miRNA expression is altered in SNEB.
- The second objective is the *in silco* identification of gene targets of miRNAs among sets of differential expressed hepatic genes previously reported in the same animals.
- The third objective is to evaluate the functional role of differentially expressed miRNAs in regulating putative target mRNAs differentially expressed under SNEB *in vitro*.

1.10 Experimental Design

An outline of the experimental design for the thesis is shown below in Figure 1.7.

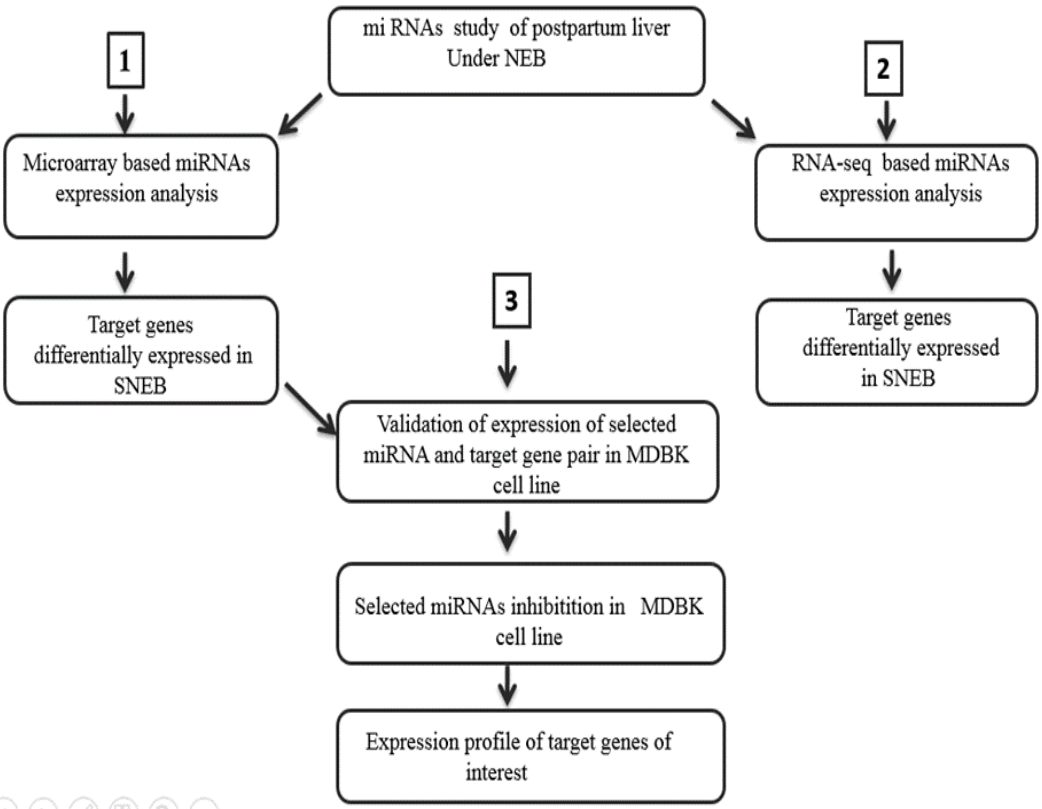


Figure 1.7 Experimental design for dairy cattle liver miRNAs expression analysis, target prediction and direct targeted repression function evaluation under SNEB.

Chapter 2

Methodologies

2.1 An overview of miRNA expression profiling techniques

miRNA expression determination is an important step in their functional elucidation. The physicochemical nature of miRNAs is especially considered in order to optimize standard mRNA expression profiling techniques for miRNA identification and expression determination (van Rooij, 2011). The GC content of miRNAs is heterogeneous and that presents a melting temperatures (Tm) adjustment challenge for probe or primer design (Benes & Castoldi, 2010; Castoldi et al., 2008). To overcome this challenge extended loop probes (Wang, Ach & Curry, 2007), stem-loop primers (Chen et al., 2005) or modified nucleotides such as locked nucleic acids (LNA) or 2'-O-(2-methoxyethyl)-derivatives are used (Beuvink et al., 2007). For accurate expression profiling it is important to distinguish the mature miRNA from its pre-processed sequence that also exists as part of a pri-miRNA and pre-miRNA. The probes are designed to be sensitive enough to discriminate members of the same miRNA family differing by only a single nucleotide (Becker et al., 2010; Benes & Castoldi, 2010; Willenbrock et al., 2009). Low throughput techniques such as Northern blotting, real-time quantitative polymerase chain reaction (RT-qPCR) and *in situ* hybridization (ISH) can be used to determine the level of individual miRNAs. The expression of large numbers of miRNAs can be profiled in parallel in a single session with high-throughput techniques including microarray analysis or deep sequencing (van Rooij, 2011). The principle of Northern blotting is to determine the relative quantity and size of RNA in a given sample (Varallyay, Burgyan & Havelda, 2008). In this technique gel electrophoresis is used for RNA size-based separation and the size-separated RNA is transferred to nitrocellulose blotting paper or membrane. Following hybridization to a labelled probe, the target miRNA is quantified by exposure to photographic film or by luminescence based techniques. LNA probes with a nucleotide chemically modified for greater affinity are used as one way to improve sensitivity (Varallyay et al., 2008). ISH is used to monitor and detect sub-cellular and tissue level miRNA accumulation and location (Silahtaroglu et al., 2007). ISH uses a labelled RNA probe complementary to the RNA of interest to localize a specific miRNA and to determine its distribution. For improved miRNA detection

in ISH LNA probes are used to for greater target affinity (van Rooij, 2011). Here we describe RT-qPCR, used for single miRNA expression validation in Papers 2 and 3.

2.1.1 RT-qPCR based miRNA expression profiling

RT-qPCR expression profiling is a two-step process. In the first step RNA is reverse transcribed into complementary DNA (cDNA). This cDNA is amplified using polymerase chain reaction and quantified in real-time in the second step (Fiedler, Carletti & Christenson, 2010). Two qPCR techniques, differing in the reverse transcription (RT) step, are commonly used to detect specific miRNAs (van Rooij, 2011). First is the TaqMan® (Applied Bio systems, UK) method that uses miRNA-specific stem-loop primers for reverse transcription. Stem-loop primers have a short single-stranded region complementary to the 3′ end of the miRNA, a double-stranded stem region and a loop region for the universal primer-binding. cDNA is subjected to standard TaqMan® assay-based real-time qPCR (with a miRNA-specific primer and a universal primer (TaqMan PCR) (Chen et al., 2005). The second SYBR Green (Life Technologies, UK) method involves addition of a common sequence poly-A tail to the 3′-end of all miRNAs to enable the use of universal reverse transcription primers (Fig. 2.1). Given their short length and the absence of a polyadenylated 3' tail, the miRNAs poly-T oligonucleotide primers are not suitable for RT of miRNA and gene-specific, stem-loop RT primers are a better option, these primers can discriminate between the mature miRNA and longer precursor miRNA (Castoldi et al., 2013; Schmittgen et al., 2008).

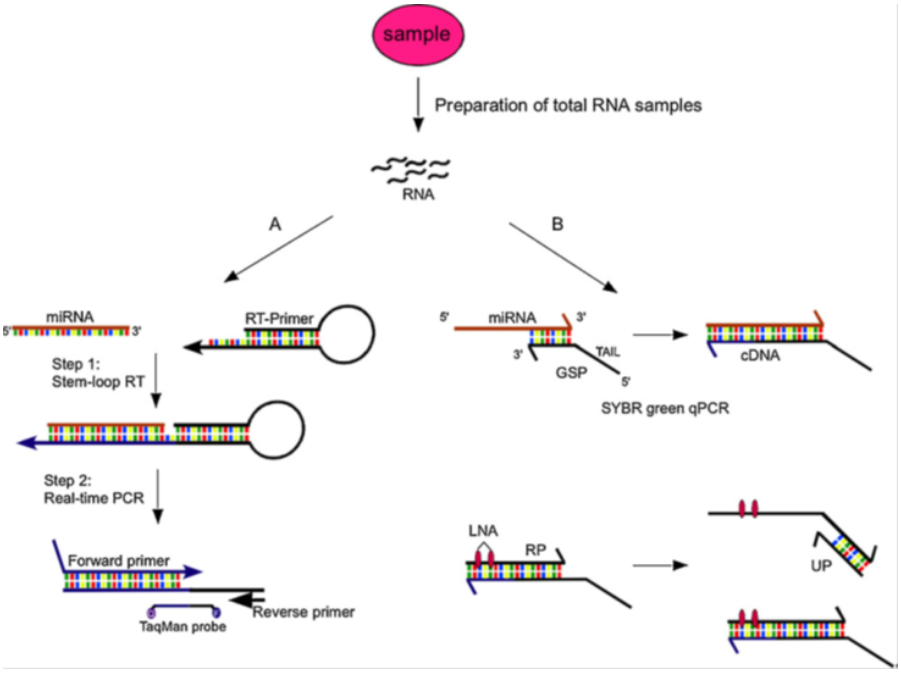


Figure 2.1 miRNA RT-PCR techniques (A) TaqMan RT-PCR (B) The SYBR Green RT-PCR. Sourced from (de Planell-Saguer & Rodicio, 2011). Stem loop primers are used in the case of Taqman based RT in order to create universal primer binding region for PCR. SYBR Green primers adds a common sequence poly-A tail to the 3′-end of miRNA for late universal primer binding during PCR.

2.1.2 miRNA expression profiling using microarrays

High-throughput techniques like microarrays and next generation sequencing are used to profile the expression of hundreds of miRNAs simultaneously in a single experiment. Microarrays are used for high-throughput measurement of miRNA expression. Microarrays have thousands of oligonucleotides, called probes, spotted to a solid surface, usually small glass slides (Cheung et al., 1999; Maskos & Southern, 1992). The probes are complementary to the sequence of interest (the target miRNAs). The target samples labelled with fluorescent dye are hybridized to microarrays and scanned for probe-level florescence intensity which is taken as being proportional to target expression (Figure. 2.2). Single-channel as well as two-channel microarrays are available. In the case of two channel array two samples can be hybridized to the same array, each sample labelled with a different fluorescent dye (Cheung et al., 1999).

miRNA expression profiling microarrays have been optimized taking into account the innate properties of miRNAs (Pritchard, Cheng & Tewari, 2012; Sato et al., 2009). Variation in the Tm of different miRNAs is overcome by designing altered length probes to optimize binding affinities to the labelled miRNAs at a set hybridization temperature. Modified oligonucleotide probes, including LNA increase the Tm to help increase the binding affinity of the captured probes and 2′-O-methyl modified nucleotides are also used to improve stability and increase hybridization affinities (Pritchard et al., 2012; Thomson et al., 2004; van Rooij, 2011).

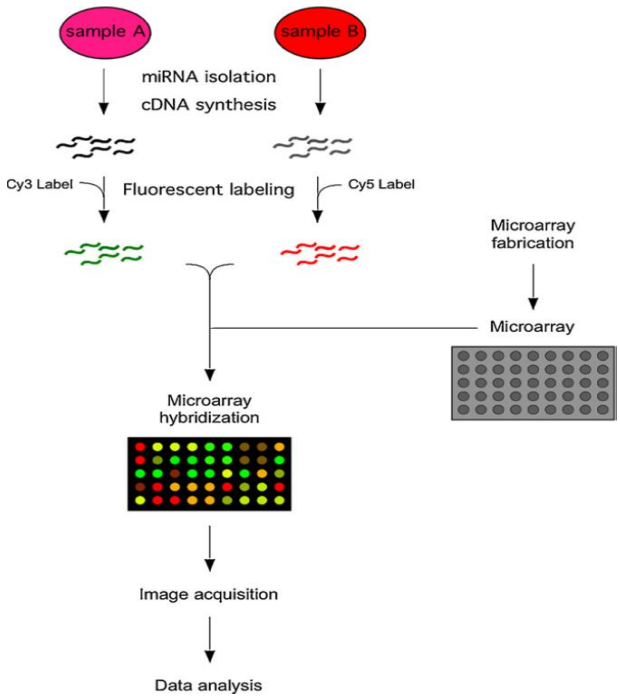


Figure 2.2 Microarray miRNA expression analysis scheme, sourced from (de Planell-Saguer & Rodicio, 2011).

Microarray platforms for miRNA expression levels include Affymetrix, Illumina, Agilent and Exiqon miRCURY LNA arrays Platforms differ depending upon the sample labelling dye used, the design of probes and the signal-detection methods applied (You et al., 2006). Affymetrix uses a one-channel system and each miRNA is typically measured by a set of 11-20 probe pairs collectively referred to as "probe set". Each probe pair is comprised of a perfect match (PM) and corresponding mismatch (MM) probe which is identical to the PM probe except for an altered middle base (van Rooij, 2011). We used an Affymetrix miRNA array for expression

profiling in Paper 2. Several microarray expression data analysis software tools are available through R, a freely available statistical programming environment, widely used by the bioinformatics community. R runs on a wide variety of UNIX platforms as well as Windows(Team, 2012) and MacOS Bioconductor is a repository for software packages for the analysis of microarrays and RNA-seq that work in the R environment (Gentleman et al., 2004). Affy is a Bioconductor package for pre-processing and analysis of expression intensity data from Affymetrix platforms (Gautier et al., 2004).

2.1.2.1 Pre-processing of microarray expression data

Pre-processing of expression data is carried out to control for different sources of variation during expression profiling (Irizarry et al., 2003; Shi et al., 2006). Typically pre-processing is done in three steps. First of all background correction is done on expression intensity values to remove background noise resulting from non-specific binding of the labelled mixture. For background correction it is assumed that the observed signal is a mix of the true signal coming from the hybridization of probe to target of interest and the background signal due to non-specific hybridization or other sources of contamination. A basic method of background correction can be subtraction of the estimated background signal from the intensity signals retrieved from the array for each probe (Ritchie et al., 2007). Robust Multi-array Average (RMA) (Irizarry et al., 2003) is a popular pre-processing algorithm which performs background correction through a global correction of all PM intensities based on the assumption that the observed intensity signal is the combination of a foreground signal and background signal with normal distribution (Irizarry et al., 2003). Microarray miRNA expression intensities are biased by technical variations such as differences in sample RNA preparation, dye labelling and hybridization efficiency across different arrays. In order to reduce this bias expression data normalization is performed. Standard mRNA expression normalization procedures are applied to miRNA data and these methods are based on the assumption that only a small proportion of probes are differentially expressed (Pradervand et al., 2009; Sarkar et al., 2009; Steinhoff & Vingron, 2006). Quantile normalization ranks all the transcripts in the data in such a manner that the gene at

a given particular rank has the same expression level across all samples. The mean expression level of the Nth ranked gene is first calculated in all the samples and this mean is then utilized as the expression level for the Nth ranked gene in each individual sample (Bolstad et al., 2003). Quantile normalization is one of the most robust methods and can also be implemented as part of the RMA pre-processing procedure (Irizarry et al., 2003). Among others, LOESS (Locally Weighted Regression and Smoothing Scatter plots) normalization transforms the expression data by applying a local regression with weighted penalties for outliers (Risso et al., 2009; Steinhoff & Vingron, 2006). Variance stabilization normalization (VSN) is another normalization procedure that calibrates sample-to-sample variations to make the variance independent of the mean intensity (Huber et al., 2002; Suo et al., 2010). Pre-processing also involves summarization across the chip of all signal intensity measures of a probe set, representing a single miRNA, into a single expression value. The simplest summarization involves calculating the mean or median value of all probes of a target transcript. Median polish summarization is a summarization method used for miRNAs expression data analysis. This method is also a part of the RMA procedure (Bolstad et al., 2003; Tukey, 1977).

2.1.2.2 Microarray Data analysis and Differential expression

Differential expression analysis is used to compare miRNA expression between one condition and another. The most commonly used Bioconductor packages for differential expression analysis of microarray data, including limma (Linear Models for Microarray Data analysis), Rank pro (Rank product) and samr (Significance Analysis of Microarrays) are briefly described below (Breitling et al., 2004; Gentleman et al., 2004; Smyth, Michaud & Scott, 2005; Tusher, Tibshirani & Chu, 2001).

The limma method is based on applying linear regression models with arbitrary coefficients and contrasts of interest to each gene in the expression data. Model fitting involves generation of a design matrix representing the miRNAs hybridized to the array and a contrast matrix for experimental conditions. The coefficients defined by the design matrix are applied to the contrasts of interest. To improve analysis accuracy, variance transformation is carried out to shrink the variance towards a common value by sharing information across genes. This replaces the

sample variance used in the modified t-test of limma called the moderated t-test (Gentleman et al., 2004; Smyth et al., 2005).

The Rank product method is a non-parametric statistical method based on ranks of fold-changes (Breitling et al., 2004). The assumption made by the Rank product method is that only a minority of genes are affected by the differences between the sample groups and, of these, most changes are independent of each other while the variance is assumed to be almost equal for all genes. Rank product uses permutation re-sampling to calculate the rank products of the genes in a set number of permutations. The rank product of the genes in the permutations is compared to the observed rank product to declare differential expression (Breitling et al., 2004).

Significance Analysis of Microarrays (SAM) is another permutation-based non-parametric method. In SAM expression level is defined as the response variable. SAM computes a test statistic to measure the relationship between each gene and the response variable and computes order statistics. SAM then carries out a set of permutations of response values and for each permutation re-computes the expected test statistic. From the set of permutations, it estimates the expected order statistics. Expected statistics for a gene are compared to observed test statistics and for a set thresh hold parameter called delta, SAM declares the genes as significantly differentially expressed. In hepatic miRNA micro array expression profile study we used SAM for differentially expression analysis (Jeanmougin et al., 2010; Tusher et al., 2001).

2.2 High-throughput sequencing of miRNAs

Expression profiling through DNA sequencing relies on determining the order of nucleotides in DNA. Massive parallel sequencing also called ’High-throughput sequencing’ (HTS), ’Next generation sequencing’ (NGS) or ‘deep sequencing’ is used to generate millions of sequences in parallel. Recently HTS methods have become a popular technology for studying miRNA expression (Soon, Hariharan & Snyder, 2013).

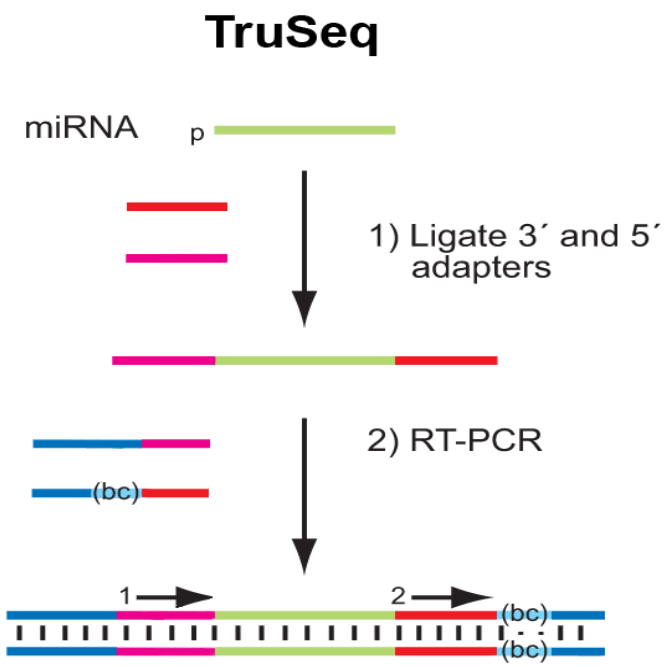


Figure 2.3. Illumina RNA library preparations. Sourced from (Van Nieuwerburgh et al., 2011).The miRNAs are ligated with adaptors and reverse transcribed. Bar code (bc) addition is for sample recognition for multiplexed sequencing.

Sequencing platforms differ on the basis of the detection chemistry used (Ansorge, 2009; Soneson & Delorenzi, 2013). Chain termination detection chemistry process involves DNA synthesis termination at different stages by using deoxyribonucleotide (dNTP), dideoxy terminator nucleotides (ddNTP) that lack a 3'-OH. During DNA synthesis from a template strand, a ddNTP causes chain termination of DNA extension when it encounters a complimentary dNTP in the template sequence, resulting in generation of extended DNA fragments of varying length. The size separation of extended fragments is carried out by electrophoresis and a sequencer is used to report the order of bases by combining information from all the extended fragments (Sanger, Nicklen & Coulson, 1977).

In contrast sequencing by synthesis involves synthesizing the complementary strand of a template DNA one base at a time, while determining the incorporated nucleotide during each synthesis step (Metzker, 2009). Pyro-sequencing is an example of this sequencing procedure where PCR amplifications of a template DNA immobilized on glass beads are carried out. Beads with multiple copies of DNA are then placed in the microscopic wells and the four deoxyribonucleotides

(dNTP) are added sequentially; when a base is incorporated in extending a DNA fragment a pyrophosphate is released. The pyrophosphate is used to make ATP which in turn helps in conversion of leuciferin to oxyluciferin resulting in light emission. The order of dNTPs that produce the chemiluminescent signals is determined to give the DNA sequence of the template (Pourmand et al., 2002; Ronaghi, 2001). For the pyro-sequencing of small RNA, including miRNAs the Genome Sequencer FLX (454 Life Science and Roche Applied Science) (Droege & Hill, 2008; Margulies et al., 2005; Pourmand et al., 2002; Ronaghi, 2001) has been used. The most popular method for sequencing by synthesis available for small RNA is the next generation sequencing with HiSeq® System 2000 (Illumina Inc., San Diego, CA, USA) and is capable of generating millions of small RNA-sequence reads from a given sample (GenomeWeb, 2014; Minoche, Dohm & Himmelbauer, 2011).

2.2.1 Library preparation and sequencing

The first step in Illumina sequencing is sample cDNA library preparation. For this purpose sample RNA fragmentation of the length of ~75 nucleotides is followed by reverse transcription to cDNA. Adaptor sequences are ligated to the cDNA to help in the hybridization to a lawn of oligonucleotides on the surface of, an optically-transparent slide, called a flow cell (Meyer & Kircher, 2010; Zhong et al., 2011) (Figure. 2.3). A complimentary copy of template cDNA is made using a DNA polymerase extension activity. This complimentary copy is subjected to PCR amplification to produce millions of clusters, each containing 1,000 of copies of a single template molecule. After cluster generation the reverse strand is removed, the remaining strands are hybridized with a sequencing primer and the flow cell is loaded with each of the four types of nucleotides fluorescently labelled with a different colour. The four nucleotides have to compete for binding sites on the template DNA. Whenever a base is incorporated it is detected by laser-powered imaging, sequencing is done simultaneously for all clusters (Figure.2.4) (Kircher, Stenzel & Kelso, 2009; Mardis, 2008).

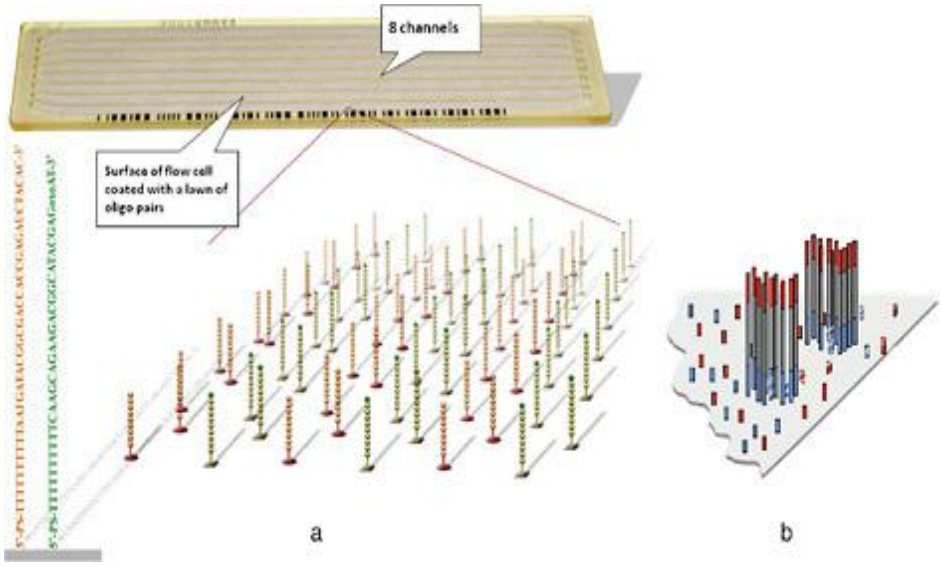


Figure 2.4 Illustration of Illumina RNA-sequencing. Sourced from (Ronaghi et al., 2010). Samples are hybridized to flow cells with the help of adaptors and transcribed to complimentary copies. PCR amplification is followed by sequencing by synthesis with sequencing primers.

2.3 Analysis of RNA-seq data

A choice of bioinformatics tools is available for next generation sequencing data analysis (Horner et al., 2010). We discuss those used for miRNA sequence data analysis. Before any analysis, sequencing quality information is necessary to determine the accuracy of sequencing plate form and eliminate poor quality reads resulting from low accuracy base-calling. A quality score is assigned to each base-call, generally used is the Phred quality score (Q score). The Phred quality scores for base-calling is the log of the error probability $Q = -10 \log P$, where P is the probability of an erroneous base call (Ewing & Green, 1998; Schmieder & Edwards, 2011).

