Leibniz-Institut für Nutztierbiologie (FBN)

MiRNAs as regulators of gene expression modulate development and energy metabolism of skeletal muscle

Inaugural-Dissertation

zur Erlangung des Grades

Doktor der Agrarwissenschaft

(Dr. agr.)

der Landwirtschaftlichen Fakultät

der Rheinischen Friedrich-Wilhelms-Universität Bonn

von

Puntita Siengdee

aus

Lampang, Thailand

Referent: Prof. Dr. Klaus Wimmers

Korreferent: Prof. Dr. Karl Schellander

Tag der mündlichen Prüfung: 27 Juni 2014

Erscheinungsjahr: 2014

Diese Dissertation ist auf dem Hochschulschriftenserver der ULB Bonn
http://hss.ulb.uni-bonn.de/diss_online elektronisch publiziert.

Dedicated to my family

MiRNAs as regulators of gene expression modulate development and energy metabolism of skeletal muscle

It is important to understand the molecular networks affecting biological properties of muscle in order to improve the efficiency of meat production and meat quality in domestic animals. The discovery of miRNA represents an important breakthrough in biology in recent years. MiRNA function identification has become one of the active research fields in muscle biology addressing muscle development, growth and metabolism. This thesis aims at the identification of miRNAs differentially expressed in skeletal muscle at various developmental stages and in pig breeds differing in muscularity. Moreover, links between miRNAs and mRNAs should be shown in order to address biofunctions affected by miRNAs in muscle. Finally, miRNAs impacted on muscle metabolism should be validated exemplarity by *in vitro* cell culture experimants. The first approach demonstrates the comprehensive miRNA expression profiles of *longissimus dorsi* (LD) during muscle development and growth. A comparative study on two distinct phenotypic pigs were performed using miRNA custom designed arrays. Two different key stages 63 and 91 days post-conception (dpc), and one adult stage (180 days post-natum) were analysed in German Landrace (DL) and Pietrain (Pi) breeds. Several potential candidate miRNAs are significantly up-regulated and associated with muscular developmental stages and breed types. The Affymetrix GeneChip porcine genome microarrays were also used to obtain the differential transcriptional profile of mRNA targets of the same animals. The combination of miRNA–mRNA expression data and Ingenuity Pathway Analysis established complex miRNA–dependent regulatory networks. A number of miRNA–mRNA interactions, that were associated to cellular growth and proliferation and lipid-metabolism functions, revealed insights into their role during skeletal muscle development and growth.

The second approach involves in muscle growth in *post mortem* pig traits (crossbred [PI×(DL×DE)] population, n = 207). The experiment integrated miRNA and mRNA expression together with network analysis by using weighted gene co-expression network analysis (WGCNA). In this part, we identified the negative miRNA-mRNA co-expression networks which revealed several biological pathways underlying the

difference of meat properties and muscle traits (i.e. glucose metabolic process, mitochondrial ribosome and oxidative phosphorylation).

In the last approach, C2C12 *in vitro* model studies revealed that miRNAs are modulated in cellular ATP production and energy metabolism processes during myogenic differentiation. Correlation analyses were performed between ATP level, miRNA and mRNA microarray expression profiles during C2C12 differentiation. Among 14 significant miRNAs as representing cellular ATP regulators involved in mitochondrial energy metabolism, miR-423-3p is a novel regulator for cellular ATP/ energy metabolism via targeting the group of mitochondrial energy metabolism genes (*Cox6a2*, *Ndufb7*, and *Ndufs5*).

In conclusion, the present study further adds a comprehensive knowledge on the systems perspective of the skeletal muscle miRNAs and their target genes regulation networks that influence on skeletal muscle starting from early muscle development to mature muscle growth.

MiRNAs regulieren die Genexpression und modulieren die Entwicklung und den Energiestoffwechsel der Skelettmuskulatur.

Das Verständnis von molekularen Netzwerken mit Einfluss auf die biologischen Eigenschaften des Muskels ist notwendig, um die Effizienz der Fleischproduktion und die Fleischqualität in Nutztieren zu verbessern. Die Erforschung von miRNAs stellt einen entscheidenden Durchbruch in der Biologie in den letzten Jahren dar. Die Identifizierung von miRNA-Funktionen wurde seit dem eines der aufstrebenden Forschungsschwerpunkte in der Muskelbiologie mit Bezug auf Muskelentwicklung, -wachstum und -stoffwechsel. Das Ziel dieser Dissertation ist die Identifizierung von miRNAs mit differenzieller Expression in der Skelettmuskulatur im Hinblick auf verschiedene Entwicklungsstadien und Schweinerassen mit unterschiedlichem Muskelansatz. Im Weiteren soll die Verknüpfung von miRNA- und mRNA-Datensätzen helfen, durch miRNA beeinflusste Biofunktionen im Muskel zu benennen. Abschließend sollen exemplarisch einige miRNAs mit Einfluss auf den Muskelmetabolismus durch in vitro Zellkulturstudien validiert werden.

Der erste Forschungsansatz lieferte umfassende miRNA-Expressionsprofile des *longissimus dorsi* (LD) während der Muskelentwicklung und des Wachstums. Dazu wurden Schweine mit unterschiedlicher phänotypischer Ausprägung unter der Verwendung von spezifisch gefertigten miRNA-Arrays vergleichend analysiert. Tiere der Deutschen Landrasse (DL) und der Rasse Pietrain (Pi) wurden zu zwei wesentlichen pränatalen Entwicklungszeitpunkten (am 63 und 91 Tag nach Empfängnis) sowie im adulten Stadium (180 Tage nach Geburt) untersucht. Für zahlreiche potentielle Kandidaten-miRNAs konnte gezeigt werden, dass diese signifikant hochreguliert sind und Assoziationen zu muskulären Entwicklungsstadien und der Rasse aufzeigten. Zusätzlich wurden porcine Genommikroarrays (Affymetrix GeneChip) verwendet um Profile der differentiell exprimierten mRNA-targets im gleichen Tier zu untersuchen. Durch die Kombination von miRNA- und mRNA-Expressionsdaten gekoppelt mit Ergebnissen aus der Analyse von regulierten Signalwegen (Ingenuity pathway analysis) konnte ein Komplex aus miRNA-abhängigen regulatorischen Netzwerken etabliert werden. Zahlreiche miRNA-mRNA-Interaktionen im Zusammenhang mit Funktionen des zellulären Wachstums, der Proliferation und des Fettstoffwechsels, ermöglichten

Einblicke in die Funktion dieser Wechselwirkungen während der Entwicklung und des Wachstums der Skelettmuskulatur.

Der zweite Forschungsansatz berücksichtigt das Muskelwachstum in relevanten *post mortem* Merkmalen (Kreuzungsrasse [Pi x (DLxDE)], n=207). Für diesen Ansatz wurden die Expressionsdaten der miRNA- und mRNA-Analysen in einem Ko-Expressionsnetzwerk integriert. Dabei wurden die Wechselwirkungen zwischen verschiedenen Komponenten berücksichtigt und gewichtet. Negative miRNA-mRNA-Ko-Expressionsnetzwerke konnten identifiziert werden. Diese deuten auf biologisch relevante Signalwegen hin, welche mit unterschiedlichen Ausprägungen der Fleischeigenschaften und Merkmalen der Muskulatur in Zusammenhang stehen (z.B. Prozesse des Glucosemetabolismus, mitochondriale Ribosomen und oxidative Phosphorylierung).

Im abschließenden Forschungsansatz konnte durch Analysen des C2C12-Muskelzellmodells gezeigt werden, dass miRNAs im Zusammenhang mit der zellulären ATP-Produktion und mit Prozessen des Energiemetabolismus im Rahmen der myogenen Differenzierung reguliert werden. Dazu wurden zum Zeitpunkt der C2C12-Zelldifferenzierung ermittelte ATP-Gehalte und miRNA- und mRNA-Mikroarray-Expressionsprofile miteinander verknüpft. Unter den 14 miRNAs, die als zelluläre ATP-Regulatoren am mitochondrialen Energiemetabolismus beteiligt sind, konnte miR-423-3p, durch den Einfluss auf Gene aus der Gruppe des mitochondrialen Energiemetabolismus (*Cox6a2*, *Ndufb7* und *Ndufs5*), als neuer Regulator für zelluläres ATP bzw. den Energiemetabolismus bestätigt werden.

Zusammenfassend liefern die vorliegenden Studien wesentliche Erkenntnisse zu systemischen Funktionen der miRNAs in der Skelettmuskulatur und verdeutlichen ihren Einfluss auf Gennetzwerke, welche die Prozesse von der frühen Muskelentwicklung bis hin zum Muskelwachstum beeinflussen.

Contents

CHAPTER 1

General introductions

1.1 Overview of miRNAs

MicroRNAs (miRNAs) are a class of single-stranded, non-coding RNAs (ncRNAs) molecules that control gene expression in animals, plants, and unicellular eukaryotes. MiRNAs function as 21–24-nucleotide (nt) in length and interact with mRNAs which contain complementary sequences, and thereby regulate transcriptional and/or post-transcriptional repression. Recently considerable evidence indicates that miRNAs can play a major role in regulating gene expression and likely influence the output of many protein-coding genes in a variety of eukaryotic systems. MiRNAs are involved in several diverse biological processes including cell determination, cell death, cancer, animal development, cell development, differentiation, apoptosis and physiological (Bartel 2004; Ambros 2004). Now the miRNA database ‘‘miRNABase’’ is the online repository for all miRNA sequences and annotation (accessible at http://www.mirbase.org/) contained more than 24,000 hairpin sequences and over 30,000 mature sequences in over 140 species (miRBase 20).

1.1.1 Biogenesis of microRNAs

The biogenesis of miRNAs in animals occurs through a multi-step process that involves several different enzymes first in the cell nucleus and finally in the cytoplasm (Fig. 1.1). In the nucleus, Primary microRNAs (pri-miRNAs) are transcribed by RNA polymerase II (pol II). Pri-miRNA transcripts consist of one or more hairpin structures of 100–1000 nt. Pri-miRNAs fold into hairpin structures containing imperfectly base paired stems and a terminal loop which harbor 7‑methylguanosine (m7G) 5’-cap, spliced and poly-A tails. Then, pri-miRNAs are further processed by microprocessor complex (RNase III complex) that mainly composed of a processing enzyme (Drosha). The pri-miRNA hairpins are recognized by RNA binding protein DiGeorge syndrome critical region gene 8 (DGCR8) in mammals or Pasha in *Drosophila melanogaster* (Filipowicz *et al.* 2008; Krol *et al.* 2010) at the base of the stem, positioning the enzyme in the correct spatial configuration for asymmetric cleavage (5’ phosphate and two nucleotide 3’ overhang).

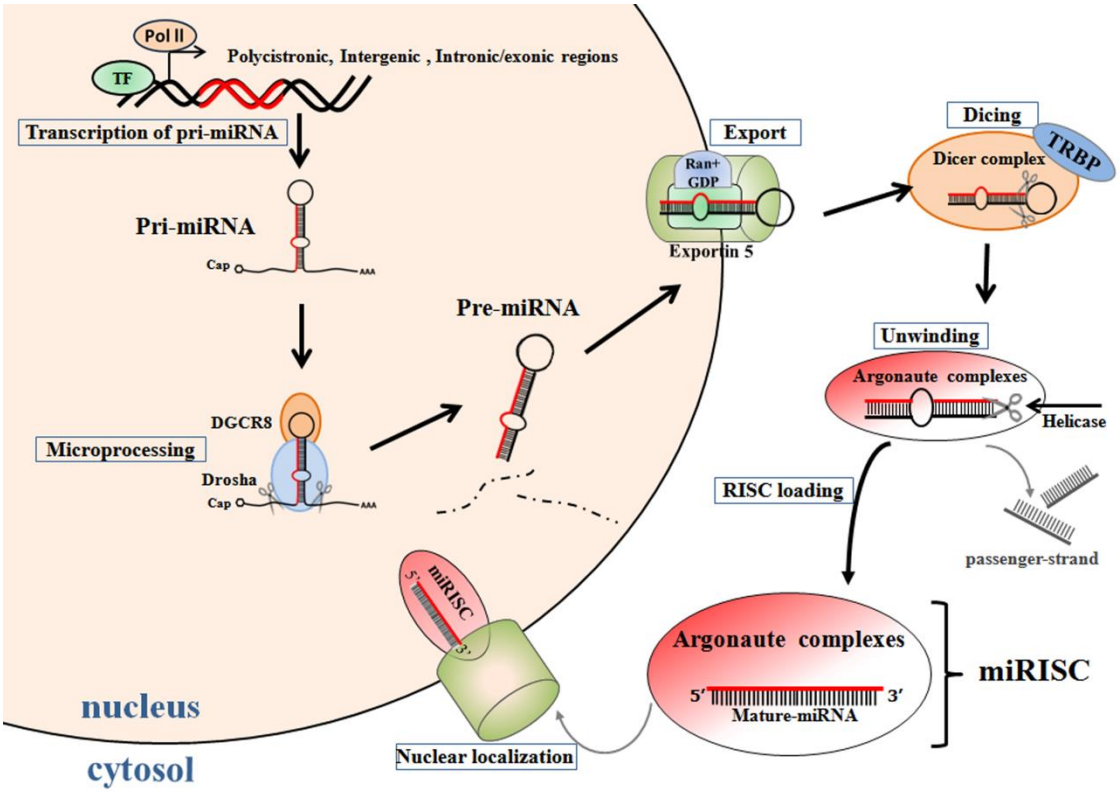

Figure 1.1: The biogenesis of miRNAs. MiRNAs are endogenously encoded in the genome. They are generally transcribed by Pol II into the primary miRNAs (pri-miRNAs) as long several 100–1000 nt. The pri-miRNAs are processed (cropping) by RNase III–type enzyme Drosha with its partner DGCR8 (also known as Pasha in invertebrates) into the precursor miRNAs (pre-miRNAs) consisting of approximately 70 nt in the nucleus. Then pre-miRNAs are exported into the cytoplasm by the nuclear export factor Exportin 5 through, a RAN-GTP-dependent process. In the cytoplasm, pre-miRNAs are further processed (dicing) by another RNase III protein Dicer-TRBP complex into the double stand ~19–25 nt long miRNA duplex structures consisting of the ‘guide strand’ (miRNA) and the ‘passenger strand’ (miRNA*). The miRNA/miRNA∗ duplexes are loaded into Argonaute effector complexes as known as RNA-induced silencing complex (RISC). One of the thermodynamically less stable at 5’-end of the two strands in the duplex will become the mature miRNA. Then, mature miRNA is preferentially retained in RISC and form miRISCs complex which regulates protein expression.

Microprocessor complex will sever one dsRNA spiral away from the attachment site (11 bps), releasing a precursor microRNA (pre-miRNA) ~60–70 nt stem loop intermediate containing a ~2 nt 3’ overhang, essential for nuclear export by exportin 5 and theirs cofactor RAN-GTP (Bartel 2004). In the cytoplasm, the RNase III enzyme

Dicer complex removes the loop region from pre-miRNAs into ~19-25 nucleotide duplex miRNAs. Dicer interacts with the double-stranded RNA-binding protein TRBP, which likely bridges the initiator and effector steps of miRNA action. Next, the duplexes are loaded into miRNA-class Argonaute effectors (in mammals, Ago1–4). MiRNA duplexes are unwound by helicase. One of the less stable which has its 5' terminus at this end will be mature miRNA or 'guide strand' (Khvorova *et al.* 2003). The other strand is often called the passenger strand or miRNA*. The mature miRNA is preferentially retained in Ago and it is incorporated into a multiple-protein nuclease complex to form the functional miRNA-induced silencing complexes (miRISCs), whereas the miRNA* is degraded (Kim 2005; Wienholds & Plasterk 2005; Kim & Nam 2006; Krol *et al.* 2010; Siomi & Siomi 2010; Yang & Lai 2011 ; Pasquinelli 2012).

1.1.2 Mechanism of miRNAs

The mature miRNAs function as components of ribonucleoprotein (RNP) complexes or RNA-induced silencing complexes (RISCs), referred to as either micro-ribonucleoproteins (miRNPs) or miRNA-induced silencing complexes (miRISCs). MiRNAs bind to complementary sequences in the 3'-UTR of target mRNA. MiRISCs regulate their target mRNA/protein by different mechanisms including, translational repression, mRNA cleavage, mRNA deadenylation or alteration of mRNA stability (Pillai *et al.* 2007; Filipowicz *et al.* 2008; Pasquinelli 2012), see in Figure 1.2. These actions are based on the degree of complementarity between miRNA and target gene in combination with Argonaute (Ago) family proteins. It is now clear that miRNAs effect on protein synthesis by mRNA destabilization or suppression of translation. MiRNAs activate the decapping and deadenylation complexes, leading to mRNA degradation or inhibition of protein translation (Meister 2007; Filipowicz *et al.* 2008; Huntzinger & Izaurralde 2011; Pasquinelli 2012).

- MiRNAs promote mRNA degradation by either of two mechanisms in Figure 1.2B). First mechanism, when miRNAs bind to complementary mRNAs with complete or nearly complete complementarity. A miRNA guide RISC-mediated endonucleolytic cleavage between nucleotides pairing 10 and 11 nt of the 5'-UTR on the miRNA. Through this process, the target mRNAs are degraded by siRNA like effect which occur much more frequently in plants than in mammals (Bartel 2004; Bracken *et al.* 2011).

Second mechanism is deadenylation and exonucleolysis of mRNA target. This is the predominant in mammals. In this case, miRNAs bind to targets with only partial complementarity (imperfect base pairing). MiRISC and the associated GW182 [glycine-tryptophan (GW) repeat-containing protein of 182 kDa] family proteins complex recruit the CCR4–NOT complex to promote the deadenylation of poly(A) tail of target mRNA. These lead to the dissociation of poly(A)-binding protein complex (PABPC) and re-localization of target mRNA to the processing bodies (P-bodies) (Mathonnet *et al.* 2007; Fabian *et al.* 2010; Pasquinelli 2012).

- Protein translational inhibition of miRNAs occurs also at imperfect base pairing condition (Fig. 1.2C). The expression of target proteins will be down-regulated through different mechanisms either at initiation or post-initiation stage of translation. Several published papers provide important supporting mechanistic insights into the repression at the initiation step by using artificial luciferase reporters with multiple 5'-end mRNA-binding site constructs (Pillai *et al.* 2005; Meister 2007; Filipowicz *et al.* 2008). The investigation revealed that miRNAs repress the mRNAs translational initiation by (i) miRNAs interfer the eIF4F complex (comprising eIF4e, eIF4G and eIF4A) to function on m7G cap recognition (refer as 'decapping'), (ii) MiRNAs obstruct the formation of the 80S ribosomal complex, and (iii) MiRNAs have a positive or negative effect on the activity of PABPC to bound at the 3'-UTR (Huntzinger & Izaurralde 2011; Pasquinelli 2012). The suppression effect was also observed at the post-initiation stage of translation by inhibiting ribosome elongation. MiRNAs caused ribosomes to get free from 3'-UTR of mRNAs (ribosome drop-off). In all protein translational inhibition processes, miRNAs repress or inhibit protein translation machinery without causing mRNA degradation (Petersen *et al.* 2006; Filipowicz *et al.* 2008; Fabian *et al.* 2010). The effect of miRNAs on protein production has been examined (Baek *et al.* 2008). The result suggested that relatively low extents of miRNAs expressions are able to alter the expression of most of the target proteins. Whereas, some of these protein targets were directly repressed by miRNAs in mRNA levels (Baek *et al.* 2008). The degree of translational repression is dependent on the number, and also positioning of miRNAs to binding in the 3'-UTR of the target (Fabian *et al.* 2010).

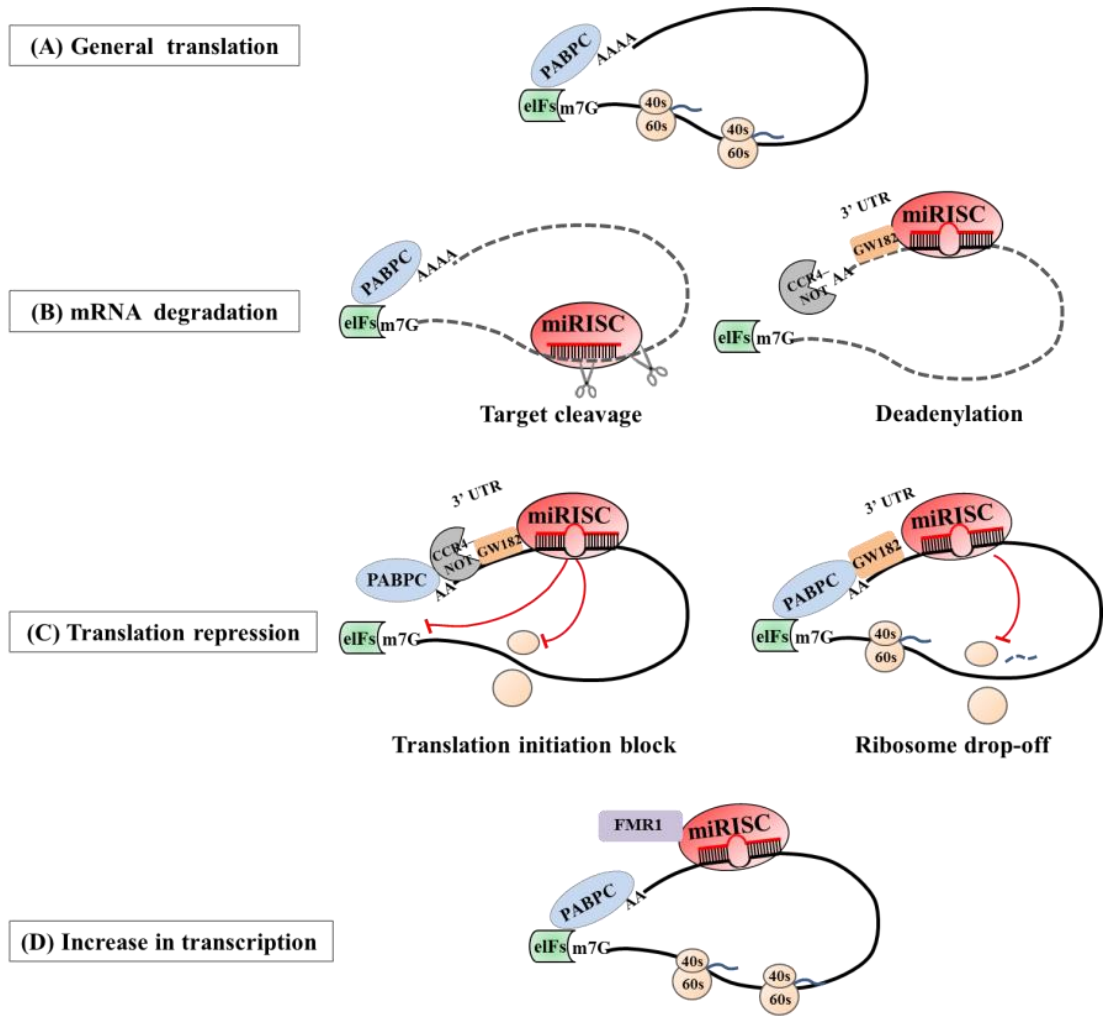

Figure 1.2: Model of miRNA mediated translational repression. (A) General outline of most eukaryotic mRNA translational initiation where the interaction of eIFs with both (m7G) cap and PABPC allows for the establishment of a closed loop. Following, recruitment of the ribosome to the 5'-end of mRNA which is required for efficient translation initiation. (B) Two mechanisms lead to mRNA target degradation: first is endonucleolytic cleavage of the target, in case, direct cleavage by Ago-2 when miRNA-mRNA paired with nearly or complete complementarity. Second mechanism is deadenylation at 3'-end mRNA target even if, imperfect pairing between miRISC and target 3'-UTR (partial complementarity). MiRISC interaction with GW182 and the CCR4–NOT complex cause mRNA deadenylation and dissociation of PABPC in P-bodies. (C) Initiation and post-initiation translational repression (partial complementarity), ribosomes drop off mRNAs when miRNAs bind to the 3'-UTR and blocking translation initiation through recruitment of CCR4–NOT by GW182. (D) MiRNAs up-regulate target expression via a mechanism involving with Ago-2 and fragile X mental retardation protein 1 (FMR1). (modified from (Pillai *et al.* 2007; Keller *et al.* 2011; Pasquinelli 2012)).

1.1.3 MiRNA target recognition

The principle of miRNA-mRNA interctions has been observed. Plant miRNAs follow the strategy of perfect or nearly complete complementarity and lead to endonucleolytic mRNA cleavage by an RNAi-like mechanism (Bartel 2004). While, in most cases, animal miRNAs imperfectly pair with their related targets as previously mentioned. The most stringent requirement of imperfect miRNA-mRNA complementarity in animals is usually composed of matched nucleotides between the 5'-end of the miRNA called the 'seed region' and the 3'-UTR of the target mRNA. This seed region starts from the second nucleotide of the mature miRNA. Positions 2–8 nt of the 5'-end of miRNA are referred to as the seed region which is most important for the miRNA-mRNA recognition and most conserved among species (Kim & Nam 2006; Lytle *et al.* 2007; Bartel 2009). Seed pairing algorithms and evolutionary conservation became a powerful tool for identifying miRNA-regulated genes. One perfectly complementary site is sufficient for the siRNA or miRNA induced cleavage of mRNA. However, in many cases, even though containing a canonical 7-8mer site, it is not enough for down-regulation of the target gene. Mismatches in the seed sequence of a miRNA or even for unknown context reasons outside of the seed sites must also play important roles for target recognition (Grimson *et al.* 2007). Some target sites with imperfect seed matching i.e. one nucleotide bulge, gaps, a single mismatch or G:U wobble bases can also be if supported by extensive complementarity to the miRNA 3'-end. The 3'-end supplementary site improve binding specificity and affinity, or it can even compensate for a mismatch of the seed by ideal pairing on miRNA nucleotides 13-16 (Doench & Sharp 2004; Brennecke *et al.* 2005). However, the effectiveness of translational repression is dependent on the number of imperfect reorganization sites by the same or several different miRNAs (Pillai *et al.* 2007). Consequently, it allows us to predict the miRNA targets by computational approaches. Computational prediction of miRNA targets are commonly used algorithms that place variable weight on: degrees of complementarity of miRNA seed region, conserved regions on miRNA site recognition among related species and thermodynamics of miRNA-mRNA heteroduplex (heteroduplex free energy (ΔG)) (Kertesz *et al.* 2007; Thomas *et al.* 2010). At present, several computational approaches have been used to successfully identify potential miRNA relevant to genes or identify potential mRNA targets for experimental validation.

Some of the most commonly (web-based or non-web-based) used computational prediction tools are available like: TargetScan (http://www.targetscan.org/) (Grimson *et al.* 2007; Friedman *et al.* 2009; Garcia *et al.* 2011), PicTar (http://pictar.mdc-berlin.de/) (Chen & Rajewsky 2006), miRanda (http://www.microrna.org) (Betel *et al.* 2008; Betel *et al.* 2010), PITA (http://genie.weizmann.ac.il) (Kertesz *et al.* 2007) and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/) (Rehmsmeier *et al.* 2004). The general description for miRNA target prediction algorithms and software is give in several reviews (Zhang *et al.* 2006 ; Betel *et al.* 2008; Thomas *et al.* 2010; Tan Gana *et al.* 2012).

1.2 Skeletal muscle growth and development

1.2.1 An overview of muscle growth and development

In domestic animal species such as the pig, muscle fibres characteristics play a key role in meat quality and quantity involving in meat tenderness, pH, water-holding capacity, color and flavor. The proportion, size and cross sectional area of skeletal muscle fibre are reported to correlate with many meat quality traits (Rehfeldt *et al.* 2000; Ryu & Kim 2005; Bérard *et al.* 2011). Increases in muscle quantity (improving growth rate and muscularity) could be achieved by controlling either the total number of muscle fibres (TNF), and/or the average size of muscle fibres (Ashmore *et al.* 1973; Picard *et al.* 2002). TNF in porcine is exclusively defined by myogenesis processes which have been restricted since prenatal stages of muscle development. The development of muscle is a complex process. Muscle fibres or myofibres are the structural units of skeletal muscle. In livestock, all muscle fibres are formed during the prenatal stage. “Myogenesis” is the term of new muscle fibres formation process, where pluripotent mesenchymal stem cells are converted into committed muscle cells. Myogenesis processes comprises many events; proliferation, differentiation and migration of myoblasts (Perry & Rudnicki 2000 ; Rehfeldt *et al.* 2011; Yan *et al.* 2012).

Myogenesis in mammal begins very early during embryonic life. The appearance of two distinct populations of myoblasts established the origin of the different types of muscle fibres (Picard *et al.* 2002). The biphasic nature of the fibre formation has been well established in the pig (Ashmore *et al.* 1973; Wigmore & Stickland 1983). The first population of fibres, called primary muscle fibres, is formed in the presumptive muscle

by cell fusion during the embryonic stage (primary myogenesis). Only a small number of these fibres are then used as a template for the attachment and formation of myoblasts to form secondary fibres which occur during the fetal stage (secondary myogenesis) (Bérard *et al.* 2011). Due to this mode of formation developing muscle fibres can be classified into two generations: primary myogenesis to develop primary fibres and secondary myogenesis to develop secondary fibres. Completing these processes the TNF was fixed. In the late foetal or early postnatal period of muscle formation, the adult muscles stem cells called satellite cells, will contribute to the third myogenic generation into new fibres when muscle damaged or injured (Picard *et al.* 2002; Rehfeldt *et al.* 2011; Yan *et al.* 2012). Recently, there is increasing evidence demonstrated that prenatal and early postnatal development of skeletal muscle has dramatic impact on postnatal development and growth and physiological function in both animals and human. Moreover, understanding mechanisms that control prenatal development and growth are critical to define strategies to reduce the incidence of developmental disorders and their long-term consequences.

1.2.2 Myogenesis of skeletal muscle cells (prenatal development)

There are two new concepts of general interest in muscle developmental biology, how myoblasts recognise and fuse with one another and how signalling and transcriptional networks contribute to muscle progenitor specification. In early vertebrate embryo (embryogenesis), skeletal muscle forms from paraxial mesoderm as the un-segmented mesoderm (in mammals), which separates into blocks of cells called somites (epithelial spheres of paraxial mesoderm). Somites are transient structures, they are extremely important in organizing the segmental pattern of vertebrate embryos. Each somite is composed of three zones around a cavity. The ventral part of the somite called the sclerotome, will contribute the cartilage and bone of the vertebrae and ribs. Whereas the dorsal part of the somite called the myotome and dermomyotome, which contain the group of cells to form the skeletal muscles (back and body) and deep dermis layer, respectively (Gilbert 2000; Brent & Tabin 2002 ; Bentzinger *et al.* 2012).