2.3.1 Sequence alignment

RNA-seq data analysis begins with the alignment of sequenced reads to a reference genome in a multistep process. First a heuristic search is carried out for a subset of possible matching locations on the reference genome followed by a more accurate alignment by evaluating all candidate alignments with the help of a local

‘alignment' algorithm (Oshlack, Robinson & Young, 2010). For fast searching, alignment tools use indexing algorithms (Fonseca et al., 2012; Horner et al., 2010; Lindner et al., 2011; Lunter & Goodson, 2010; Mu et al., 2012; Schbath et al., 2012; Yu et al., 2012). Generally used tools for short read alignment of small RNA seq data include Bowtie (Langmead et al., 2009), MAQ and Novoalign (Li & Homer, 2010; Novocraft, 2013). Novoalign and MAQ algorithms are built upon a Spaced-seeds indexing algorithm. A keys mapping values hash table is created, with values being the reference genome, in the case of Novalign, or reads, in the case of MAQ (Figure. 2.5). Novalign allocates a scoring matrix with values for all possible nucleotide matches in order to find the alignment with the highest score. Penalties are assigned for both gap-opening and gap-extension; and the best alignments with the maximum possible score are reported (Novocraft, 2013).

The Bowtie aligner implements Burrows-Wheeler transform (BWT) algorithm and FM index (fast match index) to lexographically compress the genome and efficiently index it (Burrows & Wheeler, 1994). The BWT generates a matrix of the reference genome with rows representing cycles of lexicographically ordered genome sequences. The result of BWT, the BWT sequence, is given in the last column of the matrix. For further compression and indexing of BWT sequences (Burrows & Wheeler, 1994; Langmead et al., 2009). The FM-index is efficient and facilitate fast string-matching (Schbath et al., 2012).

2.3.2 Estimating transcript expression from RNA-seq data

After alignment to the reference genome the annotated reads mapping to each genomic feature are counted for expression abundance profiling. One issue is that there always exist some reads that align to multiple locations. RNA-seq tools have different strategies to resolve this problem. HTSeq (Andres, 2013) algorithm overlaps aligned sequencing reads with the genome annotations to count reads mapped to each genomic feature, and calculates the average quality score for each

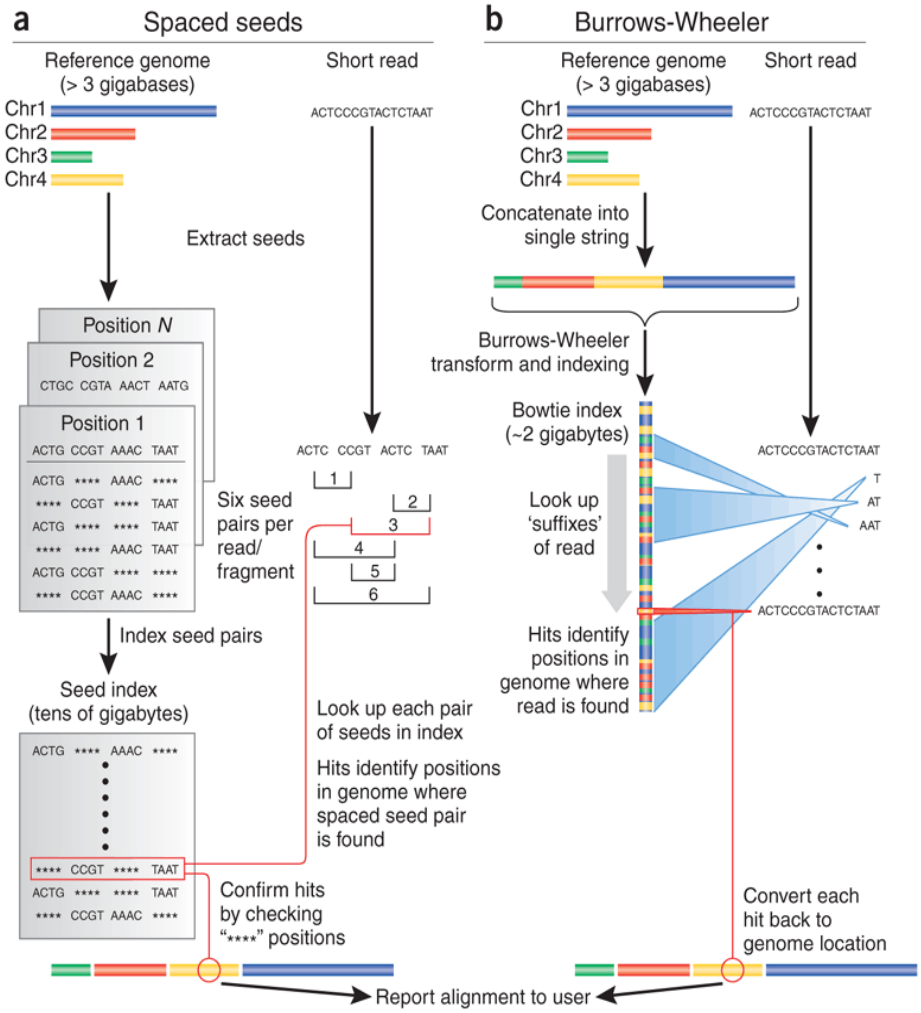


Figure 2.5 Algorithms for aligning short sequencing reads: (a) spaced-seed indexing (b) Burrows-Wheeler Transform (Langmead et al., 2009). Sequence Hash tables are created with keys (seeds) matching values corresponding to short reads and reference genome in the case of space seed indexing method. In the case of BWT the reference genomes are laxographicaly compressed and fast match index are used for fast string matching of short reads.

position in the reads, adding up the quality scores alongside the read counts. For accurate differential expression profiling only uniquely mapped reads are retained by default. However, if required, HTSeq-count also reports multi reads with more than one alignment (Anders 2010; Li, Bolser et al. 2013; Van Verk, Hickman et al. 2013). The Cufflinks (Trapnell et al., 2010) works by constructing a parsimonious set of transcripts or assemblies containing the smallest number of transcribed fragments to explain both the reference transcripts and the sequenced read for all alignments in an RNA-seq experiment. For multi-mapped reads, expression

estimates are calculated for all mapped positions. The likelihood of the sequence mapping to any one of the multiple putative positions is recalculated using information from the expression of each transcript, the fragment length and sequence bias estimations (Roberts et al., 2011; Trapnell et al., 2012; Trapnell et al., 2010).

2.3.3 Differential expression in RNA-seq data

Differential gene expression analysis of RNA-seq data comprises three main steps; normalization of read counts, parameters estimated for statistical modelling of read distribution and finally differential expression analysis (Rapaport et al., 2013; Soneson & Delorenzi, 2013; Tarazona et al., 2011). State of the art tools for differential expression analysis are DESeq, edgeR and Cuffdiff (Anders & Huber, 2010; Oshlack et al., 2010; Trapnell et al., 2012; Trapnell et al., 2010). In DESeq only a small number of genes are assumed to be differentially expressed and for data normalization a reference sample is created with values representing the geometric mean of each gene overall samples. A normalization factor for each sample library called the size factor was computed by using the median of the ratio for read counts of each gene in the sample over its respective geometric mean across all samples given in reference sample. Cuffdiff extends the process of normalization scale determination by first performing intra-condition library scaling and then between condition normalization scales are computed. Cuffdiff also attempts to account for changes in isoform levels by carrying out additional transcript-specific normalization that helps estimates the abundance of each isoform (Trapnell et al., 2012).

The trimmed means of M values (TMM) is used as scaling factor to normalize the library sizes per sample in EdgeR (Bullard et al., 2010). For TMM computation first a reference sample is selected which has the closest average expressions to the mean of all samples. The remaining samples are treated as test samples and log ratios between each test sample and the reference sample are calculated. A subset of genes is selected by removing the highest and lowest expressed genes and genes with the highest log ratio. TMM is computed from this selected subset of genes, as the weighted means of log ratios between the test sample and the reference sample (Dillies et al., 2012; Rapaport et al., 2013). The user can also opt to implement

quantile normalization, to obtain the same empirical distribution for all counts across all samples. Quantile normalization is becoming the preferred method for miRNA-seq data normalization (Bullard et al., 2010; Oshlack et al., 2010; Robinson, McCarthy & Smyth, 2010)

The distribution modelling of count data is done for statistical analysis of RNA-seq expression. A Poisson distribution model which assumes that the variance is equal to the mean is used to model expression data in many biological applications (Cai et al., 2004; Soneson & Delorenzi, 2013) but is not suitable for count data that arise from RNAseq experiments as the variance of gene expression is larger than the mean (Bullard et al., 2010). A negative binomial (NB) model proves to be the best distribution model for read counts and is preferred over the one-parameter Poisson or Geometric distributions. The NB model takes into account the true biological variation (Jacobsen, Andreasen & Mouritzen, 2011) with a dispersion factor estimated to distinguish biological variation from technical variation. The variance of the NB distribution depends on the mean as given in equation below

$$\boldsymbol{\nu = \mu + \alpha\mu^2}$$

Here the following symbols apply; variance ν, mean μ and α, the dispersion factor. The dispersion factor represents the variation of gene expression relative to its mean. Accurate estimates of the dispersion parameter are critical to the correct inference of differential expression. Two popular tools, based on the NB model, for differential expression analysis are DESeq and EdgeR (Anders & Huber, 2010; Robinson et al., 2010). In the EdgeR algorithm information is shared across genes in such a way that genes may take individual values for dispersion, but individual dispersions are stabilized towards a common dispersion value so that the dispersion factor estimate is a weighted combination of two components; a gene-specific dispersion effect and a common dispersion effect calculated from all genes. This type of shrinkage of dispersion is called trended dispersion shrinkage. DESeq on the other hand uses maximum dispersion shrinkage that is the maximum of per gene dispersion effect and the trended shrinkage dispersion. Cuffdiff uses a similar variance model for single-isoform genes as DESeq (Trapnell et al., 2012). To test for differential expression for single factor experiments, significance testing is done using a pairwise exact test, that is a modification of the Fisher's exact test adopted

for the NB distribution modelling in both edgeR and DESeq packages. Cuffdiff uses a t-test statistic for differential expression analysis (Figure. 2.6) (Anders et al., 2013).

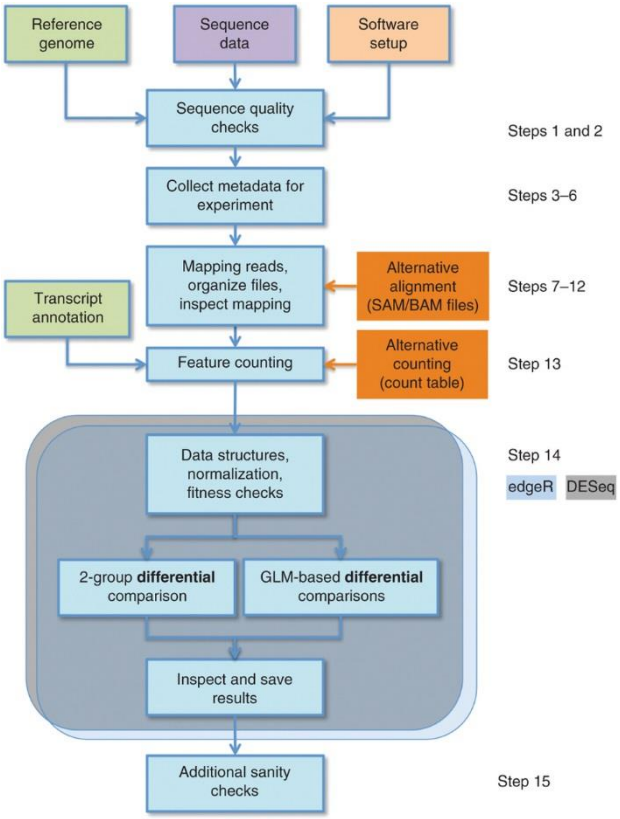


Figure 2.6 Differential expression analysis pipelines for RNA-seq count data carried out with EdgeR and DESeq. Sourced from (Anders et al., 2013). DESeq and EdgeR methods have similar procedures. Steps within the EdgeR or DESeq for differential analysis depends on the simple or complex experimental design.

2.4 miRNA mechanisms of Action

miRNAs can regulate their targets through deadenylation of mRNA leading to their subsequent degradation. The miRNA-target mRNA interaction generally occurs at the 3'UTR of the mRNA and 5'UTR of miRNA. This interaction requires complimentary base pairing of 7-nucleotides between the 2nd and 8th nucleotides at the 5'end of the miRNA called the 'seed region' and a 7-nucleotides long target-site in the 3'UTR of the mRNA. Although interaction between the miRNA seed region and the mRNA target site is required, perfect complementarity of interaction is not essential. A single miRNA can have target sites on several mRNA (Droege

& Hill, 2008). Usually multiple miRNA have target sites on the 3‘UTR of a gene and alternative transcripts of a gene with varied 3'UTR lengths could be regulated by different sets of miRNAs (Majoros et al., 2013). The existence of multiple target sites for a miRNA enhances the degree of down-regulation. Two sites of the same or different miRNAs when located close together could act in synergy (Grimson et al., 2007; Saetrom et al., 2007). Target recognition proficiency of miRNAs is partially controlled by the thermodynamics of base-pairing between target site and seed region. A stronger repression activity is reported for miRNAs that form stable seed-target duplex.

2.4.1 miRNA Target Prediction

The computational inference of miRNA-binding sites on target mRNAs is known as target prediction. A number of miRNA target prediction tools exist that take into consideration a selection of features to predict miRNA target genes. mRNA-sequence complementarily is a primary feature used in all target prediction algorithms (Cai et al., 2004; Gomes et al., 2013).

2.4.2 Target prediction tools

TargetScan is the most widely used target-prediction tool used to calculate the likelihood of an interaction between a miRNA and an mRNA (Friedman et al., 2009). According to TargetScan there are four possible types of interaction between a seed region and its target site, including;

- "8mer” seed match which refers to a complementarity of target site on mRNA to positions 2 to 8 of the miRNA followed by an "A" in the mRNA-sequence.
- "7mer-m8” refers to exact seed match complementarity of target site on mRNA to positions 2 to 8 of the miRNA
- "7mer-1A” refers to a seed match in positions 2-7 followed by an "A".

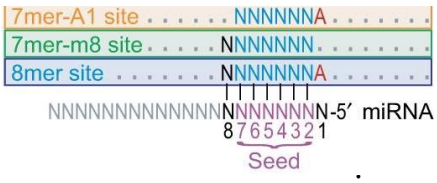
- "3' compensatory" a seed match when a pairing to the miRNA 3' region compensates for the imperfect match in the seed region (Figure. 2.7).

The TargetScan algorithm identifies sites complementary to the seed region of a miRNA on a target mRNA. Beside interaction types, TargetScan uses additional features in target prediction that help to improve the accuracy of target prediction. These include the AU content 30 nt upstream and downstream of the predicted site and the distance to the nearest end of the annotated UTR of the target gene. Nearness to other binding sites is also considered (Friedman et al., 2009; Grimson et al., 2007). A predicted efficacy called the context score, calculated by TargetScan, is a measure of all the features. Moreover, as most miRNA families are orthologous and many conserved targets sites are retained among mammals, this level of evolutionary conservation is predicted by TargetScan as the "probability of conserved targeting" (PCT).

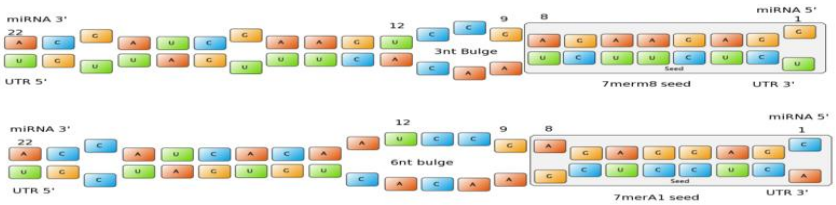
miRanda is another popular target prediction tool that uses a position-weighed local alignment algorithm for target prediction. miRanda target prediction is based on three properties: sequence complementarity, free energy of RNA-RNA duplexes and conservation of targets in related species (Griffiths-Jones et al., 2008; Lytle, Yario & Steitz, 2007).

2.5 Functional analysis of miRNAs

Targeted miRNA expression modulation is carried out to establish their regulatory role. The advantage of miRNAs for manipulation of transcriptional expression is that they are comprised of known short sequences and are conserved across species. Both gain- and loss-of-function strategies are used to study miRNA functions *in vitro* and *in vivo* (Obad et al., 2011; van Rooij, 2011). *In vivo* miRNA function analysis is beyond the scope of this thesis. *In vitro* enhancement or inhibition of endogenous miRNA of interest is carried out in order to enhance or suppress miRNA activity and determine its effect on target mRNA expression. Functional miRNA inhibition can be done using 2'-O-methyl-modified oligonucleotide and locked nucleic acid-modified oligonucleotides (Stenvang et al., 2012).



(A)



(B)

Figure 2.7 A) An illustrations of seed matching types sourced from (Friedman et al., 2009) (B). Interactions between miRNAs and their binding sites with different types of seeds. Sourced from (Niopek et al., 2011). The 3 types of complementary seed match pairing according to TargetScan include 7mer1-A, 7mer-m8 and 8 mer depending upon the degree of complementarity between seed region on miRNA and binding site on target mRNA.

In order to probe miRNA functions in cell culture, antisense oligonucleotides of miRNAs known as miRNA inhibitors are transfected into monolayers of growing cells using cloning vectors or transfection agents. miRNA inhibitors are single-stranded synthesized oligonucleotide sequences designed to have affinity to their cognate miRNA targets and are capable of reducing the level of targeted endogenous miRNA (Esau, 2008; Meister et al., 2004). Control oligonucleotides with one or more mismatches in the miRNA sequence or a scrambled sequence oligonucleotide can be used as a control to assess inhibition efficiency of inhibitor miRNA specificity. Post transfection, the target mRNA is isolated from transfected cells and the expression level is quantified through RT-qPCR to evaluate functionality of targeted inhibited miRNA (Guo et al., 2010; Stenvang et al., 2012). In this thesis we used *in vitro* miRNA inhibition in a mammalian cell line using lipid based transfection (Figure. 2.8).

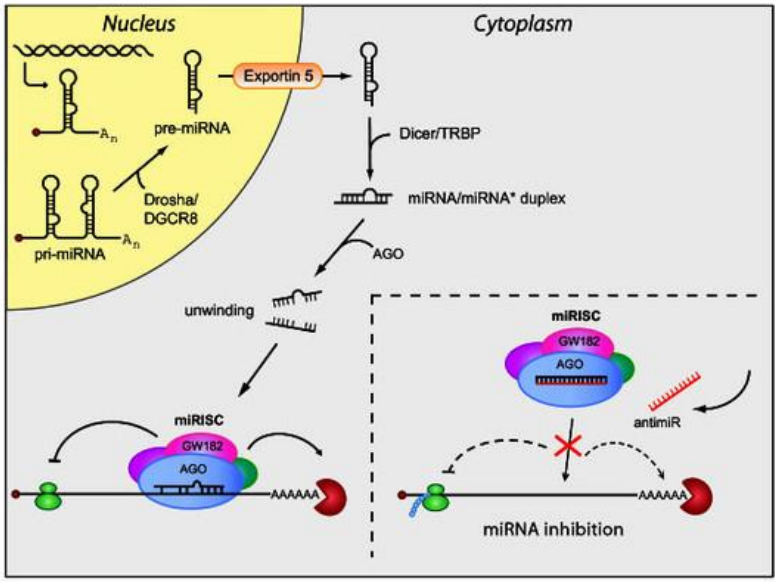


Figure 2.8 Inhibition of miRNA function by chemically modified inhibitor oligonucleotides. Sourced from (Obad et al., 2011). The pri-miRNAs are processed in to stem loop pre-miRNA followed by transportation in to the cytoplasm. Mature double stranded miRNAs are released in cytoplasm from pre-miRNAs. Single strands of mature miRNAs gets incorporated in to miRISC complex. miRNA inhibitors (antimiR) bind to miRSC-miRNAs and lead to inhibition of their function**.**

Chapter 3

Review: microRNAs in domestic livestock

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3.1 Abstract

microRNAs (miRNAs) are a class of small non-coding RNAs that bind to complementary sequences in the untranslated regions of multiple target mRNAs resulting in posttranscriptional regulation of gene expression. The recent discovery and expression- profiling studies of miRNAs in domestic livestock have revealed both their tissue specific and temporal expression pattern. In addition breed dependent expression patterns as well as single nucleotide polymorphisms (SNPs) in either the miRNA or in the target mRNA binding site have revealed associations with traits of economic importance and highlight the potential use of miRNAs in future genomic selection programmes.

3.2 Introduction

For many years it was thought that only around 2 percent of the genome was functional or coding while 98 percent of the genome or non-coding region was considered as non-functional. Recently published findings, however, from the ENCODE (Encyclopaedia of DNA Elements) project now indicate that up to 80 per cent of this non-coding region has a functional role in gene expression (Djebali et al., 2012). As the non-coding regions of genomes are being characterized and annotated their function is becoming clearer. It is apparent that the non-coding part of a genome makes a large contribution to the regulation of coding gene expression. Gene expression regulation can occur at the levels of transcription, mRNA processing, and mRNA decay and translation. One such layer of gene regulation is by non-coding RNA (ncRNA) and this mechanism is named RNA interference (RNAi) (Mello & Conte, 2004). RNAi is a normal regulatory mechanism of eukaryotic cells whereby small RNA molecules bind to their target mRNAs to silence specific genes (Almeida & Allshire, 2005; Sen & Blau, 2006). Four main types of interfering RNAs have been documented in animals, small interfering RNAs (siRNAs), repeat associated small interfering RNAs (rasiRNAs), Piwi-interacting RNAs (piRNAs) and microRNAs (miRNAs) (Ambros, 2004; Naqvi et al., 2009; Okamura & Lai, 2008). miRNAs are the most extensively studied small non coding RNAs across all species (Holley & Topkara, 2011; Le et al., 2013) and are now known to be associated with many traits and various biological networks

(Caiment etal., 2000; Hong etal., 2012; Muroya et al., 2012; Sengupta & Bandyopadhyay, 2013; Yang etal., 2010 & Zhang etal., 2009). This review will focus on recent miRNA studies in domestic animals particularly those relating to economically important traits in livestock species.

3.3 miRNA discovery, biogenesis and mechanism of action

miRNAs belong to a class of small RNAs (sRNA) about 19 to 25 nucleotides in length. They regulate gene expression in cellular processes such as signal transduction, cell cycle, differentiation and transformation and it is estimated that more than 60 percent of genes undergo direct miRNA regulation (Friedman et al., 2009). miRNAs are usually encoded in intergenic regions, but miRNAs can also exist within the introns of pre-mRNAs or be transcribed from non-coding RNA genes (Lin, Miller & Ying, 2006). Various computational as well as laboratory based methods are used for miRNAs identification and characterization. Computational methods for the identification of miRNAs consider features such as sequence composition, secondary structure (hairpin loop), thermodynamics and degree of conservation across species (An et al., 2012). miRNA expression profiling techniques include microarray hybridization (Castoldi et al., 2008), quantitative reverse transcription polymerase chain reaction (Jacobsen et al., 2011) and high-throughput sequencing (RNA seq) (Gunaratne et al., 2012).

A general miRNA data analysis pipeline has four main steps including quality assessment, normalization, annotation and differential expression analysis. Commonly used bioinformatics analysis packages based on the R statistical (Team, 2012) language are available from the Bioconductor (Gentleman et al., 2004). For putative target searches, miRNA binding sites are predicted generally in the 3`UTR of genes using one or more target prediction algorithms including miRanda (http://www.microrna.org) (Betel et al., 2008) and TargetScan (http://www.targetscan.org) (Lewis et al., 2003). Before the classification of miRNAs as regulators of gene expression, the first two members of this class of RNA named lin-4 and let-7 were discovered in the roundworm (Caenorhabditis elegans) (Lee et al., 1993; Reinhart et al., 2000), where they were found to regulate developmental timing. At present the naming convention consists of the prefix "miR" followed by a unique identifying number (e.g., miR-31) to differentiate

between miRNAs, while the genes encoding miRNAs use the same prefix but italicized (Ambros et al., 2003; Griffiths-Jones et al., 2006). Today the miRNA-mediated regulation of gene expression in various biological pathways is being studied extensively. Because of their stability in body fluids like serum, plasma, urine, and milk (Boeri et al., 2011; Courts & Madea, 2011; Ma, Jiang & Kang, 2012), miRNAs are also being considered as candidate biomarkers of various autoimmune, metabolic, cardiovascular diseases, and cancer (Alvarez & Miska, 2005; Ma et al., 2012; Rosenfeld et al., 2008; Tili et al., 2008). In addition, miRNAs are being utilized in drug discovery and developmental studies (Lindow & Kauppinen, 2012; van Rooij, Purcell & Levin, 2012; Wiggins et al., 2010). miRNA regulation also has implications in the developmental reprogramming of the cell and in stem cells research (Gangaraju & Lin, 2009) with miRNA expression perturbation techniques being applied to study their regulatory roles in mouse models and various cell lines (Park, Choi & McManus, 2010; Yan et al., 2011).

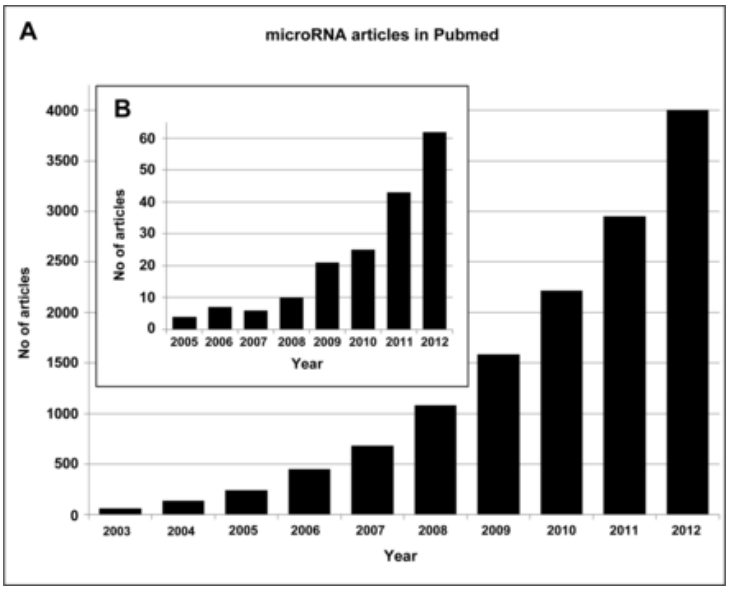

Figure 3.1 Number of references to microRNAs by year in PubMed [print publication date (PPDAT)]. (*A*) Number of references to cattle, pig, sheep, goat, chicken, and horse and microRNAs (*B*).

The latest release of miRBase (release 19, http://www.mirbase.org) (Griffiths-Jones et al., 2006), the database of published miRNA sequences and annotation, has a record of mature miRNAs for 193 species (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006; Griffiths-Jones et al., 2008; Kozomara & Griffiths-Jones, 2011). In the

last 10 years the number of publications on miRNAs in all species has increased significantly (Figure. 3.1 A). The discovery and characterization of miRNAs in domestic livestock are more recent, however (McDaneld, 2009) but are on the increase (Figure. 3.1 B). Whereas >2,000 miRNAs have been identified in human alone in miRBase, the number of mature miRNAs for cattle, sheep, pig, chicken, goat, and horse discovered to-date is much less (Table 3.1). Table 3.2 outlines some studies where miRNAs were computationally predicted or identified in various domestic livestock studies. As more miRNA discovery studies are completed the number of miRNA annotations in domestic livestock is likely to rise substantially.

Table 3.1 Number of mature miRNAs annotated in miRBase (release 19) for human and livestock species

Species	miRNAs
Human (*Homo sapiens*)	2042
Cattle (*Bos taurus*)	755
Chicken (*Gallus gallus*).	791
Horse (*Equus caballus*)	360
Pig (*Sus scrofa*)	306
Sheep (*Ovis aries*)	103
Goat (*Capra hircus*)	N.A*

3.4 miRNAs studies in domestic livestock

Studies relating to miRNAs in livestock are concerned mainly with investigating their association with traits of economic importance, especially traits associated with milk, meat and egg production and traits influencing animal productivity, fertility, embryo survival and resistance to diseases. In addition miRNAs can also be used as diagnostic tests or biomarkers for certain livestock diseases. Information on functional polymorphisms at miRNA binding sites of target genes can help improve phenotypes of interest during the process of genomic selection in various breeding programs.

3.5 Polymorphic miRNA-target interactions, miRNAs isoforms and miRNA clusters

Sequence variations like single-nucleotide polymorphisms (SNPs) can occur at miRNA binding sites on the target genes which, if functional, can alter the miRNA mediated regulation of these variant genes (Gong et al., 2012). Functional SNPs in target sites of miRNAs associated with economically important traits have been reported for livestock and some of these are listed in Table 3.3. SNPs in the primary RNAs mir-206/mir-133b cluster were reported to be associated with muscle fiber characteristics, meat quality and lean meat production in Berkshire, Landrace and Yorkshire pig breeds. SNPs in mir-206 were associated with muscle fiber percentage, drip loss, lightness and back fat thickness, while mir-133b SNPs were found to be associated with loin eye area, muscle pH and total muscle fibre number (Lee et al., 2012a). miRNAs themselves can exist as variants differing in 1 to 3 nucleotides, called isomirs. These sequence variants are most often found at the 3`end of the miRNA and mostly do not affect their functionality (Chiang et al., 2010), however, heterogeneity at the 5' end of miRNA has also been reported (Ibrahim et al., 2010; Langenberger et al., 2009; Seitz, Ghildiyal & Zamore, 2008). Isomirs have been reported in cattle (Gu, Eleswarapu & Jiang, 2007) and most isomirs were found to be 3`end variants with only a small number showing 5' end variation. While some cattle miRNAs exist as both type of isomirs, miR-125-p has two 3` end and two 5` end variants. Isomirs have also been found in the 3` end of miR-423 in a porcine muscle tissue study (Nielsen et al., 2010) while 5` end variants of miR-388 and 3` end variants of miR-96 were reported in the longissimus muscle of sheep (Zhang et al., 2013). In addition, two SNPs were identified within the primary mir-1 sequence in pigs and were found to be associated with genes involved in muscle fibre composition (Hong et al., 2012).
A further characteristic feature of miRNAs is that they tend to exist as clusters at various genomic loci and these clusters are mostly conserved among species (Altuvia et al., 2005). One study identified 60 miRNAs clusters on the cattle genome (Bartel, 2009a; Elsik, Tellam & Worley, 2009) while clusters have also been reported within specific loci on chromosome 1, 8, 12, 16 and 21 (Lo & Chen, 2009). In addition miR-23a

and miR-24 were reported to exist in the same cluster and to be co-expressed in various bovine tissues (Gu et al., 2007). In the case of the porcine genome 33 miRNAs were found to exist in 14 different clusters on nine different porcine chromosomes (Cho et al., 2010). So far, little is known about the functional implications of the clustering of miRNAs in livestock genomes.

Table 3.2 Polymorphisms in miRNA binding sites on gene targets

Specie	Study	Gene	Function	miRNAs binding site
Cattle	*(Mullen et al., 2010)*	GH21, GH24	growth hormone	miR-1306, miR-17-3p, miR-220a, c, d, miR-92-b miR-671
	(Li et al., 2011a)	HSF1	heat shock protein	miR-671, miR-484
	(Hou et al., 2012a)	BOLA-DQA2	immune response	miR-2318
Pig	*(Xu et al., 2011a)*	DRD2L, DRD2S	dopamine receptor	miR-744, miR-339-5p, miR-1307, miR-1271, miR-328
Sheep	*(Clop et al., 2006)*	GDF8 GDF8 MSTN	muscle mass protein	miR-1, miR-206 miR-1, miR-206 miR-1, miR 206
Goat	*(Zidi et al., 2010)*	CSN1S1	milk protein	miR-101

Target binding sites with functional SNPs are identified in 3`UTR s of target genes

Table 3.3 Number of miRNAs in various livestock miRNAs studies

Species	Study	Tissue	*n*
Cattle	(Gu et al., 2007)	various	54
	(Strozzi et al., 2009)	various	249
	(Long & Chen, 2009)	various	31
	(Glazov et al., 2009)	kidney	5
	(Coutinho et al., 2007)	embryo	98
	(Tripurani et al., 2010)	ovary	58
	(Huang et al., 2011)	teste	122
	(Huang et al., 2011)	ovary	136
	(Hossain et al., 2009)	ovary	38
Chicken	(Xu et al., 2011b)	embryo/adult	25
	(Glazov et al., 2008)	embryo (*day 5, 7, 9*)	349
	(Darnell et al., 2006)	embryo (*day 0.5–5*)	4
	(Hicks, Tembhurne & Liu, 2008)	embryo (*day 11*)	14
	(Yao et al., 2011)	preadipocytes	63
	(Hicks, Trakooljul & Liu, 2010)	liver	17
	(Shao et al., 2008)	various	29
Horse	(Zhou et al., 2009)	various	407
Pig	(Lian et al., 2012)	teste	15
	(Chen et al., 2012b)	adipose	140
	(Wernersson et al., 2005)	various	51
	(Kim et al., 2008)	heart, liver	14
	(Zhou et al., 2010)	muscle	16
Sheep	(Barozai, 2012)	various	172
	(Torley et al., 2011)	ovary	128
	(Sheng et al., 2011)	various	31
	(Caiment et al., 2010)	skeletal muscle	55
Goat	(Li et al., 2012)	mammary tissue	441

3.6 Expression profiles of livestock miRNAs

miRNA expression has now been profiled across a variety of tissues from livestock species. miRNAs have also been characterized in body fluids like milk and blood where they exist in micro vesicles or exosomes. miRNAs regulate a large number of genes and certain miRNAs show ubiquitous expression across various tissues suggesting that these miRNAs may play a regulatory role in biological processes vital to all cells across all tissues (Coutinho et al., 2007; Hicks et al., 2008; Jin et al., 2009a). It is evident from the literature, however, that some miRNAs exhibit tissue specific or stage specific patterns of expression. Table 3.4 provides a list of studies that have investigated miRNAs associated with tissue specific patterns of expression in livestock species. The expression patterns of miRNAs associated with important traits of livestock species are discussed below.

3.6.1 miRNAs and milk traits

A key feature of miRNAs is their high stability in a variety of body fluids including milk and blood (Hunter et al., 2008). This feature makes them potential biomarkers for various metabolic and physiological conditions (Chen et al., 2008). Milk miRNAs are isolated from micro vesicles, which are small bodies surrounded by a plasma membrane and secreted by various types of cells into body fluids (Kosaka et al., 2010). These micro vesicles play a role in the transfer of various types of RNAs and proteins and are also involved in intercellular communication (Valadi et al., 2007). The presence of several miRNAs in milk micro vesicles of cattle suggested a role for miRNAs in the gastrointestinal system as well as a possible role in the immune function of calves (Hata et al., 2010). miRNAs were screened from cattle milk at different lactation stages and it was found that 108 miRNAs exhibited increased expression in the colostrum compared to mature milk while consistent levels of expression were recorded for a set of seven miRNAs during all stages of lactation (Chen et al., 2010). It was suggested that these miRNAs could be used as quality control markers to test milk purity, as formula milk showed very low amount of these miRNAs compared to pure raw milk. miRNA abundance was also compared in exosomes isolated from porcine milk in early lactation (0 and 3 days) and later lactation (7, 14, 21 and 28 days) periods (Gu et al., 2012).

Table 3.4 Tissue-dependent expression of miRNAs in livestock species

Species	Study	Tissue*	miRNA
Cattle	(Coutinho et al., 2007)	embryo	miR-199a
		thymus	miR-150
		small intestine	miR-140
	(Jin et al., 2009a)	brain	miR-129
		liver	miR-122
		muscles	miR-1, miR-206
	(Hossain et al., 2009)	cumulus cells of ovary	miR-29a, miR-125b, miR-409, miR-503
	(Tesfaye et al., 2009)	mature oocytes	miR-496, miR-297, miR-292-3P, miR-99a, miR-410, miR-145,
		immature oocytes	miR-512-5p, miR-214
Chicken	(Cutting et al., 2012)	embryonic chicken	miR-101, miR-202-5p, miR-31
	(Wang et al., 2012)	muscle	miR-133a, miR-1
		embryo	miR-140
Pig	(Huang et al., 2013)	longissimus muscle (post gestational fetus)	miR-495
		longissimus muscle (adult)	miR-193
	(Stowe et al., 2012)	metaphase II oocytes, 8-cell embryo, blastocyst,	miR-21, miR-205, miR-195, miR-16, miR-125b, miR-205, miR-128, miR-17, miR-210, miR-92a, miR-302a, miR-129-5p
Sheep	(Luense et al., 2010)	fetal ovary	miR-497, miR-15b

Thirteen miRNAs were more abundant in the early lactation periods and found to target immune related genes. Similar findings were reported in another study where miRNA expression profiles were compared between colostrum and mature milk (Izumi et al., 2012). It was found that miR-15b, miR-27b, miR-34a, miR-106b, miR-130a, miR-155, and miR-223 exhibited higher expression in colostrum. The latter two miRNAs have anti-inflammatory roles with miR-155 associated with regulation of T immune cells differentiation and miR-223 associated with neutrophil activation. Casein, a phosphoprotein, makes up a major portion of milk protein used for cheese production (Heck et al., 2009; Masotti, Battelli & De Noni, 2012). A study of polymorphism in the 3`UTR of casein genes (CSN1S1, CSN1S2, CSN2, CSN3) in 5 breeds of goat including Murciano-Granadina, Cashmere, Canarian, Saanen, and Sahelian breeds reported a SNP in the 3`UTR of CSN1S1 in Murciano-Granadina goats that disrupts the binding site for miR-101 (Zidi et al., 2010).

3.6.2 miRNAs and fertility traits

miRNAs of livestock gonadal tissues, oocytes and sperm have been implicated in regulation of many fertility traits in livestock (Carletti & Christenson, 2009). In a cattle miRNA study expression of seven miRNAs was higher in ovarian cortex compared to other compartments of the ovary while miR-29a was more abundant in cumulus cells (Hossain et al., 2009). Similarly, in another study where miRNA expression in beef cattle ovaries was profiled, expression of miR-106 was higher in oocytes compared to cumulus oocyte complexes (COCs) and granulosa cells (Miles et al., 2012). In addition, the expression of the predicted target genes of miR-106 were decreased in oocytes compared to COCs. Higher expression of miR-205, miR-150, miR-122, miR-96, miR-146a and miR-146b-5p was reported in immature cattle oocytes compared to mature oocyte from 0 h to 22 h of oocyte maturation (Abd El et al., 2011). The same set of miRNAs also showed higher expression in early (8-cell) stage embryo compared to the blastocyst stage embryo. In addition, a set of 51 miRNAs were reported to be more highly expressed in mature oocytes compared to surrounding cumulus cells with miR-205 being the most highly expressed. It was also reported that 47 miRNAs were more highly expressed in immature oocytes compared to cumulus cells with miR-122 being the most highly

expressed (Abd El et al., 2011). Oocytes undergo various morphological changes and oocyte miRNAs expression varies with the stages of oogenesis. In one study, miR-424 and miR-10b were reported to be highly expressed in the germinal vesicle stage of cattle oocytes (Tripurani et al., 2010). In another study, where bovine oocytes at different stages of maturation were compared, 7 miRNAs (miR-496, miR-297, miR-292-3P, miR-99a, miR-410, miR-145 and miR-515-5p) were reported to be more abundant in mature oocytes while 2 miRNAs (miR-512-5p and miR-214) were more abundant in immature oocytes (Tesfaye et al., 2009). Ovarian hormones play many essential roles in reproduction. The aromatase enzyme converts androgen to ovarian estrogens and the regulation of the aromatase gene CYP19 can have major implications in ovarian development and function (Lance & Bogart, 1992). In a porcine ovarian study the aromatase gene was reported to be down regulated by miR-378 in granulosa cells during follicular development (Xu et al., 2011b). In another study, testosterone was reported to alter the expression of ovine fetal ovarian miR-497 and miR-15b at day 90 of gestation (Luense et al., 2010). In a chicken study, miR-499 and miR-1709 were found to be associated with the regulation of pleiotrophin (Lee et al., 2012a; Lee et al., 2012b), a growth hormone with a role in the development of the oviduct and formation of the egg (Muramatsu et al., 2006). The expression of gonadal miRNAs at the time of sexual differentiation was profiled in the chicken embryo study (Cutting et al., 2012) and reported sexually dimorphic expression patterns for miR-101, miR-31 and miR-202-5p. Target genes of these miRNAs are involved in important gonadal differentiation pathways associated with TGF-β (transforming growth factor beta). Expression of sheep gonadal miRNAs at gestational day (GD) 42 considered as early gestation and the mid-gestational (GD 75) stage of development were profiled from ovaries and testis (Torley et al., 2011). In that study 24 miRNAs were reported to be differentially expressed between the two sexes at GD42 and another 43 at GD75, targeting genes important in gonadal development pathways. The X chromosome also has a secondary role to play in reproduction with deregulation of certain X linked genes leading to premature ovarian failure (Toniolo & Rizzolio, 2007). One study, reported an increased expression of 24 X-linked miRNAs in porcine ovaries compared to testis which targeted genes involved in various ovarian functions (Li et al., 2011b).

miRNAs have also been found in testicular tissue and sperm (Amanai, Brahmajosyula & Perry, 2006). A deep sequencing study of conserved and pig specific miRNAs from the sexually immature (30 days) and mature (180 days) porcine testis reported higher expression of 96 conserved miRNAs and lower expression of conserved 26 miRNAs in mature testis with predicted targets of these miRNAs having potential roles in the regulation of spermatogenesis (Lian et al., 2012). In addition 10 miRNAs (including miR-153 and miR-205) were only expressed in the mature testis, whereas three miRNAs (miR-196, miR-485-3p and miR-149*) were only expressed in the immature testis. In a cattle study expression of seven miRNAs was reported to be different in the spermatozoa of high and low fertility Holstein bulls suggesting a role for miRNAs in cattle fertility (Govindaraju et al., 2012). The let-7 family of miRNA is functionally conserved across species and has been reported to play important role in various biological processes (Roush & Slack, 2008) including cell differentiation and cancer (Boyerinas et al., 2010) and in metabolism (Frost & Olson, 2011). In a comparison of normal porcine sperm with those from pigs with morphological abnormalities (Curry, Safranski & Pratt, 2011) the expression of the let-7 family members, let-7a, let-7d, let-7e and miR-22, was higher in the morphologically abnormal group whereas miR-15b was expressed at a lower level. miRNAs expression of chicken embryonic gonads was profiled at days 5.5, 6.5, and 9.5 (Bannister et al., 2011). At each time point a higher expression of miR-202* was reported in the developing testis compared to that in ovaries suggesting a role for miR-202* in sexual differentiation of testis. In addition female gonads treated with aromatase inhibitor at day 3.5 had increased expression of miR-202*. miR-202* was found to be associated with DMRT1 (doublesex and mab-3 related transcription factor 1) and SOX9 (sex determining region Y-box 9), both of which have a role in testicular development (Koopman, 2009; Vidal et al., 2001). A role for miRNAs in bovine ovarian luteal cell apoptosis has also been suggested (Ma et al., 2011). miR-378 was reported to have a higher expression in regressed corpus luteum (CL) compared to non-regressed CL and was able to decrease *IFNGR1* (interferon gamma receptor 1) protein level in early, mid, late and regressed cattle CL. Sheep miRNAs were profiled in follicular-luteal transitional stages including growing and pre-ovulatory follicles as well as from day 3 and day 9 post oestrous CL (McBride et al., 2012). Nine miRNAs had lower expression during the process of follicular-luteal transition while eight other miRNAs had

higher expression. The putative gene targets of these miRNAs were associated with processes involved in follicular differentiation. A significant increase in expression of miR-26b was reported in porcine atretic follicles which in turn target the *ATM* (ataxia telangiectasia mutated) gene involved in DNA fragmentation in granulosa cells (Lin et al., 2012). *AHCYL1* (S-adenosylhomocysteine hydrolase-like protein 1) is expressed in the oviduct and plays a role in embryonic development (Buhi, Alvarez & Kouba, 1997). In chick oviduct AHCYL1 was reported to have putative target sites in its 3`UTR for miR-124a, miR-1602, miR-1612, miR-1669, miR-1710 and miR-1782 (Jeong et al., 2012). In addition miR-124a, miR-1669, miR-1710 and miR-1782 were reported to influence the expression of AHCYL1 in vitro. In addition, miR-124a and miR-1669 were found to have binding sites on genes involved in the development and differentiation of the oviduct. In a study of equine follicular fluid, miRNAs which were present in follicle fluid were also detected in surrounding granulasa and cumulus cells suggesting a role for micro vesicles in miRNAs transfer (da Silveira et al., 2012). In addition the miRNA profile in ovarian follicular fluid was reported to vary among young and old mares (da Silveira et al., 2012). The placenta is essential for the transfer of nutrients and oxygen to the foetus in utero during pregnancy. In a porcine placental study, differential expression of miR-125b, miR- 92 b, miR-106a, miR-24, and miR-20 was reported between day 30 and 90 of gestation (Su et al., 2010).

3.6.3 miRNAs and embryonic development and survival

Early embryo survival is essential for efficient livestock production (Diskin & Morris, 2008). The miRNA expression pattern in elongated bovine Day 17 embryos following transfer as blastocysts on Day 7 after in vitro fertilization (IVP) or cloning has also been reported (Castro et al., 2010). In that study miR-103, miR-203, miR-107, miR-19b, miR-21 and miR-106 were more highly expressed in cloned embryos while miR-140, miR-106b, miR-342, miR-200c, let7-b, miR-24, miR-30d and miR-26a were more highly expressed in IVP embryos. In addition, 29 miRNA present in the cloned embryos were absent in the somatic cells used for nuclear transfer, suggesting that a reprogramming of miRNA expression occurs in bovine cloned embryos after somatic cell nuclear transfer. In another study, in which miRNA expression was profiled from bovine oocytes at Germinal Vesicle

and metaphase II stages and from early embryos at 2-cell, 4-cell, 8-cell and blastocyst stages, miR-21 and miR-130a showed an increase in expression from the 1-cell up to 8-cell stage (Mondou et al., 2012). Putative targets of these miRNAs were implicated in various development processes and suggested a role for miRNAs during the maternal-to-embryonic transition. In a developmental network study of miRNAs from early and hatched-stage cattle blastocysts, miR-127, miR-130a, miR-155, miR-196a, miR-203, miR-28, miR-29c and miR-376a were reported to be more highly expressed in hatched blastocysts while miR-218, miR-335, miR-135a and miR-449b were reported to show a lower expression (Goossens et al., 2013). In addition, a direct regulation of NOTCH1 (notch homolog 1 translocation-associated), a trans membrane protein involved in cell signalling during development (Wu et al., 2002) by miR-449B and of NANOG (nanog homeobox) a transcription factor involved in development (Luo et al., 2013) by miR-28 was confirmed. Furthermore, miR-28 was shown to regulate *NANOG* expression in response to FGF signalling. miRNAs are also reported to play a role in the germ cell differentiation in chicken (Lee et al., 2011). In that study miR-181*a was reported to be more highly expressed in primordial germ cells (PGCS) resulting in inhibition of *HOAX1* (homeoboxa1) expression and repression of NR6A1 (nuclear receptor subfamily 6 group a member 1) and as a consequence inhibition of PGCS differentiation to somatic cells. Fibroblast growth factor (FGF) plays an important role in the regulation of embryonic development (Naiche, Holder & Lewandoski, 2011; Thisse & Thisse, 2005). Transcriptional regulation of miR-206 by FGF signalling was demonstrated in chicken embryo from 1.5 days to 5 days (Sweetman et al., 2006). FGF can regulate the pluripotency of embryonic stem (ES) cells through *LIN28B* (protein lin-28 homolog B) dependant pathways (Faas et al., 2013). *LIN28B* in turn has been reported to have regulatory role in miRNA expression (Moss & Tang, 2003). It was reported that LIN28B under the influence of FGF can regulate the expression of let-7b, miR-9, miR-19b, miR-107, miR-130b and miR-218 during porcine gastrulation, however, FGF was reported to regulate miR-9, miR-107 and miR-218 expression through pathways independent of *LIN28B* also (Bobbs et al., 2012). In that study miR-130b and miR-218 were reported to have target sites in the 3`UTR of *PDGFRA* (platelet-derived growth factor receptor, alpha polypeptide) which encodes a receptor for a growth factor that plays important roles in cell migration and cell lineage decision. In one porcine study

different number of miRNAs were found to be expressed in metaphase II oocytes, 8-cell embryos and blastocysts, with 63 miRNAs expressed at the 8-cell stage compared to 88 in oocytes and 84 in blastocysts (Stowe et al., 2012). Of these, miR-18a, miR-21 and miR-24 were implicated in important cellular pathways targeting genes involved in cell differentiation, cell signalling and transcription regulation along with other important cellular processes. In addition, miR-24 expression was lower in the in vivo produced embryo from artificially inseminated post-pubertal gilts relative to in vitro fertilized embryo from oocytes of pre-pubertal gilts and cycling sows and was suggested as a biomarker for embryo quality. In a recent study seven miRNA were identified in pig COCs and early embryos including miR-574, miR-24, let-7e, miR-23B, miR-30d, miR-320 and miR- 30c (Yang et al., 2012).

3.7 Summary and conclusions

This review highlights the potential role of microRNAs in regulating economically important traits in livestock. The breed dependent variation in expression of miRNAs associated with economically important traits as well as the existence of polymorphism in miRNA target sites on economically important genes could form the basis for a selection programs in cattle, sheep, chickens and pigs. The potential use of circulating miRNAs as biomarkers for different physiological conditions and diseases could also help to improve the health and fertility of livestock and of milk quality.

Chapter 4

Alterations in hepatic miRNAs expression during negative energy balance in postpartum dairy cattle

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4.1 Abstract

4.1.1 Background

Negative energy balance (NEB), an altered metabolic state, occurs in early postpartum dairy cattle when energy demands to support lactation exceed energy intake. During NEB the liver undergoes oxidative stress and increased breakdown of fatty acids accompanied by changes in gene expression. It is now known that micro RNAs (miRNA) can have a role in mediating such alterations in gene expression through repression or degradation of target mRNAs. miRNA expression is known to be altered by metabolism and environmental factors and miRNAs are implicated in expression modulation of metabolism related genes.