In details, the primary myotome is composed of a group of committed cells named precursor cells (embryonic/myogenic precursor cells or progenitor cells) which lie between the dermomyotome and the sclerotome. Later precursor cells have proliferated into committed embryonic myoblasts (which give rise to primary fibres), foetal

myoblasts (which give rise to secondary fibres) or satellite cells (which contribute to further growth and repair of the myotome) followed by the differentiation of myoblasts into myocytes (Rehfeldt *et al.* 2011). After that myocytes fuse to form a multinucleated myotube and finally differentiating into muscle fibres (Knight & Kothary 2011 ; Bentzinger *et al.* 2012).

1.2.2.1 Myogenic factors/ transcription factors

Current investigations of muscle development during early embryogenesis are providing exciting insights into molecular genetic regulatory mechanisms as well as fundamental knowledge of the transcriptional and signaling mechanisms. Myogenic regulatory factors (MRFs) are the specific transcriptional activator factors of muscle-specific genes. MRFs are organized in hierarchical gene expression networks that are spatiotemporally induced or repressed during lineage progression (Bentzinger *et al.* 2012). Currently MRFs include myogenin, myogenic regulatory factor 4 (MRF4), myogenic differentiation (MyoD) and myogenic factor 5 (Myf5) were identify (Yan *et al.* 2012). Myf5 is the earliest MRF which expressed during embryonic development followed by MyoD, Myogenin and MRF4 respectively. MyoD and myf5 play roles in early myoblast determination while, myogenin and MRF4 are required for differentiation (summarized Fig. 1.3) (Knight & Kothary 2011 ; Bentzinger *et al.* 2012). The expressions of MRFs are under the control of a panel of positive and negative signals from surrounding tissues (Maroto *et al.* 1997; Chargé & Rudnicki 2004; Knight & Kothary 2011). Specific proteins including the regulatory proteins wingless (Wnt), paired box gene (Pax) 3, 7 and sonic hedgehog (Shh) have been found to control the expression of MRFs. Wnt signaling is very important for activating and maintaining myogenesis during myotome formation. Wnt1, expressed by neural tube, especially activates the expression of Myf5, while Wnt7a, which is expressed by dorsal ectoderm, activates MyoD. Shh expressed from the notochord and floor plate induces both Myf5 and MyoD expression. The expression of Pax 3 and Pax 7 from mesechymal stem cells induces the expression of MyoD, Myf5, and myogenin, which lead myoblasts to myogenic differentiation (Maroto *et al.* 1997; Brent & Tabin 2002 ; Bryson-Richardson & Currie 2008; Bentzinger *et al.* 2012).When myoblast cells stop proliferation themselves and switch to become terminally differentiated myocytes they express the late MRFs; myogenin and MRF4, and later muscle specific genes such as myosin heavy

chain (MHC) and muscle creatine kinase (MCK) (Chargé & Rudnicki 2004). Finally, mononucleated myocytes specifically fuse to each other to form multinucleated myofibres, which ultimately mature into contracting muscle fibres (Fig. 1.3). During the course of muscle development, muscle satellite cells remain lying between the basal lamina and the fibre plasma membrane of the developing myofibre in a quiescent un-differentiated state (Bentzinger *et al.* 2012). The expression of Pax7 is important for the specification or expansion of these satellite cell populations. Myocyte enhancer factor 2 (MEF2) factors do not have direct function on myogenic activity. MEF2 activates the function of MRFs transcription and also cooperatively associated histone acetyltransferases and deacetylases during later stages of terminal differentiation (Brent & Tabin 2002 ; Bryson-Richardson & Currie 2008).

1.2.2.2 Myogenesis timing of pig muscle

Like are other vertebrates, prenatal muscle fibres in pig were classified as either primary or secondary processes. Primary muscle fibre formation starts around ~35 days of post conception (dpc) and proceeds until around 60 dpc. Secondary fibres develop from fetal myoblasts by using the primary myofibres as a template. Secondary myofibres appear on the primary fibres surfaces (Wigmore & Stickland 1983). The first secondary fibres were seen to start and cease around 55-90 dpc (Ashmore *et al.* 1973; Lefaucheur *et al.* 1995; Rehfeldt *et al.* 1999; Wigmore & Evans 2002; Te Pas *et al.* 2005; Muráni *et al.* 2007). The time of muscle fibre formation appeared to be nearly the same in both large and small animals. Due to their formation, each primary fibre became surrounded by secondary fibres. The number of muscle fibres is prenatally determined during primary and secondary muscle fibre formation during prenatal development (Wigmore & Stickland 1983; Wigmore & Evans 2002). The adult muscle was apparent by around 100 dpc. The full gestational age of pigs is very constant at 114 days. At this time, most fibres were of a uniform size and connective tissue septa divided them into fascicles. Thereafter, the postnatal growth mainly increases in the size of the muscle fibres (Fig 1.4.) (Wigmore & Stickland 1983; Zhao *et al.* 2003; Rehfeldt *et al.* 2011).

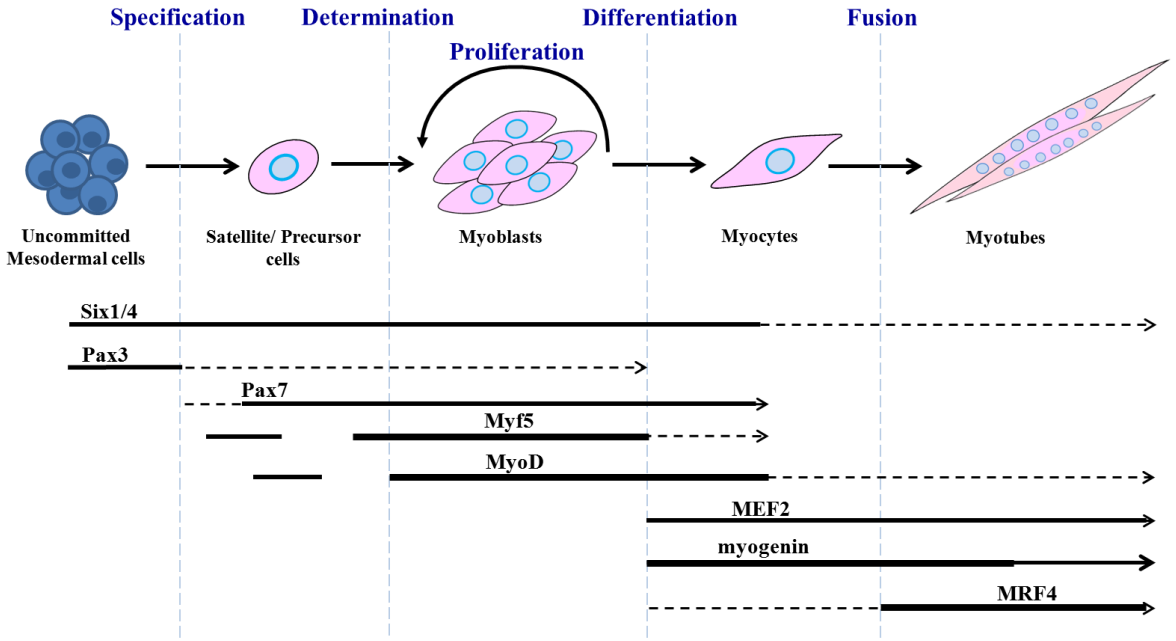

Figure 1.3: Transcription factors regulating progression through the myogenesis process.

A graphical representation of myogenesis is shown. Start from uncommitted mesodermal cells specific to form satellite (adult muscle) or precursor cells (embryo). Then precursor cells that are involved in embryonic muscle differentiation skip the quiescent satellite cell stage and directly become proliferating myoblasts. Some precursor cells remain as satellite cells in adult muscle. After that, myoblasts will differentiate into myocytes that gives rise to fuse to form a multinucleated myotube. Finally maturation of myotubes into fibres is completed by innervation. The myogenic transcription factors are required for this process in specific order. Many genes regulating myogenesis are well known. Six1/4 and Pax3/7 are master regulators of early lineage specification, whereas Myf5 and MyoD commit cells to the myogenic program. The differentiation of myoblasts into myocytes and fusion of myocytes to form myotubes, are performed by both myogenin and MRF4. Another distinctive muscle transcription factor is MEF2, which often represented with contractile protein enhancers and also cooperatively associated with MRFs. The starting point of the arrow assigned to the myogenesis-stages requirement, dashed line assigned to low expression levels, bold line assigned to high expression levels, the thickness of the lines indicates their expression levels (Adapted from (Knight & Kothary 2011 ; Bentzinger *et al.* 2012)).

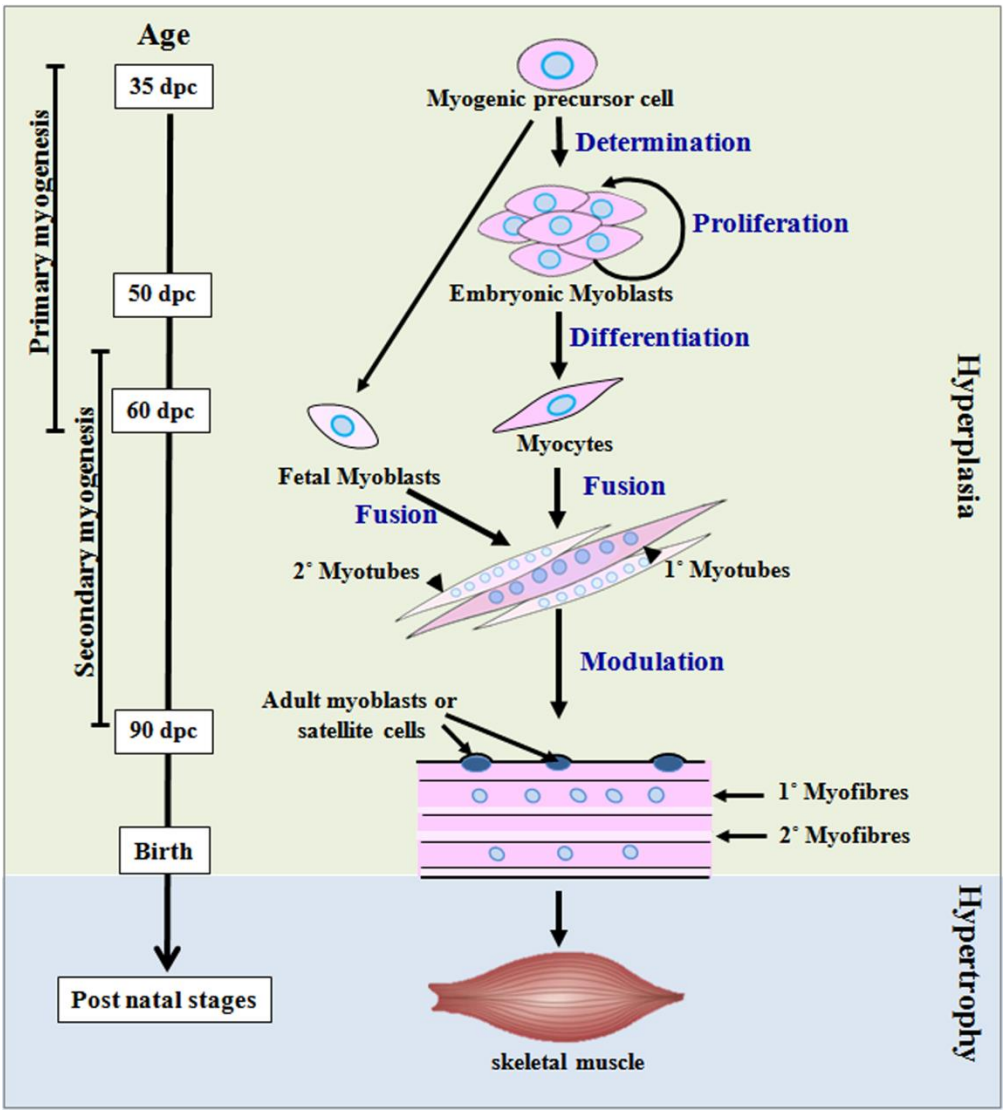

Figure 1.4: The hierarchy of porcine skeletal muscle myogenesis with developmental time. Diagram details from the embryonic state until the terminal modulation into muscle fibres and muscle growth in postnatal stage**.** Muscle precursors during development enclose embryonic myoblasts, fetal myoblasts and satellite cells. Embryonic and fetal myoblasts generate to primary and secondary fibres, respectively. Satellite cells appear at the end of gestation and are responsible for postnatal growth and regeneration. An indicative life-timing of porcine development is described. dpc, days post conception.

In the pig, a series of studies are dealing with the biological mechanisms involved in the determination of the meat quality. Many conditions including age, breed, and feeding can affect meat quality by altering the biological characteristics of

muscle development during prenatal stage (Dwyer *et al.* 1994; Picard *et al.* 2002). TNF have a positive correlation with postnatal growth potential. The littermates with a high numbers of fibres grew faster and more efficiently than littermates with a lower number of fibres. Futhermore, effects of maternal nutrition on secondary muscle fibre hyperplasia occurrs in early gestation (Dwyer *et al.* 1994; Foxcroft *et al.* 2006).

1.2.3 Skeletal muscle growth (postnatal development)

Skeletal muscle grows by both the hyperplasia and hypertrophy of muscle fibres. Hyperplasia is a process to increase in the number of fibres and basically TNF is limited at birth. Hypertrophy is the primary method for increase in individual myotube/myofibre cells size in both fibre diameter and/or length which were formed during myogenesis. Thus, normal postnatal muscle growth intended to increase in skeletal muscle mass and responded to appropriate stimuli. A recent study revealed that satellite cells only requested for the formation of new fibres and for muscle regeneration (postnatal myogenesis) upon skeletal muscle injury in adult but not appear to be necessary for muscle hypertrophy (McCarthy *et al.* 2011; Bentzinger *et al.* 2012). Postnatal muscle hypertrophy of the individual fibre cell is negatively correlated to the number of muscle fibres. Muscle cross-sectional area is positively correlated with both fibre thickness and the number of fibres (Rehfeldt *et al.* 2000; Picard *et al.* 2002). Adult skeletal muscles are composed of a mixture of myofibres. The fibre-type composition in terms of metabolic and contractile properties (Knight & Kothary 2011). Three main fibre types can be classified based metabolic characteristics and contractile fibre type: type I (slow-twitch/ oxidative), type IIA (fast-twitch/ oxido-glycolytic) and type IIB (fast-twitch glycolytic). It is generally determined that primary fibres be grown as type I slow fibres and secondary fibres mostly be grown as fast fibres (Rehfeldt *et al.* 2000; Chargé & Rudnicki 2004). In general, muscle mass increases by hypertrophy, which is associated to strength training and anaerobic training. By all case, there increased in cellular protein content. Hypertrophy controlled by the complex coordination of consequence of genetic conditions, protein composition, metabolic enzymes and contractile phenotype (Braun & Gautel 2011; Knight & Kothary 2011). Currently, numerous molecules including growth factors, different MHC isoforms and regulatory factors have been identified to govern these processes. Myostatin (MSTN), mammalian target of rapamycin (mTOR), forkhead box protein family (FOXO), or insulin-like

growth factor 1/v-akt murine thymoma viral oncogene (IGF-1/Akt) pathway are all known as a crucial regulator of skeletal muscle hypertrophy and energy metabolism (Chargé & Rudnicki 2004; Lee 2004; Favier *et al.* 2008 ; Braun & Gautel 2011; Schiaffino & Mammucari 2011).

1.2.4 Mitochondria and energy metabolism in the skeletal muscle

The main functions of the mitochondria are the production of the energy and regulation of cell metabolism. Mitochondrial functioning is necessary for cellular metabolism such as cell survival (apoptosis), calcium signalling, cellular proliferation and ATP production. During this metabolism, mitochondria produce electrical charged molecules, by folding through the electron transport chain complexes (phosphorylation) and finally combine with oxygen (oxidation) from respiration process, to synthesis ATP as the end product. The overall process is called oxidative phosphorylation which proceeds in the inner membranes of mitochondria. Chemical energy of ATP synthesis is comprised of two functionally independent of the ATP synthase, rotary motor enzymes complex units. The F1 and F0-ATP synthase units worked either in collaboration to generate ATP through mechanical rotation (Noji & Yoshida 2001). Skeletal muscle is the major energy consuming, substrate turnover and storage organ. The most of produced energy is used for the demands of muscle contraction. In skeletal muscle fibres, mitochondrial density and activity mainly varies according to fibre types and physiological functions. The main appropriate sources of energy for the muscle ATP production are glycogen, glucose, and free fatty acids (FFAs). There are three major systems responsible for the ATP resynthesis: the phosphagen system, the glycolytic system, and mitochondrial respiration (Hocquettea *et al.* 1998; Baker *et al.* 2010). Focusing in mitochondrial respiration, decarboxylation of pyruvate and β-oxidation of fatty acids results in the formation of acetyl-coenzyme A (acetyl-CoA). Metabolism of acetyl-CoA through the tricarboxylic acid cycle (TCA cycle) generates nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2). Both drive electron transport along the mitochondrial respiratory chain to produce ATP (Baker *et al.* 2010). In pig, the oxidative metabolism represents the principal source of energy during foetal life. The oxidative and glycolytic fibres can be distinguished from 21 to 28 days of postnatal life (dpn) (Lefaucheur *et al.* 1995; Picard *et al.* 2002). The difference between the fibre types or the dynamic of the phosphate groups and

metabolite transportations in the ATP production and metabolism, finally affected meat quality like tenderness and acid pH (Hocquettea *et al.* 1998; Picard *et al.* 2002). Recently there are increasing evidence show that ATP also influence myoblast and satellite cell activity during myogenesis (Ryten *et al.* 2001; Ryten *et al.* 2002; Martinello *et al.* 2011). ATP is now recognized as an important messenger molecule in cell to cell communication (cell signaling) via reacting with two families of widely distributed surface purinergic P2 receptors: ionotropic P2X and metabotropic P2Y receptors. The trophic effects of ATP on P2X and P2Y receptors have been studied in many cell and tissue types, that are involved in, cell proliferation, differentiation, and program cell death (apoptosis) (Burnstock *et al.* 2012). All of these processes are of key importance in skeletal muscle myogenesis and regeneration. Thus, ATP known to be a critical power source for viable cells and skeletal muscle activity, ATP is also expected to play an important role from the early development processes of the muscles as well.

1.3 Biological function of miRNAs in muscle growth and development and muscle metabolism

The biological functions of miRNAs have been abundantly investigating in many animal species and in many tissues or though varietal cell types. Single miRNA may target numbers of different mRNAs and on the other hand each mRNA can be targeted by number of miRNAs, resulting in complex regulatory networks. Not surprisingly, miRNAs are involved in many biological processes especially they have an important role in muscle development, growth and metabolism processes which directed affect meet quality. Recently several miRNAs have been identified by validated experimentation methods in order to known their crucial functions that are related to muscle development/myogenesis, muscle growth as well as muscle metabolism (listed in Table 1.1)

Table 1.1 Examples of microRNA and their functionally characterized

miRNA	Family	Functions	Related in muscle			Validated target proteins/ mRNAs	References
			development	growth and regeneration	Energy and metabolism		
miR-1	miR-1	induced myogenesis (muscle-specific)	✓			MyoD	(Chen *et al.* 2006; Huang *et al.* 2008)
						SRF, HDAC4	(Chen *et al.* 2006)
						MEF2	(Liu *et al.* 2007)
						Pax3	(Hirai *et al.* 2010; Goljanek-Whysall *et al.* 2011)
						Pax7	(Chen *et al.* 2010)
		modulates muscular hypertrophy		✓		IGF1, Akt, FOXO3, MSTN	(Clop *et al.* 2006 ; Elia *et al.* 2009)
miR-20a/b	miR-17	cell and tissue morphology muscle contraction/ extension of cells	✓	✓		SMAD2, SMAD4, TGFBR2, E2F1	(O'Donnell *et al.* 2005; Li *et al.* 2012b)
miR-18a/b		proliferation of muscle cells	✓			SMAD2, SMAD4, Dicer	(Li *et al.* 2012b; Tao *et al.* 2012)
miR-17-5p		proliferation of muscle cells	✓			SMAD2, SMAD4, TGFBR2, CDKN1A, E2F1	(Li *et al.* 2012b) (O'Donnell *et al.* 2005; Pullamsetti *et al.* 2012)
miR-106a		proliferation of muscle cells	✓			RB1	(Li *et al.* 2012a)
miR-103	miR-103	proliferation of muscle cells/ muscle contraction	✓			CCNE1, CDK2, CREB1, CDK6	(Takahashi *et al.* 2009; Liao & Lönnerdal 2010)
miR-107		Insulin and glucose sensitivity, adipogenesis			✓	CAV1, DMPK	(Gambardella *et al.* 2010; Trajkovski *et al.* 2011)
						PPARG, FABP4, ADIPOQ	(Xie *et al.* 2009)

Table 1.1 (continued)

miRNA	Family	Functions	Related in muscle			Validated target proteins/ mRNAs	References
			development	growth and regeneration	Energy and metabolism		
miR-133a/b	**miR-133**	proliferation of muscle cells/myoblast (muscle-specific)	✓			MyoD, SRF, HDAC4	(Chen *et al.* 2006)
						MEF2	(Liu *et al.* 2007)
						CTGF	(Chen *et al.* 2006; Duisters *et al.* 2009)
		Increase muscle cells size		✓		c-MET, HGF, LIF, IGF-1	(McCarthy & Esser 2007)
		controls brown adipose determination			✓	PRDM16	(Yin *et al.* 2013)
miR-122	**miR-122**	proliferation and differentiation of cells (liver-specific)			✓	CUTL1, SLC7A1	(Chang *et al.* 2004; Xu *et al.* 2010)
		lipid metabolism			✓	FASN, ACC2, ACC2, SCD1	(Esau *et al.* 2006)
miR-148	**miR-148**	promotes myogenic differentiation	✓			ROCK1	(Zhang *et al.* 2012a)
miR-181 a/ b	**miR-181**	Pro and terminal differentiation of muscle cells	✓			HOXA11, MyoD, MHC	(Naguibneva *et al.* 2006; Li *et al.* 2012a)
miR-188	**miR-188**	proliferation of cells	✓	✓		MMP-2,-9, TIMP-1,-3,-4, NOX-4	(Mishra *et al.* 2009)
miR-199a/b	**miR-199**	proliferation of cells	✓			NFATC, DYRK1A	(da Costa Martins *et al.* 2010)

Table 1.1 (continued)

miRNA	Family	Functions	Related in muscle			Validated target proteins/mRNAs	References
			development	growth and regeneration	Energy and metabolism		
miR-206	**miR-206**	induced myogenesis (skeletal muscle-specific)	✓			HLH, MyoD	(Kim *et al.* 2006)
						Pax3	(Hirai *et al.* 2010; Goljanek-Whysall *et al.* 2011)
						Pax7	(Chen *et al.* 2010; Dey *et al.* 2011)
		modulates muscular hypertrophy		✓		MSTN	(Clop *et al.* 2006)
miR-208a/b	**miR-208**	Regulate MHC (Type I muscle fibres specific) a cardiac-specific	✓			SOX6, PURB, SP3, CBX5	(van Rooij *et al.* 2009)
			✓			MED13 (known as Thrap1), β-MHC, MSTN	(van Rooij *et al.* 2007; Callis *et al.* 2009)
					✓	ESRRG	(Gan *et al.* 2013)
miR-21	**miR-21**	proliferation/ differentiation of muscle cells	✓			CSE, SP1	(Dong *et al.* 2012; Li *et al.* 2012a; Pullamsetti *et al.* 2012)
miR-214	**miR-214**	Skeletal muscle differentiation	✓			EZH2, MHC, PROX1, PTCH1	(Flynt *et al.* 2007; Li *et al.* 2012a)
miR-222	**miR-221**	proliferation of muscle cells	✓			CDKN1B, CDKN1C, MHC, KIT (known as c-Kit), SMA, CNN, TAGLN2 (known as SM22α)	(Esau *et al.* 2006; Cardinali *et al.* 2009; Williams *et al.* 2009)
miR-221							
miR-223	**miR-233**	Glucose uptake			✓	SLC2A4	(Lu *et al.* 2010)

Table 1.1 (continued)

miRNA	Family	Functions	Related in muscle			Validated target proteins/ mRNAs	References
			development	growth and regeneration	Energy and metabolism		
miR-25	**miR-25**	growth and proliferation of cells	✓	✓		KLF4, MYH11	(Kuhn *et al.* 2010)
miR-92a						p63 family	(Manni *et al.* 2009)
						ITGA5	(Bonauer *et al.* 2009; Manni *et al.* 2009;
mir-27 a/b	**miR-27**	Early proliferation/ differentiation	✓			Pax3	(Crist *et al.* 2009),
		negative regulator of muscle growth		✓		MSTN	(Huang *et al.* 2012; Miretti *et al.* 2013)
miR-29	**miR-29**	Glucose transport			✓	INSIG1, CAV2, SLC16A1	(He *et al.* 2007; Pullen *et al.* 2011)
miR-30	**miR-30**	differentiation of cells/ muscle contraction	✓			CTGF	(Duisters *et al.* 2009; Tao *et al.* 2012)
		lipid synthesis			✓	APOB	(Soh *et al.* 2013)
miR-320	**miR-320**	growth and proliferation of cells	✓	✓		IGF-1	(Wang *et al.* 2009)
		Regulates glycolysis			✓	PFKM	(Tang *et al.* 2012)
miR-486	**miR-486**	Skeletal muscle differentiation	✓			Pax7	(Dey *et al.* 2011)
						PI3-kinase, Akt	(Smalla *et al.* 2010)
miR-499	**miR-499**	Regulate MHC (Type I muscle fibres specific)	✓			Thrap1, β-MHC, MSTN	(Callis *et al.* 2009)
					✓	ESRRG	(Gan *et al.* 2013)
miR-99a/b	**miR-99**	proliferation of muscle cells	✓			TGF-β	(Carraro *et al.* 2009)

1.4 The objectives of the PhD thesis

As explained in the previous sections, miRNAs are involved in various processes of muscle biology. A better understanding of miRNAs and their targets regulatory networks will support the understanding of molecular mechanisms implicated in muscle. The overall objective of this study was to reveal and integrate the mRNA and miRNA expression profiles in skeletal muscle, in order to identify the regulatory networks of miRNAs and their target genes, i.e. mRNAs, which influence muscle development, structural and metabolic properties of muscle and subsequently meat and carcass traits and energy metabolism.

The objectives of the study can further be detailed for the following chapters:

Chapter 2: To investigate the differential expression of mature microRNAs at different stages of skeletal muscle development in two pig breeds (German Landrace (DL) and Pietrain (Pi)), that are known to be distinct in muscle, meat and carcass traits.

Chapter 3: To determine differentially expressed mRNA and miRNA transcripts and to elucidate biological functions of mRNA - miRNA pairs at different stages of skeletal muscle development in the two pig breeds German Landrace (DL) and Pietrain (Pi).

Chapter 4: To evaluate trait-dependent mRNA and miRNA expression by detecting groups of co-expressed genes (mRNAs) and on the hierarchically superior level of miRNAs that are correlated with organismal traits related to carcass and meat quality in commercial crossbreed pigs [Pietrain (Pi) × German Large White (DE) × German Landrace (DL)] population.

Chapter 5: To identify the differentially expressed mRNA and miRNA transcripts and to elucidate miRNA-mRNA pairs which modulate energy metabolism during the C2C12 murine myogenic differentiation.

1.4.1 The experimental design

The general strategy taken in this study is shown in **Fig. 1.5**

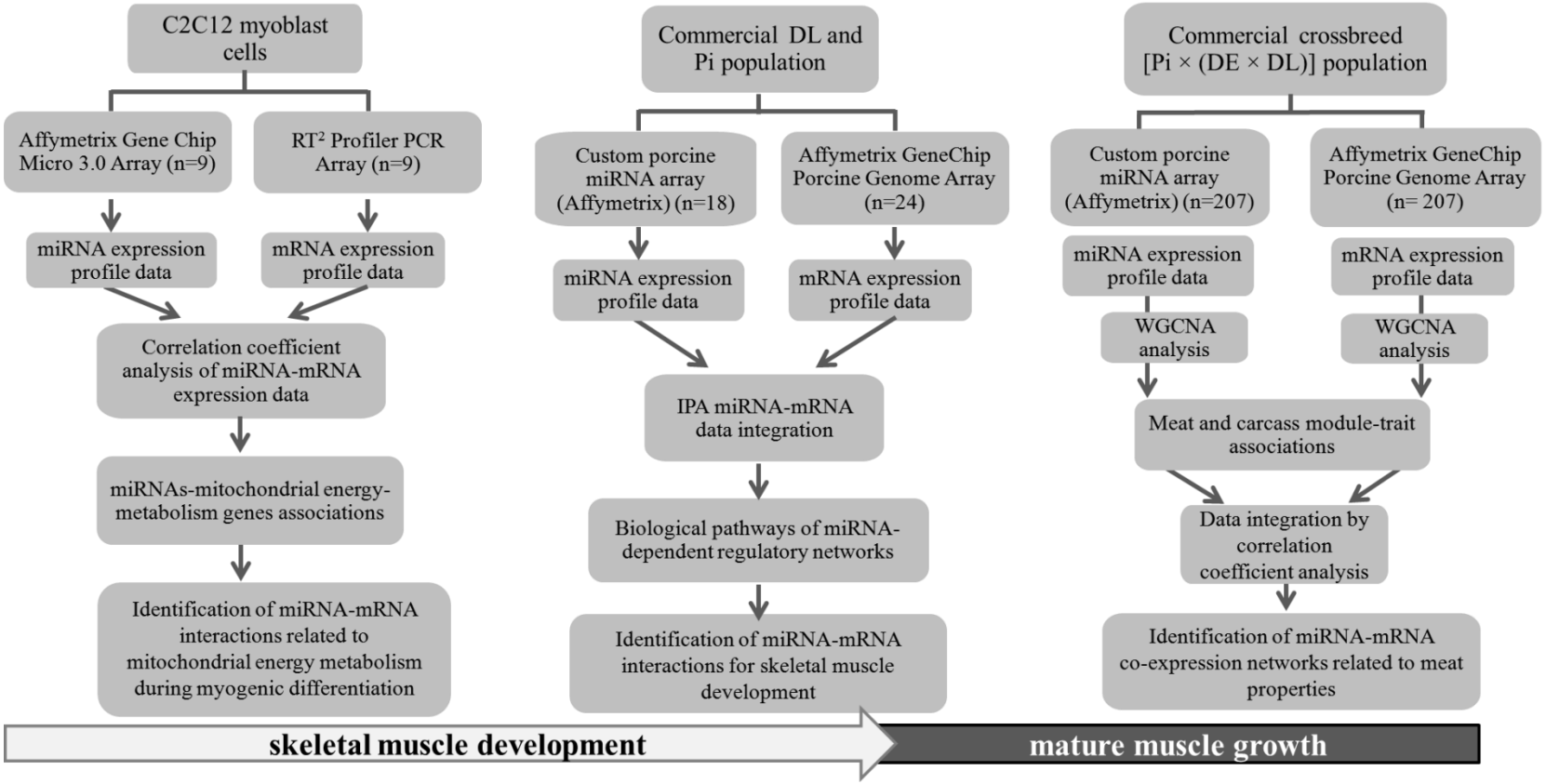

Figure 1.5: Schematic overview of the design of this study

CHAPTER 2

Pre- and postnatal muscle microRNA expression profiles of two pig breeds differing in muscularity

Puntita Siengdee, Nares Trakooljul, Eduard Murani, Manfred Schwerin, Klaus Wimmers, Siriluck Ponsuksili

Published in: Gene 561(2):190–198

Article first published online: 2015 Feb 25. DOI: 10.1016/j.gene.2015.02.035.

Pre- and postnatal muscle microRNA expression profiles of two pig breeds differing in muscularity

Abstract

MicroRNAs (miRNAs), though non-coding themselves, post-transcriptionally regulate gene expression via translational repression, degradation, or deadenylation of target mRNAs. The evolutionary conservation of miRNA functions across species suggests that miRNA-mediated gene regulation is critical in biological pathways, including muscle development. In order to elucidate miRNA functions it is first essential to develop comprehensive miRNA expression profiles. In the present study a comparative expression analysis of miRNAs expressed in the longissimus dorsi muscle at two prenatal stages (63 and 91 days post-conception (dpc)), and one adult stage (180 days post-natum) in both German Landrace (DL) and Pietrain (Pi), pig breeds was performed using a custom-designed array (Affymetrix). During the prenatal stages, miR-199 and the miR-17 family were significantly up-regulated at 63-dpc, whereas miR-1 and miR-133a were overexpressed at 91-dpc (*q-value* < 0.1). The abundance of several miRNAs was increased in the adult stage compared to 91-dpc including miR-1, miR-133, miR-22(a/b) and miR-29a. The up-regulation of some miRNAs was breed-specific, such as miR-199 and the miR-17 family which were all up-regulated in Pi pigs, while miR-133, miR-181 and miR-214 were up-regulated in DL pigs. Several pathways related to muscle development were enriched with predicted targets for the differential miRNAs by Ingenuity Pathway Analysis. The dynamic expression of porcine muscle miRNAs suggests a functional role for miRNA-mediated gene regulation during muscle development. Breed-associated regulation of miRNAs was also observed in this study suggesting that miRNAs likely contribute to phenotypic variations of pig muscle traits; however functional validation of each miRNA-target relationship is still needed.

Keywords porcine, microRNA, microarray, muscle development, skeletal muscle

Introduction

MicroRNAs (miRNAs) are short (~22 nucleotides), single-stranded, non-coding RNA molecules that regulate gene expression by translational repression, degradation, or deadenylation of target mRNAs. The binding of a miRNA-loaded RNA-induced silencing complex (RISC) to the 3' untranslated region (3'-UTR) of a target mRNA leads to the degradation of the mRNA or inhibition of protein translation depending upon the degree of complementarity [1-3]. Numerous studies have demonstrated that miRNAs regulate the expression of target genes in diverse cellular processes including differentiation, proliferation, and apoptosis and hence play important roles in an array of physiological processes including developmental timing, patterning, embryogenesis, organogenesis, cell lineage and growth control [4-7]. Similar to protein-coding genes, miRNAs show spatio-temporal expression patterns during normal cell/tissue development including muscle growth, as well as in pathology [8-10]. Indeed, altered expression patterns of miRNAs during myogenesis have been associated with muscular disorders [11].

The total number of muscle fibres in pigs is exclusively defined during myogenesis prenatally. Myogenesis involves a commitment of pluripotent mesenchymal stem cells to enter the myogenic lineage, followed by proliferation, differentiation/migration of myoblasts resulting in the formation of two morphologically/ functionally distinct types of multinucleated myotubes [12-15]. Primary muscle fibre formation starts around ~35 days of gestation and proceeds until around 60 days post-coitum (dpc). The primary myofibres serve as templates for the secondary myofibre formation during 54-90 dpc [16-18]. Postnatal growth of the muscle mainly increases in length and girth of the muscle fibres. Anyhow, around 60-90 dpc are key time points to elucidate biology of the porcine skeletal muscle development. It is of interest to elucidate regulatory pathways underlying the development using a comparative profiling of miRNAs during the key-stages in DL and Pi pigs which showed significant differences in muscle traits [19]. Moreover, comprehensive profiles of miRNA expression are important prior knowledge needed for miRNA functional studies. The miRNA expression profiles in porcine muscle during different developmental stages have been previously reported in several studies [20-23]. However, the association of miRNA expression with developmental stages and pig breed differences is relatively unknown. This information will be valuable for a

functional gene study to understand muscle biology for the improvement of pork production and it may also provide additional information interesting to biomedical research using pigs which share both physiological and genomic similarities with humans, as an animal model [24].

The aim of the present study was to determine the miRNA expression profiles in skeleton muscle at three developmental stages of two different porcine breeds. We used a custom-designed miRNA microarray to determine the muscle miRNA expression in 63- and 91-day-old foetuses which covers secondary muscle fibre formation and in adult muscle tissue. Selected differentially expressed miRNAs were validated by real-time PCR. Several miRNAs were found differentially expressed during muscle developmental stages and between breed types.

Materials and methods

Animals and tissue collection

German Landrace (DL) or Pietrain (Pi) sows on the 2nd - 4th parity mated with boars of the same breed type were obtained from two large pedigreed farms. Productive performance of these two animal resources has been previously described by Ponsuksili [19]. The sows were on a standard gestational diet program throughout the experimental period. At 63 and 91 dpc, foetuses were obtained from 5 sows of each breed type. Musculus longissimus dorsi (mld) samples were dissected from the foetuses and snap frozen in liquid nitrogen. For the adult stage (180 day postnatum), mld samples were collected from 18 pigs from each breed (the same herds as foetuses). All pigs received ad libitum standard diets according to the German performance test's recommendation (ZDS 2003). Guidelines of the Animal Protection under the German law were followed for animal care and tissue collection procedures. The experimental protocol was approved by the Animal Care Committee of the Leibniz Institute for Farm Animal Biology.

RNA isolation and pooling

Total RNA enriched for small RNA, including miRNAs was isolated from frozen tissues using a miReasy Mini kit and RNeasy MinElute Clean up kit (Qiagen) according to the manufacturer's recommendation. The RNA quality and quantity were determined

using an Agilent Small RNA kit and an Agilent 2100 Bioanalyzer (Agilent Technologies) according to the manufacturer's protocol. Six individual RNA samples, three pairs of male and female littermate foetuses obtained from 3 out of 5 sows mentioned above, were pooled in equal amounts. Sex determination of foetuses was confirmed by PCR amplification of a sex-determining region on the Y chromosome [13]. For the adult stage, six individual RNA samples were also pooled.

miRNA microarray

A total of 18 RNA pools (enriched for small RNA and miRNA), 3 for each stage and breed type, were used for miRNA microarray analysis. Briefly, 250 ng of RNA was poly(A)-tailed by ATP–poly-A-Polymerase and then end-labelled with FlashTag Biotin using a FlashTag™ Biotin RNA Labeling kit (Genisphere) according to the manufacturer's recommendations. Each biotin-labelled RNA sample was then hybridized to a custom porcine miRNA array (Affymetrix GeneChip). After hybridization (16 hours) and washing, the expression of miRNAs was detected using a Streptavidin-PE (Phycoerythrin) conjugate and the signal was recorded as an image file by a GeneChip scanner 3000. Our custom array was designed from 284 known pig miRNA (miRBase 14.0) and 391 porcine miRNA candidates obtained from a BLAST search of conserved sequences of various species mature miRNA (miRBase 14.0) against the pig genome. These 675 miRNAs were clustered into 159 miRNA families. Details of miRNA array were shown in Supplemental Table S8. The expression data including the probe information was deposited in the Gene Expression Omnibus (GEO) public repository with the GEO accession number GSE34213: GSM844738-GSM844755.

Statistical analysis

The GeneChip image file was quantified using the Affymetrix GCOS software (version 1.1.1) with the default parameters and all probe sets scaled to a target intensity of 500. Data pre-processing and normalization were performed on JMP Genomics 4 (SAS) using a Robust Multi-array Average (RMA) background correction, log2 transformation and quantile normalization methods. Differential expression was analyzed by procedure GLM using the method of least squares to fit general linear models (JMP Genomics 4).

Breed, stage and their interaction were considered as fixed effects. The resulting P-values were adjusted using a q-value method [25].

Pathway and Cluster analyses

To explore functional roles of miRNAs potentially regulating muscle development and muscle characteristics in the two pig breeds, lists of differentially expressed miRNAs for stage and breed type were analysed using Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, http://www.ingenuity.com). In addition, two-way hierarchical clustering (HCL) analysis was performed using the top 60 differentially expressed miRNAs found to be statistically significant for either breed, stage or breed-stage interaction effects on TMeV version 4.7.4 [26].

Validation of microarray result

Eight individual RNA samples for each stage and breed (3 stages X 2 breeds) were used for validation of the miRNA microarray results by stem-loop real-time PCR. Single-stranded cDNA was synthesized using 500 ng of total RNA, enriched for small RNA and miRNAs, 50 Units of Superscript III reverse transcriptase (Invitrogen) and Megaplex^{TM} Pools containing 380 stem-looped reverse-transcription (RT) primers (Applied Biosystems) according to the manufacturer's protocol. The reaction mixture was pre-incubated on ice for 5 min, followed by 40 cycles of 16°C for 2 min, 42°C for 1 min, and 50°C for 1 sec, and terminated by an incubation at 85°C for 5 min. Quantitative real-time PCR (qPCR) was performed in duplicates on a LightCycler® 480 system (Roche) using 20 ng of cDNA, a standard LightCycler 480 SYBR Green I Master Mix, a forward primer specific each miRNA and a universal reverse primer (Applied Biosystems). The PCR conditions were 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The following primers were used for qPCR: Universal PCR-reverse primer (5'-GTGCAGGGTCCGAGGT-3'); forward primers for miR-214 (5'-CGACAGCAGGCACAGACA-3'), miR-17 (5'-CCCGCAAAGTGCTTACAGT-3'), miR-20b (5'-CCCGCAAAGTGCTCACAGT-3'), miR-133a (5'-TTTGGTCCCCTTCAACCAGCT-3'); U6-forward primer (5'-CTCGCTTCGGCAGCACA-3') and U6-reverse primer (5'-AACGCTTCACGAATTTGCGT-3'). U6, a small nucleolar RNA, was used as an internal control. The relative abundance of miRNAs was calculated using a comparative

threshold cycle ΔΔCt method [27, 28]. Correlation coefficient analysis (*r*) between the qPCR and microarray data was performed using SAS version 9.2.

Results

To identify miRNAs involved in muscle development, we carried out a longitudinal comparative miRNA microarray analysis. *M. longissimus* of DL and Pi pigs at two prenatal stages (63 and 91 dpc) and an adult stage (~180 days old) were obtained from two animal resources previously found to have significant differences in performance traits [19]. Applying a custom-designed miRNA microarray, we first assessed differentially expressed miRNAs between breed types within each stage to identify changes in miRNA expression associated with breed type. Secondly, we analysed expression differences between different developmental stages within the two breeds to determine divergent ontogenetic shifts in expression patterns. The numbers of differentially expressed genes are summarized in Figure 2.1.

Differentially expressed miRNA between ontogenetic stages

A total number of 606 probes on the array passed filtering and normalization and were further analyzed. The majority of the miRNA probe sequences on the array were derived from multiple species with nearly perfect sequence matches. There were 1-3 nt variants at the 3'-ends of some probes including those for cfa-, xtr-, bta- and ssc-miR-181a. A proportion of false positives (false discovery rate) incurred from a multiple hypothesis testing was estimated using a *q-value method* [25]. A *q-value* < 0.1 was used to control a false discovery rate for a *p-value*. Comparisons of miRNA expression between two consecutive developmental stages were performed for each breed to identify miRNAs whose expression alterations are associated with ontogenetic stages. Overall three stages, as expected, miR-1 and miR-133 (muscle-specific miRNAs) gradually increased from 63 dpc to 91 dpc and were also highly expressed at the adult stage. In the prenatal stages (63- vs 91-dpc), 115 and 201 probes were differentially expressed in DL and Pi, respectively ($q < 0.1$) as shown in Figure 2.1 Of these, 54 probes were commonly found in both DL and Pi with the same direction of either up- (63-dpc < 91-dpc) or down-regulation (63-dpc > 91-dpc). Among these probes, up-regulated miRNAs at 63-dpc included the miR-17 family (miR-17/18a,b/20/93/106), miR-122, miR-181b, miR-199, miR-216, miR-217, miR-455, miR-499, miR-500 and

miR-503, whereas up-regulated miRNA at 91-dpc were miR-1, miR-23b, miR-26b, miR-133a/b and miR-181a.

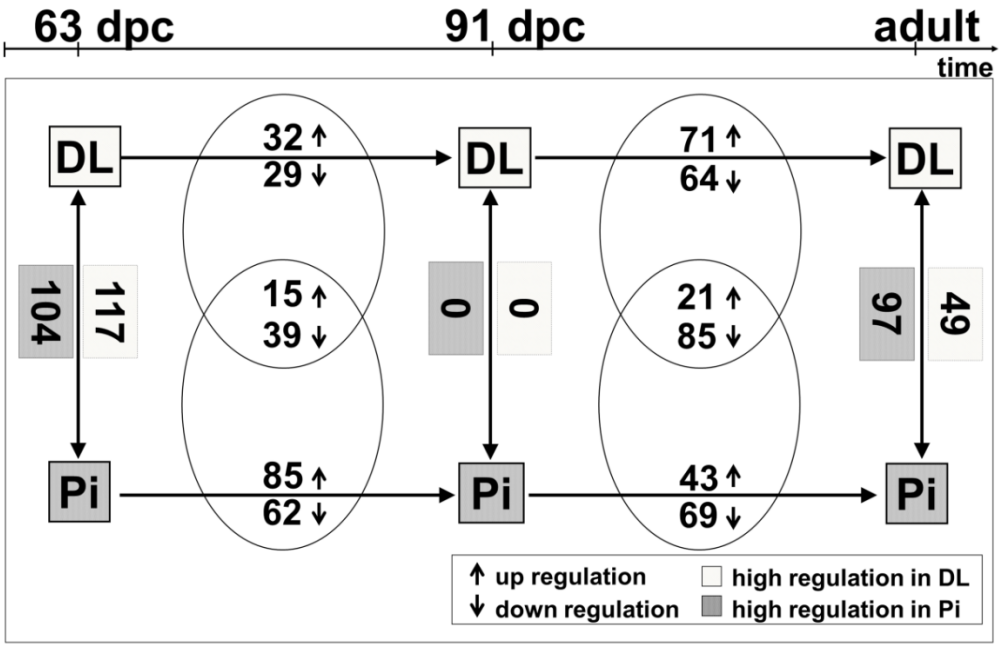

Figure 2.1: The number of significantly differentially expressed miRNA probes (*q* < 0.1) identified in *Musculus longissimus dorsi* tissue of DL vs Pi breed types at 63-dpc, 91-dpc and the adult stage (180 days old). The number of differential probes between DL and Pi within each stage is shown as vertical arrows with an open box representing up-regulation in DL pigs and closed boxes represent up-regulation in Pi pigs. The number of differential probes compared between two ontogenetic stages within each breed is indicated on the horizontal arrows with the number of up- (↑) or down-(↓) regulation and common probes identified for both DL and Pi are indicated in the overlaps.

A total of 241 and 218 probes were differentially expressed when compared between 93-dpc and the adult stage in DL and Pi, respectively (Figure 2.1). Of these, 106 probes were common to both DL and Pi with the same direction, either up- (91-dpc < adult) or down-regulation (91-dpc > adult). The up-regulated miRNA were miR-1, miR-22, miR-29, miR-30, miR-122 and miR-181c, while the down-regulated miRNA include the miR-17 family (miR-17, -18a/b, -20, -106), miR-25, miR-103, miR-107, miR-125, miR-130a/b/c, miR-199, miR-320 and miR-500.

Differentially expressed miRNA between breed types

A variance component analysis showed 221, 0, and 146 probes with significant differences of transcriptional abundance ($q < 0.1$) when compared between DL and Pi at 63-dpc, 91-dpc, and the adult stage, respectively. Among 221 probes which were differentially expressed at 63 dpc, 117 and 104 probes were up-regulated in DL and Pi, respectively (Figure 2.1). These probes represent several miRNAs including let-7, miR-23, miR-92, miR-122, miR-125, miR-133, miR-181, miR-214 and miR-320 which were up-regulated in DL, whereas the miR-17 family, miR-24, miR-27, miR-103, miR-107, miR-188 miR-199 and miR-30 were over-expressed in Pi. At 91-dpc, none of the probes passed a control of the *q-value* < 0.1 (Figure 2.1). At the adult stage, miR-30, miR-133 and miR-181 were up-regulated in DL, while let-7, the miR-17 family, miR-23, miR-24, miR-103, miR-107, miR-133, miR-143, miR-199 and miR-221 were over-expressed in Pi. Interestingly, miRNAs that were consistently up-regulated at both 63-dpc and the adult stage in the same breed include miR-133, miR-181 and miR-23 for DL and the miR-17 family, miR-24, miR-27, miR-103 and miR-107 for Pi.

Validation of differentially-expressed miRNAs by qPCR

Four differential miRNAs were selected based on their biological functions related to muscle cell growth and significant association with the experimental group (breed and developmental stage) listed in Table 2.2 miR-17, miR-20b, miR-214, and miR-133a were validated by quantitative real-time PCR as shown in Figure 2.2. A comparison of the expression data obtained from miRNA microarray and qPCR showed an agreement with a correlation coefficient (r) range from 0.57 to 0.77 suggesting reliability of the results.

Ingenuity Pathway Analysis

To explore biological pathways potentially regulated by miRNAs that showed differential expressions associated with muscle developmental stage and/or breed type, a computational target-gene prediction and gene functional classification were performed using IPA. The lists of differentially expressed miRNAs were assessed to identify potential targets based on bioinformatic predictions implemented in IPA.

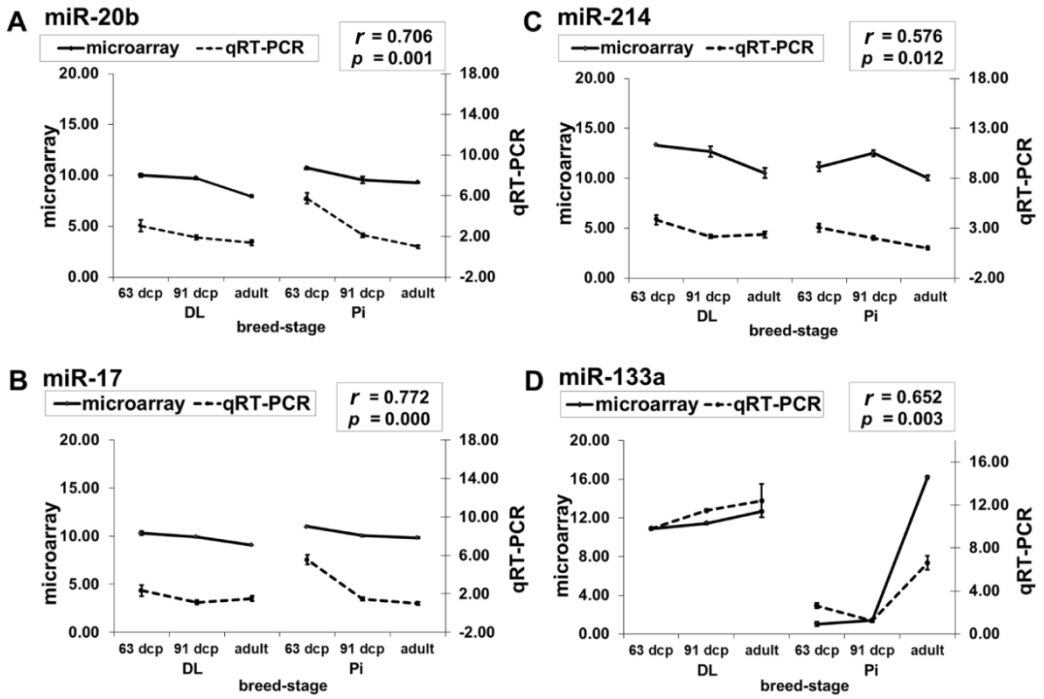

Figure 2.2: Validation of the microarray results using quantitative real-time PCR of selected miRNAs (A) miR-20b, (B) miR-17, (C) miR-214, and (D) miR-133a. Log2 transformed microarray data is plotted on the primary y-axis, while the relative expression level ($2^{-\Delta\Delta Ct}$ method) from quantitative real-time PCR is plotted on the secondary y-axis.

The Ingenuity® Knowledge Base integrates bioinformation of miRNAs and targeted genes curated in TargetScan, TarBase and miRecords that have been annotated either as computationally predicted or experimentally validated targets. Functional categorization of genes targeted by the differentially expressed miRNAs (p<0.05) is presented in Table 2.1. Genes targeted by differential miRNAs during prenatal stages (63- and 91-dpc) were enriched in important biological pathways such as cell and tissue development, cell growth and proliferation and cell cycle. Interestingly, at 63-dpc, up-regulated miRNAs in DL were associated with the functional categories "cell death" and "skeletal and muscular system development and function" which were not observed in the Pi associated gene category. Genes classified in pathways related to muscle development were more pronounced in the adult stage and targeted by miRNAs that were up-regulated in Pi. Differentially expressed miRNAs, associated biological functions and their experimentally validated targets are summarized in Table 2.2.

Cluster analysis

Cluster analysis was applied to the expression data of the top 60 differentially expressed probes using TMeV (version 4.7.4). Normalized log2 expression data of 18 samples, 2 breeds X 3 stages X 3 biological replicates, were hierarchically clustered and the results are shown as a heat map in Figure 2.3. Figure 2.1-3A shows that all samples were correctly grouped together into breed and stage according to the expression pattern of these probes which represent 34 unique miRNA species. The two prenatal stages (63- and 91-dpc) were closely clustered together and were well separated from the adult stage. Interestingly, the 63-dpc Pi was distantly grouped from 63-dpc DL and the 91-dpc DL and Pi samples which were all closely clustered together. Figure 2.1-3B and 3C demonstrate different expression patterns grouped by breed and stage, respectively.

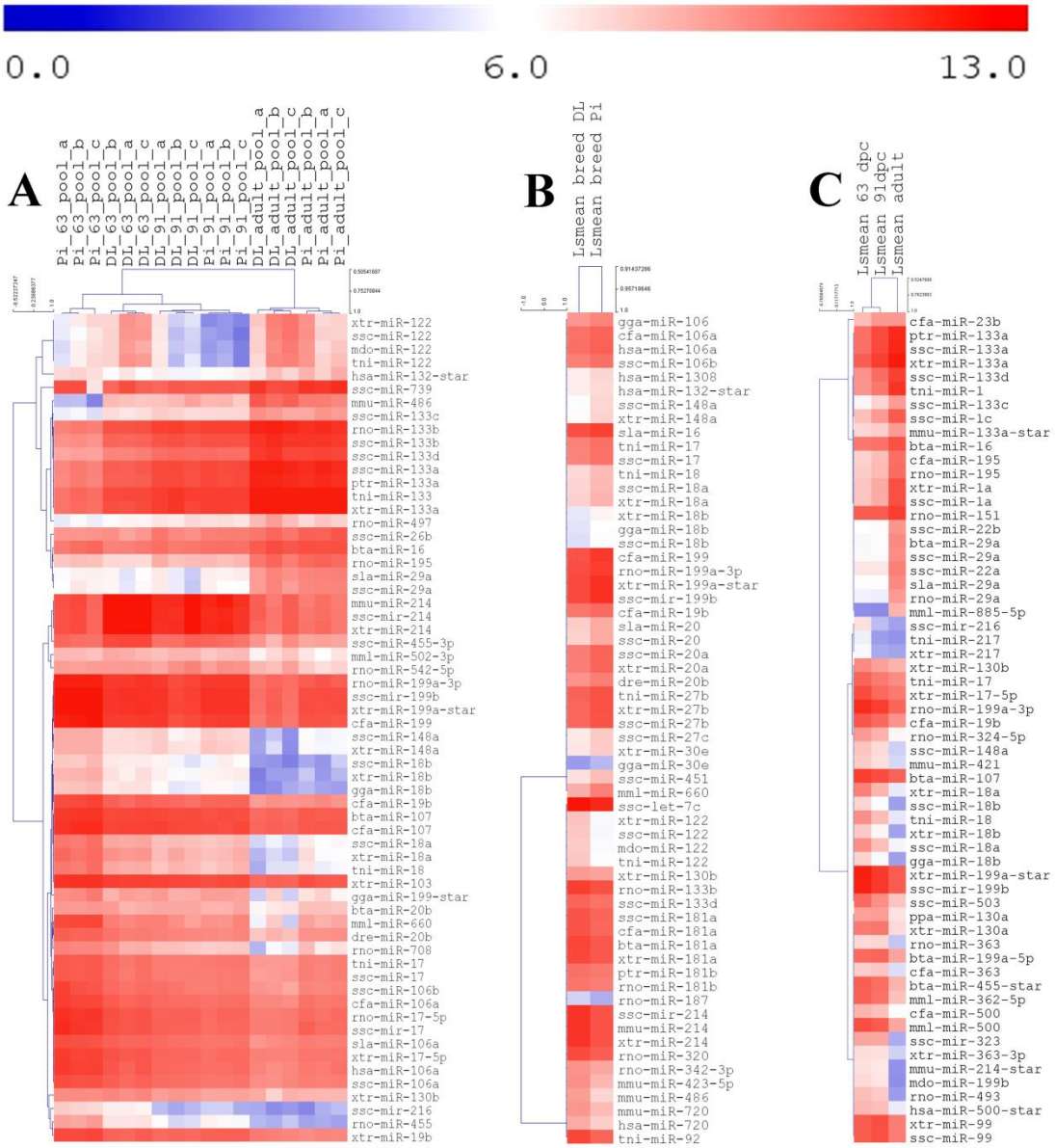

Figure 2.3: **Hierarchical cluster analysis of the top-60 differentially expressed miRNA probes** (*p-value* < 0.0001) using TMeV version 4.7.4. (A) Cluster analysis of all samples (B) Cluster analysis by breed type either DL or Pi (C) Cluster analysis by different ontogenetic stages. A pseudocolor scale bar represents the expression value for each miRNA with red, white, and blue indicating high, average, and absent expression, respectively.

Table 2.1 Functional categories (IPA) of predicted targeted genes of differentially expressed miRNA between breed type (DL vs Pi) at three ontegenetic developmental stages of muscle

Stage	**miRNA**	Functional category of predicted targets	*p-value*	No. of gene
63-dpc	**up-regulated in DL**	Connective Tissue Disorders	1.79E-21 - 2.90E-05	18
		Genetic Disorder	1.79E-21 - 3.72E-02	30
		Organismal Injury and Abnormalities	1.79E-21 - 1.63E-02	18
		Cellular Development	1.58E-11 - 4.80E-02	21
		Cellular Growth and Proliferation	1.58E-11 - 4.91E-02	24
		Cell Death	2.34E-08 - 3.84E-02	18
		Cell Cycle	4.21E-05 - 4.44E-02	7
		Connective Tissue Development and Function	4.21E-05 - 7.11E-03	6
		Skeletal and Muscular System Development and Function	2.71E-03 - 3.76E-02	6
	up-regulated in Pi	Connective Tissue Disorders	1.06E-23 - 4.22E-02	17
		Genetic Disorder	1.06E-23 - 4.80E-02	31
		Organismal Injury and Abnormalities	1.06E-23 - 2.18E-02	15
		Cellular Development	1.93E-08 - 4.16E-02	17
		Cellular Growth and Proliferation	1.93E-08 - 4.71E-02	17
		Cellular Function and Maintenance	4.09E-08 - 1.40E-02	6
		Cell Cycle	6.17E-07 - 4.71E-02	8
		Organismal Development	1.70E-09 - 3.90E-02	7
		Connective Tissue Development and Function	1.65E-05 - 6.00E-03	4
		Embryonic Development	2.13E-04 - 3.90E-02	4

Table 2.1 (continued)

Stage	miRNA	Functional category of predicted targets	p-value	No. of gene
91 dpc	**up-regulated in DL**	Developmental Disorder	6.94E-04 - 2.87E-02	6
		Cellular Development	7.65E-06 - 4.10E-02	7
		Cellular Growth and Proliferation	7.65E-06 - 4.49E-02	9
		Cellular Movement	1.57E-04 - 3.97E-02	4
		Cell Death	5.80E-04 - 3.27E-02	5
		Organismal Development	1.44E-05 - 1.44E-05	2
	up-regulated in Pi	Developmental Disorder	1.06E-06 - 4.84E-02	6
		Connective Tissue Disorders	6.15E-04 - 6.15E-04	2
		Cellular Development	4.22E-04 - 4.55E-02	3
		Cellular Growth and Proliferation	5.49E-04 - 4.92E-02	4

Table 2.1 (continued)

Stage	miRNA	Functional category of predicted targets	p-value	No. of gene
adult	**up-regulated in DL**	Cell Cycle	2.62E-06 - 9.61E-03	3
		Cellular Development	6.25E-05 - 2.03E-02	7
		Cellular Growth and Proliferation	6.25E-05 - 4.26E-02	8
		Cell Morphology	1.21E-03 - 4.83E-02	2
		Connective Tissue Development and Function	2.62E-06 - 1.07E-02	5
		Organ Morphology	4.82E-03 - 2.50E-02	2
	up-regulated in Pi	Genetic Disorder	4.36E-25 - 4.95E-02	27
		Skeletal and Muscular Disorders	4.36E-25 - 8.22E-03	17
		Developmental Disorder	3.37E-23 - 2.25E-02	14
		Connective Tissue Disorders	9.37E-22 - 8.22E-03	18
		Cellular Development	9.73E-08 - 4.39E-02	19
		Cellular Growth and Proliferation	9.73E-08 - 4.95E-02	18
		Cellular Function and Maintenance	8.57E-06 - 4.39E-02	6
		Organismal Development	5.66E-07 - 3.70E-02	8
		Embryonic Development	2.01E-04 - 3.70E-02	4
		Organ Development	2.01E-04 - 3.70E-02	4
		Tissue Development	2.01E-04 - 3.70E-02	6

Table 2.2 Differentially expressed miRNAs, associated biological functions and their experimentally validated targets

miRNA	Family	Breed-associated up-regulation			Biological function	Validated tagets (protein/ mRNA)	References
		63-dpc	91-dpc	adult			
miR-103	**miR-103**	Pi	DL	Pi	proliferation of cells/ muscle contraction	CCNE1, CDK2, CREB1, CDK6	[53, 54]
miR-107		Pi	DL	Pi			
miR-122	**miR-122**	DL	DL	ns	proliferation of cells	CUTL1, MAP3K12, LAMB2,CCNG1 cytochrome P450 family LETFs	[55]
miR-133a/b	**miR-133**	DL	ns	DL	proliferation of muscle cells/ proliferation of cardiomyocytes	SRF, MEF2, HDAC4, CTGF	[30, 37]
miR-20a/b	**miR-17**	Pi	ns	Pi	cell and tissue morphology muscle contraction/ extension of cells	TGFBR2, E2F1	[44, 45]
miR-18a/b		Pi	ns	Pi	proliferation of muscle cells	SMAD2, SMAD4, Dicer	[44, 56]
miR-17-5p		Pi	DL	Pi		TGFBR2, p21, E2F1	[44, 45, 57]
miR-106a		Pi	DL	Pi		RB1, Stat3, Mapk14	[58, 59]
miR-181	**miR-181**	DL	DL	DL	differentiation of cells	MHC, MCK, HOXA11	[50, 60]
miR-199a/b	**miR-199**	Pi	Pi	Pi	proliferation of cells	calcineurin/NFAT, Dyrk1a	[61]
miR-214	**miR-214**	DL	ns	ns	proliferation of cells	MHC, *Prox1*, *ptc1*	[39, 60]
miR-222	**miR-221**	DL	ns	ns	proliferation of muscle cells	p27(Kip1), p57(Kip2), MHC, *c-Kit*, SMA, CNN, SM22α	[62-64]
miR-221		ns	ns	Pi			
miR-24a	**miR-24**	Pi	DL	Pi	differentiation of muscle cells/ muscle contraction	c-Myc, E2F2 Myogenin, MEF2, α-actin and caveolin3	[46, 48]
miR-25	**miR-25**	Pi	ns	ns	growth and proliferation of cells	KLF4, MYH11	[65]
miR-92a		DL	ns	DL		p63 family, DeltaNp63beta, Integrin α5 subunit	[57, 66, 67]

ns means no significant difference

Discussion

To identify miRNAs that might have functional roles in porcine muscle development and therefore may contribute to the emergence of phenotypic differences, we performed a comparative miRNA expression profiling of skeletal muscle across three developmental stages in two distinct breed types, DL and Pi. The 63- and 91-dpc stages encompass secondary muscle fibre formation and are critical for muscle development that predetermines muscle characteristics in adult pigs [29,30]. The adult stage of 180 days was chosen to represent fully developed muscle tissue. Although the high throughput sequencing approach may offer more advantages for expression profiling, a microarray platform was used in the present study due to its accessibility and cost-effectiveness. Our custom array was designed from porcine miRNA sequences archived in miRBase (version 14.0) as well as those that are publicly available but not yet present in miRBase [31,20]. In addition, miRNA sequences from other species with nearly perfect matches to the pig genome were also used in the array. The rapid growth of sequencing technologies has resulted in miRNA discovery rates which greatly exceed the capacity of validation, annotation and incorporation of new miRNAs into the repository. This includes several probes in our custom array such as hsa-miR-720 and mmu-miR-720, mml-miR-660, ptr-miR-188, and rno-miR-188. The expression of muscle miRNAs in the present study generally agreed with previous reports [32,20]. Many miRNAs expressed in the present study, for example let-7, miR-17, miR-19, miR-23, miR-25, miR-27, miR-106, miR-133, miR-199 and miR-214 have been reported in numerous other studies of miRNA expression in vertebrate muscles [33-36,21,32,20,22]. These studies include miRNA expression analysis in mouse myoblast cell lines, adult mouse muscles, zebrafish skeletal muscle, mesenchymal stem cells and porcine skeletal muscle, suggesting that in addition to sequence convervation, these miRNAs are likely functionally conserved across species.