4.1.2 Results

miRNA expression was profiled in the liver of moderate yielding dairy cattle under severe NEB (SNEB) and mild NEB (MNEB) using the Affymetrix Gene Chip miRNA_2.0 array with 679 probe sets for *Bos-taurus* miRNAs. Ten miRNAs were found to be differentially expressed using the 'samr' statistical package (delta = 0.6) at a q-value FDR of < 12%. Five miRNAs including miR-17-5p, miR-31, miR-140, miR-1281 and miR-2885 were validated using RT-qPCR, to be up-regulated under SNEB. Liver diseases associated with these miRNAs include non-alcoholic fatty liver (NAFLD) and hepatocellular carcinoma (HCC). miR-140 and miR-17-5p are known to show differential expression under oxidative stress. A total of 32 down-regulated putative target genes were also identified among 418 differentially expressed hepatic genes previously reported for the same animal model. Among these, *GPR37* (G protein-coupled receptor 37), *HEYL* (hairy/enhancer-of-split related with YRPW motif like), *DNJA1*, *CD14* (Cluster of differentiation 14) and *GNS* (glucosamine (N-acetyl)-6-sulfatase) are known to be associated with hepatic metabolic disorders. In addition miR-140 and miR-2885 have binding sites on the most down-regulated of these genes, *FADS2* (Fatty acid desaturase 2) which encodes an enzyme critical in lipid biosynthesis. Furthermore, *HNF3*-gamma (Hepatocyte nuclear factor 3-gamma), a hepatic transcription factor (TF) that is involved in *IGF-1* expression regulation and maintenance of glucose homeostasis is a putative target of miR-31.

4.1.3 Conclusions

This study shows that SNEB affects liver miRNA expression and these miRNAs have putative targets in hepatic genes down-regulated under this condition. This study highlights the potential role of miRNAs in transcription regulation of hepatic gene expression during SNEB in dairy cattle.

4.2 Background

Over the past few decades improvements in milk production through genetic selection have been associated with a reduction in cow fertility and this decrease in fertility has become a major concern for dairy producers (Diskin & Morris, 2008; Lucy, 2007). Reproduction is an energetically expensive process and an altered metabolic state called NEB has been established as one of the major physiological causes of decreased fertility in high yielding dairy cattle (Butler, 2003; Diskin & Morris, 2008; Lucy, 2007). NEB is the result of increased energy demands to support lactation, coupled with lowered feed intake (Fenwick et al., 2008a; Van Knegsel et al., 2007b). Immune related and hepatic functions are known to be effected by NEB (Fenwick et al., 2008a; Fenwick et al., 2008b; McCabe et al., 2012; McCarthy et al., 2010; Patton et al., 2006; Wathes et al., 2011) and there is an increased metabolic load on the liver to overcome the energy deficit under NEB. There is a dramatic increase in hepatic oxidation of fatty acids for energy production and in addition there are extra demands on the liver to increase glycogenesis to meet the glucose requirements of milk production (McCabe et al., 2012; McCarthy et al., 2010). In order to understand the complex metabolic adjustments in postpartum dairy cattle liver during NEB, a dairy cattle model based on different milking regimes was developed (Patton et al., 2006). Earlier in a microarray gene expression study 418 hepatic genes were reported to be differentially expressed as a result of SNEB (McCarthy et al., 2010). These differentially expressed genes have roles in lipid metabolism and glycogenic processes, immune response and the somatotropic axis involved in milk production. The regulation in gene expression under SNEB, however, is yet to be fully understood.

It is now known that a class of small RNAs called miRNA, about 19 to 25 nucleotides in length, can regulate such alterations at the gene expression level. miRNAs were first discovered from the round worm *C. Elegans* almost 3 decades ago (Lee et al., 1993). Since their discovery, hundreds of miRNAs have been identified across the plant and animal kingdoms. It is estimated that whereas only 1-5% of genomic transcripts in mammals code miRNAs, up to 60% of genes are regulated by miRNAs (Friedman et al., 2009; Sun et al., 2010).

The biogenesis of miRNA is a multistep process that begins with miRNA gene transcription resulting in primary miRNA (pri-miRNA) in the nucleus, followed by the generation of around 70nt long stem–loop precursor miRNAs (pre-miRNAs) from pri-miRNA. Pre-miRNAs are then translocated to the cytoplasm where they are trimmed to remove the terminal loop and release ~22 nt long duplex mature miRNAs containing a guiding strand and a passenger strand. The guiding strand assembles into cytoplasmic RNA-induced silencing complex (*RISC)* with argonaute protein that guides the complex to their complementary mRNA targets (Kim, 2005; Krol, Loedige & Filipowicz, 2010). The miRNA target-mRNA complementary base-pair interaction generally occurs between the target site on the *3'UTR* of the mRNA and the 2nd to 8th nucleotides on the *5'UTR* of the miRNA called the 'seed region'. Generally, miRNAs repress translation through deadenylation of mRNA leading to their subsequent degradation or translation repression. miRNAs are reported to regulate a wide range of biological processes including cell cycle regulation, proliferation and differentiation as well as development, immune response, carcinogenesis and various metabolic processes (Carleton, Cleary & Linsley, 2007; Esquela-Kerscher & Slack, 2006; Hoefig & Heissmeyer, 2008). The effects of metabolites and environmental factors including hormones, cytokines and nutrients on miRNAs is well established (Becker et al., 2011; Li et al., 2009; Liu et al., 2012; Rottiers & Naar, 2012). Alterations in cattle liver miRNA expression in response to external anabolic steroids was reported and miRNAs were suggested as potential biomarker for drug abuse in cattle (Becker et al., 2011). In another study testosterone treatment was reported to alter miR-22, miR-690, miR-122, let-7a, miR-30 and let-7d expression in female rat liver (Deli et al., 2010). Increases in expression of miR-155 and miR-132 due to oxidative stress was reported in an ALD (alcholic liver disorder) mouse model (Bala et al., 2009).

Oxidative stress of hepatocytes was also reported to alter miR-199a-5p expression (Dai et al., 2013). Nutritional modulation of miRNA expression has been reported in various dietary intervention studies of metabolic disorders like obesity, diabetes and fatty liver (Kirpich et al., 2011; Tomofuji et al., 2009; Vallanat et al., 2010). A high fat diet was reported to alter the adipose tissue miRNA expressional profile of miR-19a, -92a, -92b, -101, -103, -106, -142–5p, and 296 in cattle (Romao et al., 2012). miRNAs can regulate metabolism and homeostasis of high energy metabolites as well as insulin signalling and glucose homeostasis (Esau et al., 2006; Esau et al., 2004; Jordan et al., 2011). miR-33a and miR-33b located within the sterol regulatory element-binding proteins (SREBP), key transcription regulators of genes involved in cholesterol biosynthesis and uptake, regulate cholesterol homeostasis jointly with their host genes and have roles in the regulation fatty acid metabolism and insulin signalling (Davalos et al., 2011). In addition, miR-122, a liver specific miRNA regulates hepatic fatty acid oxidation and fatty acid and cholesterol synthesis rate (Davalos et al., 2011; Esau et al., 2006; Esau et al., 2004; Jin et al., 2009b; Jordan et al., 2011; Rayner et al., 2010a). miRNAs are associated with the pathophysiology of hepatic metabolic disorders like NAFLD and NASH (non-alcoholic steatohepatitis) as well as HCC in mouse and human studies (Ahn et al., 2011; Ahn et al., 2012; Alisi et al., 2011; Yang et al., 2010). The potential role miRNAs have to play in NEB in the dairy cow remains to be elucidated.

This study set out to determine a) if hepatic miRNA expression was altered as a result of SNEB in a model of moderate yielding Holstein Friesian dairy cows in the early postpartum period, and b) to integrate hepatic miRNA and mRNA expression profiles through prediction of targets of these miRNAs among a set of previously reported differential expressed hepatic genes under SNEB from the same animal model. Elucidation of the expression patterns of miRNAs and computational identification of their putative target among genes regulated under SNEB will contribute to the understanding of the roles of miRNAs in regulating gene expression during SNEB in dairy cows.

4.3 Methods

4.3.1 Animal model

The animal model employed in this study has been described previously (Patton et al., 2006). All procedures were carried out under license in accordance with the European Community Directive, 86-609-EC. In brief, multiparous Holstein-Friesian cows were blocked according to parity, body condition score, and previous milk yield, two weeks prior to expected calving and within block were randomly allocated to two treatments; mild NEB (MNEB, n = 12) or severe NEB (SNEB, n = 12) groups based on different feeding and milking regimes. On day 2 after calving, MNEB cows were fed *ad libitum* grass silage with 8 kg per day of a 21% crude protein dairy concentration and milked once daily; SNEB cows were fed 25 kg of silage per day silage with 4 kg of crude protein per day and milked three times daily. Three times a day versus once a day milking was used to advance a state of SNEB in one group by increasing energy withdrawal with a concomitant lower need for differences in energy intake between groups. The chemical composition of silage and concentrate offered as previously described (Patton et al., 2006). For sample collection, cows were selected from each group based on extremes of energy balance (MNEB, n = 6; SNEB, n = 6). Cows were slaughtered approximately 14 days postpartum (MNEB; 13.6 ± 0.75, range 11–15; SNEB 14.3 ± 0.56, range 13–16) (McCarthy et al., 2010). Liver tissues were retrieved within 30 min of slaughter and snap frozen at -80°C. One MNEB cow was removed retrospectively as she was deemed to be ill during the experimental period.

4.3.2 RNA extraction and quality analysis

For the purpose of RNA extraction, 100 mg frozen liver tissue was directly immersed in 1 ml Trizol (Invitrogen, Stockholm, Sweden). A Precellys 24 homogeniser (Bertin Technologies, Montigny-le-Bretonneux, France) was used to homogenise the tissue (6000 rpm for 20 sec). Homogenate was left for 5 min at RT to permit the complete dissociation of nucleoprotein complexes followed by centrifugation at 12,000 g for 10 min at 4ºC. Supernatant was transferred to a 2 ml tube. 2µl of Pellet paint® NF Co-Precipitant (EMD Millipore, Darmstadt, Germany) and 200µl chloroform was added and incubated at RT for 5 min. After

centrifugation for 15 min at 4ºC and 14000 g the aqueous phase was transferred into a new tube and 500µl isopropanol was added and incubated for 5 min on ice. Following centrifugation for 10 min at 4ºC and 14000 g the supernatant was removed and the pellet washed with 500µl 75% ETOH followed by centrifugation for 10 min at 4ºC and 14000 g. A 100µl aliquot of nuclease-free water was added to the pellet and incubated at RT for 10 min. After vortexing for 1 min 10µl of 3 M sodium acetate was added. Next, 250 µl ice cold ETOH was added and incubated for 1 hr at -20ºC, followed by centrifugation at 4ºC for 10 min and 14000 g. The supernatant was removed and washed with 500µl of 75% EtOH by vortexing followed by centrifugation at 4ºC for 10 min and 14000 g. The supernatant was removed and the pellet was air-dried for 5 min at RT following which 30µl of RNase-free water was added and left for 5 min. RNA quality and quantity was assessed using a NanoDrop spectrophotometer (ND-1000; Wilmington, DE, USA) and Bioanalyzer 2100 (Agilent Technologies Ireland, Dublin, Ireland). Mean RIN values were > 8. Total RNA was stored at -80°C.

4.3.3 Gene Chip miRNAs_2.0 array hybridization

Total RNA from liver samples of 11 cows were hybridized to the Affymetrix Gene Chip miRNAs_2.0 (Affymetrix, Santa Clara, CA, USA) arrays for miRNA expression profiling. This array has miRBase v15 coverage (www.mirbase.org) with 15,644 probe sets of 131 organisms including 679 probe sets for *Bos-taurus* miRNAs. Array hybridization was carried out at ATLAS Biolabs, Berlin, Germany. Briefly, 200 ng of total RNA labelled with Flash Tag Biotin was hybridized to the miRNA Array in a Hybridization Oven 640 (Affymetrix) at 48°C for 18 h. The arrays were stained with the Fluidics Station 450 using fluidics script FS450_0003 (Affymetrix), and then scanned on a GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA, USA).

4.3.4 Statistical analysis

All statistical analyses were performed in the R statistical computing environment (Version 2.14; http://www.r-project.org) with the *samr* package from the Bioconductor project (http://www.bioconductor.org). Data quality was assessed with the *ArrayQualitymetrix* package from Bioconductor (Dee & Getts, 2012;

Kauffmann, Gentleman R Fau - Huber & Huber, 2009). One cel file from each group was discarded due to issues with hybridisation quality. The *expresso* method of the Affy package (Gautier et al., 2004) was used to pre-processes the data with quantile normalization and median polish summarization. While the chip contains probes complementary to miRNA from other species only the expression intensities of 679 cattle miRNAs were used for further analysis. The Significance Analysis of Microarray (SAM) two-class unpaired method implemented in the *samr* Bioconductor package was used to identify differentially expressed miRNAs (Tusher et al., 2001). The *samr* parameter delta 0.6 was selected that classified miRNAs as differentially expressed at a fold-change > 1.25 and a q-value < 12%.

4.3.5 RT-qPCR validation of differentially expressed miRNA

Seven candidate miRNAs differentially expressed on the microarray; including miR-17-5p, miR-31, miR-140, miR-1281, miR-2885, miR-296, and miR-671 were selected for RT-qPCR validation. miRNA expression was carried out with TaqMan miRNA RT-qPCR assays according to the manufacturer's instructions (Applied Biosystems, Dublin, Ireland). miRNA-specific reverse transcription was performed on 10 ng of purified total RNA using the TaqMan MicroRNA Reverse Transcription kit according to manufacturer's instructions. RT-qPCR reactions were performed using 1µl of cDNA (10 ng/µl) in 9µl of Taqman universal master mix containing TaqMan PCR primers and probes on a BioRad CFX96 real time PCR system (Bio-Rad, Hemel Hempstead, UK) using the following cycling parameters; 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Four biological replicates from each of the MNEB and SNEB treatment groups were used for RT-qPCR validation. Three reference miRNAs including miR-122, let-7b and *RNU6B* were tested with the software program geNorm version 3.5(Erkens et al., 2006) for calculating the gene expression stability measure (M value). *RNU6B* was found to be the most stable internal reference miRNA across treatments with an M value of 0.7. It was more stable on its own than when used in combination with the two other reference miRNA. The software package BioRad CFX manager was used for correction of the Ct values and normalization to RNU6B using the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008). Corrected Ct values were used to calculate differential expression using the PROC t-test (SAS) (Yuan et al., 2006).

4.3.6 Prediction of differentially expressed miRNAs targets among differentially expressed hepatic genes under SNEB

A target prediction algorithm for custom data, from the TargetScan database website of miRNA target prediction (Friedman et al., 2009) implemented Perl (targetscan_61_context_scores.pl), was used to identify target sites of differentially expressed miRNAs in the 3′UTRs of 418 differentially expressed genes reported previously for the same liver tissue (McCarthy et al., 2010). Using this algorithm, conserved 8mer and 7mer sites matching the seed region of each miRNA in the 3′UTRs of differentially expressed hepatic genes were identified. Non-conserved sites and sites with mismatches in the seed region that were compensated by conserved 3′ pairing were also included (Friedman et al., 2009). Predictions were ranked based on the *context + scores* of the sites that represent the predicted efficacy of targeting. Figure 4.1 gives the analysis pipe line for miRNA expression. GO (Gene Ontology) biological processes associations of 3 conserved miRNAs (miR-31, miR 17-5p and miR-140) were retrieved using FAME (functional assignment of miRNAs via enrichment) software (Ulitsky, Laurent & Shamir, 2010). No data was available for miR-1281 and miR-2885 in the FAME database. The FAME algorithm makes direct inference of a specific miRNA function using enriched subsets of the target genes sharing a common biological process or pathway. The GO biological processes associated with down-regulated putative targets were retrieved from the UniProt Gene Ontology Annotation (GOA) database (http://www.ebi.ac.uk/GOA).

4.4 Results

Ten out of 679 mature bovine miRNAs represented on the miRNA Gene Chip miRNA_2.0 array were found to be up-regulated in SNEB cows. Table 4.1 lists the miRNAs that showed differential expression between MNEB and SNEB groups. RT-qPCR results confirmed the differential expression of 5 out of the 7 miRNAs tested. The expression of miR17-5p, -1281, -140, 2885 and -31 were consistent between microarray and RT-qPCR whereas miR-296 and miR-671 were not significant. Table 4.2 lists the differentially expressed miRNAs validated by RT-qPCR. Putative targets of these five miRNAs were identified in the set of

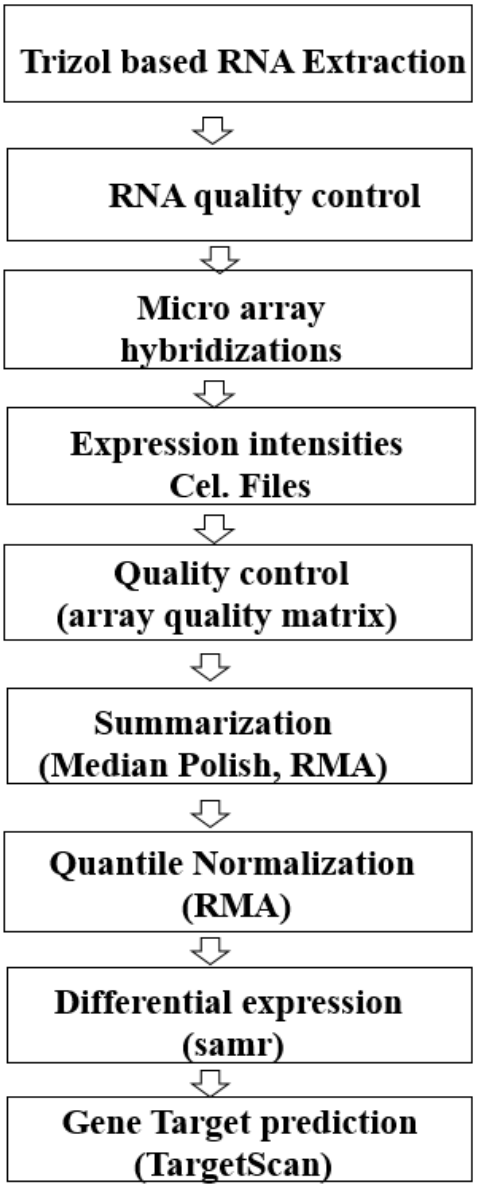

Figure 4.1 Micro-array based miRNA expression analysis and target prediction pipe line.

418 (230 down- and 188 up-regulated) hepatic genes reported previously in the same animal model. Among the 418, 67 (35 up-regulated and 32 down-regulated) genes were found to have target sites in the 3′UTRs of the up-regulated miRNAs (Table 4.3).

Table 4.1 Differentially expressed postpartum dairy cattle hepatic miRNAs under NEB

miRNA	miRBase accession	Mean SNEB	Mean MNEB	**q-value (%)	*Fold change
bta-miR-31	MI0004762	11.79±0.11	10.95±0.15	0.00	1.79
bta-miR-1281	MI0010466	7.16±0.43	6.39±0.1	11.97	1.71
bta-miR-2483	MI0011545	6.27±0.35	5.62±0.41	11.97	1.56
bta-miR-2885	MI0013058	10.26±0.23	9.63±0.1	0.00	1.55
bta-miR-296	MI0009786	8.197±0.40	7.59±0.29	11.97	1.52
bta-miR-2316	MI0011336	6.255±0.25	5.7±0.30	11.97	1.40
bta-miR-140	MI0005010	10.68±0.20	10.27±0.1	11.97	1.33
bta-miR-17-5p	MIMAT0003815	10.45±0.23	10.07±0.07	11.97	1.30
bta-miR-671	MI0009887	6.51±0.18	6.140±0.17	11.97	1.29
bta-miR-2455	MI0011512	6.91±0.18	6.55±0.16	11.97	1.28

*Differential expression analysis was done for microarray expression intensities of 679 bovine miRNAs with SAM (significance analysis of microarrays) method at delta parameter of 0.6. ** q FDR < 12%.

MNEB= Mild negative energy balance and SNEB= severe negative energy balance group groups

Table 4.2 Differentially expressed liver miRNAs under SNEB using RT-qPCR

miRNA	SNEB† Mean	MNEB† Mean	Fold change*	**p- value
bta-miR-31	23.885±1.05	25.925±1.05	4.11	0.033
bta-miR-1281	22.682±0.26	24.315±1.24	3.10	0.042
bta-miR-2885	29.157±0.36	30.70±0.36	2.91	0.006
bta-miR-17-5p	25.96±0.90	28.175±0.68	4.62	0.008
bta-miR-140	23.89±1.06	26.035±1.03	3.90	0.030
bta-miR-296	33.78±0.19	33.55±0.59	0.85	0.490
bta-miR-671	34.27±2.54	34.16±0.48	0.92	0.930

* Fold change values are derived from back-transformed Ct-values.

**Differential expression analysis was carried out with PROC t-test (SAS) after normalization of Ct-values to reference gene RNU6B using $2^{-\Delta\Delta Ct}$ method. †SNEB= Severe negative energy balance, MNEB= mild negative energy balance.

All of the five differentially expressed miRNAs validated by RT-qPCR in this study were up-regulated however there was no significant enrichment among the down-regulated putative targets genes using the Fisher test for enrichment analysis. Overall miR-140 was found to have the most putative targets among differentially expressed genes under SNEB while miR-17-5p had the second highest number of targets. As the consensus is that up-regulated miRNAs result for the most part in down-regulation of their target genes, we further selected only down-regulated genes as putative affected targets for subsequent analysis to investigate the role of differentially expressed miRNAs under SNEB. As miRNAs are thought not to switch off their targets completely but rather fine-tune their expression, all down-regulated genes were included irrespective of fold-change. Figure 4.2 presents the subset of the putative down-regulated targets for these up-regulated miRNAs. miR-140 had the highest number of putative down-regulated target genes followed by

miR-17-5p. miR-31 was the most up-regulated miRNA under SNEB while miR-1281 had all but one of its putative target genes down-regulated under SNEB. miR-2885 has *FADS2* as its only putative down-regulated target under SNEB and *FADS2* is also a putative target of miR-140. *DSG1* (desmoglein1) and *FXR1* (fragile X mental retardation, autosomal homolog 1) are both shared targets of miR-17-5p and miR-31 while *GNS* (glucosamine-N-acetyl-6-sulfatase) is a shared target of mir-140 and miR-31 (Figure. 4.3). Table 4.4 lists the bio-types of putative targets down-regulated under SNEB. The important GO biological processes associated with down-regulated putative targets are given in Table 4.5 and additional information is in Supplementary Table 4.1. The GO biological functions associated with three conserved miRNAs are given in Supplementary Table 4.2. miR-31 is associated with the GO biological processes of cell growth, amino acid transport and response to stress. miR-17-5p is associated with GO biological processes include regulation of cell cycle and protein metabolism. miR-140 is associated with GO biological processes of cell proliferation, polysaccharide metabolism and signal transduction, while both miR-31 and miR-140 are associated with response to nutritional level.

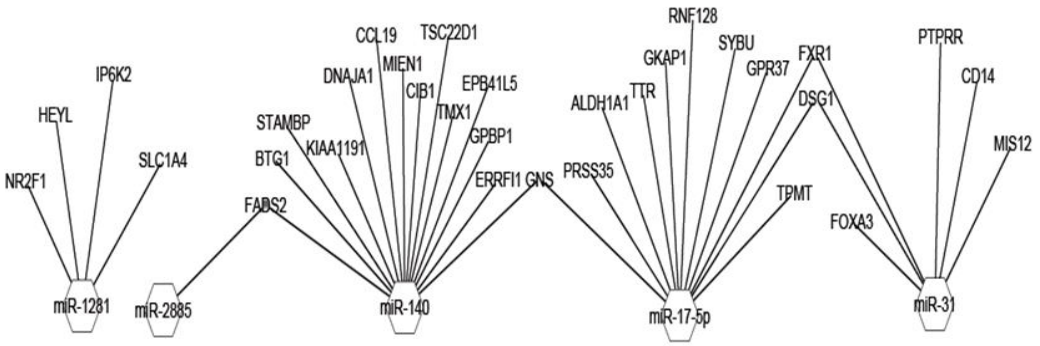

Figure 4.2 Differentially expressed miRNAs under SNEB and their shared targets hepatic genes. Hexagons represent the up-regulated miRNAs with edges leading to nodes representing their putative targets down-regulated under SNEB. Nodes with two edges represent putative targets shared by two up-regulated miRNAs.

miR-140	FC	mir17-5p	FC	bta-miR-31	FC	mir2885	FC	miR-1281	FC
FADS2	-19.60	DSG1	-2.10	DSG1	-2.10	FADS2	-19.60	HEYL	-2.27
CCL19	-1.50	GPR37	-1.84	FOXA3	-2.04	RNF4	1.19	SLC1A4	-2.20
BTG1	-1.44	SYBU	-1.72	PTPRR	-1.48	LPCAT3	1.25	IP6K2	-1.43
TSC22D1	-1.41	PRSS35	-1.50	MIS12	-1.42	BIN1	1.47	NR2F1	-1.19
KIAA1191	-1.32	TTR	-1.32	CD14	-1.39	TUFT1	2.05	POLR3GL	1.15
DNAJA1	-1.31	FXR1	-1.26	FXR1	-1.26	SCARB1	2.62		
MIEN1	-1.28	RNF128	-1.24	NHEJ1	1.27	SLC25A34	3.29		
EPB41L5	-1.25	TPMT	-1.24	ARG1	1.32				
ERRFI1	-1.25	GKAP1	-1.21	SEC61A2	1.41				
CIB1	-1.15	ALDH1A1	-1.12	OAT	1.59				
GNS	-1.12	GNS	-1.12	HSF2BP	1.80				
STAMBP	-1.09	CMPK1	1.15	SLC25A48	1.93				
TMX1	-1.09	HMGN4	1.17						
GPBP1	-1.08	RNF4	1.20						
DPYD	1.12	BNIP3L	1.20						
MINA	1.13	SLC27A4	1.25						
SNAPIN	1.15	CNN1	1.29						
POLR3GL	1.15	ARG1	1.32						
HMGN4	1.17	AADAT	1.33						
SUMO3	1.18	CPQ	1.86						
THG1L	1.21	IL1A	2.03						
ADTRP	1.22								
DENND2D	1.26								
REEP5	1.28								
MBL2	1.35								
PDRG1	1.38								
ACP2	1.38								
HDC	1.45								
RCAN3	1.52								
TUFT1	2.04								
SCARB1	2.61								
SLC25A34	3.29								
CPT1B	5.52								

Figure 4.3 Predicted miRNA gene targets with fold-change under SNEB Colour index fold change in SNEB compared to MNEB groups of target hepatic genes of differentially expressed miRNA from the NEB cattle model : (blue >= 2, green <2 and >1, yellow >=-1 and <-1.25, orange >=-1.25 and < -2 and red >=-2).

Table 4.3 Putative target genes of up-regulated hepatic miRNAs differentially expressed under SNEB

Down-regulated putative targets		Up-regulated putative targets	
Symbol	Gene name	Symbol	Gene name
ALDH1A1	aldehyde dehydrogenase 1 family, member A1	*AADAT*	aminoadipate aminotransferase
BTG1	B-cell translocation gene 1, anti-proliferative	*ACP2*	Acid phosphatase 2, lysosomal
CCL19	chemokine (C-C motif) ligand 19	*ADTRP*	Androgen-Dependent TFPI-Regulating Protein
CD14	CD14 molecule	*ARG1*	Arginase, liver
CIB1	Calcium and integrin binding 1 (calmyrin)	*BIN1*	Bridging integrator 1
DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1	*BNIP3L*	BCL2/adenovirus E1B 19 kDa interacting protein 3-like
DSG1	desmoglein 1	*CMPK1*	cytidine monophosphate (UMP-CMP) kinase 1, cytosolic
EPB41L5	Erythrocyte membrane protein band 4.1 like 5	*CNN1*	Calponin 1, Basic, Smooth Muscle
ERRFI1	ERBB receptor feedback inhibitor 1	*CPQ*	carboxypeptidase Q
FADS2	Fatty acid desaturase 2	*CPT1B*	carnitine palmitoyltransferase 1B (muscle)
FOXA3/ HNF3-gamma	Forkhead box A3	*DENND2D*	DENN/MADD domain containing 2D
FXR1	Fragile X mental retardation, autosomal homolog 1	*DPYD*	dihydropyrimidine dehydrogenase
GKAP1	G kinase anchoring protein 1	*HDC*	histidine decarboxylase
GNS	glucosamine (N-acetyl)-6-sulfatase	*HMGN4*	high mobility group nucleosomal binding domain 4
GPBP1	GC-rich promoter binding protein 1	*HSF2BP*	heat shock transcription factor 2 binding protein
GPR37	G protein-coupled receptor 37 (endothelin receptor type B-like)	*IL1A*	interleukin 1, alpha
HEYL	hairy/enhancer-of-split related with YRPW motif-like	*LPCAT3*	lysophosphatidylcholine acyltransferase 3
IP6K2	inositol hexakisphosphate kinase 2	*MBL2*	mannose-binding lectin (protein C) 2, soluble (opsonic defect)
KIAA1 191	KIAA1 191	*MINA*	MYC induced nuclear antigen
MIEN1	Migration and invasion enhancer 1Bottom of Form	*NHEJ1*	nonhomologous end-joining factor 1
MIS 12	MIND kinetochore complex component, homolog (S. pombe)	*OAT*	ornithine aminotransferase (gyrate atrophy)
NR2F1	nuclear receptor subfamily 2, group F, member 1	*PDRG1*	p53 and DNA-damage regulated 1
PRSS35	protease, serine, 35	*POLR3GL*	polymerase (RNA) III polypeptide G (32kD)-like
PTPRR	protein tyrosine phosphatase, receptor type, R	*RCAN3*	RCAN family member 3
RNF 128	ring finger protein 128	*REEP5*	receptor accessory protein 5

Table 4.4 Intracellular location and bio-types of down-regulated putative gene targets of up-regulated miRNAs

Symbol	Entrez gene name	Location	Type
KIAA1191	KIAA1191	Cytoplasm	Other
MIEN 1	Migration and invasion enhancer 1	Cytoplasm	Other
MIS 12	MIS 12, MIND kinetochore complex component, homolog (S. pombe)	Nucleus	Other
NR2F1	Nuclear receptor subfamily 2, group F, member 1	Nucleus	Ligand-dependent nuclear receptor
PRSS35	Protease, serine, 35	Extracellular space	peptidase
PTPRR	Protein tyrosine phosphatase, receptor type, R	Plasma membrane	phosphatase
RNF 128	ring finger protein 128, E3 ubiquitin protein ligase	Cytoplasm	Enzyme
SLC1A4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	Plasma membrane	Transporter
STAMBP	STAM binding protein	Nucleus	Enzyme
SYBU	syntabulin (syntaxin-interacting)	Unknown	Other
TMX1	thioredoxin-related transmembrane protein 1	Cytoplasm	Enzyme
TPMT	thiopurine S-methyltransferase	Cytoplasm	Enzyme
TTR	Transthyretin1	Cytoplasm	Transporter
ALDH1A1	aldehyde dehydrogenase 1 family, member A1	Cytoplasm	enzyme
BTG1	B-cell Translocation gene 1, anti-proliferative	Nucleus	Transcription Regulator
CCL19	chemokine (C-C motif) ligand 19	Extracellular space	Cytokine
CD14	CD14 molecule	Plasma Membrane	Transmembrane receptor
CIB1	Calcium and integrin binding 1 (calmyrin)	Nucleus	Other
DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1	Nucleus	Other
DSG1	desmoglein 1	Plasma membrane	Membrane protein
EPB4 1 L5	erythrocyte membrane protein band 4.1 like 5	Plasma membrane	Membrane protein
ERRFI1	ERBB receptor feedback inhibitor 1	Cytoplasm	Other
FADS2	Fatty acid desaturase 2	Plasma membrane	Enzyme
FOXA3/*HNF3-gamma*	forkhead box A3	Nucleus	Transcription regulator
FXR1	Fragile X mental retardation, autosomal homolog 1	Cytoplasm	Other
GKAP1	G kinase anchoring protein 1	Cytoplasm	Other
GNS	Glucosamine (N-acetyl)-6-sulfatase	Cytoplasm	Enzyme
GPBP1	GC-rich promoter binding protein 1	Nucleus	Transcription regulator
GPR37	G protein-coupled receptor 37 (endothelin receptor type B-like)	Plasma membrane	G-protein coupled receptor
HEYL	Hairy/enhancer-of-split related with YRPW motif-like	Nucleus	Transcription regulator
IP6K2	inositol hexakisphosphate kinase 2	Cytoplasm	kinase

Table 4.5 GO biological Processes associated with miRNAs

miRNA	* Evidence code	GO term	p-value	q-value	z-score	enrichment factor	Targets	HG p-value
mir-17-5p								
	ISO,IDA	regulation of cell cycle	0.0001	0.0648	4	1.63	42	0.00068
	IDA	cell cycle arrest	0.0002	0.0486	4.03	2.34	15	0.00512
	ISO	negative regulation of progression through cell cycle	0.0002	0.0324	3.77	1.73	31	0.00060
	ISO,IDA	ER-nuclear signaling pathway	0.0003	0.0405	4.01	3.58	5	0.06127
	ISO	protein kinase cascade	0.001	0.081	3.23	1.44	49	0.00043
	ISO	intracellular signaling cascade	0.0014	0.094	3.14	1.25	124	0.00010
	IDA	regulation of GTPase activity	0.0026	0.1271	3.06	1.93	15	0.00383
	ISO,IMP	sterol metabolism	0.0027	0.125	3.2	2.33	9	0.05208
	ISO,IMP	cholesterol metabolism	0.0029	0.1174	3.1	2.35	8	0.06675
miR-31								
	IEA	amino acid transport	0.0011	0.0734	4.34	5.4	4	0.02223
	IEA	carboxylic acid transport	0.0012	0.0641	4.03	4.69	5	0.01089
	IDA	cell growth	0.0098	0.124	2.64	2.37	8	0.02002
	IDA	regulation of cell growth	0.037	0.2823	2	2.13	6	0.05510
	IDA	negative regulation of growth	0.0448	0.3236	1.98	2.49	4	0.04053
mir-140								
	IMP	positive regulation of cell proliferation	0.0037	0.5681	3.21	2.2	14	0.00087
	IMP	regulation of cell proliferation	0.0045	0.4545	2.85	1.73	22	0.00087
	IEA	response to nutrient levels	0.0449	0.3887	2.05	2.95	3	0.07195

*Evidence code for GO terms: IEA= inferred from electronic annotation, IDA= inferred from direct assay, IMP = inferred from mutant phenotype and ISO = inferred from sequence orthology.

4.5 Discussion

Dairy cows enter a state of NEB postpartum when the energy supply is prioritised towards the mammary tissues for milk production while the energy intake is reduced. The principal physiological response to the energy deficit is mobilization of non-esterified fatty acids (NEFA) from adipose tissue. NEFAs are broken down in the liver to release energy and any incomplete breakdown results in production of *BHB* (β-hydroxybutyrate) or *TAGs* (triacylglycerides). Accumulation of *TAGs*, *BHB* and NEFAs results in the onset of oxidative stress in the liver that can lead to fatty liver or lipidosis(McCarthy et al., 2010). In addition components of the somatotrophic axis like *IGF* (insulin like growth factor) and *GH* (growth hormones) that have roles in the control of milk production are also affected by SNEB (Fenwick et al., 2008a; McCabe et al., 2012; McCarthy et al., 2010; Morris et al., 2009). Reduced *IGF1* expression is also negatively correlated with fertility in dairy cows (Taylor et al., 2004). Previous nutritional supplementation of animals in NEB with lipogenic and glycogenic nutrients partly succeeded in compensating for energy partitioning between mammary tissues and the rest of the body (Van Knegsel et al., 2007a; Van Knegsel et al., 2007b). However, the regulation of such alterations is yet to be explored. In this study, five miRNAs were altered in response to SNEB. Two of these miRNAs have been reported to be altered under oxidative stress in humans (Read et al., 2012; Yang et al., 2010) and miR-17-5p has been associated with oxidative stress in HCC (Yang, Yin et al. 2010). In addition alterations in miR-140 expression under chemical and oxidative stress has also been reported (Read et al., 2012). Three of the miRNAs, miR- 17, miR-31 and miR-140, found to be up-regulated under SNEB have been previously associated with hepatic disorders (Hill-Baskin et al., 2009; Michelotti, Machado & Diehl, 2013; Shan et al., 2013; Shan et al., 2009) and miR-140 has been associated with NAFLD. Two of these miRNAs have been reported to be altered under oxidative stress in humans and miR-17-5p has been associated with oxidative stress in HCC (Yang et al., 2010). A number of the putative down-regulated targets of the differentially expressed miRNAs in this study are known to have roles in hepatic disorders. These include the miR-17-5p target *GPR37* (G protein-coupled receptor 37) a member of the G-protein coupled receptor-1 family associated with NAFLD (Sharma et al., 2006). The miR-1281 target *HEYL* (hairy/enhancer-of-split related with YRPW

motif-like) is associated with HCC (Huang et al., 2006; Xie & Proud, 2013). The miR-140 target *DNJA1* that has a role in lipid intake is associated with the oxidative stress response of high fat induced NAFLD (Kirpich et al., 2011; Vallanat et al., 2010). The miR-31 target, *CD14* (Cluster of differentiation 14) involved in transmission and release of polysaccharides, is also implicated in ALD, hepatic cholestasis and hepatic fibrosis (Kolios, Valatas & Kouroumalis, 2006; Seki & Brenner, 2008). The miR-17-5p target *GNS* (glucosamine (N-acetyl)-6-sulfatase) that metabolises glucosamine sulphate has been implicated in hepatocellular dysplasia, cirrhosis of the liver and hepatic fibrosis (Roy & Finegold, 2010). Moreover, the miR-17-5p target *TTR*, a carrier protein for transport of lipid soluble vitamins from the liver to the circulation is also reduced under SNEB. Reduced *TTR* expression has been reported in a study of periparturient period Holstein and Jersey dairy cows in NEB (Rezamand et al., 2007). In addition we were able to postulate a role for miR-31 in SNEB through its putative target hepatic transcription factor *FOXA3* also known as *HNF3-gamma*. Bovine *IGF-I* has binding sites for *HNF3-gamma* in its promoter region (Eleswarapu et al., 2009). While another recent human study reported target sites of miR-31 on the 3′UTR of *IGF-1* (Mosakhani et al., 2010). *HNF*-gamma is also known to be involved in glucose homeostasis in hepatocytes through regulation of *GLUT2* (glucose transporter 2) (Costa et al., 2003; Shen et al., 2001). However, the most interesting putative target of all was the gene encoding the lipogenesis enzyme *FADS2* which is critical for long-chain polyunsaturated fatty acids biosynthesis (Lattka et al., 2010). In this study we found binding sites for two up-regulated miRNAs miR-140 and miR-2885 in the 3′UTR of *FADS2*, whereas miR-140 has been previously implicated in the regulation of *FADS2* homolog *FADS1* (Park et al., 2011).

4.6 Conclusion

This study suggests a role for hepatic miRNAs in lipid and energy metabolism through the identification of a subset of their putative targets associated with important metabolic processes. Moreover hepatic miRNAs associated with genes involved in the somatotropic axis can be a possible link between reduced reproductive performance and SNEB in the early postpartum dairy cow. Further direct functional studies of selected miRNA-mRNA putative pairs in the liver of

such models will help to improve our understanding of the molecular mechanisms and pathways involved in SNEB in postpartum dairy cattle.

Chapter 5

The miRNAome of the postpartum dairy cow liver under negative energy balance

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5.1 Abstract

5.1.1 Background

Negative energy balance (NEB) is an altered metabolic state in high yielding cows that occurs during the first few weeks postpartum when energy demands for lactation and maintenance exceed the energy supply from dietary intake. NEB can, in turn, lead to metabolic disorders and to reduced fertility. Alterations in the expression of more than 700 hepatic genes have previously been reported in a study of NEB in postpartum dairy cows. miRNAs (micro-RNA) are known to mediate many alterations in gene expression post transcriptionally. To study the hepatic miRNA content of postpartum dairy cows including their overall abundance and differential expression under mild NEB (MNEB) and severe NEB (SNEB) short read RNA sequencing was carried out. To identify putative targets of differentially expressed miRNAs among differentially expressed hepatic genes reported previously in dairy cows under SNEB computational target identification was employed.

5.1.2 Results

Our results indicate that the dairy cow liver expresses 53 miRNAs at a threshold of 10 reads per million (RPM). Of these, 10 miRNAs accounted for >95% of the miRNAome (miRNA content). Of the highly expressed miRNAs, liver-specific miR-122 constitutes 75% followed by liver-enriched miR-192. Five out of thirteen let-7 miRNA family members are also among the highly expressed miRNAs. miR-143, down-regulated under SNEB, was found to have 16 putative up-regulated gene targets associated with SNEB including *SLC2A12* (solute carrier family 2 (facilitated glucose transporter, member 12) and LRP2 (low density lipoprotein receptor-related protein 2), which have roles in glucose and lipid metabolism and are up-regulated under SNEB.

5.1.3 Conclusions

This is the first liver miRNA-seq profiling study of moderate yielding dairy early postpartum. Tissue specific miR-122 and liver enriched miR-192 are two of the

most abundant miRNAs in the postpartum dairy cow liver. miR-143 is significantly down-regulated under SNEB and putative targets of miRNA-143 which are up-regulated under SNEB, include genes with roles in liver glucose and lipid metabolism.

Keywords: micro RNA, RNA-seq, liver, negative energy balance, nutrition, Dairy cattle

5.2 Background

5.2.1 miRNAs

miRNAs are among the most abundant and extensively studied classes of small non-coding RNA. miRNAs are estimated to regulate the expression of up to 60% of mammalian protein coding genes (Friedman et al., 2009) and are associated with many economically important traits in domestic livestock (Fatima & Morris, 2013). The biosynthesis of miRNA is comprised of multiple stages. The initial stage involves transcription of the miRNA gene to produce primary miRNAs, several hundred base-pairs in length, followed by the generation of 70 nucleotide-long hairpin shaped pre-miRNAs. Finally Dicer-mediated processing results in the mature ~22bp miRNA (Lee et al., 2002). miRNA-mediated interference involves imperfect base-pairing of miRNAs, typically to the 3' UTR of the target mRNA. miRNAs have been reported to regulate gene expression in various biological pathways including those involved in metabolism and energy homeostasis (Dumortier, Hinault & Van Obberghen, 2013). In addition, miRNAs have been implicated in a wide variety of diseases (Ming et al., 2012; Pandey et al., 2009). The regulatory role of miRNAs in liver disease such as hepatitis, diet induced non-alcoholic fatty liver; alcoholic and non-alcoholic hepatocellular carcinoma and cirrhosis have been reported in humans and demonstrated in mouse liver studies (Alisi et al., 2011; Bala et al., 2009; Tsai et al., 2012). In the case of beef cows both liver specific and ubiquitously expressed miRNAs have been reported (Gu et al., 2007; Jin et al., 2009a). In another cow liver study miRNAs were suggested as biomarkers for anabolic steroid abuse screening (Becker et al., 2011).

5.2.2 Negative energy balance

The postpartum period is of critical importance for the general and reproductive health of dairy cows (Sheldon, 2004). During the early postpartum period energy expenditure for maintenance and lactation exceeds energy intake from nutrition resulting in NEB (Wathes et al., 2009)NEB increases the risk of other metabolic disorders such as ketosis and fatty liver and animals are prone to infectious diseases, including mastitis, due to reduced immunity (Liu et al., 2010). NEB has profound effects on the liver which undergoes many physiological and biochemical changes to counteract the energy imbalance (Wathes et al., 2009). An energy balance model of early lactation dairy cows was developed previously (McCarthy et al., 2010; Patton et al., 2006) resulting in two groups of cows with MNEB and SNEB. Alterations in gene expression in the liver associated with SNEB have been reported in two previous studies (McCabe et al., 2012; McCarthy et al., 2010).

In the first study 418 genes were found to be differentially expressed due to SNEB using microarray-based expression profiling (McCarthy et al., 2010). In the second study, using RNA-seq, 413 genes were shown to be differentially expressed (McCabe et al., 2012) with 72 differentially expressed genes in common between the two studies. Both of these studies report alteration of genes involved in lipid metabolism the main biological process altered during SNEB. The mechanisms, however, mediating these alterations in transciptome expression are yet to be fully understood. In a microarray-based and RT-qPCR validated study of liver miRNAs from this NEB model, five miRNAs were found to be up-regulated under SNEB (Fatima et al., 2014). Microarray expression profiling, however, is limited to the pre-defined probe sets on the array and only relative expression of miRNAs can be measured. On the other hand with RNA sequencing (RNA-seq) it is possible to measure absolute expression of all putative miRNAs in a sample (Gunaratne et al., 2012). As miRNA activity and function depend on abundance (Ebert & Sharp, 2012), accurate quantification of miRNA abundance is important (Brown et al., 2007; Sarasin-Filipowicz et al., 2009). miRNA sequencing is based on short read RNA-seq and involves generation and high-throughput sequencing of 50bp cDNA reads derived from the small RNA fraction of total RNA (Garber et al., 2011).

The objectives of this study were to (i) carry out an in-depth global analysis of the hepatic miRNA content of postpartum dairy cows including their overall abundance

and differential expression under MNEB and SNEB using RNA sequencing and (ii) to computationally identify putative targets of differentially expressed miRNAs among differentially expressed hepatic genes reported previously in dairy cows under SNEB.

5.3 Methods

5.3.1 NEB model

A NEB dairy cow model developed previously was used. In this model differential feeding and milking regimes were used to produce two groups of Holstein Friesian cows; MNEB and SNEB (McCarthy et al., 2010; Patton et al., 2006). Briefly, MNEB cows were fed a*d libitum* grass silage with 8 kg/day of a 21% crude protein dairy concentrate and milked once daily. SNEB cows were fed 25 kg/day silage with 4 kg/day concentrate and milked thrice daily.

5.3.2 Liver Tissue Collection for miRNA analysis

Cows were slaughtered and the entire liver was removed within 15 to 30 min. Samples weighing approximately 1 g were dissected, rinsed in RNAse-free phosphate buffer, snap-frozen in liquid nitrogen and stored at -80ºC. Liver tissue samples from 4 animals from each group were used for miRNA library preparation.

5.3.3 RNA Extraction and Library Preparation

Total RNA enriched for small RNA was extracted from each of the 8 samples using the mirVana™ miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, 1 mg of frozen liver tissues was lysed in 500 µl lysis/binding solution followed by the addition of 50 µl of miRNA homogenate. Following vortexing, the solution was incubated on ice for 10 min. 500 µl of acid-phenol chloroform was added to the solution followed by vortexing for ~60 sec. Phase separation of the filtrate was carried out at 10,000 g for 5 min at room temperature. The aqueous phase was transferred to a new tube with the addition of 1/3 volume of 100 % ethanol and mixed by vortexing. Samples were passed through a glass-fibre filter cartridge by centrifugation for ~30 sec at 10,000 g and the filtrate was collected. A 2/3 volume of 100 % ethanol was added to the

filtrate and mixed by vortexing. The filtrate was passed through a new filter cartridge at 10,000 g for 30 seconds. The flow-through from this step was discarded and the filter washed with 700 µl Wash Solution 1 provided in the kit. The flow-through was again discarded and the filter was centrifuged for 1 min. The filter was washed with 500 µl Wash Solution 2 provided in the kit, the flow-through discarded and the filter centrifuged for 1 min. This step was repeated once. Finally, to elute RNA from the filter, 50 µl of 95°C nuclease-free water was applied for 1 min to filter followed by a 30 sec centrifugation. The RNA extract was stored at −80°C. An Agilent RNA 6000 Nano Kit and the 2100 Bioanalyzer was used to measure total RNA integrity (Agilent Technologies, Colorado Springs, CO, USA) and the Agilent Small RNA Kit (Agilent Technologies) was used for miRNA quantification. Small RNA libraries were constructed at BGI (Shenzhen, China) and sequenced on an Illumina HiSeq2000 platform, using one sample per lane at a sequencing depth of 20M bases.

5.3.4 Data Analysis Pipeline

FASTQC v0.10.0 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to carry out preliminary quality control of the 8 FASTQ files. The 3′ adaptor sequences were trimmed with the Cutadapt v1.1 (https://code.google.com/p/cutadapt). After trimming, reads shorter than 18 nucleotides were discarded. The FASTQ quality filter v0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/) was applied to trim low quality bases from reads. Phred scores were calculated and reads where at least 50 % of the bases had a Phred score < 20 were discarded. The filtered reads were further trimmed at their ends to remove low quality bases (Phred score <20). Sequence reads were then aligned to the bovine genome (UMD3.1) using Novoalign version 2.07.11 (http://www.novocraft.com) with the option of "m" miRNA mode. Only uniquely aligned reads were retained. HTSeq version 0.5.3p3 (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html) was used to annotate uniquely aligned reads using Ensembl (v66) bovine gene and miRNA annotation.

5.3.5 Differential Expression Analysis

Quantile normalisation was used to normalise count data across (Bullard et al., 2010) libraries. The Bioconductor package EdgeR (v2.4.6) (Oshlack et al., 2010) which uses a negative binomial model to account for both biological and technical variability was used to identify differentially expressed miRNAs between the two groups using moderated tag wise dispersions. A Benjamini and Hochberg (Benjamini, Hochberg & Series, 1995) corrected P value cut-off of <0.05 was applied to correct for multiple testing.

5.3.6 RT-qPCR validation of differentially expressed miRNAs

RT-qPCR was used for technical validation of differentially expressed miRNA using TaqMan miRNA assay (Applied Biosystems, Dublin, Ireland). Gene-specific reverse transcription was performed on 10 ng of purified total RNA using the TaqMan MicroRNA Reverse Transcription kit according to manufacturer's instructions (Applied Biosystems, Dublin, Ireland). RT-qPCR reactions were performed using 1µl of cDNA (10ng/µl) in 9µl of Taqman universal master mix containing TaqMan PCR primers and probes on a BioRad CFX96 real time PCR system (Bio-Rad, United Kingdom) using the following cycling parameters: 95°C for 10min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. A total of 4 biological replicates from each of the MNEB and SNEB treatment groups were used for RT-qPCR validation. From our experience with microarray hepatic miRNA expression validation using the same tissue samples, *RNU6B* was chosen as the most stable internal reference miRNA (Fatima et al., 2014). miRNA expression levels were recorded as Ct values, i.e., the number of PCR cycles at which the fluorescence signal is detected above the threshold value. The software package BioRad CFX manager was used for correction of the Ct values and normalization to RNU6B using the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008). Corrected Ct values were used to calculate differential expression using the PROC t-test (SAS) (Yuan et al., 2006). A P<0.05 was deemed to be significant.