In this study, two muscle-specific miRNAs, miR-1 and miR-133 were observed to be up-regulated during prenatal developmental stages and also abundantly expressed at the 180 days postnatal stage. Co-expression of miR-1 and miR-133 has been observed to modulate skeletal muscle proliferation and differentiation in vitro cultured myoblasts and *in vivo* frog embryos [33]. MiR-1 directly targets HDAC4, a transcriptional repressor of muscle gene expression to promote myogenesis, while miR-133 down-regulates SRF to enhances myoblast proliferation. The functional roles of

muscle-specific miRNAs, especially miR-1 and miR-133 in muscle development have been extensively reviewed in model organisms including mice [37] . Recently, it has been shown that miR-1 and miR-206, a closely related miRNA, are crucial regulators for myogenic differentiation in C2C12, a mouse myoblast cell line. Several genes known to play roles in skeletal muscle development and/or differentiation have been experimentally validated as direct targets of miR-1 and miR-206 including Meox2, Fzd7, MAP4K3, Smarcd2 and Smarcb1 [38].

It is noteworthy that miR-17, miR-18a/b, miR-20, miR-93 and miR-106 all of which belong to the miR-17 family and miR-17-92 cluster were up-regulated at 63-dpc, down-regulated at 91-dpc and then up-regulated again at the adult stage. This dynamic regulation was observed to be associated with breed type: these miRNAs were consistently higher expressed in Pi pigs compared to DL pigs. This is of interest because DL and Pi pigs are considerably different in numerous traits including growth rate, body composition, muscularity and fat content: Pi pigs are leaner and have a lower ration of fat/meat content compared to DL pigs [39,19]. The miR-17 family has been demonstrated to regulate cell growth and development via down-regulation of several key genes. It has been experimentally confirmed that transforming growth factor, beta receptor II (TGFBR2) is a direct target of both miR-17 and miR-20a and SMAD2 and SMAD4 are regulated by miR-18a [40]. Together the miR-17-92 cluster may inhibit proliferation inhibition and collagen synthesis in palatal mesenchymal cells induced by the transforming growth factor, beta 1-pathway (TGFB-pathway). Moreover, miR-17 and miR-20a are known to establish a regulatory network with two transcription factors, c-MYC and E2F1, which modulate cell proliferation, growth and apoptosis [41].

c-MYC up-regulates the expression of E2F1 to promote cell cycle progression and simultaneously activates the expression of miR-17 and miR-20a which serve to tightly control proliferation by directly targeting E2F1.

Several other miRNAs were also found up-regulated in Pi pigs including miR-24 and miR-26. MiR-24 has been linked to the inhibition of skeletal muscle differentiation by transforming TGFB, which can regulate the expression of miR-24 via modulation of SMAD3, a transcriptional regulator [42]. During retina morphogenesis and development, miR-24a plays an important role in regulating apoptosis [43]. A functional study of miR-26 suggests it is an inhibitor of post-mitotic cell proliferation as its over-expression increased G1-cell arrest [44].

MiRNAs up-regulated in DL pigs at 63-dpc and/or the adult stage include miR-122, miR-133, miR-181, and miR-214. MiR-122 has been suggested to be a regulator of cholesterol and fatty-acid metabolism. Inhibition of miR-122 function by injection of miR-122- specific antisense oligonucleotides in mice showed a significant reduction of both plasma cholesterol levels and liver fatty-acid and cholesterol synthesis rates as well as an accompanying increase of hepatic fatty-acid oxidation [45]. MiR-214 is involved in the determination of muscle cell fate during somitogenesis in zebrafish by targeting of suppresor of fused (Su(fu)) gene, a negative regulator of the signaling pathway [35]. MiR-181 likely influences muscle phenotypes via the down-regulation of homeobox protein HOXA11, in the early stage of muscle differentiation [46]. In addition, the direct down-regulation of connective tissue growth factor (CTGF) by miR-133 and miR-30 is associated with hypertrophy of cardiac myocytes [47].

Genetic variations can alter the expression and function of miRNAs and target genes. Bioinformatics analyses of vertebrate genomes identified a number of polymorphisms within the seed region of miRNAs that might influence its function and ultimately contribute to phenotypic variations of complex traits [48]. Recently, 12 SNPs segregating in Berkshire, Landrace and Yorkshire have been identified in the genomic region of the miR-206/ miR-133b cluster and shown to be associated with muscle fibre characteristics, lean meat production and meat quality traits [49]. It has been proposed that these SNPs somehow influence the processing and maturation of the miRNAs. It is of interest that the expression of some miRNAs was associated with breed type (DL or PI) which differs in several muscle traits. An association analysis between SNPs and miRNA transcriptome may shed light into a new genetic factor underlying muscle traits. It is evident that miRNAs have functional importance in numerous biological processes. It is of interest whether the differentially expressed miRNAs identified in the present study significantly influence the muscle phenotypic variations observed between DL and Pi breeds. It is likely that a set of differential miRNAs can concurrently target a large spectrum of mRNAs and pathways since an individual miRNA targets numerous gene targets. The IPA analysis showed that predicted targets of the differentially expressed miRNAs were enriched for in cellular growth and development as well as cell proliferation and the cell cycle. Altogether suggests that a dynamic expression pattern of miRNAs is involved in porcine muscle development.

Conclusions

The present study identified differentially expressed miRNAs at different muscle developmental stages of two pig breeds divergent in muscle traits. The results presented here provide a primary evidence of miRNAs that could have a significant influence on muscle traits. Breed and/or stage differences of miRNA expression observed in this study further suggest that miRNAs could play a key role and have an impact in phenotypic variations in the porcine muscle; however underlying genetic factors and a functional validation of each miRNA-target relationship still remain to be determined.

Acknowledgements

The authors thank Joana Bittner and Annette Jugert for their excellent technical assistance. This research was supported by German Research Foundation (Deutsche Forschungsgemeinschaft, DFG; PO 753/7-1, WI 1754/10-14)

References of chapter 2

1. Filipowicz W, Bhattacharyya SN, Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 9:102-114

2. Winter J, Jung S, Keller S, Gregory RI, Diederichs S (2009) Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol 11:228 - 234

3. Pasquinelli AE (2012) MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. Nat Rev Genet 13 (4):271-282

4. Ambros V (2004) The functions of animal microRNAs. Nature 16 (431):350-355

5. Boehm M, Slack F (2005) A Developmental Timing MicroRNA and Its Target Regulate Life Span in C. elegans. Science 23 (5756):1954-1957

6. Calin GA, Croce CM (2006) MicroRNA-Cancer Connection: The Beginning of a New Tale. Cancer Res 66 (15):7390-7394

7. Cuellar TL, McManus MT (2005) MicroRNAs and endocrine biology. J Endocrinol 187:327-332

8. Bushati N, Cohen SM (2007) microRNA Functions. Annu Rev Cell Dev Biol 23:175-205

9. Williams AH, Liu N, Rooij Ev, Olson EN (2009) MicroRNA control of muscle development and disease. Curr Opin Cell Biol 21:461-469

10. Chen J-F, Callis TE, Wang D-Z (2009) microRNAs and muscle disorders. J Cell Sci 122:13-20

11. Eisenberga I, Eranb A, Nishinod I, Moggioe M, Lampertie C, Amatof AA, Lidovb HG, Kangb PB, Northi KN, Mitrani-Rosenbaumj S, Flanigank KM, Neelyl LA, Whitneyl D, Beggsb AH, Kohanec IS, Kunkel LM (2007) Distinctive patterns of microRNA expression in primary muscular disorders. PNAS 104 (43):17016–17021

12. Perry R, Rudnicki M (2000) Molecular mechanisms regulating myogenic determination and differentiation. Frontiers in Bioscience 5:750-767

13. Muráni E, Murániová M, Ponsuksili S, Schellander K, Wimmers K (2007) Identification of genes differentially expressed during prenatal development of skeletal muscle in two pig breeds differing in muscularity. BMC Dev Biol 7:109

14. Yan X, Zhu M-J, Dodson MV, Du M (2012) Developmental Programming of Fetal Skeletal Muscle and Adipose Tissue Development. J Genomics 1:29-38

15. Rehfeldt C, Te Pas MFW, Wimmers K, Brameld JM, Nissen PM, Berri C, Valente LMP, Power DM, Picard B, Stickland NC, Oksbjerg N (2011) Advances in research on the prenatal development of skeletal muscle in animals in relation to the quality of muscle-based food.I. Regulation of myogenesis and environmental impact. Animal 5 (5):703-717

16. Te Pas MFW, Cagnazzo M, De Wit AAC, Priem J, Pool M, Davoli R (2005) Muscle transcriptomes of Duroc and Pietrain pig breeds during prenatal formation of skeletal muscle tissue using microarray technology Archiv Tierzucht / Archives Animal Breeding 48 (Special Issue):141-147

17. Lefaucheur L, Edom F, Ecolan P, Butler-Browne GS (1995) Pattern of muscle fiber type formation in the pig. Dev Dyn 203 (1):27-41

18. Wigmore P, Evans D (2002) Molecular and cellular mechanisms involved in the generation of fiber diversity during myogenesis. Int Rev Cytol 216:175-232

19. Ponsuksili S, Murani E, Walz C, Schwerin M, Wimmers K (2007) Pre- and postnatal hepatic gene expression profiles of two pig breeds differing in body composition: insight into pathways of metabolic regulation. Physiol Genomics 29:267-279

20. Huang T-H, Zhu M-J, Li X-Y, Zhao S-H (2008) Discovery of Porcine microRNAs and Profiling from Skeletal Muscle Tissues during Development. PLoS ONE 3 (9):3225

21. Nielsen M, Hansen JH, Hedegaard J, Nielsen RO, Panitz F, Bendixen C, Thomsen B (2009) MicroRNA identity and abundance in porcine skeletal muscles determined by deep sequencing. Anim Genet 41 (2):159-168

22. McDaneld TG, Smith TP, Doumit ME, Miles JR, Coutinho LL, Sonstegard TS, Matukumalli LK, Nonneman DJ, Wiedmann RT (2009) MicroRNA transcriptome profiles during swine skeletal muscle development. BMC Genomics 10:77

23. Xie SS, Huang TH, Shen Y, Li XY, Zhang XX, Zhu MJ, Qin HY, Zhao SH (2010) Identification and characterization of microRNAs from porcine skeletal muscle. Anim Genet 41 (2):179-190

24. Bode G, Clausing P, Gervais F, Loegsted J, Luft J, Nogues V, Sims J (2010) The utility of the minipig as an animal model in regulatory toxicology. J Pharmacol Toxicol Methods 62 (3):196-220

25. Storey J, Tibshirani R (2003) Statistical significance for genomewide studies. Proc Natl Acad Sci U S A 100 (16):9440-9445

26. Saeed AI, Bhagabati NK, Braisted JC, Liang W, Sharov V, Howe EA, Li J, Thiagarajan M, White JA, Quackenbush J (2006) TM4 microarray software suite. Methods Enzymol 411:134-193

27. Tesfaye D, Worku D, Rings F, Phatsara C, Tholen E, Schellander K, Hoelker M (2009) Identification and Expression Profiling of MicroRNAs During Bovine Oocyte Maturation Using Heterologous Approach. Mol Reprod Dev 76:665-677

28. Livaka KJ, Schmittgen TD (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods 25 (4):402-408

29. Rehfeldt C, Stickland NC, Fiedler I, Wegner J (1999) Environmental and genetic factors as sources of variation in skeletal muscle fibre number. Basic Appl Myol 9 (5):235-253

30. Bérard J, Kalbe C, Lösel D, Tuchscherer A, Rehfeldt C (2011) Potential sources of early-postnatal increase in myofibre number in pig skeletal muscle. Histochem Cell Biol 136 (2):217-225

31. Reddy AM, Zheng Y, Jagadeeswaran G, Macmil SL, Graham WB, Roe BA, Desilva U, Zhang W, Sunkar R (2009) Cloning, characterization and expression analysis of porcine microRNAs. BMC Genomics 10:65

32. Zhou B, Liu HL, Shi FX, Wang JY (2010) MicroRNA expression profiles of porcine skeletal muscle. Anim Genet 41 (5):499-508

33. Chen J-F, Mandel EM, Thomson JM, Wu Q, Callis TE, Hammond SM, Conlon FL, Wang D-Z (2006) The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nature Genet 38 (2):228 - 233

34. Kim HK, Lee YS, Sivaprasad U, Malhotra A, Dutta A (2006) Muscle-specifi c microRNA miR-206 promotes muscle differentiation. J Cell Biol 174 (5):677-687

35. Flynt AS, Li N, Thatcher EJ, Solnica-Krezel L, Patton JG (2007) Zebrafish miR-214 modulates Hedgehog signaling to specify muscle cell fate. Nature Genet 39:259 - 263

36. Chen J-H, Wei W-J, Xiao X, Zhu M-J, Fan B, Zhao S-H (2008) Expression analysis of miRNAs in porcine fetal skeletal muscle on days 65 and 90 of gestation. Asian-Aust J Anim Sci 21 (7):954-960

37. Sokol N (2012) The role of microRNAs in muscle development. Curr Top Dev Biol 99:59-78

38. Goljanek-Whysall K, Pais H, Rathjen T, Sweetman D, Dalmay T, Münsterberg A (2012) Regulation of multiple target genes by miR-1 and miR-206 is pivotal for C2C12 myoblast differentiation. J Cell Sci 125:3590-3600

39. Rehfeldt C, Fiedler I, Dietl G, Ender K (2000) Myogenesis and postnatal skeletal muscle cell growth as influenced by selection. livprodsci 66:177-188

40. Li L, Shi J, Zhu G, Shi B (2012) MiR-17-92 cluster regulates cell proliferation and collagen synthesis by targeting TGFB pathway in mouse palatal mesenchymal cells. J Cell Biochem 113 (4):1235-1244

41. O'Donnell K, Wentzel E, Zeller K, Dang C, Mendell J (2005) c-Myc-regulated microRNAs modulate E2F1 expression. Nature 435 (7043):839-843

42. Sun Q, Zhang Y, Yang G, Wang J, Chen YX, Zhang Y, Cao G, Sun U, Zhang P, Fan M, Shao N, Yang X (2008) Transforming growth factor-b-regulated miR-24 promotes skeletal muscle differentiation. Nucleic Acids Res 36 (8):2690–2699

43. Walker JC, Harland RM (2009) microRNA-24a is required to repress apoptosis in the developing neural retina. Genes Dev 23:1046-1051

44. Lal A, Navarro F, Maher CA, Maliszewski LE, Yan N, O'Day E, Chowdhury D, Dykxhoorn DM, Tsai P, Hofmann O, Becker KG, Gorospe M, Hide W, Lieberman J (2009) miR-24 Inhibits Cell Proliferation by Targeting E2F2, MYC, and Other Cell-Cycle Genes via Binding to "Seedless" 3′UTR MicroRNA Recognition Elements. Mol Cell 35 (5):610-625

45. Esau C, Davis S, Murray SF, Xian Yu X, Pandey SK, Pear M, Watts L, Booten SL, Graham M, McKay R, Subramaniam A, Propp S, Lollo BA, Freier S, Bennett CF, Bhanot S, Monia BP (2006) miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. Cell Metab 3:87-98

46. Naguibneva I, Ameyar-Zazoua M, Polesskaya A, Ait-Si-Ali S, Groisman R, Souidi M, Cuvellier S, Harel-Bellan A (2006) The microRNA miR-181 targets the homeobox protein Hox-A11 during mammalian myoblast differentiation. Nat Cell Biol 8:278 - 284

47. Duisters RF, Tijsen AJ, Schroen B, Leenders JJ, Lentink V, van der Made I, Herias V, van Leeuwen RE, Schellings MW, Barenbrug P, Maessen JG, Heymans S, Pinto YM, Creemers EE (2009) miR-133 and miR-30 Regulate Connective Tissue Growth Factor Implications for a Role of MicroRNAs in Myocardial Matrix Remodeling. Circulation Res 104:170-178

48. Zorc M, Skok D, Godnic I, Calin G, Horvat S, Jiang Z, Dovc P, Kunej T (2012) Catalog of microRNA seed polymorphisms in vertebrates. PLoS One 7 (1):e30737

49. Lee J, Kim J, Lim K, Hong J, Hong K, Lee Y (2012) Effects of polymorphisms in the porcine microRNA MIR206 / MIR133B cluster on muscle fiber and meat quality traits. Anim Genet. doi:doi: 10.1111/j.1365-2052.2012.02362.x.

50. Liao Y, Lönnerdal B (2010) Global MicroRNA Characterization Reveals That miR-103 Is Involved in IGF-1 Stimulated Mouse Intestinal Cell Proliferation. PLoS One 5 (9):e12976

51. Takahashi Y, Forrest A, Maeno E, Hashimoto T, Daub C, Yasuda J (2009) MiR-107 and MiR-185 Can Induce Cell Cycle Arrest in Human Non Small Cell Lung Cancer Cell Lines. PLoS One 4 (8):e6677

52. Xu H, He J, Xiao Z, Zhang Q, Chen Y, Zhou H, Qu L (2010) Liver-enriched transcription factors regulate microRNA-122 that targets CUTL1 during liver development. Hepatology 52 (4):1431-1442

53. Tao J, Wu D, Li P, Xu B, Lu Q, Zhang W (2012) microRNA-18a, a member of the oncogenic miR-17-92 cluster, targets Dicer and suppresses cell proliferation in bladder cancer T24 cells. Mol Med Rep 5 (1):167-172

54. Pullamsetti S, Doebele C, Fischer A, Savai R, Kojonazarov B, Dahal B, Ghofrani H, Weissmann N, Grimminger F, Bonauer A, Seeger W, Zeiher A, Dimmeler S, Schermuly R (2012) Inhibition of microRNA-17 improves lung and heart function in experimental pulmonary hypertension. Am J Respir Crit Care Med 185 (4):409-419

55. Jiang Y, Wu Y, Greenlee A, Wu J, Han Z, Li X, Zhao Y (2011) miR-106a-mediated malignant transformation of cells induced by anti-benzo[a]pyrene-trans-7,8-diol-9,10-epoxide. Toxicol Sci 119 (1):50-60

56. Carraro G, El-Hashash A, Guidolin D, Tiozzo C, Turcatel G, Young BM, De Langhe SP, Bellusci S, Shi W, Parnigotto PP, Warburton D (2009) miR-17 family of microRNAs controls FGF10-mediated embryonic lung epithelial branching morphogenesis through MAPK14 and STAT3 regulation of E-Cadherin distribution. Dev Biol 333 238-250

57. Cheng AM, Byrom MW, Shelton J, Ford LP (2005) Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. Nucleic Acids Res 33(4):1290-1297

58. da Costa Martins P, Salic K, Gladka M, Armand A, Leptidis S, el Azzouzi H, Hansen A, Coenen-de Roo C, Bierhuizen M, van der Nagel R, van Kuik J, de Weger R, de Bruin A, Condorelli G, Arbones M, Eschenhagen T, De Windt L (2010) MicroRNA-199b targets the nuclear kinase Dyrk1a in an auto-amplification loop promoting calcineurin/NFAT signalling. Nat Cell Biol 12 (12):1220-1227

59. Liu X, Cheng Y, Zhang S, Lin Y, Yang J, Zhang C (2009) A necessary role of miR-221 and miR-222 in vascular smooth muscle cell proliferation and neointimal hyperplasia. Circ Res 104(4) :476-487

60. Cardinali B, Castellani L, Fasanaro P, Basso A, Alemà S, Martelli F, Falcone G (2009) Microrna-221 and Microrna-222 Modulate Differentiation and Maturation of Skeletal Muscle Cells. PLoS One 4 (10):e7607

61. Davis B, Hilyard A, Nguyen P, Lagna G, Hata A (2009) Induction of MicroRNA-221 by Platelet-derived Growth Factor Signaling Is Critical for Modulation of Vascular Smooth Muscle Phenotype. J Biol Chem 284 (6):3728-3738

62. Kuhn AR, Schlauch K, Lao R, Halayko AJ, Gerthoffer WT, Singer CA (2010) MicroRNA Expression in Human Airway Smooth Muscle Cells: Role of miR-25 in Regulation of Airway Smooth Muscle Phenotype. Am J Respir Cell Mol Biol 42(4) :506-513

63. Manni I, Artuso S, Careccia S, Rizzo M, Baserga R, Piaggio G, Sacchi A (2009) The microRNA miR-92 increases proliferation of myeloid cells and by targeting p63 modulates the abundance of its isoforms. FASEB 23 (11):3957-3966

64. Bonauer A, Carmona G, Iwasaki M, Mione M, Koyanagi M, Fischer A, Burchfield J, Fox H, Doebele C, Ohtani K, Chavakis E, Potente M, Tjwa M, Urbich C, Zeiher AM, Dimmeler S (2009) MicroRNA-92a Controls Angiogenesis and Functional Recovery of Ischemic Tissues in Mice. Science 324 (5935):1710-1713

CHAPTER 3

Transcriptional profiling and miRNA-dependent regulatory network analysis of longissimus dorsi muscle during prenatal and adult stages in two distinct pig breeds

Puntita Siengdee, Nares Trakooljul, Eduard Murani, Manfred Schwerin, Klaus Wimmers, Siriluck Ponsuksili

Published in: Animal Genetics (2013) 44(4):398-407

Article first published online: 2013 Mar 19 DOI: 10.1111/age.12032

Transcriptional profiling and miRNA-dependent regulatory network analysis of longissimus dorsi muscle during prenatal and adult stages in two distinct pig breeds

Summary

MicroRNAs (miRNAs) and mRNAs establish a complex regulatory network influencing diverse biological pathways including muscle development and growth. Elucidating miRNA-dependent regulatory networks involved in muscle development could provide additional insights into muscle traits largely predefined during prenatal development. The present study aimed to determine differentially expressed transcripts and functional miRNA-mRNA relationships associated with different stages of skeletal muscle development in two pig breeds, German Landrace (DL) and Pietrain (Pi) distinct in muscle characters. A comparative transcriptional profiling of *longissimus dorsi* (LD) muscle tissues from foetuses at 35, 63 and 91 days post conception (dpc) as well as adult pigs (180 days post natum, dpn) were performed using the Affmetrix GeneChip porcine genome microarray. Differential expression patterns were identified to be associated with muscularly developmental stages and breed types. The integration of miRNA expression data and IPA pathway analysis revealed several miRNA-dependent regulatory networks related to muscle growth and development. The present results provide insights into muscle biology for further improvement of porcine meat quality.

Key words: Microarray, muscle development, skeletal muscle, pig, microRNA

Introduction

The formation of muscle fibres (myogenesis) is a primarily prenatal process in mammalian species determining muscle growth and meat quality. Muscle growth is influenced by the number, size and type of muscle fibres. Muscle fibres are formed during prenatal stages and the total number of muscle fibres is mainly predefined before birth with the total number of myofibres being almost fixed at around 90 days of gestation (dg), whereas contractile and metabolic maturations mostly occur during the first three postnatal weeks (Picard *et al.* 2002). Understanding the prenatal development of skeletal muscle is therefore critical for improvement of meat quality because of its dramatic impacts on postnatal development and growth.

In the pig, formation of the total number of fibres (TNF) involves two major waves; a primary generation (myoblasts start differentiating into primary myofibres) from ~35-60 days post conception (dpc) and a secondary generation (secondary myofibres develop from new myoblasts using the primary myofibres as a template) from ~54-90 dpc (Rehfeldt *et al.* 1999). However, only recent counting of number of muscle fibres from birth to weaning in M. semitendinosus cross section suggested a postnatal increase may be related to both elongation of existing muscle fibres and genesis of tertiary myofibres (Bérard *et al.* 2011). Anyhow, around 35-, 60- and 90 dpc are key-time points to elucidate the biology of the prenatal skeletal muscle development. Relationships between muscle mass, fibre number and structure have been well demonstrated in comparative studies between pig breeds differing in muscle traits (Muráni *et al.* 2007). German Landrace (DL) and Pietrain (Pi) pigs considerably contrast in growth rate, body composition, muscularity and fat content: DL is more obese and Pi is more lean (Rehfeldt *et al.* 2000; Ponsuksili *et al*. 2007).

MicroRNAs (miRNAs) are short (~20-30 nucleotides) single-stranded, non-coding RNA molecules which are highly conserved in closely-related species. MicroRNAs repress gene expression post-transcriptionally via hybridization to their target mRNAs at the 3'-untranslated region (3'-UTR) (Kim & Nam 2006). The miRNAs-dependent gene regulation has been shown to play vital roles in developmental processes and cellular functions (Chen *et al.* 2009; Williams *et al.* 2009). Transcriptome analyses of different muscle fibre types and/or muscle tissues at different developmental stages in pigs have revealed several regulatory genes involving in myogenesis including growth factors, regulatory proteins, receptors, and transcription factors (Lin & Hsu 2005; Te

Pas *et al*. 2005; Cagnazzo *et al.* 2006; Muráni *et al.* 2007; Te Pas *et al.* 2007; Davoli *et al.* 2011). In addition, an increasing evidence have shown a dynamic change of miRNA expression during developmental stages of the porcine skeleton muscle (Huang *et al.* 2008; Chen *et al*. 2008 ; McDaneld *et al.* 2009 ; Xie *et al.* 2010; Zhou *et al.* 2010). Only a few reports have demonstrated the miRNA-dependent regulatory networks of the muscle in pigs. This study aims to identify differentially expressed genes by a comparative transcriptional profiling of porcine skeletal muscle at developmental stages 35-, 63- and 91-day-old foetuses as well as at 180 days postnatal in adult pigs for DL and Pi breed types and to explore miRNA-dependent regulatory networks by an integration of miRNA profiling data derived from the same samples with the identified differentially expressed genes using Ingenuity Pathways Analysis (IPA) software. The results of the present study showed that differential expression patterns are associated with development and growth stage as well as pig breed and these transcriptional signatures are potentially regulated by the differential expression of miRNAs in muscle tissue. Our study provided additional insights into muscle molecular biology for further improvement of meat quality.

Materials and Methods

Animals and tissue collection

At each prenatal stage of 35, 63 and 91 dpc fetuses were obtained from 5 sows per breed, German Landrace (DL) or Pietrain (Pi). Sows in their second, third or fourth parity were selected from two large herdbook farms. The average performance of animals has been previously reported (Ponsuksili *et al.* 2007). Sows were mated to boars of the same breed (DL or Pi) and slaughtered at 35, 63 or 91 days of pregnancy. Uteri were taken out of the abdomen and fetuses were obtained immediately. The longissimus muscle tissues were isolated from fetuses, snap frozen in liquid nitrogen and stored at -80 °C. The *longissimus dorsi* (LD) muscle samples of 36 adult pigs (180 days of age on average) of the two breeds were obtained from the same herds. All pigs were fed ad libitum according to the guidelines of the German performance test (ZDS 2003). Animal care and tissue collection procedures followed the guidelines of the

German Law of Animal Protection and the experimental protocol was approved by the Animal Care Committee of the Leibniz Institute for Farm Animal Biology.

Total RNA isolation

Total RNA was isolated from the LD muscle tissue using Tri-Reagent according to the manufacturer's protocol (Sigma-Aldrich, Taufkirchen, Germany) followed by on-column DNase treatment using the RNeasy Mini kit (Qiagen, Hilden, Germany). The quality and quantity of the RNA samples was checked and measured using a NanoDrop ND-1000 Spectrophotometer (PEQLAB, Erlangen, Germany) and an Agilent 2100 Bioanalyzer for RNA (Agilent Technologies, Santa Clara, CA).