5.3.7 miRNA Target Predictions

TargetScan Release 6.2 (www.targetscan.org) was used to predict targets of differentially expressed miRNAs among the two datasets of up-regulated hepatic genes previously reported FOR the liver tissues of the same cows and animal model (McCabe et al., 2012; McCarthy et al., 2010). Figure 5.1 gives the analysis pipeline used. Gene Ontology(GO) biological processes associations of conserved miRNAs were retrieved using FAME (functional assignment of miRNAs via enrichment) software (Ulitsky et al., 2010). The FAME algorithm makes direct inference of a specific miRNA function using enriched subsets of the target genes sharing a common biological process or pathway.

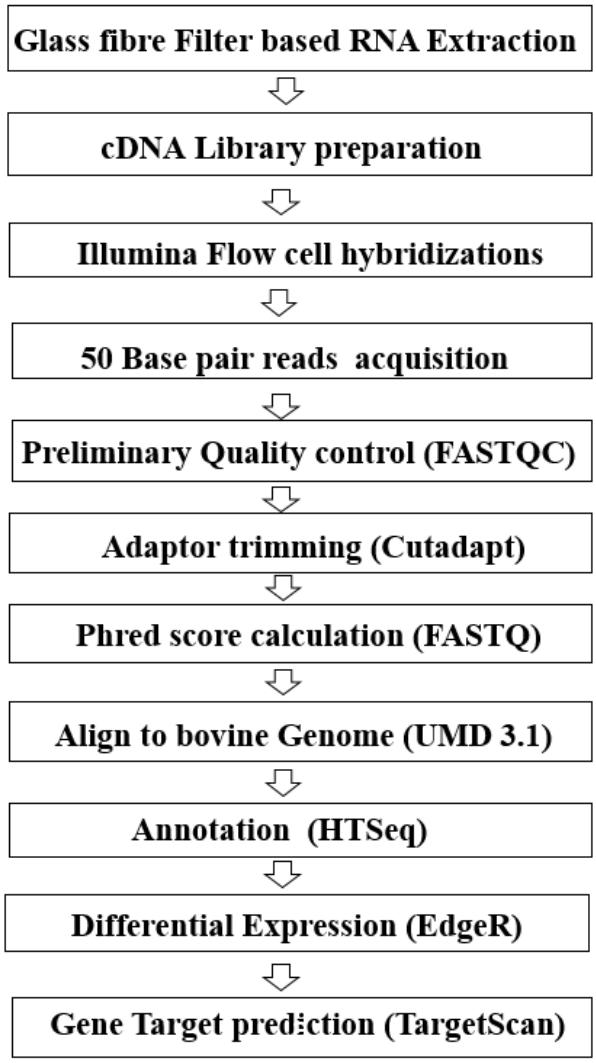

Figure 5.1 miRNA analysis from RNA-seq data pipeline

5.4 Results

In total, more than 300 million reads were sequenced from 8 cDNA libraries. Of these, >292 million reads passed quality control filters and just over 200 million reads aligned uniquely to Ensembl annotated genes in the UMD_3.1 assembly of the bovine genome (Table 5.1). A summary of the data is provided in Table 5.1. 99% of reads aligned to known miRNAs (Table 5.2). The UMD_3.1 build of the bovine genome has 26,618 annotated genes and miRBase version 16 includes 792 *Bos Taurus* miRNAs including both pre and mature miRNAs. There were 479 miRNAs for which at least one read was observed in each of the 8 samples in our dataset. Out of these 53 miRNAs were expressed above a mean threshold of 10 reads per million (RPM) across all 8 samples (Figure 5.2 and Table 5.3).

The 10 most highly expressed miRNAs accounted for more than 95% of all miRNAs expressed. The liver miRNA expression profile was dominated by miR- 122, which accounted for 75% of all highly expressed miRNAs. This was followed by miR-192 which accounted for 8% and the let 7 family member miR-3596 which accounted for over 7% of the most abundantly expressed miRNAs. The remaining 7 most highly abundant miRNAs were four members of the let 7

family (let-7c, let-7i, let-7g and let-7f-2) which accounted for ~3% while miR-140 accounted for ~1.5% and both miR-29 and miR-423 made up ~0.5% each (Figure 5.2). The most highly expressed liver miRNAs are all evolutionary conserved and include 5 members of the 13-member let-7 family (let-7c, let-7i, let-7g, let-7f-2 and miR-3596). These miRNAs were located across 9 different chromosomes with both let7-i and miR-3596 located on chromosome 5. There was no evidence of clustering of highly expressed miRNAs in a particular genomic region. All are mature RNAs except for let-7f-2 a stem-loop miRNA. The sequences and genomic coordinates of highly expressed miRNAs are given in Table 5.3. One miRNA, miR-143 was differentially expressed between the SNEB and MNEB groups. miR-143 was 3.2-fold down-regulated in the SNEB group and was the 11th most

abundantly expressed miRNA. The direction and magnitude of miR-143 regulation was confirmed (2.4-fold down-regulated) following RT-qPCR validatio n (Supplementary Table 5.1). In order to identify genes that may be affected by the down-regulation of miR-143 in SNEB, we computationally predicted miRNA targets among the 3'UTRs of a combined set of 757 hepatic genes that have been previously reported to be differentially expressed in the liver tissues of the same animals (McCabe et al., 2012; McCarthy et al., 2010). A total of 34 genes reported to be differentially expressed under SNEB were found to be putative targets of miR- 143 and 16 of these were up-regulated under SNEB (Table 5.4 and Table 5.5). The functional categories of the 16 up-regulated putative targets are given in Table 5.6. The GO biological processes associated with up-regulated target are given in Table 5.7. The GO biological processes associated with miR-143 are given in Table 5.8.

5.5 Discussion

The postpartum increase in milk production in high yielding dairy cows is accompanied by increased nutritional and energetic demands. This results in 10-12 weeks of NEB in all high-yielding dairy cows (Garber et al., 2011). Previous reports have shown that SNEB affects the expression of multiple genes in the liver, including genes involved in lipid and glucose metabolism and homeostasis (McCabe et al., 2012; McCarthy et al., 2010). Hepatic miRNAs have been reported to play a role in hepatic functions and disorders in human and mouse (Kaur et al., 2011). In addition miRNAs are reported to be associated with energy metabolism through their role in modulation of glucose and lipid homeostasis in metabolism and metabolic disorders (Chen et al., 2012a; Rottiers & Naar, 2012). The miRNA profile of MNEB and SNEB dairy cow liver two weeks postpartum in this study shows some degree of similarity with hepatic miRNA profiles of other animals including human, mouse, rat, and beef cows.

Table 5.1 Read statistics for SNEB and MNEB postpartum dairy cow liver miRNA-seq data

*Sample	Processed Reads	Reads after QC	Reads Aligning to †UMD3.1.68 †	Reads Aligning Uniquely to UMD3.1.68	Reads Aligning to Genes
5N	42,701,190	42,060,747	39,510,950	28,788,096	28,526,343
7N	35,531,177	33,034,345	29,929,732	22,008,536	21,617,227
9N.	39,733,845	35,308,457	32,324,504	24,940,747	24,682,635
10N	37,834,521	34,658,341	31,140,804	22,754,746	22,477,046
2P	35,079,188	32,996,280	30,024,759	23,042,362	22,809,423
3P	44,401,783	43,107,430	40,269,999	30,931,375	30,757,376
4P	40,699,727	32,868,631	29,571,511	23,373,573	23,050,065
5P	38,751,887	38,079,095	36,361,987	34,268,099	33,963,761
Sum	**314,733,318**	**292,113,326**	**269,134,246**	**210,107,534**	**207,883,876**
Average	**39,341,665**	**36,514,166**	**33,641,781**	**26,263,442**	**25,985,485**

†UMD3.1.68 = Ensembl v68 annotation of the UMD3.1 assembly of the bovine genome. *S = SNEB, M=MNEB

Table 5.2 Percentage distribution of RNA biotypes among annotated reads from miRNA-seq

Gene Biotype	% Distribution
miRNA	99.06
misc_RNA	0.02
mt-rRNA	0.08
mt-tRNA	0.04
protein_coding	0.59
Pseudogene	0.001
Retrotransposed	0.0003
rRNA	0.00036
snoRNA	0.198
snRNA	0.0012

Abbreviation: miRNA = microRNA, mt-tRNA = mitochondrail transfer RNA, Rrna = Ribosomal RNA, snoRNA = small nucleolar RNA, snRNA = small nuclear RNA and mt-rRNA = mitochondrial ribosomal RNA

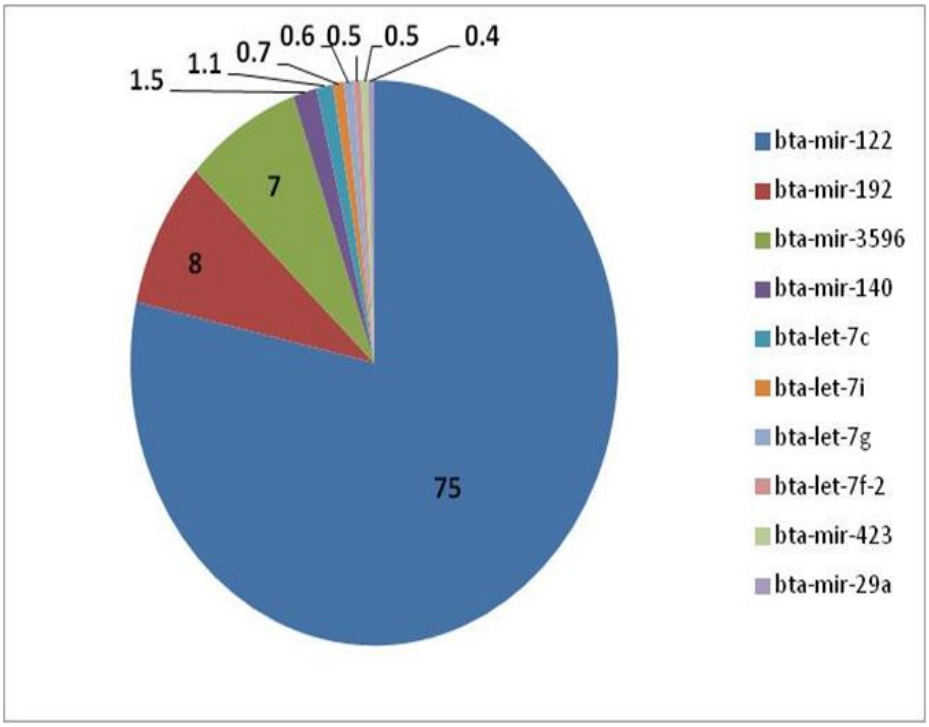

Figure 5.2 The 10 most abundant hepatic miRNAs in the postpartum dairy cow

Table 5.3 Highly expressed liver miRNAs in postpartum dairy cows

miRNA	Sequence	miR family	Coordinates (UMD3.1)
bta-miR-122	UGGAGUGUGACAAUGGUGUUUG	miR-122	chr24: 58095642-58095726 [+]
bta-miR-192	CUGACCUAUGAAUUGACAGCCAG	miR-192	chr29: 43731660-43731765 [+]
bta-miR-3596	AACCACACAACCUACUACCUCA	let 7	chr5: 117120188-117120270 [-]
bta-miR-140	UACCACAGGGUAGAACCACGGA	miR-140	chr18: 37088137-37088230 [+]
bta-miR-423	AAGCUCGGUCUGAGGCCCCUCAGU	miR-423	chr19: 21799484-21799577 [+]
bta-let-7g	UGAGGUAGUAGUUUGUACAGUU	let 7	chr22: 49189340-49189422 [+]
bta-let-7c	UGAGGUAGUAGGUUGUAUGGUU	let 7	chr1: 19930459-19930542 [-]
bta-let-7i	UGAGGUAGUAGUUUGUGCUGUU	let 7	chr5: 51209081-51209164 [-]
bta-miR-29a	CUAGCACCAUCUGAAAUCGGUUA	miR-29	chr4: 95402319-95402382 [-]
bta-let-7f-2	UGUGGGAUGAGGUAGUAGAUUGUAUAGUUUUAGGGUCAUACCCCAUCUUGGAGAUAACUAUACAGUCUACUGUCUUUCCCACG	let 7	chrX: 96383532-96383614 [-]

Table 5.4. Up-regulated putative hepatic gene targets of miR-143 in postpartum dairy cattle in SNEB

Human Gene Name	Fold Change†	FDR††
SCGB1A1	10.27	0.019035
C6orf146	7.86	7.47E-05
PLB1	5.58	1.48E-06
CHRM1	4.3	0.004222
SLC2A12	1.97	0.001349
PLAUR	3.46	0.015094
CCDC113	3.34	0.005373
LRP2	3.28	0.015161
TUFT1	3.03	7.01E-12
SULT1C2	2.90	0.008312
SERPINE1	2.86	8.58E-12
ARHGEF40	2.85	1.34E-06
SLC22A16	2.66	1.75E-07
CTGF	2.11	0.017083
GPCPD1	1.06	3.12E-06
C6orf145	2.1	1.02E-06

† Fold changes were calculated using EdgeR statistical procedure.
††FDR were calculated using Benjamini Hochberg method.

Table 5.5 Base Mean expression for Up-regulated target genes of miR-143 from RNA –seq study (Mc Cabe et al., 2012)

Human Gene Name	Base Mean† (MNEB+SNEB)	Base Mean (MNEB)	Base Mean (SNEB)	Fold Change	Log2 Fold Change	P value	adjusted (BH) P value	resVar (MNEB)	resVar (SNEB)
PLB1	29.1185	8.326674	46.44502	5.57786	2.479712	5.93E-09	1.48E-06	0.277636	0.344567
CHRM1	21.56216	7.700423	33.1136	4.300232	2.104414	5.23E-05	0.004222	0.854318	1.023651
SLC2A12	5.087202	1.960092	7.693127	3.924881	1.972649	0.001349	0.057254	0.118218	0.127172
PLAUR	29.10871	12.44103	42.99845	3.456179	1.789178	0.000251	0.015094	0.429457	0.520239
CCDC113	17.06415	7.492157	25.0408	3.342269	1.740828	7.04E-05	0.005373	0.2205	0.256329
LRP2	11.33265	5.051188	16.56721	3.279864	1.713636	0.000255	0.015161	0.190552	0.214477
TUFT1	261.1597	123.9149	375.5305	3.030553	1.599581	8.53E-15	7.01E-12	0.094163	0.130408
SULT1C2	47.53053	23.32172	67.70454	2.903068	1.537578	0.000126	0.008312	0.042281	0.052478
SERPINE1	362.7678	180.0529	515.0302	2.860438	1.516236	1.17E-14	8.58E-12	0.46681	0.653116
ARHGEF40	155.8692	77.65421	221.0483	2.846572	1.509226	5.26E-09	1.34E-06	0.171222	0.229892
SLC22A16	56.18415	29.49551	78.42468	2.658868	1.410812	5.53E-10	1.75E-07	6.184935	7.732543
C6orf145	88.66879	50.60869	120.3855	2.378753	1.250205	1.02E-06	0.000156	0.428256	0.549958
CTGF	85.52957	53.25352	112.4263	2.111152	1.078031	0.000301	0.017083	0.18246	0.232251
GPCPD1	147.1713	92.02309	193.1282	2.098692	1.069491	1.34E-08	3.12E-06	0.298524	0.393327
C6orf146	11.43379	2.410559	18.95315	7.862554	2.974998	4.33E-07	7.47E-05	0.154758	0.180922

† Base mean ' =A measure of read abundance, i.e. expression level of target genes according to edgeR algorithm,

resVar = Residual variance

Table 5.6 Functional categories of up-regulated putative target hepatic genes of miR-143

ID	Entrez Gene Name	Type(s)
ARHGEF40	Rho guanine nucleotide exchange factor (GEF) 40	Enzyme
CCDC113	coiled-coil domain containing 113	other
CHRM1	cholinergic receptor, muscarinic 1	G-protein receptor
CTGF	connective tissue growth factor	Growth factor
C6orf146	family with sequence similarity 217, member A	other
GPCPD1	Glycerophosphocholine phosphodiesterase GDE1 homolog (S. cerevisiae)	Enzyme
LRP2	Low density lipoprotein receptor-related protein 2	Transporter
PLAUR	plasminogen activator, urokinase receptor	Transmembrane receptor
PLB1	phospholipase B1	Enzyme
C6orf145	PX domain containing 1	Signalling protein
SCGB1A1	secretoglobin, family 1A, member 1 (uteroglobin)	Cytokine
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	Enzyme
SLC22A16	solute carrier family 22 (organic cation/carnitine transporter), member 16	Transporter
SLC2A12	solute carrier family 2 (facilitated glucose transporter), member 12	Transporter
SULT1C2	sulfotransferase family, cytosolic, 1C, member 2	Enzyme
TUFT1	tuftelin 1	Enzyme

Table 5.7 GO terms associated with target genes of miR-143 down-regulated under SNEB

Symbol	GO Identifier	Evidence code†	GO Term Name
LRP2	GO:0006629	TAS	lipid metabolic process
CHRM1	GO:0006464	TAS	cellular protein modification process
CTGF	GO:0001558	IEA	regulation of cell growth
GPCPD1	GO:0006071	IEA	glycerol metabolic process
PLAUR	GO:0006501	TAS	C-terminal protein lipidation
PXDC1	GO:0007154	IEA	cell communication
SCGB1A1	GO:0000122	IEA	negative regulation of transcription from RNA polymerase II promoter
SERPINE1	GO:0010951	ISO, IDA	negative regulation of endopeptidase activity
SLC2A12	GO:0055085	TAS	transmembrane transport
SULT1C2	GO:0006805	TAS	xenobiotic metabolic process
TUFT1	GO:0030282	NAS	bone mineralization
ARHGEF40	GO:0035023	IEA	regulation of Rho protein signal transduction

†Abbreviations for Evidence code**:** TAS = Traceable Author Statement, IEA = Inferred from Electronic Annotations, ISO= Inferred from Sequence Orthology, IDA= Inferred from Direct Assay, NAS= Non-traceable Author Statement

5.5.1 Abundantly expressed miRNAs in postpartum dairy cow liver

This the first liver miRNA-seq profiling study of moderate yielding dairy cows in first 2 weeks postpartum. The most dominant among the ten highly abundant miRNAs in this study, miR-122 is a liver-specific conserved miRNA. This dominance of expression by miR-122 is consistent with previous studies in dairy (Gu et al., 2007) and beef cows (Jin et al., 2009a) where miR-122 was reported to be only expressed in liver, when compared with other tissues, and constituted more

than 57 % of all the miRNA reported in liver (Jin et al., 2009b). In addition, miR-122 has been reported to account for 70 % of the total miRNAs in human liver (Jopling, 2012). Tissue specific roles of miR-122 are well established in human and miR-122 has been implicated in the hepatic disorders hepatocarcinogenesis and hepatocellular carcinoma (Hou et al., 2011; Tsai et al., 2012; Zeng et al., 2010). Furthermore, the antagonism of miR-122 was reported to result in disruptions of cholesterol and lipid metabolism in mice (Tsai et al., 2012). The second most abundant miRNA in this study miR-192 has been associated with cellular responses to glucose stimulus (Pogribny et al., 2010) and was also reported to be one of the most highly expressed after miR-122 in human liver miRNA studies (Hou et al., 2011; Li et al., 2013b) and in a mouse liver study (Wang et al., 2009). miR-192 unlike miR-122 is not a liver specific miRNA and has also been reported to have important roles in human and bovine renal tissues and functions (Chung et al., 2010; Coutinho et al., 2007; Hossain et al., 2009) Five of the thirteen members of the let-7 family are also highly expressed in this study including let-7c, let-7i, let-7g and miR-3596 and let-7-f. The let-7 family members have been associated with hepatic development and disorders as well as glucose and insulin metabolism (Ma et al., 2013). Over-expression of let-7c in human liver was implicated in hepatocytes oxidant injury (Hou et al., 2012b) while let-7g was demonstrated to suppress HCC metastasis in a mouse model (Ji et al., 2010). miR-140 which is also among the highly expressed miRNAs in this study has been reported to be abundantly expressed in human and mouse liver with implications in liver function and disorders (Ferland-McCollough et al., 2010; Fowell et al., 2010; Zheng et al., 2010).

5.5.2 Differentially expressed miRNAs in post- partum dairy cow liver under SNEB

miR-143, a highly expressed miRNA in this study, is down-regulated under SNEB. miR-143 is not liver-specific, however its tissue-enriched expression has been reported in human (Zhang et al., 2009) chicken (Trakooljul et al., 2010), rat (Fowell et al., 2010) and mouse (Jordan et al., 2011; Ono, 2011) liver. In the case of human, miR-143 has been implicated in hepatocellular carcinoma. In the rat study, miR-143 was demonstrated to regulate activation and proliferation of myofibroblastic hepatic stellate cells (HSC) while in the mouse study miR-143 was shown to

regulate glucose metabolism in vitro, and was associated with hepatic insulin regulation. In addition, miR-143 has been found to be ubiquitously expressed in bovine tissues including liver (Gu et al., 2007) and has also been associated with bovine intramuscular fat proliferation and differentiation (Li et al., 2011a) and high back fat deposition in crossbred beef cattle (Jin et al., 2010).

5.5.3 Some important predicted gene targets of miRNA miR-143

Of the 16 putative targets of miR-143 which have been reported previously to be up-regulated under SNEB, two (*LRP2* and *SLC2A12*) have roles in lipid and glucose metabolic processes. These genes are of particular interest because the metabolism of lipids and glucose is altered during SNEB (Grummer, 1993; Harfoot & Hazlewood, 1993). *LRP2* is implicated in lipid metabolism through its role as a receptor for sterols, steroid hormones bound to carrier proteins (Willnow, Hammes & Eaton, 2007) like lipoproteins (May et al., 2007) and apolipoprotein M of liver (Dahlback & Nielsen, 2006; Dahlback & Nielsen, 2009; Faber et al., 2006). A study of miRNA based modulation of obesity also reported *LRP2* as a putative target of miR-130a, with roles in lipid metabolism (Perri et al., 2012). *SLC2A12* is a facilitative glucose transporter that mediates a bidirectional and energy-independent process of glucose transport across cell membrane (Gude et al., 2005; Pujol-Gimanez et al., 2012). *SLC2A12* is an insulin-responsive glucose transporter (Karim, Adams & Lalor, 2012) and it over expression was reported to improve glucose clearance rate in mice (Yuan et al., 2006).

5.6 Conclusions

From a global examination of miRNA expression in the liver of postpartum dairy cattle ten highly expressed miRNAs dominate. Tissue specific miR-122 and liver enriched miR-192 are two of the most abundant miRNAs in the postpartum dairy cow liver. These two genes are good candidates as potential mediators of the metabolic effects of NEB. miR-143 is significantly down-regulated under SNEB and putative targets of miRNA-143 which are up-regulated under SNEB, include genes with roles in liver glucose and lipid metabolism

Table 5.8 GO terms associated with miR-143 based on FAME miRNA gene ontology algorithm

GO term Confidently predicted functions (FDR<0.1):	Evidence code†	p-value	q-value	FAME Enrichment factor	p-value
carboxylic acid transport	ISO	0.0001	0.0367	5.71	0.0049
positive regulation of small GTPase mediated signal transduction	IDA	0.0001	0.0183	8.55	0.0043
Rac protein signal transduction	IGI	0.0001	0.0122	16.02	0.0033
regulation of small GTPase mediated signal transduction	IDA	0.0013	0.0795	2.54	0.0023
regulation of Rho protein signal transduction	ISO	0.0024	0.0979	3.21	0.0082
Less confident functions (FDR>0.1, p-value<0.05):					
Rho protein signal transduction	ISO	0.0033	0.1211	2.89	0.010
symbiosis, encompassing mutualism through parasitism	ISO	0.0044	0.1451	4.42	0.0143
regulation of JNK cascade	ISO	0.005	0.1529	5	0.0043
amine transport	NA	0.0055	0.1442	3.72	0.0260
zinc ion transport	ISO	0.0058	0.1263	6.11	0.0121
phospholipid transport	IEA	0.0086	0.1569	5.36	0.006
response to virus	IDA	0.0086	0.1494	3.95	0.122
di-, tri-valent inorganic cation homeostasis	IDA, IGI	0.0093	0.1492	3.14	0.082
Transport	TAS	0.0105	0.1613	1.32	0.102
physiological response to stimulus	IMP,ISO	0.0106	0.1563	1.61	0.23
positive regulation of enzyme activity	ISO	0.0114	0.155	2.01	0.057

†Abbreviations for Evidence code: IEA = inferred from electronic annotation; ISO = inferred from sequence orthology; IMP = inferred from mutant phenotype; IDA = inferred from direct assay; IGI = inferred from genetic interaction.

Chapter 6

miR-2885 inhibition for functional analysis

6.1 Abstract

6.1.1 Background

Up-regulation of bovine micro RNA (miRNA) miR-2885 in the liver of postpartum cattle liver was reported to be associated with severe negative energy balance (SNEB) (Fatima et al., 2014). miR-2885 has putative gene target sites on the most down-regulated gene *FADS2* (fatty acid desaturase 2) which encodes an enzyme critical in lipid biosynthesis (Fatima et al., 2014; McCarthy et al., 2010). *FADS2* is the most down-regulated gene under SNEB. miR-2885 contains target site in the 3'UTR of *FADS2* at position 400 to 406. The objective of this study was to investigate whether the putative miR-2885 binding sites in the *FADS2* 3'UTR are active.

6.1.2 Objectives

To investigate whether the putative miR-2885 binding sites in *FADS2* 3'UTR are active, miR-2885 inhibition was performed on a bovine kidney cell line and the expression of *FADS2* was quantified using RTPCR.

6.1.3 Results

Inhibition of miR-2885 in MDBK cells led to a 5-fold increase in *FADS2* expression.

6.2 Introduction

Negative energy balance (NEB) is an altered metabolic state in high yielding cows that occurs during the first few weeks postpartum, when energy demands for lactation and maintenance exceed the energy supply from dietary intake. SNEB gives rise to pleiotropic changes in hepatic gene expression including those involved in fatty acids metabolism (McCabe et al., 2012; McCarthy et al., 2010), Cell viability is dependent on the supply of the essential fatty acids (EFAs) linoleic and α-linolenic acid. *FADS2* is a lipogenic enzyme is a member of the fatty acid desaturase (*FADS*) gene family that encodes desaturase enzymes to catalyse

desaturation of fatty acids during their biogenesis *FADS2* catalyses the conversion of essential fatty acids (EFA) to 3- and 6-polyunsaturated fatty acids (PUFAs) (Stoffel et al., 2008). *FADS2* was found to be most down-regulated gene in dairy cow liver under SNEB postpartum (McCarthy et al.,2010). miR-2885 was reported to be up-regulated under SNEB and identified to have putative binding site on *FADS2* (McCarthy et al., 2010).

6.2.1 miRNAs

miRNAs are small non-coding RNAs around 19 to 22 nucleotides long. miRNAs are considered as important regulators of gene expression underlying various biological processes including cell cycle regulation, proliferation, development, immune response and metabolism (Mattick, 2003). In addition, miRNAs have been implicated in many traits of economic importance in domestic animals (Fatima et al., 2014).

miRNAs were first discovered two decades back in the round worm *C. Elegans* and were reported to regulate *lin-14,* a gene critical for larval transition from L3 to L4 (Lee et al., 1993). miRNA biogenesis is a multistep process that starts with genes encoding miRNAs getting transcribed by RNA polymerase II into a primary miRNA (pri-miRNA). The pri-miRNA is then processed by Drosha in the nucleus into a ~70 nt pre-miRNA. Pre miRNA is exported to the cytoplasm by Exportin where Dicer cleaves it to yield a ~20 nt long mature miRNA/miRNA* duplex. The mature miRNA guiding strand is transported to a silencing complex (RISC) containing the argonaute (AGO) protein (Tran & Hutvagner, 2013) before being guided to the target mRNA.

In mammals, only 6-7 nucleotides of the miRNA sequence typically, have exact matches with the miRNA binding site on the 3'UTR of the target mRNA (Friedman et al., 2009). After complimentary binding, miRNAs generally leads to a decrease in target mRNA expression or in some cases to its complete degradation (Bartel, 2009b). The targeted role of miRNAs on gene expression is evaluated through gain- and loss-of-function experiments. miRNA function loss can be achieved by either using knockout mice with missing key genes involved in the miRNA biogenesis process, such as Dicer, Drosha and Ago2, or suppressing miRNAs activity with miRNA sponges and miRNA inhibitors. A miRNA sponge is a synthetic miRNA

decoy target with multiple binding sites inserted into its 3′ UTR complimentary to the seed region of all members of a miRNA family to 'soaks up' their activity by complimentary annealing. miRNA sponges are expressed inside the cell from transfected plasmids or from chromosomal insertions. miRNA inhibitor are synthetic oligonucleotides that carries complimentary sites for endogenous mature miRNA and is introduced in to the cells for complimentary binding to mature miRNAs to suppress their activity. The effect of miRNA inhibition can be assessed through quantification of target mRNAs through RT-qPCR and their encoded proteins (Ebert, Neilson & Sharp, 2007; Kluiver et al., 2012; Stenvang et al., 2012). Role of miRNA in lipid metabolism has been validated through functional-loss studies (Karolina & Jeyaseelan, 2013). Inhibition of miR-33a and miR-33b was reported to increase the expression of *ABCA1* (ATP-binding cassette transporter member 1) in mouse liver and induced an increase in plasma HDL (High-density lipoprotein) levels in African green monkeys (Rayner et al., 2011a; Rayner et al., 2010b). Targets of miR-33a and miR-33b were found to be involved in fatty acid oxidation and fatty acid synthesis through sterol regulatory element binding transcription factor 1 (*SREBF1*), fatty acid synthase (*FASN*), ATP citrate lyase (*ACLY*) and acetyl-CoA carboxylase-α (*ACACA or ACC1*). miR-122 inhibition in mouse liver resulted in a reduction in hepatic fatty acid synthesis and an increase in hepatic fatty acid oxidation (Gerin et al., 2010). In other studies of mice and in African green monkeys, inhibition of mir-122 caused a reduction in total plasma cholesterol (Zampetaki & Mayr, 2012b).

In this study we postulate a potential regulatory role for miR-2885 in SNEB through the regulation of *FADS2* gene expression and examined this functional role of miR-2885 in a Madin-Darby Bovine Kidney (MDBK) cell line. The MDBK cell line was chosen to study this interaction between hepatic miRNA-target gene pairs as it was previously characterized to express ~70% of bovine liver transcripts and is a good proxy for bovine liver, with genes mainly involved in basic cellular functions, signalling and metabolic pathways (Bionaz et al., 2008). MDBK cell lines have previously been used for studying bovine fatty acid metabolism (Bionaz et al., 2008; Litherland et al., 2010).

6.3 Methods

6.3.1 Cell line propagation and transfection

MDBK cell-line (Sigma-Aldrich, St. Louis, MO, USA) monolayers were seeded in 6-well plates at a density of $3x10^5$ cells per well in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) with 10% foetal bovine serum (FBS), 1% non-essential amino acids (NEAA) and 1 % penicillin/streptomycine (Pen/Strep) at 37°C. After 24 hours medium was removed, the monolayer washed with 1 ml phosphate buffered saline (PBS) and 2 ml of fresh medium added to each well. The transfection mixture containing miRNAs inhibitor for miR-2885 or negative control inhibitor at a final concentration of 400 nM (mirVana™, Life Technologies, United Kingdom) were added drop wise using HiPerFect Transfection reagent (Qiagen, Manchester, United Kingdom) and cells were left to incubate for 24 hrs at 37°C.

6.3.2 Extraction and isolation of RNA from MDBK cell line

After 24 hrs of transfection medium was removed and 1 ml trypsin solution (1:250 U/mg) was added to each well and approximately 1 million cells were harvested. Cells were centrifuged at 450 **g** for 5 min and after removal of trypsin were suspended in 200 µl PBS and 400 µl of the Lysis/Binding Buffer from the High-pure RNA extraction kit (Roche Applied Science, West Sussex, UK). The contents were mixed by vortexing and homogenized by pipetting up and down. The sample was transferred to the upper reservoir of the filter insert and was centrifuged for 15 sec at 8000g. The flow-through was discarded and 100 µl mixture of 90 µl DNAse incubation buffer and 10 µl DNAse was added to the upper reservoir of the filter insert followed by incubation for 15 min at room temperature. A 500 µl aliquot of Wash Buffer I was added to the upper reservoir of the filter tube assembly and centrifuged for 15sec at 8000g. The flow-through was discarded and 500µl of Wash Buffer II was added to the upper reservoir of the filter tube assembly and centrifuged for 15 sec at 8000g. The flow-through was discarded and 200µl of Wash Buffer II was added to the upper reservoir of the filter tube assembly and centrifuged for 2 min at 10,000 g. The filter tube was transferred into a clean, sterile 1.5 ml micro centrifuge tube. A 50 µl of elution Buffer was added to the upper

reservoir of the filter tube the tube assembly was centrifuged for 1 min at 8000g to elute the RNA.

6.3.3 Real Time- qPCR

First strand cDNA Synthesis was carried out with RevertAid H Minus First strand synthesis kit (Thermo Scientific, Epsom, UK). Briefly, 1µg of total RNA in 12 µl nuclease-free water and 1 µl oligo (dT) primer were added to a sterile 2ml tube on ice. Following addition of 4 µl of 5X Reaction Buffer, 1 µl RiboLock RNase inhibitor (20 U/µl), 2 µl of 10 mM dNTP Mix and 1 µl RevertAid H Minus M-MuLV Reverse Transcriptase (200 U/µl). The reaction mix was centrifuged gently and cDNA synthesis was carried out for 60 min at 42°C. The reaction was terminated by heating at 70°C for 5 min.

6.3.4 PCR Amplification of First Strand cDNA

A 0.5µl aliquot of a 10 µM forward and reverse primer solution was added to 2.5 µl of QuantiFast SYBR Green PCR master mix (Qiagen, Manchester, United Kingdom) per reaction with nuclease free water to make up a 4 µl reaction mix. A 4 µl aliquot of reaction mix was added per PCR plate-well followed by 1 µl of cDNA. PCR was carried out at initial heat activation for 5 min at 95°C followed by two-step cycling (35–40 cycles), denaturation for 10 s at 95° and a combined annealing/extension at 60°C for 30s *FADS2* expression was normalized to the housekeeping gene *ß-Actin*. *ß-Actin* was more stably expressed gene compared to other candidate housekeeping genes including *GAPDH* and *RSP9*. The software package BioRad CFX manager was used for correction of the Ct values and normalization to *ß-Actin* using the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008). Corrected Ct values were used to determine differential expression using the Wilcoxon rank test (Yuan et al., 2006). A $P<0.05$ value was deemed to be significant.

6.4 Results

Inhibition of miR-2885 in MDBK cells led to a 5-fold increase in *FADS2* expression. Ct value and fold change are given in Table 6.1. Table 6.2 lists the Ct values for target *FADS2*, house keeping gene *Beta-Actin* and candidate house keeping genes under miR-2885 inhibition and negative control inhibition. Biological processes associated with *FADS2* are listed in Table 6.3. The primer sequences of *FADS2, ß-Actin, GAPDH and RSP9* are given in Supplementary Table 6.1

Table 6.1 *FADS2* expression profile after inhibition of miR-2885 in MDBK cell line

miR-2885 inhibitor Ct values	Negative control Inhibitor Ct values	Fold change†	P values
20.44	22.89	5.16	3.55E-05
20.52	22.91		
20.64			

MDBK cells 24 hours after transfection with miR-2885 inhibitor. Ct values for are normalized with respect to *beta-Actin.*

† Fold-change is based on back-transformed Ct values.

Table 6.2 Ct values of *FADS2* and *Beta-Actin* for miR -2885 inhibitor and negative control inhibitor transfection

Inhibitor miRNA treatment	*FADS2*	*β- Actin*	*RSP9*	*GAPDH*
antimiR-2885 replicate 1	20.44	14.53	17.07	17.26
antimiR-2885 replicate 2	20.52	14.19	16.37	15.10
antimiR-2885 replicate 3	20.64	14.07	15.37	16.65
negative control inhibitor replicate 2	22.89	13.95	15.52	15.83
negative control inhibitor replicate 3	22.91	13.83	15.77	17.21

MDBK cells 24 hrs after transfection with miR-2885 inhibitor and negative control inhibitor in 3 replicates from a 6 well plate.

6.5 Discussion

A dramatic reduction (19-fold) in *FADS2* expression from postpartum dairy cattle liver and an up-regulation of mir-2885 was previously reported under SNEB. miR-2885 has binding sites in the *FADS2* 3'UTR. *FADS2* is a desaturase enzyme critical in lipid biosynthesis and is associated with metabolic disorders, nutrition induced ketosis and NAFLD (non-alcoholic fatty liver disease) (Stoffel et al., 2008; Videla et al., 2004). A SNP in the transcriptional regulatory region of *FADS2* was reported to cause expression inhibition and was associated with *FADS2* deficiency in an abnormal lipid metabolism condition in human (Lattka et al., 2010).

Modulation of *FADS2* expression through nutrition and transcription factors are known (Desert et al., 2008) The nutritional regulation of *FADS2* expression was reported in rat liver, where *FADS2* expression was found to be inversely related to maternal dietary fat intake. Altered *FADS2* expression under feed restriction in periparturient dairy cow liver has also been reported (Hiller et al., 2012; Loor et al., 2007). The transcriptional factors (TFs) *SREBP1* (Sterol regulatory element-binding protein 1), *SREBP2* (Sterol regulatory element-binding protein 2) and *PPARα* (Peroxisome proliferator-activated receptor alpha) are known to modulate the expression of *FADS2* (Geay et al., 2010; Lattka et al., 2010).

Recently miRNAs have been implicated in expression regulation of fatty acid metabolism pathways enzymes (Rayner et al., 2011b; Rayner et al., 2010a). 20miR-33a and b have been reported to regulate fatty acid oxidation enzymes in hepatic cell lines (Rayner et al., 2011b; Rayner et al., 2010a). miR-122, miR-33 and miR-193 are known to regulate the expression of fatty acid synthetic enzymes (Zampetaki & Mayr, 2012a). miR-140-5p and miR-150 binding sites have been identified on *FADS1AT1* (Fatty Acid desaturase 1 alternative transcript 1) (Park et al., 2012). In our study functionality of miR-2885 binding sites in the *FADS2* 3'UTR was analyzed through miRNA inhibition. For this purpose inhibition of miR- 2885 in MDBK cells was carried out followed by *FADS2* expression profile. We found an increase in the expression of *FADS2* after miR-2885 inhibition.

6.6 Conclusions

This study shows for the first time that *FADS2* the most highly down-regulated hepatic gene under severe NEB can be functionally regulated by miR-2885 one of the most up-regulated miRNA. Dysregulation of the miRNA-target gene relationship of miR-2885 and *FADS2* could be a likely mechanism to ameliorate or eliminate the negative impact of NEB in the postpartum dairy cow.

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Table 6.3 GO terms associated with *FADS2*

Molecular Function	Evidence code†	Biological Process	Evidence code	Cellular Component
Stearoyl-CoA 9-desaturase activity (GO:0004768)	IEA	Unsaturated fatty acid biosynthesis (GO:0006636)	IEA	Endoplasmic reticulum (GO:0005789)
Iron ion binding (GO:0005506)	IEA	Unsaturated fatty acid metabolism (GO:0033559)	TAS	Integral to plasma membrane (GO:0005887)
Heme binding (GO:0020037)	IEA	Alpha-linolenic acid metabolism (GO:0036109)	TAS	Membrane (GO:0016020)
		Linoleic acid metabolism (GO:0043651)	IEA	
		Small molecule metabolism (GO:0044281)	TAS	

†Abbreviations for Evidence code: IEA = Inferred from electronic annotation and TAS = traceable author statement

Chapter 7

General Discussion

7.1 General Discussion

miRNAs play important roles in various processes including reproduction, immunology, feed efficency and milk production in livestock species. One of the current areas of interest in livestock miRNA research is reproduction and embryo survival. Differential expression of miRNA associated with these traits have been reported in cattle, sheep, pig, goat and chicken. In the context of dairy cattle and milk production, miRNAs have potential roles as biomarkers for pathogens conditions and milk quality. Research into role and implications of miRNAs in improving these traits will continue to contribute to the potential production and further efficiency in the dairy cattle. The decline in dairy cow fertility contributes significantly to economic loss in this sector. Postpartum NEB is known to have deleterious effects on the health and fertility of dairy cattle. The analysis of miRNAs in postpartum NEB in the liver of dairy cattle in this thesis highlights the potential impact of this altered metabolic condition on the expression of liver miRNAs. In the first miRNA microarray expression analysis we found five miRNA differentially expressed under SNEB. The metabolic stress associated with NEB that arises in general as a result of increased milk production and limited feed intake during NEB of postpartum period is considered a potential cause of differential expression of miRNAs. In order to evaluate the miRNA-mRNA interactions, we further identified a total of 67 putative miRNAs targets, among differentially expressed liver genes reported previously from this model. The integration of miRNA-mRNA expression data highlights the potential contributions of miRNAs in regulation of target genes involved in fatty acid metabolism, somatotropic axis and fertility. All five differentially expressed miRNAs in this study are up-regulated under SNEB. Only 34 out of a total 67 target genes are down-regulated. We did not find any significant enrichment for putative targets down-regulated under SNEB among the putative targets genes. Thus up-regulated miRNAs have both up and down-regulated putative targets. Overall miRNA mediated regulation seems to be confounded.

This can be attributed to several factors. One of them relates to the degree of accuracy of target prediction by TargetScan algorithm (Witkos et al., 2011). Previously experimental validation studies of putative targets have proved that the rate of false positives in target prediction databases is > 20 % (Marin & Vanicek, 2011). Moreover several factors can contribute to the effective down-regulation of true targets. Firstly, where miRNAs binding sites overlap on shared targets, it lead to dilution of effective targeting. Secondly, imperfect complementary binding of miRNA to target mRNAs leads to suppression of target that is not of a significant magnitude. Thirdly, in order to facilitate interaction between miRNA and the 3′UTR, accessibility of target site in the region corresponding to the seed is restricted. Complications of RNA structure and RNA-binding proteins structure can affect the site accessibility (Grimson et al., 2007). The suboptimal level of free energy required to make the complementary site accessible, the 'opening energy' and the 'hybridization energy' between the miRNA and the 3′UTR also effects the extent of miRNA mediated regulation (Lewis et al., 2003). Furthermore in addition to miRNAs, expression regulation involves other regulatory mechanisms like TFs can confound the regulatory impacts of miRNAs.

Quantitative Real-time PCR procedure was used to further validate the results of differential expression analysis of miRNAs from both RNA-seq and micro array expression studies. For an accurate qRT-PCR validation, the selection of appropriate reference genes is required (Johnson et al., 2014). It is recommended that more than one candidate miRNAs from expression data or others small RNAs are tested for stability of expression across all conditions. Several expression analysis tools are available to help identify the most stabily expressed house-keeping genes across all samples in the experimental conditions. In this thesis we tested two of the highly and stably expressed miRNAs in our data set; miR-122 and let-7b along with widely used small RNA *RNU6B* as candidate house-keeping genes. We found *RNU6B* to have the most stable expression on its own compared to being used in combination with other two reference genes across all samples.

In addition to micro arrays we used RNA-seq to profile overall picture of liver miRNA content in both MNEB and SNEB groups postpartum. In the RNA-seq study ten liver miRNA represented the 95 percent of all expressed miRNAs, these are dominated by the liver specific miR-122. Here miR-122 represented ~ 75% of all expressed liver miRNAs. In this study only one miRNA, miR-143 that is known to be associate with the fatty acid biosynthesis and also the adipose development was found to be differentially expressed under SNEB. From the microarray study total ten miRNAs were found to be differentially expressed under SNEB condition. The difference may have been due in part to the fact that most of the sequencing reads in the RNA-seq study were associated with a small number of miRNAs possibly limiting the ability of RNA-seq to accurately estimate the abundance of more lowly expressed miRNAs. Less abundant miRNAs would not be affected to the same extent in a micro-arrays based approach which has probes designed to correspond to the known miRNAs. In this study, one additional miRNA miR-1247, would have been declared differentially expressed were it not excluded from the analysis due to having read counts below the lower threshold criterion of 10 reads per million. In addition, in this study a cut-off of 18 nts in length was used in the analysis, whereas in the microarray study, miR-1281 which was found to be differentially expressed is 17 nts in length. Small sample size, within group variance and still to be improved and optimized well developed methods for statistical analysis to take in to consideration the overexpressed genes (Git et al., 2010).

The functional role of miR-2885 which has binding sites on *FADS2* was validated in a MDBK cell line. Several factor are considered to achieve maximum inhibitor activity. These include amount of miRNA inhibitor used, the transfected cell line type, the transfection reagent and the length of time of exposure of cells to transfection mix. All these factors need to be optimized (Kim & Eberwine, 2010) for accurate results. After miRNA inhibition the quantification of target miRNA expression is not a reliable estimation of its functional impact. Therefore it is recommended that the functional effect of inhibited miRNA sholud be evaluated through quantification of the target gene expression post-transfection (Nikcevic et al.,2003).

In this study we report that miR-2885 inhibition results in an increase in *FADS2* expression. Deregulation of the miRNA-target-gene relationship between miR- 2885 and its target *FADS2* could be a likely mechanism to ameliorate or eliminate the negative impact of SNEB in the postpartum dairy cow.

In conclusion postpartum SNEB alters hepatic miRNAs expression. The putative target genes of these miRNAs are known to be involved in lipid biosynthesis and somatotrophic axis. This indicates that miRNAs are a part of a yet to be discovered larger regulatory network modulating the expression of hepatic genes under SNEB in postpartum dairy cattle. The regulatory role of miRNAs can be explored to help improve energy balance.

7.2 Future work

In this section further potential scope of research in this area of miRNA mediated regulation of NEB is discussed.

7.2.1 The combined effect of up-regulated miRNAs on common target genes

miRNAs share common target genes, as 3'UTRs of a gene can carry binding sites for several miRNAs. The magnitude of the contribution of more than one miRNAs, working synergistically to regulate a shared target gene, can be profiled simultaneously through *in vitro* miRNA inhibition (Hashimoto, et al., 2013). From our data set, the contribution of miR-140 and miR-2885 in the shared target, *FADS2* expression inhibition can be profiled on a bovine cell line.

Proposed Protocol

- Cells seeded at a recommended density per well will be transfected with miRNA inhibitors, at a final equal concentration each of miR-2885 and miR-140.

- RNA extraction will be carried out 48 h after transfection. Levels of target mRNA (*FADS2*) expression will be examined (qRT-PCR).

- Target protein extracts will be analyzed using the western blotting.

7.2.2 Synergetic Cooperation of miRNAs with Transcription Factors

TFs mediate gene expression regulation at the transcription level. TFs physically bind to specific binding sites (motifs) called transcription factor binding sites (TFBSs) in the target gene regulatory regions (e.g. promoters, enhancers regions). In the cases where both TF and miRNAs share the same target gene, their complimentary roles in regulation of target gene expression can be validated simultaneously (Chen et al., 2012).

Proposed Protocol

- TFBSs will be retrieved for the target genes from the UCSC genome browser.

- For the *in vitro* functional validation of role of TF and miRNA mediated regulation, mutation will be introduced in the promoter region sequence in the TFBS on target genes.
- Transfection vectors carrying the mutated promoter and 3´ UTR of a target gene will be designed. Vectors and miRNA inhibitors will be transfected into selected cell line.

- Post transfection cells will be collected and target gene expression will be evaluated.
- Target proteins expression will be analyzed using western blotting.

7.2.3 SNPs in Target site

Selective post-transcriptional regulation of target genes carrying polymorphic miRNA binding site can lead to identification of a SNP-dependent post-transcriptional regulatory mechanism (Clop et al., 2006). We can identify polymorphisms across candidate target genes down-regulated under SNEB for miRNA differentially expressed under SNEB.

Proposed Protocol

- Cattle miRNA sequences will be downloaded from miRBase.
- 3'UTRs for each gene will be retrieved from Ensemble.
- SNP will be identified in the binding site of 3 UTR of target genes.
- Selected miRNA complementary binding site sequences and polymorphic sequences encompassing miRNA binding sites in target genes will be sub cloned into the luciferase based expression vector. This expression vector will be designed by an introduced cleavage site in the 3'UTR of the firefly luciferase coding region.
- Cells will be transfected with the expression vector.
- Cells will be also be transfected with miRNA inhibitor.
- Post-transfection cells will be collected and the luciferase activity will be measured.
- Total RNA will be isolated and RTPCR reaction performed for target gene expression analysis.
- Expression quantification of target protein will be done using western blotting.

7.2.4 Alterations in miRNA expression after NEFA supplementation in cell line

Increase in NEFA concentration level and their β-oxidation is known to cause lipotoxicity resulting from increased reactive oxygen species (ROS) production, leading to alterations in miRNAs expression. Lowering of NEFAs supplementation is known to restores the normal expression of miRNAs (Plaisance et al. 2014). We can investigate the effects of NEFA supplementation on target miRNA expression in a selected cell line *in vitro.*

Proposed Protocol

- Cells will be treated with various concentrations of NEFAS like PA (palmatic acid; C16:0), SA (stearic acid; C18:0), OA (oleic acid; C18:1, n −9), LA (linoleic acid; C18:2, n−6), AA (arachidonic acid; C20:4, n−6), EPA (eicosapentaenoic acid; C20:5, n−3) and DHA (docosahexaenoic acid; C22:6, n−3) for 24 h.
- The alterations in miRNA expression will be determined by micro array analysis.
- Target protein expression analysis will be done using western blot.

7.2.5 Mouse model for the identification of miRNA determining energy expenditure and homeostasis.

Animal models can be used to further explore the *in vivo* role for miRNA in metabolic regulation under differential nutritional conditions (Vora et al., 2013).