Microarray hybridization and data analysis

A pooled RNA was generated from an equal amount of six RNA samples extracted from three pairs of male and female littermate of different sows/litters which were assigned to each experimental condition as mentioned above. Sex determination of the 35-dpc fetuses was performed using a PCR amplification of a sex-determining region on the Y chromosome (Muráni *et al.* 2007). Three RNA pools for each stage and breed (2 breeds×4 stages×3 biological replicates) were used for cDNA and biotin-labeled cRNAs syntheses and chip hybridizations according to the manufacturer's protocol. A total of 24 Affymetrix GeneChip porcine Genome Arrays (Affymetrix, St. Clara, USA) each containing 24,123 probe sets were utilized. The biotin-labeled cRNA was fragmented and hybridized to an array for 16 hours at 45 °C. Hybridization, washing, and scanning of the arrays were performed on a Fluidics Station 450. The expression data was quality controlled and preprocessed using the Affymetrix GCOS 1.1.1 software with global scaling to a target signal of 500.

Data pre-processing, normalization and statistical analysis

The Expression Console software (Affymetrix, Santa Clara, CA, USA) was used for GC-RMA normalization and log 2 transformations. The assignments of present or absent calls of probe-sets were using the MAS5 algorithm. The transcripts that were expressed less than 50 % of the pools within breed and stage were discards. For a second filtering step standard deviations were calculated for each probe set over all subsets of arrays. Probe sets with a small standard deviation (SD $\leq$ 0.25) across the

experimental conditions were filtered out due to their uninformative signals to increase detection power via a reduction of the number of tested hypotheses in multiple-testing adjustments (Bourgona *et al.* 2010) The expression data was deposited in the Gene Expression Omnibus public repository (GEO accession number GSE38518: GSM944473-GSM944496).

Statistical analyses were performed using the procedure in JMP Genomics 4 (SAS Institute, Cary, NC). Breed, stage and their interaction (breed×stage) were treated as fixed effects. Essentially the SAS MIXED procedure fits linear models on a row-by-row basis to pre-normalized data. The adjusting for multiple tests across the Type 3 tests for all of the fixed effects was calculated using the post hoc Tukey-Kramer test. For controlling false discovery rate, we choose the FDR according to Benjamin and Hochberg, 1995.

IPA pathways analyses

The identified differentially expressed genes between the DL and Pi breed at each stage were analyzed using the Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems®, http://www.ingenuity.com). Essentially, IPA associated gene annotation (GO) terms to the gene lists, functionally categorized genes into functional groups and statistically identified overrepresentation for each group using B-H Multiple Testing Correction (FDR) at $p < 0.05$. IPA integrated the Ingenuity Knowledge Base and the submitted gene expression profiles to generate biological relevant gene regulatory networks.

To identify miRNA-dependent regulatory networks, differentially expressed miRNAs of the same samples at 63 and 91 dpc and the adult stage of 180 days were identified from miRNA microarray data (GEO accession number GSE34213: GSM844738-GSM844755). Details of miRNA microarray analysis are available in Supplemental Method. The resulting list of differential miRNAs was then analyzed using the microRNA Target Filter in IPA to identify their potential mRNA targets based on TargetScan, TarBase, miRecords, and the Ingenuity® Knowledge Base. The expression data of differential mRNA from the present study was integrated with the miRNA expression using the expression pairing function (IPA) to obtain miRNA-mRNA relationships with expression changes in opposite direction. The miRNA-mRNA

interacting networks were further extracted for biological pathways related to muscle development and growth, cellular growth and proliferation and lipid-metabolism.

Validation of microarray results using Quantitative real-time PCR

Twelve individual RNA samples from each breed and stage (2 breeds x 4 stages) were used for quantitative real-time PCR (qPCR) to validate the microarray results. Total (DNase treated) RNA was reverse-transcribed into cDNA using 200 U of SuperScript II and Oligo(dT) in a total volume of 10 µl reaction. The qPCR was performed in a 10 µl reaction containing 5.0 µl of LightCycler 480 SYBR Green I Master (Roche), 600 nM of each primer and 2 µl of cDNA (20 ng) using the LightCycler 480 system (Roche, Mannheim, Germany). All reactions were performed in duplicates. The PCR program was 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, 60 °C for 10 sec and 72 °C for 15 sec. Seven genes were selected (*MDH1, PRDM5, PDHA1, IGF1, IGF2, CYP19A1* and *SMPX*) and the primer sequences are available in Table S1. A standard curve was generated for each gene using serial dilutions of specific PCR product, for the absolute quantification method. The expression value was normalized to two reference genes, Hypoxanthin-Phosphoribosyl-Transferase 1 (*HPRT1*) and Ribosomal Protein S3 (*RPS3*). Correlation coefficient analysis (*r*) between the microarray and qPCR data was performed using SAS version 9.2 (SAS Institute, Cary, NC).

Results

Differentially expressed genes

The Affymetrix GeneChip porcine genome array was used to profile the transcriptional difference of LD muscle at four developmental stages (35, 63, and 91 dpc and 180 dpn) for DL and Pi. To simplify the statistic model, we first compared expression profiles between the two breed types at each developmental stage. Then we analysed gene expression profile between two adjacent ontogenetic stages for each breed.

A total number of 14,573 out of 24,123 probes on the microarray passed our filtering and were used for further analyses. The number of differentially expressed genes (FDR ≤ 0.05) identified in a comparison between DL and Pi at each stage is shown (vertical arrows) in Figure 3.1. The highest number of 3276 differential probes was observed at the adult stage. Of these 1787 and 1489 probes showed higher transcript abundance in

DL and Pi, respectively. Among these, probes representing *CYP7B1 DDX3X, ALB, EIF2S3, EIF1AX, TMSL3, CASQ2, FGB* and *HSPA2* differed more than six-fold. At 35 dpc, 1422 probes were differentially expressed. At 63 dpc, 970 probes significantly altered between the two breed types. At 91 dpc, there were 1787 differentially expressed probes and 367 probes changed more than two-fold. A complete list of all differentially expressed genes between DL and Pi is available in Table S2-S5. A total of 21, 20, 24 and 30% of expressed probes on the array were differentially altered in either DL or Pi at 35, 63 and 91 dpc and adult stages, respectively.

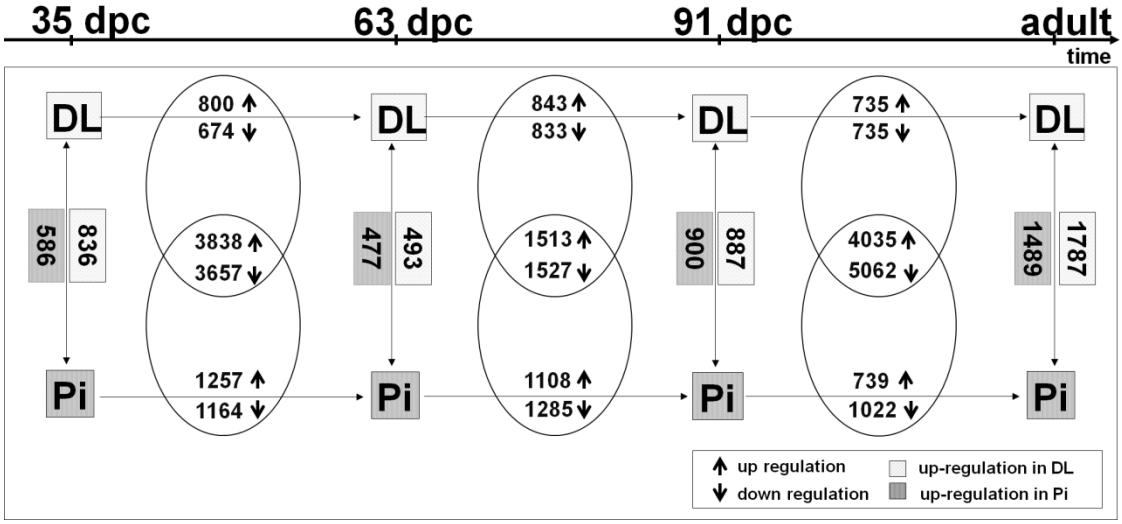

Figure 3.1: The number of differentially expressed probes (FDR < 0.05) identified in *longissimus dorsi* (LD) muscle tissue between DL and Pi breed types at 35, 63 and 91 dpc as well as adult stage (180 days old). The numbers of up-regulated genes associated with either DL or Pi at each stage are shown on vertical arrows. The number of differentially expressed probes determined between ontogenetic stages in either DL or Pi is depicted on horizontal arrows and probes commonly identified in both breed types are shown in the overlaps. Arrow head shows up- (↑) or down- (↓) regulations.

We then compared the expression profile between two adjacent ontogenetic stages within each breed. Three comparisons (period I: 35 vs 63 dpc; period II: 63 vs 91 dpc and period III: 91 dpc vs adult) for each breed type were investigated. Figure 3.1 shows the number of differentially expressed probes identified between two consecutive ontogenetic stages (horizontal arrows). In Pi, the number of 9916, 5433 and 10858 probes were declared differentially expressed at period I, II and III, respectively (FDR < 0.05). There were 8969, 4716 and 10567 differential probes in DL at period I, II and III, respectively. A comparison of the gene lists showed 7495, 3040 and 9097 probes

commonly identified in both Pi and DL at period I, II and III, respectively. These genes that showed the same shift of abundance along stages in both breeds, thus, can be assumed to reflect ontogenetic processes that do not contribute to breed differences. In this regard differentially expressed probes exclusive to either DL or Pi are of interest (Figure 3.1).

Validation of differentially expressed genes by qPCR

Seven genes *MDH1, PRDM5, PDHA1, IGF2, CYP19A1, SMPX* and *IGF* were selected based on their functions in muscle development for a qPCR assay to validate the Affymetrix gene expression result. Results from microarray and qPCR showed good agreement with the coefficient of correlation (*r*) ranging from 0.44 to 0.92 among all validated genes (Figure3.2).

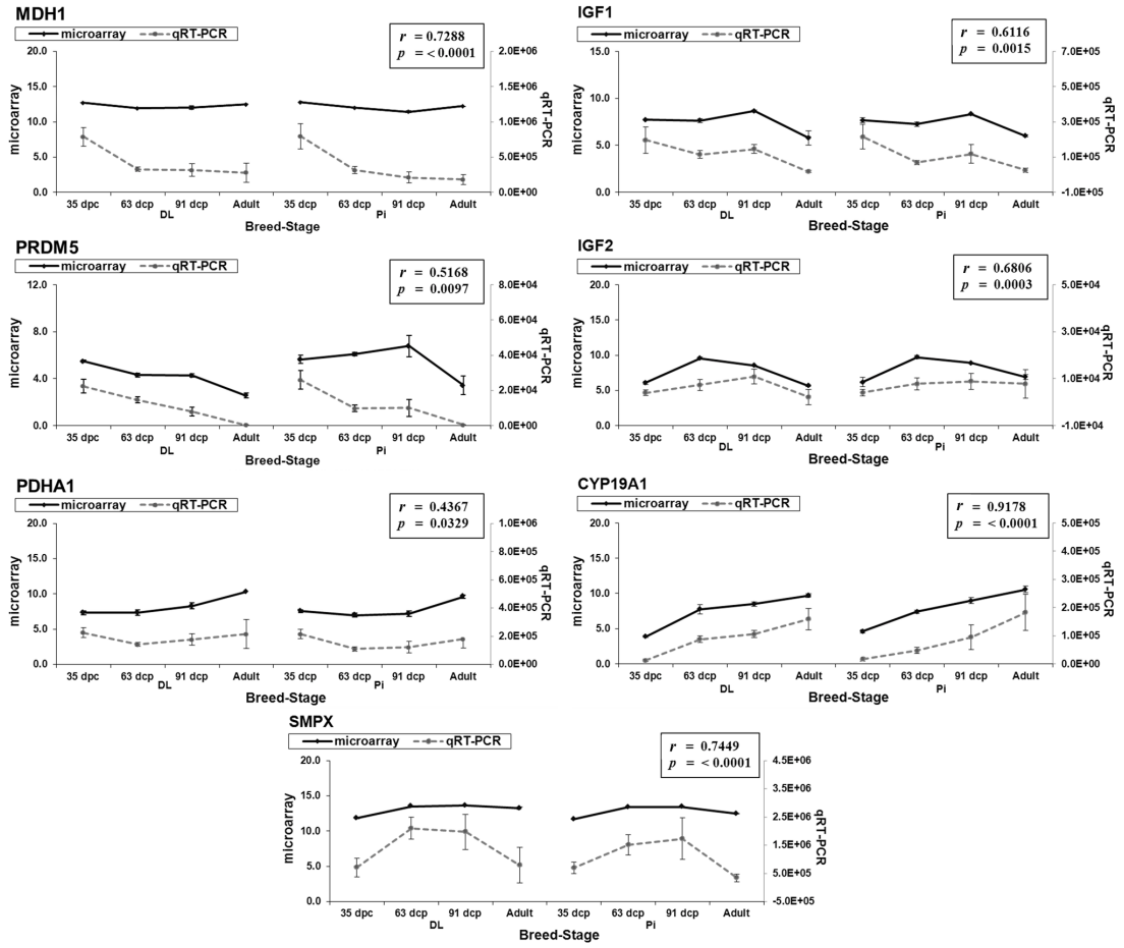

Figure 3.2: Validation of the microarray results using Real-time PCR method in 7 genes; *MDH1, PRDM5, PDHA1, IGF2, CYP19A1, SMPX* and *IGF*. Microarray results (on the primary Y-axis) and QPCR (on the secondary Y-axis) for each gene are depicted in the same graph with *r* and p-value.

Ingenuity Pathways Analysis

To gain further insight into molecular function of differentially expressed genes, we interrogated our gene list using IPA. Lists of up-regulated genes in each stage and breed were used in this analysis. GO categories related to muscle growth and development including “cellular growth and proliferation”, “cellular function and maintenance”, “cell death”, “cell cycle”, “cell morphology” and “lipid metabolism” were overrepresented with different significant levels associated with breed and stage (Figure 3.3). A complete list of IPA result is available in Table S6. It is noteworthy that the number of up-regulated genes differed between DL and Pi for the biological terms “muscular development and function”, “cellular growth and proliferation” and “lipid metabolism” (Table 3.1). The number of up-regulated genes associated with “muscle development and function” and “cellular growth and proliferation” is higher in DL than Pi at all prenatal stages except for the adult stage which is much higher in Pi. There were more lipid-metabolism related genes identified in DL than in Pi at 91 dpc and adult stages. In addition, top canonical pathways regulating cell growth and development such as PTEN, EIF2, mTOR and p70S6K signalling were identified. A complete list of the identified canonical pathways is available in Table S7.

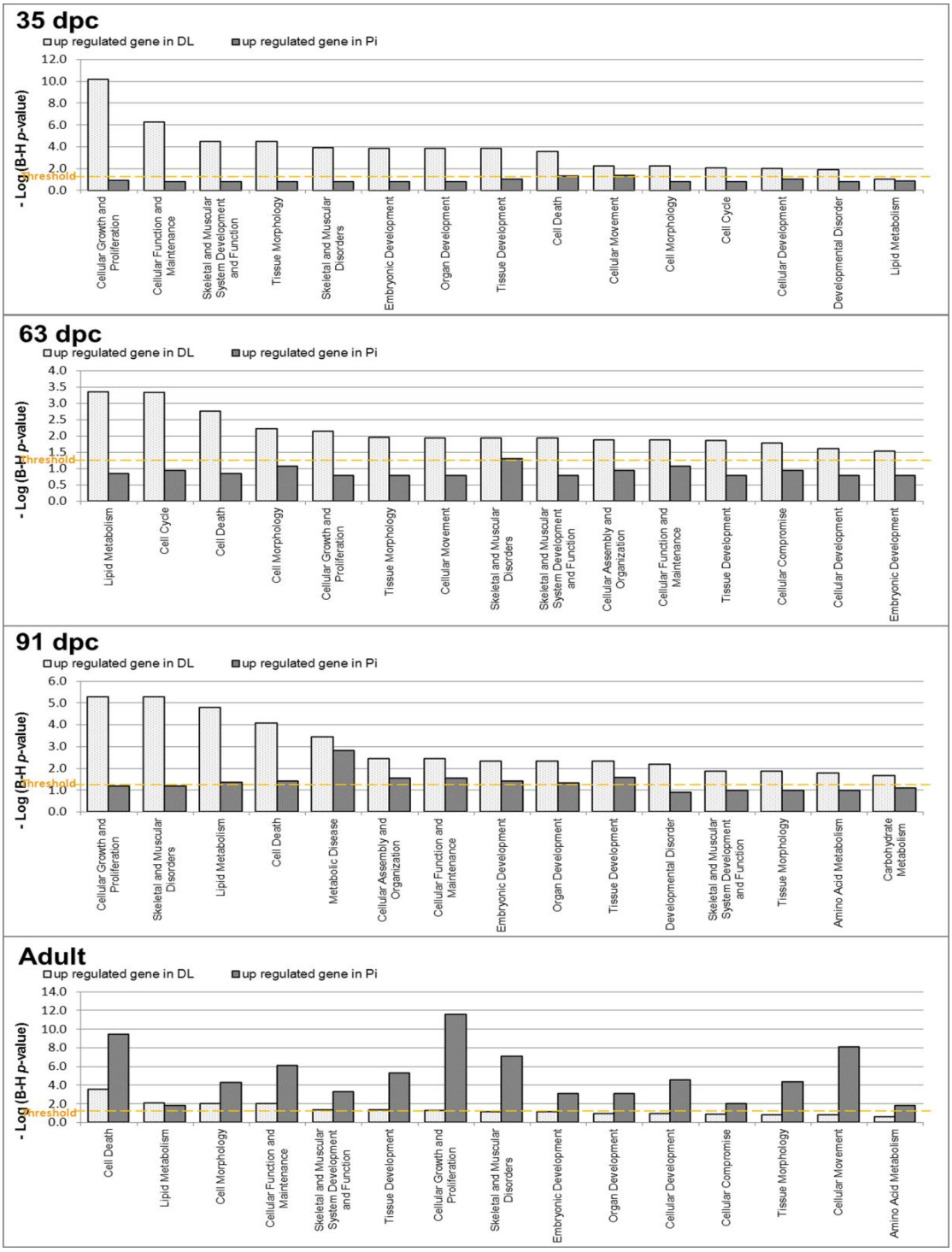

Figure 3.3: IPA analysis of differentially expressed genes identified biological pathways associated with porcine skeletal muscle development stages in either DL (light box) or Pi (gray box) breed types. Only fifteen biological pathways related to muscle development and growth were selected. Log p-value is depicted on the Y-axis with threshold B-H Multiple Testing Correction $P < 0.05$ and pathway names are on the X-axis.

Table 3.1. The number of up-regulated genes in either DL or Pi at each stages associated with three selected functional categories derived from IPA analysis

Stage	Breed	Skeletal and Muscular System Development and Function		Cellular Growth and Proliferation		Lipid Metabolism	
		No. genes	B-H correction p-value*	No. genes	B-H correction p-value*	No. genes	B-H correction p-value*
35 dpc	DL	95	3.64E-05-1.52E-01	195	6.84E-11-1.52E-01	13	9.23E-02-1.52E-01
	Pi	29	1.60E-01-1.92E-01	104	1.26E-01-1.92E-01	11	1.41E-01-1.92E-01
63 dpc	DL	36	1.18E-02-9.25E-02	108	7.24E-03-9.25E-02	30	4.59E-04-9.25E-02
	Pi	14	1.70E-01-2.03E-01	61	1.70E-01-2.03E-01	37	1.46E-01-2.03E-01
91 dpc	DL	58	1.43E-02-1.67E-01	189	5.41E-06-1.67E-01	57	1.57E-05-1.67E-01
	Pi	23	1.10E-01-1.76E-01	109	6.68E-02-1.76E-01	22	4.45E-02-1.76E-01
adult	DL	59	4.89E-02-2.88E-01	102	5.37E-02-2.88E-01	77	8.98E-03-2.88E-01
	Pi	116	5.51E-04-2.97E-02	322	2.65E-12-3.92E-02	29	1.62E-02-3.25E-02

***** B-H correction p-value = Benjamin-Hochberg correction for multiple testing was applied by the IPA.

Analysis of microRNA-dependent regulatory network

Analysis of the miRNA microarray data identified 221, 44 and 152 miRNAs differentially expressed at 63 and 91 dpc and 180 day adult stage, respectively. The expression of both differentially expressed miRNAs and mRNAs at each stage was then analyzed to determine the miRNA-mRNA relationship using the microRNA Target Filter tool in IPA. The expression of the miRNAs and mRNA targets was matched using the IPA pairing function. Several miRNA-mRNA pairs in our dataset showed either positive (the same direction) or negative (the opposite direction) expression correlations. A total number of 217, 145 and 343 miRNA-mRNA pairs (both direction) were identified at 63, 91 dpc and adult stage, respectively (Table S8). These accounted for 30, 9 and 17 miRNA families and 76, 82 and 159 target genes at these three stages, respectively. At 63 dpc, a total number of 111 miRNA-mRNA interactions with a negative correlation of gene expression were identified. These comprised of 55 mRNAs targeted by 26 miRNA families (let-7, miRNA-1/206, -103/107, -105, -124, -125, -128, -129, -133, -16/195/497, -181, -18/4735, -199a-3p, -199a-5p, -19, -17/20/106 , -216, -218, -24, -30, -320, -324, -374, -455, -9 and -32/92). At 91 dpc, 9 miRNA families (miR-1/206, -103/107, -125, -181 -199a-3p, -17/20/106, -24, -30 and -374) were identified to regulate 51 mRNAs and generate 77 interaction pairs with a negative

expression correlation. Furthermore, 16 miRNA families (miR-103/107, -125, -129, -133, -16/195 /497, -181, -18/4735, -194, -199a-3p, -199a-5p, -17/20/106, -218, -24, -30, -628 and -32/92) were found related to 144 mRNAs generating 178 negatively correlated expression patterns in adult stage. Interestingly, miR-24, miR-30, miR-199a-3p, miR-103/ 107, miR-125, miR-181and miR-17 families (-17/ 20/ 106) were differentially expressed at all three stages studied. MiR-30 and miR-199a-3p were up-regulated in the Pi breed throughout muscle development stages while, miR-181was overexpressed in DL. A list of important genes associated with cell growth and proliferation and muscle development is given in Table 3.2 together with their potential miRNAs derived from an expression pairing analysis in IPA. To explore the miRNA-dependent regulatory network, we further extracted miRNA-mRNA pairs with a negative correlation and generated a miRNA-dependent regulatory network focusing on biological function "cell growth and proliferation" and "lipid-metabolism" (Figure 3.4). These two categories were selected because of their close relations with muscle development.

Table 3.2. Expression pairing analysis (IPA) of differentially expressed genes and potential miRNAs in *longissimus dorsi* tissues

Function	Gene	Potential miRNA[a]
muscle contraction/ extension of cells	***KPNA3***	miR-103, -16, -24, -20
growth of cells	***AKT1***	miR-374
	CALM1	miR-133a, -181a, -218, -92a
proliferation of cells	***TSC1***	miR-128, -320b, -92a, let-7a
	PIK3R1	miR-103, -129, -16, -17-5p, -20
growth and proliferation of cells	***TGFBR2***	miR-103, -17-5p, -19b, -216, -9
	NTRK2	miR-103, -17-5p, -374
	JARID2	miR-125b, -181a, -30e-5p, -92a
	RAP1B	miR-181a, -24, -30e-5p, -92a
synthesis/ oxidation of fatty acid	***ACSL4***	miR-129, -16, -17-5p, -181b, -19b, -374
development of muscle	***ELAVL1***	miR-125b, -129, -19b, -324-5p, -9
length of myotube	***BCL2***	miR-1, -16, -17-5p, -181b, -20

[a] Lists of miRNAs potentially targeting the 3'-UTR of the differentially expressed genes with expression changes in an opposite direction.

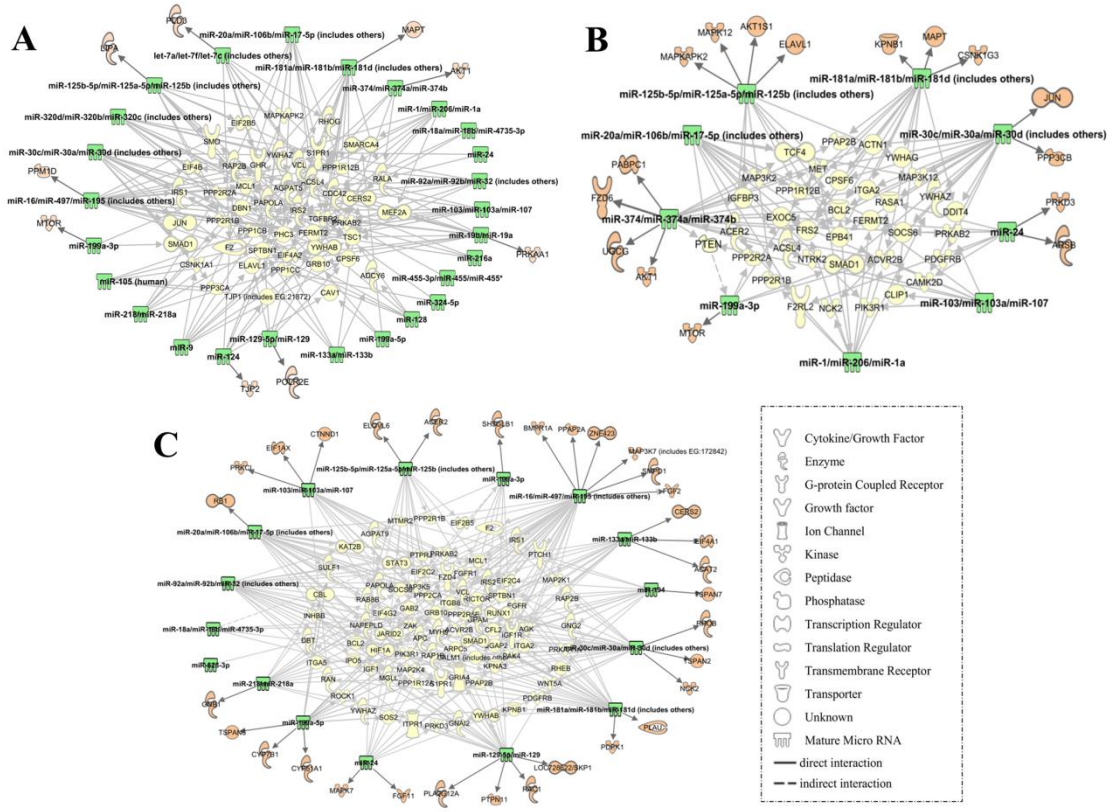

Figure 3.4: IPA microRNA Target Filter analysis revealed miRNA-dependent regulatory networks associated to cellular growth and proliferation and lipid-metabolism **functions.** MiRNA-mRNA interactions with negative-correlations were extracted and used to generate miRNA-dependent regulatory networks using IPA pathway designer. MiRNA-mRNA regulatory networks (A) at 63 dpc comprising 26 miRNAs, 55 mRNAs and 111 interaction pairs (B) at 91 dpc with 9 miRNAs, 51 mRNAs and 77 interaction pairs (C) at adult stage with 16 miRNAS, 144 mRNAs and 178 interaction pairs. Nodes representing miRNAs are colored by green. Light-orange nodes mRNAs connected to miRNA by bold edges are regulated solely by a single miRNAs. The mRNAs regulated by more than two miRNAs are grouped and shown in yellow color nodes and all their edges colored by gray. Edges represent the relationship between two nodes.

Discussion

Our LD muscle transcriptome analyses of these critical stages showed a number of differentially expressed genes and provided a comprehensive list of transcriptional differences underlying muscle development and growth and the muscular distinction between the two pig breed types. The IPA analysis identified overrepresentation of the molecular function and cellular processes categories. It is noteworthy that during

prenatal period, the number of identified up-regulated genes overrepresented in the category muscle growth and development is higher in the DL than in the Pi breed type (Table 1). However, this number is inversely observed in the adult stage: the number of up-regulated genes is higher in Pi than in DL. This result suggests that at early stage of muscle fibre formation is unique and complex in these two breed types. Interestingly, our results showed a higher number of lipid-metabolism associated genes for DL breed type at almost all stages compared to Pi. Lipid deposition within myofibres occurs as early as during myogenesis (Karunaratne *et al.* 2005). The hepatic expression profile from our previous study also showed an association between the obese DL breed type and genes in lipid metabolism pathways (Ponsuksili *et al.* 2007).

GO and IPA pathways analyses (Table 1 and Table S6) identified several candidate genes related to muscle growth and development including *TNNC2* and *AKT1*. Both genes are directly involved with the regulation of the myoblast determination factor (MyoD) and have been identified to be excellent model genes for skeletal muscle development studies (Li *et al.* 2008; Rotwein & Wilson 2009). Troponin C type 2-fast (*TNNC2*) is a fibre-specific gene expressed during myoblast differentiation and skeletal muscle development in fast twitch skeletal muscle. *TNNC2* encodes the Troponin C subunit that binds calcium ions leading to the inhibitory of actin filaments and ultimately contraction of skeletal muscle (Chen *et al.* 2011). The v-akt murine thymoma viral oncogene homolog 1 also known as a serine/threonine protein kinase (*AKT1*) is a critical mediator in myogenic differentiation (Sumitani *et al.* 2002; Rotwein & Wilson 2009) and plays an important role determining size and number of type II muscle fibres (Goncalves *et al.* 2010). Insulin-like growth factor 2 (*IGF2*) is functionally classified in "Cellular Growth and Proliferation" as well as "Skeletal and Muscular System Development and Function" pathways. It has been reported that *IGF2* modulates the transcription activity of MyoD, which in turn regulates the expression of numerous muscle-specific genes (Wilson & Rotwein 2006; Lobjois *et al.* 2008). Moreover, *IGF2* is well known as a genetic factor which plays a stimulatory role in the initiation of skeletal myogenic programing and is used as a biomarker for muscle mass and fat deposition (Van Laere *et al.* 2003).