Proposed Protocol

- Mice will be randomized in to three groups. The *ad libitum*-fed control or a normal diet fed group, HFD (high-fat diet fed) group and DER (dietary restriction) group.

- Body weights will be recorded, daily food intake for each mouse will be calculates and energy expenditure will be assessed.
- Blood concentration level for insulin, *IGF-1*, NEFA, BHB and glucose will be calculated.
- Mice will be sacrificed and liver tissue samples will be taken for RNA isolation and gene expression analysis.
- miRNA micro array expression analysis will be carried out.
- Target gene expression will be profiled through RT-PCR.
- Proteins expression analysis will be carried out using western blotting.

Chapter 8

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Chapter 9

Appendices

Appendix A

Detailed of GO biological processes associated with differentially expressed miRNAs and putative target genes down-regulated under SNEB

Supplementary Table 4.1. GO biological processes associated with putative target genes down-regulated under SNEB

Symbol	Evidence Code	ENTREZ GENE Name	GO Biological Process
TTR		Transthyretin1	
	TAS		extracellular matrix organization
	TAS		photo transduction, visible light
	TAS		retinoid metabolic process
	IEA		retinol metabolic process
	IEA		transport
TPMT		Thiopurine S-methyltransferase	
	TAS		methylation
	TAS		nucleobase-containing compound metabolic process
	TAS		small molecule metabolic process
	TAS		xenobiotic metabolic process
TMX1		Thioredoxin-related transmembrane protein 1	
	NAS		DNA replication
	TAS		ER to Golgi vesicle-mediated transport
	NAS		cell proliferation
	IEA		cell redox homeostasis
	NAS		leukocyte activation
	IDA		negative regulation of apoptotic process
	NAS		positive regulation of growth
	NAS		positive regulation of transcription, DNA-dependent
	NAS		response to stress
	NAS		signal transduction
STAMBP		STAM binding protein	
	TAS		JAK-STAT cascade
	IMP		mitotic cytokinesis
	IMP		negative regulation of Ras protein signal transduction
	IEA		negative regulation of apoptotic process
	IMP		negative regulation of phosphatidylinositol 3-kinase cascade
	TAS		positive regulation of cell proliferation
	IMP		protein deubiquitination
	IEA		proteolysis
	TAS		slc1a4

SLC1A4		Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	
	IDA		L-alanine transport
	IDA		L-cystine transport
	IDA		NOT L-cystine transport
	IDA		L-serine transport
	TAS		amino acid transport
	IDA		chloride transport
	IMP		cognition
	TAS		glutamine transport
	IDA		hydroxyproline transport
	TAS		ion transport
	IDA		proline transmembrane transport
	IDA		proline transport
	NAS		synaptic transmission, glutamatergic
	IDA		threonine transport
	TAS		transmembrane transport
RFP128		Ring finger protein 128, E3 ubiquitin protein ligase	
	IEA		negative regulation of cytokine biosynthetic process
PTPRR		Protein tyrosine phosphatise, receptor type, R	
	IEA		in utero embryonic development
			protein dephosphorylation
NR2F1		Nuclear receptor subfamily 2, group F, member 1	forebrain development
	IEA		gene expression
	TAS		intracellular receptor signaling pathway
	TAS		negative regulation of transcription from RNA polymerase II promoter
	IDA		neuron migration
	IEA		signal transduction
	TAS		transcription initiation from RNA polymerase II promoter
MIS12		MIS 12, MIND kinetochore complex component, homolog (S. pombe	
	IEA		cell division
	IMP		chromosome segregation
	IDA		kinetochore assembly
	IEA		mitosis
	TAS		mitotic cell cycle

MIEN 1		Migration and invasion enhancer 1	
	IEA		apoptotic process
	IMP		cell redox homeostasis
	IDA		negative regulation of apoptotic process
	IEA		positive regulation of cell migration
	TAS		positive regulation of filopodium assembly
BTG1		B-cell translocation gene 1,anti-proliferative	
	NAS		cell migration
	NAS		negative regulation of cell growth
	IDA		negative regulation of cell proliferation
	IMP		positive regulation of angiogenesis
	IMP		positive regulation of endothelial cell differentiation
	IMP		positive regulation of fibroblast apoptotic process
	IDA		positive regulation of myoblast differentiation
	NAS		positive regulation of myoblast differentiation
	NAS		regulation of transcription, DNA-dependent
ALDH1A1		Aldehyde dehydrogenase 1 family, member A1	
	TAS		cellular aldehyde metabolic process
	TAS		ethanol oxidation
	TAS		positive regulation of Ras GTPase activity
	IEA		retinol metabolic process
	TAS		small molecule metabolic process
	TAS		xenobiotic metabolic process
CL19		Chemokine (C-C motif) ligand 19	
	ISS		T cell costimulation
	ISS		activation of JUN kinase activity
	TAS		cell communication
	ISS		cell maturation
	TAS		cellular calcium ion homeostasis

	IDA		dendritic cell chemotaxis
	IDA		establishment of T cell polarity
	IEA		immune response
	ISS		immunological synapse formation
	NAS		inflammatory response
	ISS		interleukin-12 secretion
	ISS		mature dendritic cell differentiation
	IDA		myeloid dendritic cell chemotaxis
	IDA		negative regulation of leukocyte apoptotic process
	ISS		positive regulation of Cdc42 GTPase activity
	IDA		positive regulation of ERK1 and ERK2 cascade
	IDA		positive regulation of I-kappaB kinase/NF-kappaB cascade
	IDA		positive regulation of JNK cascade
	IDA		positive regulation of NF-kappaB import into nucleus
	ISS		positive regulation of Ras GTPase activity
	ISS		positive regulation of T cell proliferation
	ISS		positive regulation of T-helper 1 cell differentiation
	IDA		positive regulation of cell motility
	ISS		positive regulation of chemotaxis
	ISS		positive regulation of dendritic cell antigen processing and presentation
CD14		CD14 molecule	
	TAS		I-kappaB kinase/NF-kappaB cascade
	TAS		MyD88-dependent toll-like receptor signaling pathway
	TAS		MyD88-independent toll-like receptor signaling pathway
	TAS		TRIF-dependent toll-like receptor signaling pathway

	TAS		apoptotic process
	TAS		cell surface receptor signaling pathway
	IDA		cellular response to lipopolysaccharide
	IDA		cellular response to lipoteichoic acid
	IEA		inflammatory response
	TAS		innate immune response
	TAS		phagocytosis
	IEA		positive regulation of cytokine secretion
	IEA		positive regulation of endocytosis
	IDA		positive regulation of tumor necrosis factor production
	IEA		response to heat
	TAS		toll-like receptor 2 signaling pathway
	TAS		toll-like receptor 3 signaling pathway
	TAS		toll-like receptor 4 signaling pathway
	TAS		toll-like receptor TLR1:TLR2 signaling pathway
	TAS		toll-like receptor TLR6:TLR2 signaling pathway
	TAS		toll-like receptor signaling pathway
CIB1		Calcium and integrin binding 1 (calmyrin)	
	IMP		apoptotic process
	TAS		cell adhesion
	IDA		cellular response to DNA damage stimulus
	TAS		double-strand break repair
	IDA		endomitotic cell cycle
	TAS		extrinsic apoptotic signaling pathway
	IMP		negative regulation of cell proliferation
	IDA		positive regulation of calcineurin-NFAT signaling cascade

	IGI		positive regulation of establishment of protein localization to plasma membrane
DNAJA1		DnaJ (Hsp40) homolog, subfamily A, member 1	
			androgen receptor signaling pathway
			protein folding
			response to heat
			response to unfolded protein
			sperm motility
			spermatogenesis
DSG1		Desmoglein 1	
	IEA		apoptotic process
	IEA		calcium-dependent cell-cell adhesion
	IEA		cell-cell adhesion
	TAS		cell-cell junction assembly
	IEA		cellular component disassembly involved in execution phase of apoptosis
	IEA		homophilic cell adhesion
	IEA		maternal process involved in female pregnancy
	IEA		protein stabilization
	IEA		response to progesterone stimulus
ERRFI1		ERBB receptor feedback inhibitor 1	
	ISS		lung alveolus development
	ISS		lung epithelium development
	ISS		lung vasculature development
	IDA		negative regulation of epidermal growth factor-activated receptor activity
	ISS		negative regulation of epidermal growth factor-activated receptor activity
	IDA		negative regulation of protein autophosphorylation
	TAS		positive regulation of Rho GTPase activity
	ISS		regulation of keratinocyte differentiation
	TAS		response to stress

	ISS		skin morphogenesis
FADS2		Fatty acid desaturase 2	
	TAS		alpha-linolenic acid metabolic process
	TAS		linoleic acid metabolic process
	TAS		small molecule metabolic process
	IEA		unsaturated fatty acid biosynthetic process
	TAS		unsaturated fatty acid metabolic process
FOXA3		Forkhead box A3	
HNF3-gamma	TAS		cellular glucose homeostasis
	IEA		cellular response to starvation
	IEA		chromatin modification
	IBA		embryo development
	TAS		endocrine pancreas development
	IBA		negative regulation of transcription from RNA polymerase II promoter
	IBA		neuron fate specification
	IBA		pattern specification process
	IBA		positive regulation of neuron differentiation
	IDA		positive regulation of transcription from RNA polymerase II promoter
	IBA		regulation of hormone levels
	IBA		regulation of sequence-specific DNA binding transcription factor activity
	IEA		spermatogenesis
	IBA		tissue development
FXR1		Fragile X mental retardation, autosomal homolog 1	
	TAS		apoptotic process
	IEA		cell differentiation
	IEA		muscle organ development
	IBA		negative regulation of translation
GKAP1		G kinase anchoring protein 1	
	ISS		signal transduction
GNS		Glucosamine (N-acetyl)-6-sulfatase	
	TAS		carbohydrate metabolic process

	TAS		glycosaminoglycan catabolic process
	TAS		glycosaminoglycan metabolic process
	TAS		keratan sulfate catabolic process
	TAS		keratan sulfate metabolic process
	TAS		small molecule metabolic process
GPBP1		GC-rich promoter binding protein 1	
	IEA		positive regulation of transcription, DNA-dependent
	IEA		transcription, DNA-dependent
gpr37			
	TAS		G-protein coupled receptor signaling pathway
HEYL		Hairy/enhancer-of-split related with YRPW motif-like	
	IDA		
	TAS		Notch signaling pathway
	ISS		Notch signaling pathway
	ISS		atrioventricular valve morphogenesis
	ISS		cardiac epithelial to mesenchymal transition
	IEA		cardiac ventricle morphogenesis
	ISS		cellular response to BMP stimulus
	ISS		endocardial cushion morphogenesis
	IEA		epithelial to mesenchymal transition involved in endocardial cushion formation
	ISS		glomerulus development
	IDA		mesenchymal cell development
	IDA		negative regulation of androgen receptor activity
	IDA		negative regulation of androgen receptor signaling pathway
	ISS		negative regulation of transcription, DNA-dependent
	ISS		outflow tract morphogenesis
	IDA		positive regulation of neuron differentiation

	IEA		positive regulation of transcription from RNA polymerase II promoter
	ISS		proximal tubule development
	IEA		pulmonary valve morphogenesis
	ISS		skeletal muscle cell differentiation
	IDA		ventricular septum morphogenesis
IP6K2		Inositol hexakisphosphate kinase 2	
	TAS		cytokine-mediated signaling pathway
	TAS		inositol phosphate metabolic process
	IMP		negative regulation of cell growth
	IEA		phosphate ion transport
	IDA		phosphatidylinositol phosphorylation
	IMP		positive regulation of apoptotic process
	TAS		small molecule metabolic process
	TAS		type I interferon-mediated signaling pathway

TAS= Traceable Author Statement, IMP = inferred from mutant phenotype, IDA= inferred from direct assay, IEA= inferred from electronic annotations ISS = Inferred from Sequence or Structural Similarity IBA Inferred from Biological aspect of Ancestor
NAS= Non-traceable Author Statement

Supplementary Table 4.2: FAME Go biological processes associated with Up-regulated miRNAs under SNEB

miRNA	Evidence code	GO term	p-value	q-value	z-score	enrichment factor	Targets	HG p-value
mir-17-5p								
	ISO,IDA	regulation of cell cycle	0.0001	0.0648	4	1.63	42	0.000682
	IDA	cell cycle arrest	0.0002	0.0486	4.03	2.34	15	0.005124
	ISO	negative regulation of progression through cell cycle	0.0002	0.0324	3.77	1.73	31	0.000604
	ISO,IDA	ER-nuclear signaling pathway	0.0003	0.0405	4.01	3.58	5	0.061275
	ISO	protein kinase cascade	0.001	0.081	3.23	1.44	49	0.000432
	IDA	unfolded protein response	0.0012	0.09	3.68	3.93	3	0.148206
	ISO	intracellular signaling cascade	0.0014	0.094	3.14	1.25	124	0.000101
	IDA	regulation of GTPase activity	0.0026	0.1271	3.06	1.93	15	0.003838
	ISO,IMP	sterol metabolism	0.0027	0.125	3.2	2.33	9	0.052088
	ISO,IMP	cholesterol metabolism	0.0029	0.1174	3.1	2.35	8	0.066757
	IDA	phosphate metabolism	0.003	0.1124	2.71	1.24	91	9.16E-05
	IDA	positive regulation of cellular metabolism	0.0041	0.1476	2.69	1.28	64	8.85E-05
	ISO	positive regulation of metabolism	0.0052	0.162	2.62	1.27	67	0.000112
	IGI,IMP	di-, tri-valent inorganic cation homeostasis	0.0054	0.1576	2.77	1.99	10	0.3262
	IEA	regulation of small GTPase mediated signal transduction	0.0054	0.1507	2.77	1.57	28	2.72E-05
	IDA	Transcription	0.0054	0.1458	2.55	1.13	214	3.94E-07
	IMP	negative regulation of phosphate metabolism	0.0061	0.1581	2.97	3.07	3	0.202438

	ISO,TAS	regulation of transcription	0.0075	0.1676	2.39	1.12	210	3.60E-07
	ISO	protein modification	0.0076	0.1631	2.46	1.16	160	1.71E-05
	IGI,IMP,ISO	metal ion homeostasis	0.0088	0.1728	2.58	1.85	11	0.274285
	ISO,IGI,IMP	regulation of cellular metabolism	0.0089	0.1706	2.37	1.11	235	3.43E-07
	ISO,IMP	regulation of metabolism	0.0096	0.1728	2.36	1.11	247	3.45E-07
	ISO,TAS	protein amino acid dephosphorylation	0.0104	0.1736	2.4	1.58	19	0.003511
	ISO	positive regulation of biosynthesis	0.0109	0.1758	2.52	2.02	9	0.126439
	ISO	Homeostasis	0.0143	0.2163	2.22	1.42	26	0.268694
	IMP	Phosphorylation	0.015	0.2167	2.23	1.22	73	0.000764
	IMP	regulation of phosphorylation	0.0153	0.2148	2.32	1.71	11	0.094397
	IDA	regulation of proteolysis	0.0154	0.2123	2.61	3.12	3	0.078464
	IGI,IMP	cell ion homeostasis	0.0155	0.2099	2.3	1.69	12	0.341117
	IMP	glucose homeostasis	0.0159	0.2109	2.5	2.56	5	0.033488
	ISO	protein amino acid phosphorylation	0.0178	0.2255	2.14	1.22	68	0.000219
	IGI,IMP,IDA	ion homeostasis	0.0186	0.2318	2.21	1.64	13	0.328259
	ISO,IDA	regulation of cell growth	0.0187	0.228	2.19	1.54	19	0.020886
	ISO,1DA	glycosphingolipid metabolism	0.0199	0.2339	2.4	2.81	3	0.123098
	ISO,IDA	small GTPase mediated signal transduction	0.0227	0.2536	2.03	1.28	48	0.002701
	ISO,IDA	signal transduction	0.0229	0.2479	1.98	1.1	226	0.001202
	ISO,IMP	regulation of hormone secretion	0.0313	0.2935	2.09	2.27	4	0.094266

		positive regulation of amino acid metabolism	0.0325	0.3013	2	1.87	6	0.075973
	IDA,ISO,TAS	enzyme linked receptor protein signaling pathway	0.0336	0.3071	1.87	1.24	46	0.00058
	IMP	regulation of phosphate metabolism	0.0365	0.3196	1.87	1.54	11	0.147173
	ISO,IMP,ISS	positive regulation of protein metabolism	0.0386	0.3244	1.86	1.6	10	0.148064
	IMP	cholesterol homeostasis	0.0451	0.3438	1.91	2.27	3	0.17482
	ISO,IMP,IDA	calcium ion homeostasis	0.0472	0.3556	1.8	1.71	7	0.494794
	IMP	negative regulation of physiological process	0.0479	0.3564	1.68	1.13	104	0.007844
	ISO,IMP	regulation of ion transport	0.0485	0.3492	1.91	2.4	4	0.192943
miR-31								
	IEA	amino acid transport	0.0011	0.0734	4.34	5.4	4	0.022233
		carboxylic acid transport	0.0012	0.0641	4.03	4.69	5	0.010893
	IDA	cell growth	0.0098	0.124	2.64	2.37	8	0.020023
		response to stress	0.0231	0.2127	2.11	1.55	18	0.173549
	IDA	regulation of cell growth	0.037	0.2823	2	2.13	6	0.055108
	IDA	negative regulation of growth	0.0448	0.3236	1.98	2.49	4	0.040537
	IDA	Growth	0.0463	0.325	1.8	1.7	9	0.093659
mir-140								
	IMP	positive regulation of cell proliferation	0.0037	0.5681	3.21	2.2	14	0.000876
	IMP	regulation of cell proliferation	0.0045	0.4545	2.85	1.73	22	0.000871
	IEA	response to nutrient levels	0.0449	0.3887	2.05	2.95	3	0.071954
	IEA	activation of protein kinase activity	0.0452	0.38	2.01	2.9	3	0.1387

Appendix B

Supplementary data for RNA-seq miRNA expression analysis

Supplementary Table 5.1. RT-qPCR results for differentially expressed miRNA under SNEB

miRNA	Sequence	**Fold change**	**p value**
mir-143	UGAGAUGAAGCACUGUAGCUCG	- 2.39	0.027

Corrected Cts for SNEB (26.58) and MNEB (25.32) were calculated using RNU6B as endogenous control.

Differential expression analysis was carried out with PROC t-test (SAS) after normalization of Ct-values to reference gene RNU6B

Appendix C

Primer sequences for RT-PCR expression analysis

Supplementary Table 6.1. RTPCR primers sequences for *FADS2* and house-keeping gene *beta-Actin*

Gene	Forward	Reverse
Beta-Actin	GTCCTCTCCCAAGTCCACAC	CGTTTGAAATGTCCGTTTGTAGAT
FADS2	AGCCCATTGAGTACGGCAAG	TACAAAGGGATGAGCAGCGG
RSP9	CCTCGACCAAGAGCTGAAG	CCTCCAGACCTCACGTTTGTTC
GAPDH	TTGTGATGGGCGTGAACC	CCCTCCACGATGCCAAA

Appendix D

List of Abbreviations

ΔΔCt	Delta delta cyclic threshold
µL	micro litre
GNS	Glucosamine N-acetyl-6-sulfatase
3'UTRs	3 PRIME untranslated region
5'UTR	5 prime untranslated region
IDA	Inferred from Direct Assay
AA	Arachidonic acid
ABCA1	ATP-binding cassette transporter member 1
ACACA	C1 acetyl-CoA carboxylase-a
ACLY	ATP citrate lyase
AGO	Argonaute protein
AHCYL1	Adenosylhomocysteinase-like 1
ALD	Alcoholic liver disease
ATM	Ataxia telangiectasia mutated
BGI	Beijing Genomics Institute
BHB	Beta hydroxybutyrate
BWT	Burrow wheeler transformation
CD14	cluster of differentiation 14
cDNA	complementary DNA
CL	corpus luteum
COCs	cumulus oocyte complex

CSN2	Beta-casein gene 2
CSN3	Beta-casein gene 3
Ct	Cyclic threshold Cytochrome
CYP19	P450, Family 19
ddNTP	Di-deoxyribo nucleotide triphosphate
DEG	Differentially expressed genes
DER	Dietary energy restriction
DHA	Docosahexaenoic acid
DMEM	Modified Eagle's Medium
DMI	dry matter intake
DMRT1	Doublesex and mab-3 related transcription factor 1
DNJA1	DnaJ Hsp40 homolog, subfamily A, member 1
dNTP	Deoxyribonucleotide triphosphate
DRD2L	Dopamine receptor D2 like
DSG1	Desmoglein 1 Essential
EFAs	fatty acids
ENCODE	The Encyclopedia of DNA Elements
EPA	Eicosapentaenoic acid
ETOH	Ethanol
FADS1	Fatty acid desaturase 1
FADS1AT1	Fatty Acid desaturase 1 alternative transcript 1
FADS2	Fatty acid desaturase 2
FAME	Functional Assignment of MicroRNAs via Enrichment
FASN	Fatty acid synthase,
FBS	Foetal bovine serum

FGF	Fibroblast growth factor
FM-index	Fast match index
FOXA3	Forkhead box A3
FXR1	Fragile X mental retardation, autosomal homolog
G	Gravity force
GD	Gestational day
GDF8	Myostatin
GH21	Growth harmone 21
GH24	Growth harmone 24
GLUT2	Glucose transporter 2
GOA	Gene ontology annotation
GPR37	G protein-coupled receptor 37 endothelin receptor type B-like
HCC	Hepatocellular carcinoma
HDL	High-density lipoprotein
HEYL	Hairy/enhancer-of-split related with YRPW motif-like
HFD	High-fat diet
HNF3-gamma	Hepatic nuclear factor 3 gamma
HOAX1	Homeobox 1
HSC	Hepatic stellate cells
HSF1	Heat shock factor 1
HTS	High throough put sequencing
IBA	Inferred from Biological aspect of Ancestor
IEA	Inferred from Electronic Annotation
IFNGR1	Interferon gamma receptor 1
IGF-1	Insulin like growth factor 1

IGI	Inferred from Genetic Interaction
IMP	Inferred from Mutant Phenotype
IPI	Inferred from Physical Interaction
ISH	In situ hybridization
ISO	Inferred from Sequence Orthology
ISS	Inferred from Sequence or Structural Similarity
LA	Linoleic acid
LC-PUFAs	Long chain polyunsaturated fatty acids
let-7	Lethal-7
limma	Linear Models for Microarray Data analysis
LIN28B	Lin-28 homolog B
lin-4	Abnormal cell LINeage family member
LNA	Locked Nucleic Acid
LOESS	Locally weighted scatterplot smoothing
LRP2	Low density lipoprotein receptor-related
LXR/RXR	protein 2 Liver X receptor-retinoid X receptor
MDBK	Madin-Darby Bovine Kidney
min	Minute
miRNAs	micro RNA
MNEB	Mild negative energy balanace
MSTN	Myostatin
NAFLD	Non alcoholic fatty liver disease
NANOG	Nanog homeobox
NAS	Non-traceable Author Statement
NASH	Nonalcoholic Steatohepatitis
NB	Negative bionamial

ncRNA	non coding RNA
NEAA	Non-essentiaL amino acids
NEB	Negative energy balance
NEFAs	Non estrified fatty caids
NEL	Net energy for lactation
ng	nano gram $=10^{-9}$ gram
NGS	Next Generation Sequencing
NR	Not Recorded
nts	Nucleotides
OA	Oleic acid
oligo	Oligo-deoxy-thymine nucleotides
P	Probability
PA	Palmitic acid
PCT	Probability of conserved targeting
PDGFRA	Platelet-derived growth factor receptor alpha
Pen/Strep	Penicillin/streptomycine
PGCS	Primordial germ cells
PBS	Phosphate buffered saline
piRNA	Piwi RNA
PPARa	Peroxisome proliferator-activated receptor alpha
pre-miRNA	Precursor micro RNA
pri-miRNA	Primary micro RNA
PUFAs	polyunsaturated fatty acids
Q	Quality socore
Rank Pred	Rank product
rasiRNA	Repeat associated small interfering RNA

RIN	RNA integrity number
RISC	RNA silencing comple
RMA	Robust Multi-array Average
RNAi	Ribonucleic acid interference
RNA-seq	Ribo nucleic acid sequencing
ROS	Reactive oxygen species
RPM	Reads per million
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SA	Stearic acid
SAM	Significant analysis of micro array
Samr	Significance Analysis of Microarrays R
SAS	Statistical analysis software
sec	Seconds
SLC2A12	Solute Carrier Family 2 Facilitated Glucose Transporter, Member12
SNEB	Severe negative energy balance
SNPs	Single nucleotide Polymorphosim
SOX9	SRY sex determining region Y-box 9
SREBF1	Sterol regulatory element bindingtranscription factor 1
SREBP	Sterol Regulatory Element-Binding Proteins
SREBP1	Sterol regulatory element-binding protein 1
SREBP2	Sterol Regulatory Element-Binding Proteins2
sRNA	Small ribonucleic acid
stRNA	Small temporal RNA
TAG	Triacyl Glyceride
TAS	Traceable Author Statement

TF	Transcription factor
TFBSs	Transcription factor binding sites
TGF-ß	Transforming growth factor, beta
TMM	Trimmed means of M values
TTR	Transthyretin1
UFL	unité fourragère lait
VSN	Varience stabilize normalization