The present result showed that the majority of miRNAs simultaneously target several mRNAs and over 80% of an individual mRNA is targeted by more than one miRNA. This phenomenon tremendously adds complexity to gene regulatory networks (Lee *et*

al. 2009; Williams *et al.* 2009). It has been estimated that 60% of all human genes are regulated by miRNAs (Friedman *et al.* 2009). In this study, we focused on a regulatory network involved in cell growth and proliferation and the lipid-metabolism since they are related to muscle development and growth. Analysis of miRNA-dependent gene regulation at 63 dpc, six genes *ACSL4, ELAVL1, PHC3, PRKAB2, TGFBR2* and *TSC1* showed the highest number of miRNA-mRNA interactions. *ACSL4* has been reported to be involved in fatty acid metabolism and lipid biosynthesis and to play an important role determining intramuscular fat content and meat quality in pigs (*Mercadé et al.* 2006; Ruść *et al.* 2011). *TGFBR2* which was also more abundant in DL than Pi is known to drive fibroblasts differentiation into myofibroblasts (Inamoto *et al*. 2010). *TGFBR2* has been experimentally observed to be directly targeted by miR-17/20a cluster (especially: miR-17 and miR-20a). Mixed miRNA mimics or miRNA inhibitors of miR-17-92 components transfection affected the TGF-b pathway and resulted in induced proliferation inhibition and cell growth arrest in mouse embryo cell line (Li *et al*. 2012), keratinocyte cell lines (Schultz *et al.* 2011) and in another study in mice neuroblastoma cell (Mestdagh *et al.* 2010). At 91 dpc, *ACSL4, BCL2, FRS2* and *NTRK2* established several miRNAs interactions and were found to be down-regulated in the DL breed type. These genes were reported to play important roles in early stage of skeletal muscle growth and development (Biral *et al.* 2002; Hoch & Soriano 2006; Kerr *et al.* 2009). At the adult stage, several genes including *ACVR2B, CALM1, JARID2, KPNA3, PIK3R1, RAB8B, RAP1B* and *ZAK* were targeted by a number of miRNAs. *CALM1* was down-regulated in DL. The gene has been implicated to play a role in muscle contractions and transduction controlling of slow fibre gene expression specifically in skeletal muscle (Frey *et al.* 2000). MiR-133 and miR-1 targeting on the 3'UTRs *CALM1* genes led to down-regulation of the genes (Ikeda *et al.* 2009).

Conclusions

In the present study, we identified a number of differentially expressed genes between DL and Pi breed types at critical stages of skeletal muscle growth and development. We used IPA to functionally categorize these genes and to extract important biological pathways underlying porcine muscle development. We further integrated miRNA expression profile to our microarray results and identified several miRNA-dependent regulatory networks. Our results showed that a number of identified differentially

expressed genes are potentially regulated by miRNAs. This suggests a complex miRNA-dependent regulatory network during muscle development. The present result further adds a comprehensive knowledge on the regulation of transcriptome during muscle development in pigs.

Acknowledgements

The authors thank Joana Bittner and Annette Jugert for an excellent technical assistance. This research was supported by German Research Foundation (Deutsche Forschungsgemeinschaft, DFG; PO 753/7-1 and WI 1754/10-14). The authors declare no conflict of interest.

References of chapter 3

Benjamin Y. & Hochberg Y. (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. Series B (Methodological) 57, 289-300.

Bérard J., Kalbe C., Lösel D., Tuchscherer A. & Rehfeldt C. (2011) Potential sources of early-postnatal increase in myofibre number in pig skeletal muscle. Histochemistry and Cell Biology 136, 217-25.

Biral D., Jakubiec-Puka A. & Betto R. (2002) Expression of Bcl-2 Family Proteins in Recovering and Regenerating Muscles. Basic and Applied Myology 12, 43-6.

Bourgona R., Gentleman R. & Huber W. (2010) Independent filtering increases detection power for high-throughput experiments. Proceedings of the National Academy of Sciences of the United States of America 107, 9546-51.

Cagnazzo M., te Pas M.F.W., Priem J., de Wit A.A.C., Pool M.H., Davoli R. & Russo V. (2006) Comparison of prenatal muscle tissue expression profiles of two pig breeds differing in muscle characteristics. Journal of Animal Science 84, 1-10.

Chen H., Wang D., Xu G., Wu T. & Zheng C. (2011) Cloning, Structural Analysis and Expression of Fast Twitch Skeletal Muscle TroponinC2 (TNNC2) Gene in Goat. In: IPCBEE (ed. by Baby S), pp. 283-91. IACSIT Press, Singapore, singapore.

Chen J.-F., Callis T.E. & Wang D.-Z. (2009) microRNAs and muscle disorders. Journal of Cell Science 122, 13-20.

Chen J.-H., Wei W.-J., Xiao X., Zhu M.-J., Fan B. & Zhao S.-H. (2008) Expression analysis of miRNAs in porcine fetal skeletal muscle on days 65 and 90 of gestation. Asian - Australasian Journal of Animal Sciences 21, 954-60.

Davoli R., Braglia S., Russo V., Varona L. & te Pas M.F.W. (2011) Expression profiling of functional genes in prenatal skeletal muscle tissue in Duroc and Pietrain pigs. Journal of Animal Breeding and Genetics 128, 15-27.

Frey N., Richardson J. & Olson E. (2000) Calsarcins, a novel family of sarcomeric calcineurin-binding proteins. Proceedings of the National Academy of Sciences of the United States of America 97, 14632-7.

Friedman R.C., Farh K.K.H., Burge C.B. & Bartel D.P. (2009) Most mammalian mRNAs are conserved targets of microRNAs. Genome research 19, 92–105.

Goncalves M.D., Pistilli E.E., Balduzzi A., Birnbaum M.J., Lachey J., Khurana T.S. & Ahima R.S. (2010) Akt Deficiency Attenuates Muscle Size and Function but Not the Response to ActRIIB Inhibition. PLoS ONE 5, 1-12.

Hoch R.V. & Soriano P. (2006) Context-specific requirements for Fgfr1 signaling through Frs2 and Frs3 during mouse development. Development 133, 663-73.

Huang T.-H., Zhu M.-J., Li X.-Y. & Zhao S.-H. (2008) Discovery of Porcine microRNAs and Profiling from Skeletal Muscle Tissues during Development. PLoS One 3, 3225.

Ikeda S., He A., Kong S. et al. (2009) MicroRNA-1 Negatively Regulates Expression of the Hypertrophy-Associated Calmodulin and Mef2a Genes. Molecular and Cellular Biology 29, 2193-204.

Inamoto S., Kwartler C.S., Lafont A.L. et al. (2010) TGFBR2 mutations alter smooth muscle cell phenotype and predispose to thoracic aortic aneurysms and dissections. Cardiovascular Research 88, 520-9.

Karunaratne J.F., Ashton C.J. & Stickland N.C. (2005) Fetal programming of fat and collagen in porcine skeletal muscles. Journal of Anatomy 207, 763-8.

Kerr B., Garcia-Rudaz C., Dorfman M., Paredes A. & Ojeda S. (2009) NTRK1 and NTRK2 receptors facilitate follicle assembly and early follicular development in the mouse ovary. Reproduction 138, 131-40.

Kim V.N. & Nam J.-W. (2006) Genomics of microRNA. Trends in Genetics 22, 165-73.

Lee Y.-B., Bantounas I., Lee D.-Y., Phylactou L., Caldwell M.A. & Uney J.B. (2009) Twist-1 regulates the miR-199a/214 cluster during development. Nucleic Acids Research 37, 123–8.

Li L, Shi JY, Zhu GQ & Shi B (2012) MiR-17-92 Cluster Regulates Cell Proliferation and Collagen Synthesis by Targeting TGFB Pathway in Mouse Palatal Mesenchymal Cells. Journal of Cellular Biochemistry 113, 1235-44.

Li Y., Chen Y., Li J. et al. (2008) Molecular characterization, expression profile and polymorphisms of the porcine TNNC2 gene. Hereditas 145, 274-82.

Lin C.S. & Hsu C.W. (2005) Differentially transcribed genes in skeletal muscle of Duroc and Taoyuan pigs. Journal of Animal Science 83, 2075-86.

Lobjois V., Liaubet L., SanCristobal M., Gle´nisson J., Fe`ve K., Rallie`res J., Le Roy P., Milan D., Cherel P. & Hatey F. (2008) A muscle transcriptome analysis

identifies positional candidate genes for a complex trait in pig. Animal Genetics 39, 147-62.

McDaneld T.G., Smith T.P., Doumit M.E., Miles J.R., Coutinho L.L., Sonstegard T.S., Matukumalli L.K., Nonneman D.J. & Wiedmann R.T. (2009) MicroRNA transcriptome profiles during swine skeletal muscle development. BMC Genomics 10, 77.

Mercadé A., Estellé J., Pérez-Enciso M., Varona L., Silió L., Noguera J.L., Sánchez A. & Folch J.M. (2006) Characterization of the porcine acyl-CoA synthetase long-chain 4 gene and its association with growth and meat quality traits. Animal Genetics 37, 219-24.

Mestdagh P., Boström A., Impens F. et al. (2010) The miR-17-92 microRNA cluster regulates multiple components of the TGF-β pathway in neuroblastoma. Molecular Cell 40, 762-73.

Muráni E., Murániová M., Ponsuksili S., Schellander K. & Wimmers K. (2007) Identification of genes differentially expressed during prenatal development of skeletal muscle in two pig breeds differing in muscularity. BMC Developmental Biology 7, 109.

Picard B., Lefaucheur L., Berri C. & Duclos M. (2002) Muscle fibre ontogenesis in farm animal species. Reproduction Nutrition Development 42, 415–31.

Ponsuksili S., Murani E., Walz C., chwerin M. & Wimmers K. (2007) Pre- and postnatal hepatic gene expression profiles of two pig breeds differing in body composition: insight into pathways of metabolic regulation. Physiological Genomics 29, 267-79.

Rehfeldt C., Fiedler I., Dietl G. & Ender K. (2000) Myogenesis and postnatal skeletal muscle cell growth as influenced by selection. Livestock Production Science 66, 177-88.

Rehfeldt C., Stickland N.C., Fiedler I. & Wegner J. (1999) Environmental and genetic factors as sources of variation in skeletal muscle fibre number. Basic and Applied Myology 9, 235-53.

Rotwein P. & Wilson E.M. (2009) Distinct Actions of Akt1 and Akt2 in Skeletal Muscle Differentiation. Journal of Cellular Physiology 219, 503-11.

Ruść A., Sieczkowska H., Krzęcio E., Antosik K., Zybert A., Koćwin-Podsiadła M. & Kamiński S. (2011) The association between acyl-CoA synthetase (ACSL4)

polymorphism and intramuscular fat content in (Landrace × Yorkshire) × Duroc pigs. Meat Science 89 440-3.

Schultz N., Marenstein D., De Angelis D., Wang W., Nelander S., Jacobsen A., Marks D., Massagué J. & Sander C. (2011) Off-target effects dominate a large-scale RNAi screen for modulators of the TGF-b pathway and reveal microRNA regulation of TGFBR2. Silence 14, 3.

Sumitani S., Goya K., Testa J.R., Kouhara H. & Kasayama S. (2002) Akt1 and Akt2 Differently Regulate Muscle Creatine Kinase and Myogenin Gene Transcription in Insulin-Induced Differentiation of C2C12 Myoblasts. Endocrinology 143, 820-8.

Te Pas M.F.W., Cagnazzo M., De Wit A.A.C., Priem J., Pool M. & Davoli R. (2005) Muscle transcriptomes of Duroc and Pietrain pig breeds during prenatal formation of skeletal muscle tissue using microarray technology Archiv Tierzucht / Archives Animal Breeding 48, 141-7.

Te Pas M.F.W., Hulsegge I., Coster A., Pool M.H., Heuven H.H. & Janss L.L. (2007) Biochemical pathways analysis of microarray results: regulation of myogenesis in pigs. BMC Developmental Biology 7, 66.

Van Laere A.S., Nguyen M., Braunschweig M. et al. (2003) A regulatory mutation in IGF2 causes a major QTL effect on muscle growth in the pig. Nature 425, 832-6.

Williams A.H., Liu N., Rooij E.v. & Olson E.N. (2009) MicroRNA control of muscle development and disease. Current Opinion in Cell Biology 21, 461-9.

Wilson E.M. & Rotwein P. (2006) Control of MyoD Function during Initiation of Muscle Differentiation by an Autocrine Signaling Pathway Activated by Insulin-like Growth Factor-II. The Journal of Biological Chemistry 281, 29962-71.

Xie S.S., Huang T.H., Shen Y., Li X.Y., Zhang X.X., Zhu M.J., Qin H.Y. & Zhao S.H. (2010) Identification and characterization of microRNAs from porcine skeletal muscle. Animal Genetics 41, 179-90.

Zhou B., Liu H.L., Shi F.X. & Wang J.Y. (2010) MicroRNA expression profiles of porcine skeletal muscle. Animal Genetics 41, 499-508.

Supporting information

Additional supporting information may be found in the online version of this article.

http://onlinelibrary.wiley.com/doi/10.1111/age.12032/full

The following materials are available for this article:

Filename	Description
age12032-sup-0001-AppendixS1.pdf	**Appendix S1.** Supplemental methods
age12032-sup-0002-TableS1.pdf	**Table S1.** The primer sequences used in real-time RT-PCR
age12032-sup-0003-TableS2.pdf	**Table S2.** Differentially expressed probes identified in porcine LD muscle between DL and Pi breed types at 35 dpc
age12032-sup-0004-TableS3.pdf	**Table S3.** Differentially expressed probes identified in porcine LD muscle between DL and Pi breed types at 63 dpc
age12032-sup-0005-TableS4.pdf	**Table S4.** Differentially expressed probes identified in porcine LD muscle between DL and Pi breed types at 91 dpc
age12032-sup-0006-TableS5.pdf	**Table S5.** Differentially expressed probes identified in porcine LD muscle between DL and Pi breed types at adult stage
age12032-sup-0007-TableS6.pdf	**Table S6.** Up-regulated genes between DL and Pi breed types at each stage associated with significant IPA categories
age12032-sup-0008-TableS7.pdf	**Table S7.** Up-regulated genes between DL and Pi breed types at each stage associated with IPA top canonical
age12032-sup-0009-TableS8.pdf	**Table S8.** MicroRNA–mRNA interactions derived from IPA microRNA targets filter analysis in selected categories ‘cell growth and proliferation’ and ‘lipid metabolism’

CHAPTER 4

Correlated mRNAs and miRNAs from co-expression and regulatory networks affecting porcine muscle finally meat properties

Ponsuksili, Yang Du, Frieder Hadlich, Puntita Siengdee, Eduard Murani, Manfred Schwerin, and Klaus Wimmers

Published in: BMC Genomics (2013) 14:533-550
DOI: 10.1186/1471-2164-14-533.

Correlated mRNAs and miRNAs from co-expression and regulatory networks affecting porcine muscle finally meat properties

Abstract

BACKGROUND: Physiological processes aiding the conversion of muscle to meat involve many genes associated with muscle structure and metabolic processes. MicroRNAs regulate networks of genes to orchestrate cellular functions, in turn regulating phenotypes.

RESULTS: We applied weighted gene co-expression network analysis to identify co-expression modules that correlated to meat quality phenotypes and were highly enriched for genes involved in glucose metabolism, response to wounding, mitochondrial ribosome, mitochondrion, and extracellular matrix. Negative correlation of miRNA with mRNA and target prediction were used to select transcripts out of the modules of trait-associated mRNAs to further identify those genes that are correlated with post mortem traits.

CONCLUSIONS: Porcine muscle co-expression transcript networks that correlated to *post mortem* traits were identified. The integration of miRNA and mRNA expression analyses, as well as network analysis, enabled us to interpret the differentially-regulated genes from a systems perspective. Linking co-expression networks of transcripts and hierarchically organized pairs of miRNAs and mRNAs to meat properties yields new insight into several biological pathways underlying phenotype differences. These pathways may also be diagnostic for many myopathies, which are accompanied by deficient nutrient and oxygen supply of muscle fibres.

Introduction

Muscle is the major energy consuming and storage organ. An imbalance of nutrients, energy, and oxygen supply-and-demand in muscle cells is evident following cardiac muscle or skeletal muscle attack, injury, or damage. The consequences of these imbalances depend on muscle structure and metabolism and, thus, the muscle's entire complement of proteins and their expression patterns. Similar changes, i.e., termination of nutrient and energy supplies and anoxia, also occur in muscle cells *post mortem*. Indeed, these changes underlie the conversion of muscle to meat in food production. The physiological processes accompanying the change of muscle to meat involve expression of many genes associated with muscle structure and metabolic features [1,2]. Genes active in the muscle could therefore potentially have pathogenetic effects by disturbing muscle energy and oxygen homeostasis *in vivo,* as well as conferring traits related to meat quality *post mortem*.

Meat quality is complex and is affected by genetic and environmental factors as well as slaughtering procedures [3]. The conversion of muscle to meat is important not only as an economic factor in pork production, but also because these events mimic pathological processes associated with muscle injury or damage in humans. *Post mortem* traits for meat quality and carcass are influenced by a complex network of gene interactions in muscle; therefore, elucidating the relationships between genes and how these genes, in turn, influence meat quality and carcass traits is critical for developing a comprehensive understanding of the muscle to meat conversion as well as muscle pathologic processes including muscle atrophy, dystrophy, and hypoxia. Additionally, pigs share many genomic and physiological similarities with humans and, therefore, provide a good model to study the genetic determination of complex traits and as a biomedical model [4,5].

Recent advances in functional genomic screening, which can help determine molecular processes underlying phenotypic differences [6-8], have identified roles for microRNAs (miRNAs) in regulation of myogenesis [9-11] and adipogenesis [12-14]. miRNAs are small, non-coding RNA molecules of approximately 22 nucleotides. The primary miRNA transcript has a stem-loop structure that is recognized and cleaved via RNA processing enzymes to produce a double-stranded duplex. miRNAs target mRNA transcripts via base-pair complementarity, typically in the 3′ untranslated region [15,16], but also in the coding sequence [17]. This targeting can induce transcript

cleavage, degradation, destabilization, or repression of translation, thereby modulating protein levels. It has been recently shown that reduction of transcript level account for most of the regulatory, repressive effects of miRNAs [18]. Target genes that are regulated by miRNAs through degradation of their respective transcripts consequently show negative correlation of their mRNA with the miRNA regulator. Moreover, one miRNA can target several — even hundreds of — genes. Therefore, a unique approach for identifying miRNA-mRNA regulatory modules was recently introduced, whereby paired miRNA-mRNA expression profiles were constructed to predict putative target genes of miRNAs [19,20].

Many studies used the network analysis for dissecting the complex traits [21,22]. Weighted gene co-expression network analysis (WGCNA) [23] has been successfully applied in a variety of different settings [24-28]. WGCNA groups genes into modules based on their co-expression across a set of samples and finally relates these modules to the traits of interest in order to elucidate relevant modules or genes.

In order to identify groups of co-expressed genes (mRNAs) and on the hierarchically superior level of miRNAs that are correlated with organismal traits related to carcass and meat quality, we first applied weighted gene co-expression network analysis (WGCNA) and subsequently we adapted the paired expression profile approach. We identified co-expression networks regulated by miRNA after filtering of negatively-correlated miRNA-mRNA pairs and predicting target genes. The integration of miRNA and mRNA expression analyses as well as network analysis enabled us to interpret the differentially-regulated genes from a systems perspective, yielding new insight into several biological pathways underlying phenotypic differences.

Results

Meat quality and carcass traits phenotypes

Elucidating the relationships between genes and how these genes, in turn, influence muscle metabolic and structural properties is critical for developing a comprehensive understanding of the muscle to meat process as well as muscle pathologic and regenerative processes related to muscle atrophy, dystrophy and hypoxia. In total, 207 performance-tested crossbred pig [PI×(DL×DE)] samples were used to investigate meat quality and carcass traits. Descriptions of 7 carcass traits and 13 meat quality traits, as

well as means and standard deviations, analysed in this study are shown in Table 4.1. High correlation coefficients were found between the same biochemical and biophysical parameters measured at different positions and at different time points *post mortem*, like fatness traits or pH. The other cluster covers the traits drip loss, protein content, and conductivity. This cluster was negatively correlated to pH or fatness (supplementary Figure 4.1).

Table 4.1 Measured carcass and meat quality traits

Traits	Definitions of traits	Mean±SD (N=207)
loin eye area (LEA) [cm^2]	area of *M. longissimus dorsi* (Mld) at $13^{th}/14^{th}$ rib	52.96±5.7
fat area (FA) [cm^2]	fat area on Mld at $13^{th}/14^{th}$ rib	14.97±3.2
meat to fat ratio (MFR)	ratio of meat and fat area	0.29±0.1
fat depth at shoulder (FDS) [cm]	depth of fat and skin on muscle, mean of 3 measures at thickest point	3.44±0.4
fat depth at tenth rib (FDTR) [cm]	depth of fat and skin on muscle, mean of 3 measures at thinnest point	1.92±0.4
loin fat depth at loin (FDL) [cm]	depth of fat and skin on muscle, mean of 3 measures at thinnest point	1.34±0.4
average back fat (ABF) [cm]	mean value of shoulder fat depth, back fat tenth rib and loin fat depth	2.23±0.4
Drip loss (DL) %	% of weight loss of Mld collected at 24 h *post mortem*, held for 48 h at 4°C	5.37±2.2
LF24MLD	Conductivity in Mld at $13^{th}/14^{th}$ rib 24 h post mortem	5.35±2.2
LF45MLD	Conductivity in Mld at $13^{th}/14^{th}$ rib 45 min *post mortem*	4.98±1.6
Intramuscular fat content (MLDIMF) %	Intramuscular fat content of Mld at $13^{th}/14^{th}$ rib	0.79±0.4
Protein content (MLDP) %	Protein content of Mld at $13^{th}/14^{th}$ rib	23.65±0.5
Water content (MLDW) %	Water content of Mld at $13^{th}/14^{th}$ rib	74.7±0.6
Ash content (MLDA) %	Ash content of Mid at $13^{th}/14^{th}$ rib	1.06±0.1
meat colour (OPTO)	meat colour 24 h *post mortem* in Mld at $13^{th}/14^{th}$ rib; OPTO star	68.56±6.4
IMP24MLD	Impedance of Mld at 24 h *post mortem*	44.63±15.6
pH45MLD	pH value in Mld at $13^{th}/14^{th}$ rib 45 min *post mortem*	6.15±0.3
pH24MLD	pH value in Mld at $13^{th}/14^{th}$ rib 24 h *post mortem*	5.48±0.1
pH45MSM	pH value in *M. semimembranosus* (Msm) at 45 min *post mortem*	6.24±0.3
pH24MSM	pH value in *M. semimembranosus* (Msm) at 24 h *post mortem*	5.53±0.1

Gene co-expression network construction for mRNA

To investigate the role of transcriptional networks in muscle, we performed a weighted gene co-expression network analysis (WGCNA) using expression data from *M. longissimus dorsi* necropsies of the 207 performance-tested crossbred pigs [PI×(DL×DE)]. Expression analysis using GeneChip Porcine Genome Arrays (Affymetrix) containing 24,123 probe sets identified 11,191 probe sets with consistent expression according to MAS5 analysis; these were used for further analysis. Using WGCNA, residuals derived from the mixed-model analysis of expression levels of 11,191 probe sets were used for constructing the muscle transcriptional network.

WGCNA grouped genes into 22 modules based on patterns of co-expression. Each module was labelled with a unique color identifier and was characterized for enrichment of genes of specific gene ontology (GO) categories (Table 4.2). To represent the gene expression profiles of the highly correlated genes inside a given module, we used the first principal component, which is referred to as the module eigengene (ME). We tested each ME for correlation with meat and carcass traits.

Module-trait associations of mRNA

Sets of genes (modules) with common expression patterns that were associated with particular traits were identified based on the correlation between ME and organismal phenotype. We identified five modules that significantly associated with meat quality. Modules dark-turquoise and orange were correlated positively to pH traits and negatively to drip loss ($ME_{[dark\text{-}turquoise]}$: pH24MLD r=0.34, $p=5.3\times10^{-7}$, DL r=-0.19, $p=5.6\times10^{-3}$; $ME_{[orange]}$: pH24MLD r=0.32, $p=3.7\times10^{-6}$, DL r=-0.31, $p=5.8\times10^{-6}$) (Figure 1). Module dark-turquoise (31 annotated genes) was highly enriched for genes belonging to the cluster “glucose metabolic process” (GO: 0006006) and the KEGG-pathway “insulin signaling” with an enrichment score (ES) of 2.65. Module orange (26 annotated genes) was enriched for transcripts of the functional annotation clusters “response to wounding”, “defense response” and “inflammatory response” (ES=2.42). Modules red, black, and tan were correlated negatively to pH traits and positively to drip loss ($ME_{[red]}$: pH45MLD r=-0.22, $p=1.8\times10^{-3}$, DL r=0.20, $p=3.9\times10^{-3}$; $ME_{[black]}$: pH45MLD r=-.23, $p=8.8\times10^{-4}$, DL r=0.16, $p=1.8\times10^{-2}$; $ME_{[tan]}$: pH45MLD r=-0.22, $p=1.4\times10^{-3}$, DL r=0.19, $p=6.9\times10^{-3}$) (Figure 4.1).

Table 4.2 List of the top GO terms in the most significant DAVID functional clusters for each muscle network module

Module	Top term	No. of genes in ME	% count[1]	Top term *P-value*
blue	GO:0044451~nucleoplasm part	524	8.97	1.32E-11
light-green	GO:0044429~mitochondrial part	97	59.79	3.24E-54
dark-orange	GO:0006414~translational elongation	24	41.67	1.37E-14
grey60	GO:0030163~protein catabolic process	105	12.38	3.10E-04
magenta	GO:0046907~intracellular transport	208	10.58	3.43E-05
red	GO:0005761~mitochondrial ribosome	315	5.08	4.41E-16
black	GO:0005739~mitochondrion	436	27.06	4.88E-46
salmon	GO:0006414~translational elongation	137	45.99	4.93E-107
green	GO:0032446~protein modification by small protein conjugation	246	4.07	7.90E-05
dark-grey	GO:0030036~actin cytoskeleton organisation	37	16.22	8.94E-05
tan	GO:0031012~extracellular matrix	154	31.17	7.96E-39
midnightblue	GO:0042060~wound healing	122	7.38	5.38E-05
pink	GO:0000323~lytic vacuole	265	10.57	2.78E-16
dark-turquoise	GO:0006006~glucose metabolic process	31	16.13	3.00E-04
purple	GO:0006986~response to unfolded protein	143	6.38	8.28E-08
light-yellow	GO:0006954~inflammatory response	85	14.12	7.94E-07
orange	GO:0009611~response to wounding	26	23.08	1.37E-03
brown	GO:0031981~nuclear lumen	1436	15.48	2.72E-27
dark-red	GO:0031981~nuclear lumen	183	21.86	8.14E-10
cyan	GO:0044265~cellular macromolecule catabolic process	182	13.19	1.61E-06
dark-green	GO:0019941~modification-dependent protein catabolic process	38	13.16	2.93E-02
grey	GO:0008219~cell death	3616	23.84	7.09E-17

[1](number of genes in term/number of genes in ME)×100)

Further, modules red (315 annotated genes), black (436 annotated genes), and tan (154 annotated genes) were enriched for genes of the top functional annotation clusters of "mitochondrial ribosome", "mitochondrion", and "extracellular matrix" with ES of 10.23, 15.15, and 27.05, respectively. Only one module ($ME_{[dark\text{-}orange]}$) showed association with traits related to fatness (Figure 4.1).

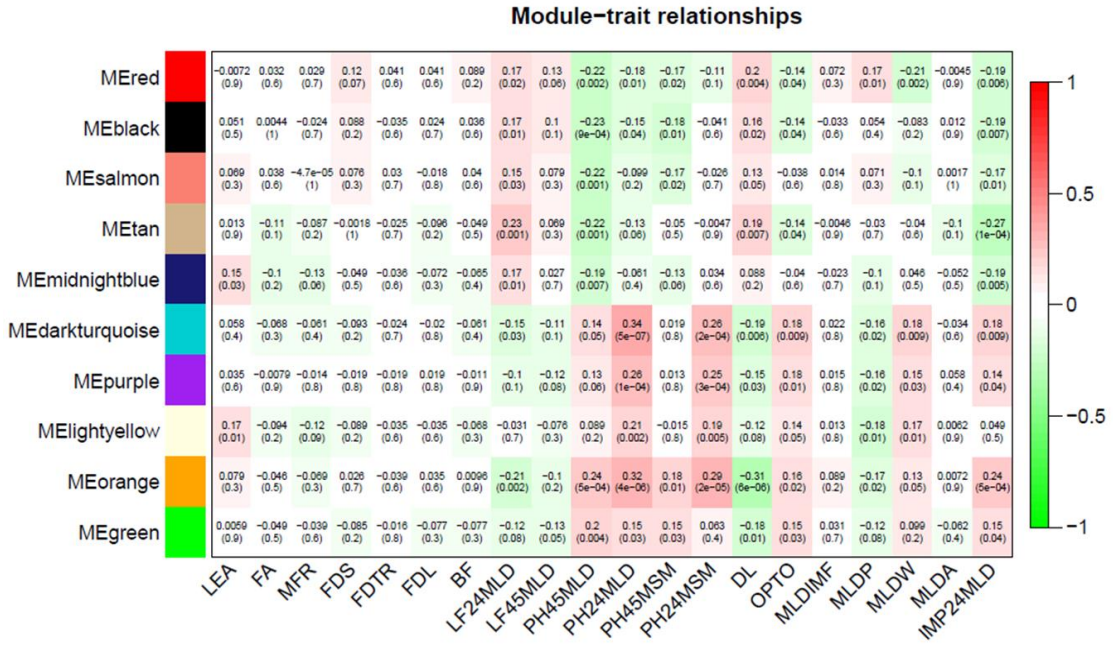

Figure 4.1: Correlation matrix of module eigengene values obtained for mRNAs and phenotypes. Weighted gene co-expression network analysis (WGCNA) groups genes into modules based on patterns of gene co-expression. Each of the modules was labelled with a unique color as an identifier. Twenty-two modules were identified; each module eigengene was tested for correlation with meat and carcass traits. Within each cell, upper values are correlation coefficients between module eigengene and the traits; lower values are the corresponding p-values.

Co-expression networks and module-trait associations for miRNA

Transcriptional networks of muscle microRNAs were studied with a WGCNA using miRNA expression data on *M. longissimus dorsi* from the same animals as above. Residuals, derived from the analysis of expression levels after correction for systematic effects according to the mixed model, were used for constructing the muscle miRNA transcriptional networks, i.e., modules. Nine modules were identified (Figure 4.2). Only 2 modules were associated with meat quality at a significance level of $p<0.05$. Module purple was correlated positively to LF24MLD at p=0.03 and negatively to pH24MLD and IMP24MLD at p=0.04 and p=0.03, respectively. Module purple consisted of 8 miRNA families (miR-17, miR-30. miR221, miR-185, miR-324, miR362, miR-500, and miR-542). Module blue was positively correlated to pH45MLD and IMP24MLD at p=0.02 and p=0.01, respectively. Module blue comprised 29 miRNA families (let-7, miR-15, miR-17, miR-31, miR-95, miR-103, miR-105, miR-122, miR-124, miR-130, miR-138, miR-154, miR-184, miR-185, miR-197, miR-202, miR-204, miR-212, miR-

214, miR-320, miR-326, miR-335, miR-346, miR-383, miR-467, miR-491, miR-744, miR-1224, and miR-1296).

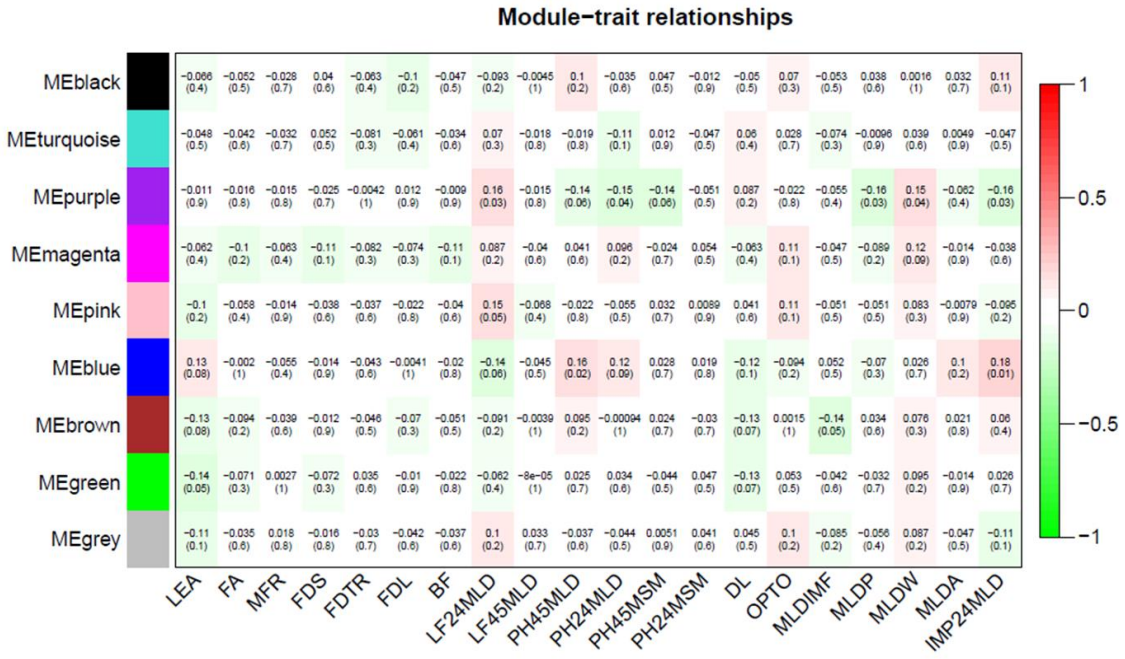

Figure 4.2: Correlation matrix of module eigengene values obtained for miRNAs and phenotypes. Weighted gene co-expression network analysis (WGCNA) groups miRNA into modules based on patterns of their co-expression. Each of the modules was labelled with a unique color as an identifier. Nine modules were identified; each module eigengene was tested for correlation with meat and carcass traits. Within each cell, upper values are correlation coefficients between module eigengene and the traits; lower values are the correspondent p-value.

Individual miRNA expression profiles and correlated traits

In addition to miRNA modules, the expression of 675 individual miRNAs probe sets, which corresponded to 513 unique sequences belonging to 159 miRNA families, were profiled and examined for correlation with meat and carcass traits. In total, 225 miRNA-phenotype pairs revealed correlations at $p<0.01$. Table 4.3 lists the top 20 miRNAs with highly significant correlations to phenotypes ($p<0.005$).

Table 4.3 Correlation coefficient of carcass and meat quality traits with abundance of individual miRNAs and the positions of miRNAs

Trait	miRNA_family	r	p-value	Chromosome	Position (bp)
DL	miR_184	-0.23	1.35E-03	7	5.38E+07
DL	miR_142	0.23	1.77E-03	12	3.59E+07
DL	miR_23	-0.21	4.42E-03	10	3.29E+07
DL	miR_181	-0.20	4.62E-03	10	2.74E+07
IMP24MLD	miR_217	-0.25	6.01E-04	3	8.87E+07
IMP24MLD	miR_184	0.25	6.41E-04	7	5.38E+07
IMP24MLD	miR_221	-0.24	8.12E-04	X	4.34E+07
LF24MLD	miR_221	0.25	4.49E-04	X	4.34E+07
MLDP	miR_185	-0.21	3.86E-03	14	5.58E+07
MLDIMF	miR_467	0.24	1.03E-03	9	1.29E+08
OPTO	miR_1827	0.24	1.03E-03	1	2.33E+08
PH24MLD	miR_133	-0.27	1.86E-04	6	9.92E+07
PH24MLD	miR_217	-0.26	2.36E-04	3	8.87E+07
PH24MLD	miR_181	0.25	4.74E-04	10	2.74E+07
PH24MLD	miR_130	-0.23	1.26E-03	2	1.31E+07
PH24MSM	miR_133	-0.24	8.24E-04	6	9.92E+07
PH24MSM	miR_363	0.23	1.39E-03	X	1.17E+08
FDS	miR_103	-0.24	6.65E-04	17	3.65E+07
FDS	miR_107	-0.24	7.63E-04	14	1.11E+08
FDS	miR_17	-0.24	8.61E-04	X	1.17E+08

Endogenous correlation of expression profiles between miRNA and mRNA

We performed pairwise correlation coefficient analysis to evaluate association of expression levels between 675 miRNA probe sets and 11,191 mRNA probe sets. Among the 7,553,925 Pearson correlation coefficients, we detected significant correlation in 5,933 miRNA-mRNA pairs at p-values $\leq 8.97 \times 10^{-5}$ (FDR=0.1). The 5,933 pairs comprised 408 miRNA probe sets belonging to 128 miRNA families that were correlated with 2,296 mRNA probe sets. Of these 5,933 pairs, 4,005 and 1,928 pairs showed positive and negative correlations, respectively. Positive correlations tended to be more dramatic than negative correlations; the correlation between miR-122 and VTN was the most significant (FDR=4.33×10^{-11}). The most significant negative correlation was between miR-154 and LOC387820 (FDR=1.1×10^{-5}). The most frequently involved miRNA family was miRNA-221, which was correlated with 616 mRNAs. In total 96 miRNA families showed significantly negative correlations with groups of up to 253 genes. We evaluated GO classification for each miRNA-correlated gene set of more than 50 genes (Table 4.4). The most striking findings were from gene sets that were negatively correlated with miR-23, miR-30, miR17, miR154, and miR-

132. For miR-23 and miR-17 the set of negatively correlated genes was highly enriched for genes belonging to the clusters "translation" (GO:0006412) and "translational elongation" (GO:0006414). The set of genes negatively correlated with miR-30 was enriched for "cytoskeletal protein binding" (GO:0008092). The set of genes negatively correlated with miR-154 was enriched for "threonine-type peptidase activity" (GO:0070003), while miR132-correlated genes were enriched for "proteasome complex" (GO:0000502).

Table 4.4 List of the top GO terms in the most significant DAVID functional clusters of genes negatively-correlated with the listed miRNAs.

miRNA	Top term	No. of genes[1]	% count[2]	Top term *P-value*
miR-23	GO:0006412~translation	253	6.14	2.25E-11
miR-30	GO:0008092~cytoskeletal protein binding	96	14.03	1.83E-03
miR-17	GO:0006414~translational elongation	87	24.69	1.60E-25
miR-154	GO:0070003~threonine-type peptidase activity	72	5.88	3.98E-05
miR-132	GO:0000502~proteasome complex	64	4.84	1.63E-02

[1]number of genes with negative correlation to the respective miRNAs

[2](number of genes in term/number of genes in ME)×100)

Integration of negative correlation of miRNA and mRNA with module-trait association and target prediction

A total of 1,928 pairs of miRNAs and mRNAs that showed negative correlations at p-values $\leq 8.97 \times 10^{-5}$ (FDR=0.1) belonged to 1,073 mRNA probe sets (929 gene) and 264 miRNA probe sets (96 miRNA families). Of these, 286 pairs were assigned to modules dark-turquoise, red, black, and tan, which showed correlation with traits related to meat quality (Figure 4.3). However, no genes in module orange were negatively correlated with miRNA at FDR < 0.1. Only one gene (*CREM*) in the module dark-turquoise was negatively correlated with miR-153 (r=-0.34 $p=2.11 \times 10^{-06}$ FDR=0.02). In module black, 69 genes were negatively correlated with 21 miRNA families, totaling 140 miRNA-mRNA pairs. In total, 101 and 43 pairs of miRNAs and mRNAs were identified in modules red (22 miRNAs and 52 genes) and tan (17

miRNAs and 26 genes). Known genes belonging to modules dark-turquoise, red, black, and tan and their negatively-correlated miRNAs are shown in Table 4.5.

Out of 1,928 pairs of miRNAs and mRNAs that showed negative correlations at p-values $\leq 8.97 \times 10^{-5}$ (FDR=0.1), 62 pairs were assigned to modules blue and purple, which correlated with meat quality (Figure 4.4). These 62 pairs of miRNAs and mRNAs belonged to 14 miRNA families and 40 genes (Table 4.6).

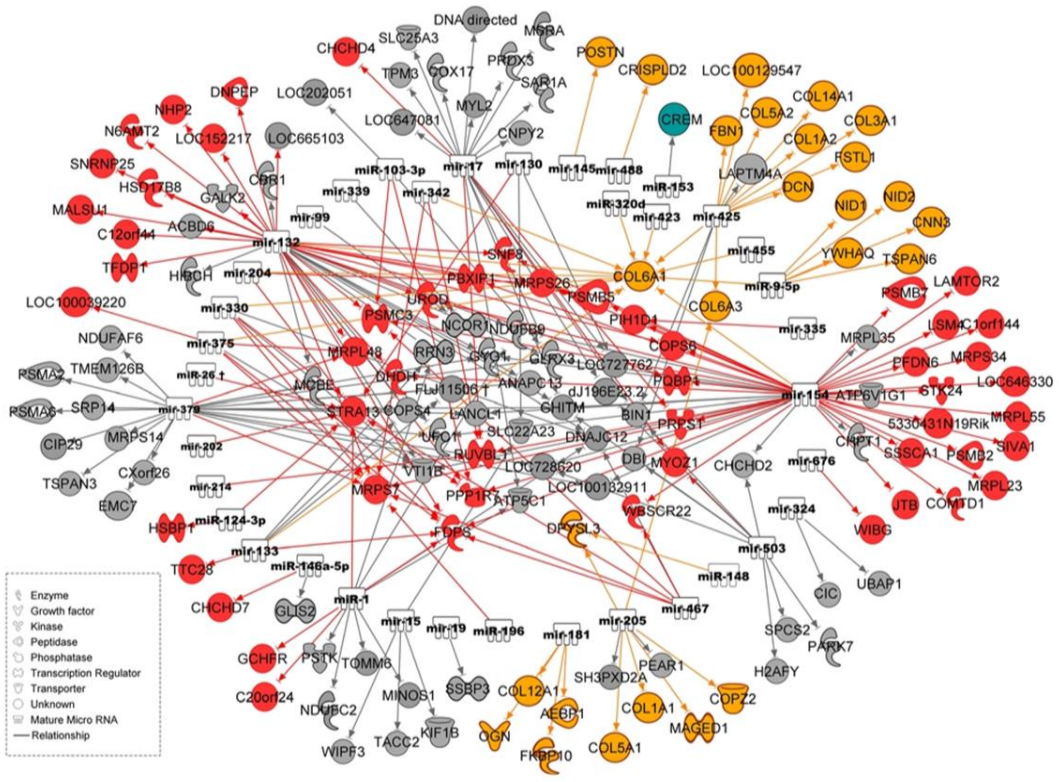

Figure 4.3: Regulatory network of negatively-correlated mRNAs and miRNAs. Genes in modules dark-turquoise, orange, red, black, and tan that were significantly associated with meat quality and were negatively correlated with various miRNAs as indicated by the arrows. Colors of symbols of mRNA encoded proteins indicate the assignment to the respective module (grey=black).

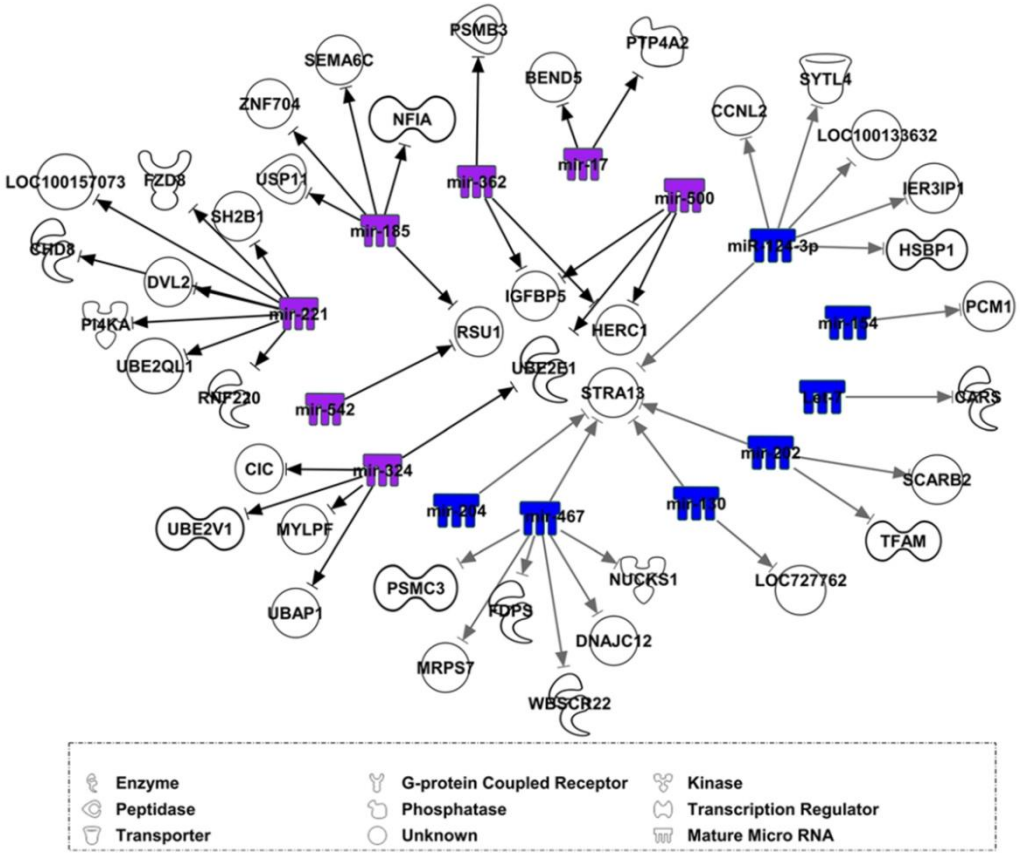

Figure 4.4: Regulatory network of negatively-correlated miRNAs and mRNAs. miRNAs in modules blue and purple that were significantly associated with meat quality and were negatively correlated with various mRNAs as indicated by the arrows. Colors of symbols of miRNAs indicate the assignment to the respective module.

Further, TargetScan and RNAhybrid were used to scan miRNA and mRNA sequences (porcine RefSeq), to obtain additional evidence for their functional link; these sequences corresponded to 1,928 pairs of negatively-correlated miRNAs and mRNAs. In total, 474 pairs of miRNA and mRNA were confirmed by either of the two *in silico* prediction methods: 331, 195, and 32 miRNA-mRNA pairs were predicted by RNAhybrid, TargetScan, or both methods, respectively. The 474 miRNA-mRNA pairs covered 121 probe sets of miRNAs (65 miRNA families) and 331 targets probe sets (297 genes). When focusing on genes of the trait-correlated modules dark-turquoise, orange, red, black, and tan, 73 out 286 pairs of miRNAs and mRNAs were confirmed with at least one *in silico* method. These 73 pairs comprised 26 miRNA families and 51 genes (Tables 4.5 and 4.6 in bold).

Table 4.5 Genes belonging to the modules dark-turquoise, red, black, and tan and their negatively-correlated miRNAs

Modules	miRNA family	Genes within modules negatively correlated with miRNA[1]
Dark-turquoise	miR-153	*CREM*
Black	miR-1	*NDUFB9, ATP5C1, DNAJC12, NDUFC2, TOMM6,* ***RRN3****, PSTK, C1orf151*
	miR-103	*GLRX3, LOC202051*
	miR-130	***SLC22A23****, LOC727762*
	miR-132	*NDUFB9, ACBD6, GHITM, DNAJC12, GLRX3,* ***CBR1****, ANAPC13, RRN3, HIBCH, MCEE, GYG1, UFC1, COPS4, GALK2, LOC665103,* ***VTI1B***
	miR-133	***RRN3****, COPS4, FLJ11506*
	miR-146	*GLIS2*
	miR-15	***KIF1B****, TACC2, Wipf3, SLC22A23*
	miR-154	*NDUFB9,* ***GHITM****, GLRX3, LOC100132911, CHPT1, GYG1, UFC1, LANCL1, LOC728620, ATP6V1G1, MRPL35, VTI1B, CHCHD2*
	miR-17	*SLC25A3, PRDX3, GHITM,* ***SAR1A****, ATP5C1, COX17, BIN1, GLRX3, MYL2,* ***TPM3****, LOC647081, CNPY2,* ***dJ196E23.2****,* ***LOC727762****, MSRA*
	miR-181	
	miR-19	*SSBP3*
	miR-205	*SH3PXD2A, PEAR1*
	miR-324	***CIC****,* ***UBAP1***
	miR-330	*DNAJC12,* ***VTI1B***
	miR-339	***Ncor1***
	miR-375	***VTI1B***
	miR-379	*C15orf24, GHITM, CIP29, TSPAN3,* ***GLRX3****,* ***PSMA2****, ANAPC13, C8orf38, SRP14, CXorf26, LOC100132911, MRPS14, MCEE, GYG1, UFC1,* ***LANCL1****,* ***LOC728620****, COPS4,* ***PSMA6****, FLJ11506, VTI1B, TMEM126B*
	miR-425	*BIN1, DBI, LAPTM4A*
	miR-467	*DNAJC12*
	miR-503	*SPCS2, GHITM,* ***BIN1****, PARK7, DBI, LOC728620,* ***H2AFY****,* ***dJ196E23.2,*** ***CHCHD2***
	miR-99	*Ncor1*
Red	miR-1	*FDPS, GCHFR, C20orf24, STRA13*
	miR-103	*PPP1R7,* ***PSMC3***
	miR-124	***HSBP1, STRA13***
	miR-130	***STRA13***
	miR-132	***SNRNP25****,* ***PPP1R7****, PSMC3,* ***FDPS****, N6AMT2, MRPS26, PSMB5, Snf8,* ***DHDH****, COPS6, HSD17B8, PQBP1, MRPS7, C7orf30, TFDP1, NHP2,* ***LOC152217****, PBXIP1, DNPEP,* ***C12orf44****,* ***UROD****,* ***MRPL48***
	miR-133	*FDPS,* ***TTC28***
	miR-146	*CHCHD7*
	miR-154	***PSMB2****, WBSCR22, PPP1R7,* ***PSMC3****, LSM4, FDPS, PIH1D1, MRPS26. 5330431N19Rik, PSMB5, Snf8,* ***JTB****, DHDH, COPS6,* ***PSMB7****, LOC646330, MRPS34,* ***PQBP1****,* ***SIVA1****,* ***MRPS7****, STK24, RUVBL1, MRPL23, PFDN6, SSSCA1, PBXIP1, PRPS1, MRPL55, COMTD1, UROD, ROBLD3, C1orf144*
	miR-17	*MYOZ1, CHCHD4, RUVBL1, PRPS1*
	miR-196	*MRPS7*
	miR-202	*STRA13*
	miR-204	*MRPS26,* ***STRA13***
	miR-214	***RUVBL1***
	miR-26	*STRA13*
	miR-330	***PPP1R7****,* ***FDPS****,* ***MRPS7***
	miR-335	*PIH1D1*

Red	miR-342	***MRPS7***, *RUVBL1*
(continued)	miR-375	***FDPS***, ***LOC100039220***, ***MRPS7***, *MRPL48*
	miR-379	*MRPS7, MRPL48*
	miR-467	*WBSCR22, PSMC3, FDPS, MRPS7, STRA13, STRA13*
	miR-503	*MYOZ1*
	miR-676	*WIBG*
tan	miR-132	***COL6A1***
	miR-133	*COL6A1*
	miR-145	***POSTN***
	miR-148	***DPYSL3***
	miR-154	*COL6A1*
	miR-181	*COL12A1, FKBP10,* ***Aebp1****, OGN*
	miR-204	*COL6A1*
	miR-205	***COL1A1***, *COL6A3,* ***DPYSL3***, ***MAGED1***, *DPYSL3,* ***COPZ2***, *COL5A1*
	miR-320	***COL6A1***
	miR-330	***COL6A1***
	miR-342	*COL6A1*
	miR-375	***COL6A1***
	miR-423	***COL6A1***
	miR-425	*DCN, COL3A1,* ***FBN1****, COL6A3, LOC100129547, COL5A2, COL1A2,* ***COL6A1****, FSTL1,* ***COL14A1***
	miR-455	*COL6A1*
	miR-488	*CRISPLD2*
	miR-9	***NID2****, COL6A1, YWHAQ, CNN3, NID1, TSPAN6*

[1]genes predicted as potential targets of the corresponding miRNAs are shown in bold.

Table 4.6 MicroRNAs belonging to modules blue and purple and their negatively-correlated genes

Modules	**miRNA family**	**Genes within modules negatively correlated with miRNA[1]**
Blue	Let-7	***CARS***
	miR-124	***STRA13***, *IER3IP1,* ***HSBP1***, ***CCNL2***, *LOC100133632, SYTL4*
	miR-130	***STRA13,*** *LOC727762*
	miR-154	*PCM1*
	miR-202	*SCARB2, STRA13, TFAM*
	miR-204	***STRA13***
	miR-467	*STRA13, MRPS7, STRA13, NUCKS1, PSMC3, FDPS, DNAJC12, WBSCR22*
Purple	miR-17	*BEND5, PTP4A2, BEND5*
	miR-185	*SEMA6C, Nfia, RSU1, USP11, SEMA6C,* ***ZNF704***
	miR-221	*RNF220, CHD8, PI4KA,* ***FLJ25076****, DVL2,* ***FZD8****,* ***Sh2b1****, LOC100157073*
	miR-324	***CIC***, ***UBE2E1***, ***UBE2V1***, ***MYLPF***, ***UBAP1***
	miR-362	***IGFBP5****, HERC1, PSMB3*
	miR-500	***IGFBP5****, UBE2E1, HERC1*
	miR-542	*RSU1*

[1]genes predicted as potential targets of the corresponding miRNAs are shown in bold.

Discussion

Here, we present an integrative approach to identify transcriptomic differences that may contribute to variation of the kinetics of metabolic processes under diminished oxygen and nutrition supply that is evident during muscle conversion to meat. The speed and extend of the switch from aerobic to anaerobic ATP production, until final total failure of energy production, and of protein degradation processes largely affect meat quality [29]. In order to identify functional networks of genes contributing to these processes an approach was used based on a multi-level integration of weighted gene co-expression network analysis (WGCNA) of mRNA and miRNA with mRNA-miRNA pair correlation and miRNA target prediction.

mRNA abundance and co-expression networks linked to muscle and meat properties

We used transcriptional network analysis to identify co-expression modules (dark-turquoise, orange, red, black, and tan) that correlated to meat quality phenotype. These modules were highly enriched for genes involved in “extracellular matrix”, “glucose metabolic process”, and "mitochondrion” (“oxidative phosphorylation” KEGG_PATHWAY); i.e. processes affecting structural and metabolic properties, oxidative phosphorylation that depends on oxygen supply. When oxygen is limited (*post mortem* or during prolonged vigorous exercise) the glucose metabolism occurs by anaerobic respiration, a process that is independent of the mitochondria. A shift from aerobic to anaerobic metabolism - favouring the production of lactic acid - results in a pH decline *post mortem* and thereby influence the meat quality [29]. So the biological process of mitochondria as well as the way of glucose metabolism play a significant role in the muscle cell and finally impact on meat quality. Indeed, mitochondrial dysfunction resulting in decreased cellular energy production is also responsible for a variety of human myopathies and cardiomyopathies [30-33].

Genes assigned to the GO category of “extracellular matrix” encode proteins belonging to the myofibrillar scaffold. The characteristics of the myofibrillar scaffold and the kinetics of their fragmentation were associated with tenderness and water-holding capacity of meat. In particular, the proteolysis of muscle proteins affects the shrinkage of myofibrils, the development of pores in the cell membranes, so called drip channels, and the non-covalent binding of water molecules [34,35]. Collagens are major

constituents of the extracellular matrix (ECM). In our study there were many collagen genes that are reported to be correlated with various muscle disorders [36-39]. For example, collagen type VI (COLVI), an important component of skeletal muscle ECM, is involved in maintaining tissue integrity [40]. *Col6a1*$^{-/-}$ mice show a complete absence of collagen VI chains and display a myopathic phenotype, abnormal mitochondria, and increased apoptosis of muscle fibres [41,42].

miRNA abundance and co-expression networks linked to muscle and meat properties

Our finding of the relevance of mitochondrial metabolic pathways, including oxidative phosphorylation, and muscle structural protein composition to *post mortem* processes affecting meat quality is in line with our previous results obtained in other populations [1,6,7]. Additionally, while the previous studies focused on trait-associated mRNA expression, here another hierarchical level in the regulatory network relevant to processes occurring under conditions of insufficient oxygen, energy, and nutrient supplies is provided. In fact, miRNA was integrated into this study as a regulator molecule of muscle transcripts. miRNAs with identical seed sequences (the same family) [43] or that are closely located on the same chromosome (the same cluster) [44,45] have similar expression trends. This was confirmed in our study, where most of the modules consisted of the same families of miRNAs or miRNAs located on the same chromosome. In this study, marginal association of miRNA co-expression modules to organismal traits was found compared to mRNA co-expression modules. On the one hand, co-expressed miRNAs of the same family or cluster might not regulate the same trait. On the other hand, this may be caused by indirect regulation of organismal traits by miRNAs via their effect on mRNA transcripts. Accordingly, individual miRNA correlation to phenotypes was also considered. Recent studies have revealed key roles for miRNAs in the regulation of skeletal muscle differentiation, and changes in miRNA expression are associated with various skeletal muscle disorders [46-48]. In this study, several miRNAs were correlated with carcass and meat quality traits. This includes miR-221, previously identified in studies of myotube maturation and in the maintenance of the myofibrillar organization [49] and found to contribute to muscle pathogenetic mechanisms [50]. Interestingly, miR-133, which showed highest correlation with pH24MLD and pH24MSM, has been widely studied for roles in the regulation of

skeletal muscle development, including in proliferation and myogenesis [10,51] as well as muscle disorders [47]. Recently, a study reported that mice with genetic deletions of miR-133a-1 and miR-133a-2 developed adult-onset centronuclear myopathy in type II (fast-twitch) myofibres, which was accompanied by impaired mitochondrial function, fast-to-slow myofibre conversion, and disarray of muscle triads [52]. These are changes of muscle structure and metabolism that also impact meat quality. In addition to its well established role in translation (table 4), miR-23a was also recently identified as a key regulator of skeletal muscle differentiation and is predicted to target multiple adult fast myosin heavy chain (Myh) genes, including *Myh1*, *2*, and *4* [53]. For fat traits, miR-103 and miR-107 were highly correlated (table 3). This is consistent with previous reports of miR-103 being involved in adipogenesis, lipid metabolism, and adipocyte differentiation [54,55] and of miR-103/107 being involved in glucose homeostasis and insulin sensitivity [56]. In this study we showed for the first time more complex correlations among miRNAs and between miRNAs and *post mortem* organismal phenotypes in swine, while also confirming previous studies in human and mouse muscle as well as C2C12 myoblasts.

Links between miRNA and mRNA that relate to muscle and meat properties

This study also sought to evaluate to what extent the co-expression modules of trait-associated mRNAs are themselves regulated by miRNAs. A regulatory link between miRNA and mRNA and a functional link to the organismal phenotype was suspected if (1) the mRNA belonged to either one of the co-expression modules associated with the traits (i.e., $ME_{dark\text{-}turquoise}$, ME_{orange}, ME_{red}, and ME_{tan}), (2) mRNA abundance was significantly negatively correlated with its miRNA regulator, and (3) the mRNA was predicted to be a target gene of the respective miRNA. Therefore, RefSeqs of the genes with present calls from the 3´-IVT-Affymetrix arrays were explored to predict the targets of miRNAs by either seed sequence complementarity [57] or by thermodynamics-based modeling of RNA:RNA duplex interactions [58]. Currently, no publicly-accessible database covers porcine miRNAs and their predicted target genes. Moreover, annotation of porcine genes is not yet finalized. Accordingly, the target predictions should be interpreted with caution.

Interestingly, no miRNA regulator was identified by negative correlation or target prediction in the module orange, which was enriched for genes related to “response to

wounding", "inflammatory response", and "defense response". Genes assigned to biofunctions related to response to exogenes stimuli, change their transcription rate immediately due to many factors. As suggested by finding no correlated miRNAs – their regulation of transcription obviously occurs without major involvement of miRNA. However, many genes in the module orange were previously confirmed as transcriptional regulators in myogenesis or were located in QTL regions for muscle fibre traits like BTG2, EGR1, ANKRDS1 and FOS [59,60]. Interestingly, the genes in module orange like Egr1, FOS and JUN that are associated with oxidative stress response were found upregulated in muscle in response to mechanical ventilation and immobilization in a porcine model for critical illness myopathy (CIM) [61,62].

Co-expressed genes in module dark-turquoise were significantly associated with meat quality and based on the current knowledge of gene functions some links among them are suggested to be relevant For example, one member of this module, *CREM*, is a transcription factor binding to cAMP-responsive elements (CREs) in the promoters of various genes. This transcription factor plays important roles in various organismal functions [63-67]. Crem inactivation or knockout has been shown to increase the rate of apoptosis in testis tissue [68,69]. The main cellular change associated with apoptosis processes also occur during post-mortem [70]. Post mortem biochemical processes in muscle lead to pH decline. A high expression of CREM being positively correlated with pH at 24 hours, may indicate a slowdown of apoptosis related post mortem processes paralleling anaerobic metabolic processes that led to a decrease of pH. This indicates that the abundance of CREM transcripts in muscle plays a significant role in meat quality. Further, CREM and miR-153 were highly negatively correlated (FDR<0.01) which is known to induce apoptosis in a glioblastoma cell line DBTRG-05MG [71]. Thus a consistent link of affetcs on apoptosis and mRNA-miRNA interaction is obvious. miR-153 also inhibits the protein kinase B (PKB/Akt) pathway by reducing the protein level of insulin receptor substrate-2 (IRS2) [72].As recently shown, miR-135a targets IRS2 levels by binding to its 3'UTR and this interaction regulates skeletal muscle insulin signaling [73]. Insulin signalling plays a pivotal role in the regulation of glucose uptake by skeletal muscle [74]. The glucose uptake in skeletal muscle has a large effects on meat characteristics [75,76]. In our study, Irs-2 also belonged to module dark-turquoise and was negatively correlated with waterholding capacity related traits like DL (p=0.004) and positively with pH (pH24MLD, p=7.05E-07; pH24MSM,

p=1.30E-03; pH45MLD, p=3.39E-03). Thus another plausible functional link of members of the module dark-turquioseand miRNAs can be shown.

Supply energy for physiological functions and play a significant role in the regulation of other cellular events including apoptosis, calcium homeostasis, and production of reactive oxygen species. Mitochondrial metabolism is affected by miRNA regulation [77]. Here, we found many miRNAs being negatively correlated to target genes of modules red, black, which were enriched for genes related to mitochondrial pathways. Indeed, miR-338 modulates energy metabolism, oxidative phosphorylation, and mitochondrial functions [78,79], and miR-15b, -16, -195, and -424 decrease cellular ATP levels in cardiomyocytes [80]. Additionally, miR-181c can enter and target the mitochondrial genome, ultimately causing electron transport chain complex IV remodeling and mitochondrial dysfunction [81]. Here we found miR-181 was correlated with DL and pH24MLD as well as S100 calcium binding protein A6 (S100A6). Further, mitochondrial genes like *Kif1b*, *Atp6v1g1*, *Atp5c1*, *Park7*, *Chchd2*, *Ruvbl1*, *Mrps7*, and *Mrpl48* were highly negatively correlated with, and some of them were predicted as targets of, miR-15, -154, -17, -503, -214, -330, -342, and -375.

Many genes of module tan were assigned in the GO category “extracellular matrix” including *Col1a1*, *Col1a2*, *Col3a1*, *Col5a1*, *Col5a2*, *Col6a1*, *Col6a3*, *Col12a1*, *Col14a1*, *Crispld2*, *Ddn*, *Fbn1*, *Nid1*, *Nid2*, *Ogn*, and *Postn*. *These genes* were negatively correlated with, and some of them were predicted as targets of, miRNA. Interestingly, *Col6a1* was found as a target for many miRNAs including miR-132, miR-205, miR-320, miR-330, miR-375, miR-423, and miR-425. Moreover, miR-205 was identified as a regulator of *Col1a1*, *Maged1*, and *Dpysl3*. Gandellini et al. (2012) [82] reported that miR-205 controls the deposition of laminin-332 and its receptor integrin-β4 as well as participates in a network involving ΔNp63α, which is essential for maintenance of the basement membrane in prostate epithelium. Similarly, other miRNAs, including miR-29, miR-133, and miR-30, are involved in the regulation of development and maintenance of extracellular matrix of bone and muscle [83,84].

Much evidence suggests that a group of miRNAs (cluster and family) may contribute to the regulation of a set of common targets [46,85,86], and are, therefore, associated with phenotypes. WGCNA was used here to group miRNA products and revealed 2 modules associated with meat quality (purple and blue). The miRNA from these modules were negatively correlated to mRNAs, and some of these were predicted as targets. Most

miRNAs in module purple were related to genes in the categories "ubiquitin" or "protein catabolic process" (*Ube2e1, Ube2b1, Ubap1, Igfbp5, Herc1, Psmb3, Flj25076*). Differential expression of genes of the ubiquitin system depending on muscle and meat quality was previously shown [1]. Recently association of genes of the ubiquitin system with meat quality was shown [87]. In particular, miR-324 was highly negatively correlated and predicted to target *Ube2e1, Ube2v1, Bap1, Lpf* and *Cic*.

Most previous muscle mRNA and miRNA expression studies focused on cardiac muscle or skeletal muscle injury [50,88]. In these injuries, the degree of damage results from an imbalance of energy, nutrients, and oxygen supply-and-demand in muscle cells. Similarly, nutrient, energy, and oxygen depletion occurs *post mortem*. Many changes in expression associated with muscle injury would therefore overlap with *post mortem* processes in conversion of muscle to meat, and vice versa. In this regard, functional annotation of mRNA co-expression and trait-correlated expression identified key *post mortem* pathways and functions, including glucose metabolic process, mitochondrial metabolic pathways, and muscle structural components, involved in muscle-to-meat conversion that will be relevant to muscle injury as well.

In this study, for the first time, expression and co-expression of miRNAs functioning as a fine-tuning of mRNA transcription and translation was integrated with mRNA transcript abundance measures and phenotypic data on meat quality. By this an additional hierarchical level, i.e. miRNA affecting mRNA, was considered in the molecular regulation of muscle-to-meat conversion. miRNAs are necessary for proper skeletal and cardiac muscle development and function, and have a profound influence on multiple myopathies, such as hypertrophy, dystrophy, and conduction defects. Consequently, an expression biomarker panel (whether from mRNA or miRNA) derived from this study may not only be predictive for quality of meat *post mortem*, but also for many muscle pathologic processes including muscle atrophy, dystrophy, and hypoxia [89,90]. The abundance of mRNAs and their fine-tuning by corresponding miRNAs in molecular pathways related to mitochondrial metabolic balance and oxidative stress, cell proliferation and differentiation, as well as muscle structural protein composition play an important role in these myopathies and meat maturation. Functional studies of the interactions among and between mRNAs and miRNAs will provide additional experimental data for validation of the relationships on the level of mRNAs, miRNAs and organismal phenotype that were stressed in this study.

Material and Methods

Animals, tissue collection, and phenotyping

Animal care and tissue collection procedures followed the guidelines of the German Law of Animal Protection, and the experimental protocol was approved by the Animal Care Committee of the FBN. This study was based on trait measurement and expression profile association analyses done with 207 performance-tested pigs from commercial herds of the crossbreed Pietrain × (German Large White × German Landrace). Animals were raised and slaughtered under standardized conditions in the experimental facilities of the Leibniz Institute for Farm Animal Biology (FBN). Sample collection was performed thoroughly after exsanguination, tissue samples were rapidly dissected, snap-frozen in liquid nitrogen and stored at -80 ˚C. The average age of the pigs at sampling was ~180 days. Technological parameters of meat quality, i.e., pH-value, conductivity, and colour, were measured by using Star-series equipment (Rudolf Matthaeus Company, Germany). Measures of pH and conductivity were at 45 min *post mortem* (pH45) and 24 h *post mortem* (pH24), in both *M. longissimus dorsi* between $13^{th}/14^{th}$ rib (pH45MLD, pH24MLD, LF45MLD, LF24MLD) and the ham (*M. semimembranosus*) (symbol: pH24MSM, LF24MSM). Muscle colour was measured at 24 h *post mortem* by Opto-Star (Matthaeus, Klausa, Germany). Drip loss was scored based on a bag method with a size-standardized sample from the *M. longissimus dorsi* collected at 24 h *post mortem* and weighed, suspended in a plastic bag, held at 4 °C for 48 h, and re-weighed [91]. To determine cooking loss, a loin cube was taken from the *M. longissimus dorsi*, weighed, placed in a polyethylene bag, and incubated in water at 75 °C for 50 minutes. The bag was then immersed in flowing water at room temperature for 30 minutes, and the solid portion was re-weighed. Thawing loss was determined similarly after at least 24 h freezing at -20 °C. Drip loss, cooking loss, and thawing loss were calculated as a percentage of weight loss based on the start weight of a sample. Shear force was measured using Instron-4310 equipment, and average values of four replicates were used for analyses.

Customized miRNA microarrays design

Our custom porcine miRNA array was designed from 284 pig miRNAs obtained from the miRBASE (miRBase 14.0). Because miRNAs are highly conserved between

closely-related species [10], we could predict novel porcine miRNA candidates by inter-species alignments requiring 100% mature miRNA similarity. Accordingly, we used previously known miRNA sequences from humans and mice, as well as other species, to perform BLAST searches against the porcine genome database porcine; 391 miRNA candidates were identified. In total, 675 miRNAs probe sets, corresponding to 513 unique sequences belonging to 159 miRNA families, were used for hybridisation with the target samples described above. Microarray data related to all samples were deposited in the Gene Expression Omnibus public repository (GEO accession number: GSE41294: GSM1013731-GSM1013920).

Customized microarrays, pre-processing, and normalization of miRNA

Total miRNA was isolated with Qiagen miReasy Mini kit and RNeasy MinElute Clean up kit (Qiagen, Hilden, Germany) according to manufacturer's protocol for small RNA. Quality and quantity of isolated total RNA and miRNA were determined using an Agilent 2100 Bioanalyzer for RNA (Agilent Technologies, Santa Clara, CA). Affymetrix customized microarrays from our porcine miRNA candidate dataset were used. Targets for hybridisation were prepared from miRNA with the FlashTag™ Biotin RNA Labeling Kit for Affymetrix GeneChip miRNA arrays (Genisphere, Hatfield, PA, USA) according to manufacturer’s recommendations. Briefly, 250 ng of miRNA of each individual were poly(A)-tailed using ATP–poly-A-Polymerase, then FlashTag Biotin end-labelled. After hybridisation of biotin-labelled complementary RNA, chips were washed and processed to detect biotin-containing transcripts by Streptavidin-PE (Phycoerythrin) conjugate, then were scanned on GeneChip scanner 3000 7G (Affymetrix, Santa Clara, US). Data were extracted from the images, and spots were quantified and processed by quality filtering. Hybridization quality was assessed in all samples by using JMP Genomics 5 utilizing Robust Multi-array Average (RMA) background correction and log2 transformations. To acquire the expression value, data were normalized between chips using the quantile normalization method.

Whole-genome expression profiling (mRNA)

Gene expression profiling of *M. longissimus dorsi* samples of pigs was conducted with the same animals (207) as for miRNA. In brief, total RNA was isolated using TRI Reagent (Sigma, Taufkirchen, Germany) and used for target preparation for microarray hybridisation. According to Affymetrix protocols, 500 ng of total RNA were reverse-transcribed into cDNA, transcribed into cRNA, and labelled using Affymetrix One cycle synthesis and labelling kit (Affymetrix, UK) to prepare antisense biotinylated RNA targets. Quality of hybridisation was assessed in all samples following manufacturer's recommendations. Data were analysed with the Affymetrix GCOS 1.1.1 software, using global scaling to a target signal of 500. Data were processed with MAS5.0 to generate cell intensity files (present or absent). Quantitative expression levels of present transcripts were estimated using the PLIER algorithm (Probe Logarithmic Intensity Error) for normalization that was implemented in Expression Console (Affymetrix). Based on BLAST comparison of the Affymetrix porcine target sequences with the porcine genome sequence (Ensembl_Sscrofa_10), 20,689 of the 24,123 probe sets on the Affymetrix Porcine GeneChip were localized and annotated [92,93]. Microarray data related to all samples were deposited in the Gene Expression Omnibus public repository [GEO accession number: GSE32112: GSM796045-GSM796251].

Pre-processing of phenotype and expression data

Phenotypes and expression levels were adjusted for systematic effects by analysis of variance performed with the procedure "Mixed" of the SAS software package (SAS version 9.1 SAS Institute, Cary, NC) before analysing their correlation and by using co-expression network. Sex and RYR genotype was used as a fixed effect, "sire" and "slaughter day" as random effects, and "carcass weight" as a covariate. Subsequently, the residuals of log2-transformed expression intensities (miRNA and mRNA) and muscle phenotype were used for further analysis. Pearson correlation of miRNA expression level and gene expression level was calculated using 190 individuals; correction for multiple testing was done by controlling the FDR level (q-value according to Storey and Tibshirani, 2003, [94]) at 10%.

Weighted Gene Co-expression Network Analysis (WGCNA)

A weighted gene co-expression network was constructed for 207 muscle biopsies using the blockwise Modules function from the WGCNA package in R [23]. Residuals of gene expression, after correcting the effect, were used for WGCNA. The blockwise Modules function allows the entire dataset of 11,191 probe sets by mRNA and 675 miRNA to be utilised in the construction of the weighted gene co-expression network.

Extremely outlying individuals were removed from the following analysis based on hierarchically clustered using the average linkage function, and common Euclidean distance. Pearson correlation matrix of all gene-gene comparisons were calculated across all microarrays. Adjacency matrix was then calculated using the correlation matrix of the expression sets. Finally, the Topological Overlap Matrix (TOM) was converted from the adjacency matrix and used to derive a TOM-based distance matrix for the hierarchical clustering of expressions. In the next step, modules of expression profiles, (i.e. sets of genes with high topological overlap) were formed based on hierarchical clustering, with empirically specified minimal module size (30 for gene expression, 10 for miRNA). According to the WGCNA methodology, rather than traditional distance or correlation based similarity measures, it utilizes the topological overlap matrix $\Omega = [\omega_{ij}]$ (TOM),

$$\omega_{ij} = \frac{a_{ij} + \sum_u a_{iu} a_{uj}}{\min\{\sum_u a_{iu}, \sum_u a_{ju}\} + 1 - a_{ij}} \quad , \quad a_{ij} = |cor(x_i, x_j)|^{\beta}$$

where x_i and x_j are the gene expression profiles of the *i-th* and *j-th* gene and α_{ij} is the adjacency. TOM based distance matrix is a robust and powerful measurement in building co-expression network. Selection of appropriate value for the power ß were derived according to the pickSoftThresholding function of the WGCNA package [23,95]. Accordingly, by manually inspecting the fit of the scale free topology model with the candidate β-values for each set of expression profile, minimal β-values giving a coefficient of determination R^2 higher than 90% were adopted.

Modules were further merged based on the dissimilarity between their "eigengenes", which were defined as the first principal component of each module. Genes that were not assigned to another module were assigned to the grey module. A threshold of 0.2 for the dissimilarity as recommended by the WGCNA author was used. Module–trait associations were estimated using the correlation between the module eigengene and the phenotype, which allows easy identification of expression set (module) highly

correlated to the phenotype. For each expression profile, Gene Significance (GS) was calculated as the absolute value of the correlation between expression profile and each trait; module membership (MM) was defined as the correlation of expression profile and each module eigengene, enabling further identification of key players in the regulation network.

Gene ontology and pathway enrichment analysis

We performed a gene ontology (GO) enrichment analysis for network modules using the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/ [96,97]. Each analysis was performed using the functional annotation clustering option. Functional annotation clustering combines single categories with a significant overlap in gene content and assigns an enrichment score (ES, defined as the -log10 of the geometric mean of unadjusted *p-value*s for each single term in the cluster) to each cluster, making interpretation of the results more straightforward. To assess the significance of functional clusters, we created 22 sets of 11,191 probe sets corresponding to 8,036 genes (size of the average module identified in this study).

Predicting porcine targets of miRNAs by RNA hybrid and TargetScan

We used two methods to predict the targets of porcine miRNA. First, we predicted targets using the computational software RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid), which detects the most energetically favourable hybridisation sites of a small RNA within a large RNA [98]. Here, we tested the miRNA probe sets with the following parameters: number of hits per target = 1, energy cutoff = −25 kcal/mol, and maximal internal or bulge loop size per side = 4. Most targets found were located on the 3′-UTR of genes. Second, TargetScan (http://www.targetscan.org) was used to detect target gene candidates based on seed complementarity on UTR database 6.0 and our porcine RefSeq transcript with our miRNA seed sequence [99]. TargetScan was applied considering both conserved and non-conserved targets. The porcine RefSeq transcripts, which derived from 11,191 probe sets that showed consistent expression in porcine muscle, were used as input targets for RNAhybrid and TargetScan.

Authors' Contributions

SP and KW conceived the study. SP analysed the microarray data and drafted the manuscript; YD made the WGCNA; FH helped in target prediction analysis. PS, EM, and MS helped in sampling and data collection and drafting the manuscript; KW discussed and contributed to data interpretation and helped in drafting the manuscript.

Acknowledgements

The authors thank Annette Jugert and Joana Bittner for excellent technical help. This research was supported by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG; FOR 753).

Additional file 1: Figure S1

Dendrogram representing the correlation coefficients between meat quality and carcass traits.

http://www.biomedcentral.com/content/supplementary/1471-2164-14-533-S1.pdf

References of chapter 4

1. Ponsuksili S, Murani E, Phatsara C, Schwerin M, Schellander K, Wimmers K: Porcine muscle sensory attributes associate with major changes in gene networks involving CAPZB, ANKRD1, and CTBP2. Funct Integr Genomics 2009, 9:455-471.

2. Ponsuksili S, Murani E, Phatsara C, Schwerin M, Schellander K, Wimmers K: Expression quantitative trait loci analysis of genes in porcine muscle by quantitative real time RT-PCR compared to microarray data. Heredity 2010, 105(3)309-317.

3. Sellier P: The future role of molecular genetics in the control of meat production and meat quality. Meat Sci 1994, 36(1-2):29-44.

4. Bode G, Clausing P, Gervais F, Loegsted J, Luft J, Nogues V, Sims J. The utility of the minipig as an animal model in regulatory toxicology. J Pharmacol Toxicol Methods 2010, 62(3):196–220.

5. Groenen MA, Archibald AL, Uenishi H, Tuggle CK, Takeuchi Y, Rothschild MF, Rogel-Gaillard C, Park C, Milan D, Megens HJ, et al.: Analyses of pig genomes provide insight into porcine demography and evolution. Nature 2012, 491(7424):393-398.

6. Ponsuksili S, Jonas E, Murani E, Phatsara C, Srikanchai T, Walz C, Schwerin M, Schellander K, Wimmers K: Trait correlated expression combined with expression QTL analysis reveals biological pathways and candidate genes affecting water holding capacity of muscle. BMC Genomics 2008a, 9:367.

7. Ponsuksili S, Murani E, Phatsara C, Jonas E, Walz C, Schwerin M, Schellander K, Wimmers K: Expression Profiling of Muscle Reveals Transcripts Differentially Expressed in Muscle That Affect Water-Holding Capacity of Pork. J Agric Food Chem 2008b, 56:10311-10317.

8. Ponsuksili S, Murani E, Brand B, Schwerin M, Wimmers K: Integrating expression profiling and whole-genome association for dissection of fat traits in a porcine model. J Lipid Res 2011, 52(4):668-678.

9. Callis TE, Deng Z, Chen JF, Wang DZ: Muscling through the microRNA world. Experimental Biology and Medicine 2008, 233(2):131–138.

10. Huang TH, Zhu MJ, Li XY, Zhao SH: Discovery of Porcine microRNAs and Profiling from Skeletal Muscle Tissues during Development. PLoS One 2008, 3(9):e3225.

11. van Rooij E, Liu N, Olson EN: MicroRNAs flex their muscles. Trends in Genetics 2008, 24(4):159–166.

12. Walden TB, Timmons JA, Keller P, Nedergaard J, Cannon B: Distinct expression of muscle-specific microRNAs (myomirs) in brown adipocytes. J Cell Physiol 2009, 218(2):444–449.

13. Xie H, Lim B, Lodish HF: MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity. Diabetes 2009, 58(5):1050-1057.

14. Ortega FJ, Moreno-Navarrete JM, Pardo G, Sabater M, Hummel M, Ferrer A, Rodriguez-Hermosa JI, Ruiz B, Ricart W, Peral B, Fernández-Real JM: MiRNA expression profile of human subcutaneous adipose and during adipocyte differentiation. PLoS One 2010, 5(2):e9022.

15. Kim VN, Han J, Siomi MC: Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 2009, 10(2):126–139.

16. Winter J, Jung S, Keller S, Gregory RI, Diederichs S: Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol 2009, 11(3):228–234.

17. Fang Z, Rajewsky N: The impact of miRNA target sites in coding sequences and in 3′UTRs. PLoS ONE 2011, 6(3):e18067.

18 Guo H, Ingolia NT, Weissman JS, Bartel DP: Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature 2010, 466(7308):835-840.

19. Peng X, Li Y, Walters KA, Rosenzweig ER, Lederer SL, Aicher LD, Proll S, Katze MG: Computational identification of hepatitis C virus associated microRNA-mRNA regulatory modules in human livers. BMC Genomics 2009, 10:373.

20. Liu H, Brannon AR, Reddy AR, Alexe G, Seiler MW, Arreola A, Oza JH, Yao M, Juan D, Liou LS, Ganesan S, Levine AJ, Rathmell WK, Bhanot GV: Identifying mRNA targets of microRNA dysregulated in cancer: with application to clear cell Renal Cell Carcinoma. BMC Syst Biol 2010, 4:51.

21 Chen Y, Zhu J, Lum PY, Yang X, Pinto S, MacNeil DJ, Zhang C, Lamb J, Edwards S, Sieberts SK, et al.: Variations in DNA elucidate molecular networks that cause disease. Nature. 2008, 452:429-435.

22 Gargalovic PS, Imura M, Zhang B, Gharavi NM, Clark MJ, Pagnon J, Yang WP, He A, Truong A, Patel S, et al.: Identification of inflammatory gene modules based

on variations of human endothelial cell responses to oxidized lipids. Proc Natl Acad Sci U S A. 2006, 103:12741-12746.

23. Langfelder P, Horvath S: WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics 2008, 9(1):559.

24. Saris CG, Horvath S, van Vught PW, van Es MA, Blauw HM, Fuller TF, Langfelder P, DeYoung J, Wokke JH, Veldink JH, van den Berg LH, Ophoff RA: Weighted gene co-expression network analysis of the peripheral blood from Amyotrophic Lateral Sclerosis patients. BMC Genomics 2009, 10:405.

25. Plaisier CL, Horvath S, Huertas-Vazquez A, Cruz-Bautista I, Herrera MF, Tusie-Luna T, Aguilar-Salinas C, Pajukanta P: A systems genetics approach implicates USF1, FADS3, and other causal candidate genes for familial combined hyperlipidemia. PLoS Genet 2009, 5(9):e1000642.

26. Farber CR: Identification of a gene module associated with BMD through the integration of network analysis and genome-wide association data. J Bone Miner Res 2010, 25(11):2359-2367.

27. DiLeo MV, Strahan GD, den Bakker M, Hoekenga OA: Weighted correlation network analysis (WGCNA) applied to the tomato fruit metabolome. PLoS One 2011, 6(10):e26683.

28. Lancu OD, Darakjian P, Malmanger B, Walter NA, McWeeney S, Hitzemann R: Gene networks and haloperidol-induced catalepsy. Genes Brain Behav 2012, 11(1):29-37.

29 Rosenvold K, Andersen HJ: Factors of significance, for pork quality - a review. Meat science 2003, 64:219-237.

30. Figueiredo PA, Mota MP, Appell HJ, Duarte JA: The role of mitochondria in aging of skeletal muscle. Biogerontology 2008, 9(2):67-84.

31. Romanello V, Guadagnin E, Gomes L, Roder I, Sandri C, Petersen Y, Milan G, Masiero E, Del Piccolo P, Foretz M, Scorrano L, Rudolf R, Sandri M: Mitochondrial fission and remodelling contributes to muscle atrophy. EMBO J 2010, 29(10):1774-1785.

32. Dorn GW: Mitochondrial dynamics in heart disease. Biochim Biophys Acta 2012, PMID: 22450031.

33. Irwin MH, Parameshwaran K, Pinkert CA: Mouse models of mitochondrial complex I dysfunction. Int J Biochem Cell Biol 2012, PMID: 22903069.

34 Bee G, Anderson AL, Lonergan SM, Huff-Lonergan E: Rate and extent of pH decline affect proteolysis of cytoskeletal proteins and water-holding capacity in pork. Meat Sci 2007, 76(2):359-365.

35 Huff-Lonergan E, Lonergan SM: Mechanisms of water-holding capacity of meat: The role of postmortem biochemical and structural changes. Meat Sci 2005, 71(1):194-204.

36 Camacho Vanegas O, Bertini E, Zhang RZ, Petrini S, Minosse C, Sabatelli P, Giusti B, Chu ML, Pepe G: Ullrich scleroatonic muscular dystrophy is caused by recessive mutations in collagen type VI. Proc Natl Acad Sci USA 2001, 98(13):7516-7521.

37. Lampe AK, Bushby KM: Collagen VI related muscle disorders. J Med Genet 2005, 42(9):673-685.

38. Merlini L, Martoni E, Grumati P, Sabatelli P, Squarzoni S, Urciuolo A, Ferlini A, Gualandi F, Bonaldo P: Autosomal recessive myosclerosis myopathy is a collagen VI disorder. Neurology 2008, 71(16):1245-1253.

39. Gualandi F, Urciuolo A, Martoni E, Sabatelli P, Squarzoni S, Bovolenta M, Messina S, Mercuri E, Franchella A, Ferlini A, Bonaldo P, Merlini L: Autosomal recessive Bethlem myopathy. Neurology 2009, 73(22):1883-1891.

40. Allamand V, Briñas L, Richard P, Stojkovic T, Quijano-Roy S, Bonne G. ColVI myopathies: where do we stand, where do we go? Skelet Muscle 2011, 23(1):30.

41. Irwin WA, Bergamin N, Sabatelli P, Reggiani C, Megighian A, Merlini L, Braghetta P, Columbaro M, Volpin D, Bressan GM, Bernardi P, Bonaldo P: Mitochondrial dysfunction and apoptosis in myopathic mice with collagen VI deficiency. Nat Genet 2003, 35(4):367-371.

42. Grumati P, Coletto L, Sabatelli P, Cescon M, Angelin A, Bertaggia E, Blaauw B, Urciuolo A, Tiepolo T, Merlini L, Maraldi NM, Bernardi P, Sandri M, Bonaldo P: Autophagy is defective in collagen VI muscular dystrophies, and its reactivation rescues myofiber degeneration. Nat Med 2010, 16(11):1313–1320.

43. Friedman RC, Farh KKH, Burge CB, Bartel DP: Most mammalian mrnas are conserved targets of micrornas. Genome Res 2009, 19(1): 92-105.

44. Altuvia Y, Landgraf P, Lithwick G, Elefant N, Pfeffer S, Aravin A, Brownstein MJ, Tuschl T, Margalit H: Clustering and conservation patterns of human microRNAs. Nucleic Acids Res 2005; 33(8): 2697–2706.

45. Tanzer A, Stadler PF: Molecular evolution of a microrna cluster. J Mol Biol 2004, 339(2): 327-335.

46. Williams AH, Liu N, van Rooij E, Olson EN: Micro¬RNA control of muscle development and disease. Curr Opin Cell Biol 2009, 21(3):461–469.

47. Eisenberg I, Eran A, Nishino I, Moggio M, Lamperti C, Amato AA, Lidov HG, Kang PB, North KN, Mitrani-Rosenbaum S, Flanigan KM, Neely LA, Whitney D, Beggs AH, Kohane IS, Kunkel LM: Distinctive patterns of microRNA expression in primary muscular disorders. Proc Natl Acad Sci USA 2007, 104(43):17016–17021.

48. Eisenberg I, Alexander MS, Kunkel LM: miRNAS in normal and diseased skeletal muscle. J Cell Mol Med 2009, 13(1):2–11.

49. Cardinali B, Castellani L, Fasanaro P, Basso A, Alema S, Martelli F, Falcone G: Microrna-221 and microrna-222 modulate differentiation and maturation of skeletal muscle cells. PLoS One 2009, 4(10):e7607.

50. Greco S, Perfetti A, Fasanaro P, Cardani R, Capogrossi MC, Meola G, Martelli F: Deregulated MicroRNAs in Myotonic Dystrophy Type 2. PLoS One 2012, 7(6):e39732.

51. Xie SS, Huang TH, Shen Y, Li XY, Zhang XX, Zhu MJ, Qin HY, Zhao SH: Identification and characterization of microRNAs from porcine skeletal muscle. Anim Genet 2010, 41(2): 179-190.

52. Liu N, Bezprozvannaya S, Shelton JM, Frisard MI, Hulver MW, McMillan RP, Wu Y, Voelker KA, Grange RW, Richardson JA, Bassel-Duby R, Olson EN: Mice lacking microRNA 133a develop dynamin 2–dependent centronuclear myopathy. J Clin Invest 2011, 121(8):3258-3268.

53. Wang L, Chen X, Zheng Y, Li F, Lu Z, Chen C, Liu J, Wang Y, Peng Y, Shen Z, Gao J, Zhu M, Chen H: MiR-23a inhibits myogenic differentiation through down regulation of fast myosin heavy chain isoforms. Exp Cell Res 2012, 318(18):2324-2334.

54. Li G, Wu Z, Li X, Ning X, Li Y, Yang G: Biological role of microRNA-103 based on expression profile and target genes analysis in pigs. Mol Biol Rep 2011, 38(7):4777-4786.

55. McGregor RA, Choi MS: microRNAs in the regulation of adipogenesis and obesity. Curr Mol Med 2011, 11(4):304-316.

56. Trajkovski M, Hausser J, Soutschek J, Bhat B, Akin A, Zavolan M, Heim MH, Stoffel M: MicroRNAs 103 and 107 regulate insulin sensitivity. Nature 2011, 474(7353):649-653.

57. Grimson A, Farh KKH, Johnston KW, Garrett-Engele P, Lim LP, Bartel DP: MicroRNA Targeting Specificity in Mammals: Determinants beyond Seed Pairing. Molecular Cell 2007, 27(1):91-105.

58. Mazière P, Enright AJ: Prediction of microRNA targets. Drug Discovery Today 2007, 12(11–12):452–458.

59. Rajan S, Chu Pham Dang H, Djambazian H, Zuzan H, Fedyshyn Y, Ketela T, Moffat J, Hudson TJ, Sladek R: Analysis of early C2C12 myogenesis identifies stably and differentially expressed transcriptional regulators whose knock-down inhibits myoblast differentiation. Physiol Genomics 2012, 44(2):183-197.

60. Reiner G, Heinricy L, Müller E, Geldermann H, Dzapo V: Indications of associations of the porcine FOS proto-oncogene with skeletal muscle fibre traits. Anim Genet 2002, 33(1):49-55.

61. Banduseela VC, Ochala J, Chen YW, Göransson H, Norman H, Radell P, Eriksson LI, Hoffman EP, Larsson L: Gene expression and muscle fiber function in a porcine ICU model. Physiol Genomics 2009, 39(3):141-159.

62. Banduseela VC, Chen YW, Göransson Kultima H, Norman HS, Aare S, Radell P, Eriksson LI, Hoffman EP, Larsson L: Impaired autophagy, chaperone expression and protein synthesis in response to critical illness interventions in porcine skeletal muscle. Physiol Genomics 2013, PMID:23572537.

63. Don J, Stelzer G: The expanding family of CREB/CREM transcription factors that are involved with spermatogenesis. Mol Cell Endocrinol 2002, 187(1-2):115-124.

64. Müller FU, Lewin G, Baba HA, Bokník P, Fabritz L, Kirchhefer U, Kirchhof P, Loser K, Matus M, Neumann J, Riemann B, Schmitz W: Heart-directed expression of a human cardiac isoform of cAMP-response element modulator in transgenic mice. J Biol Chem 2005, 280(8):6906-6914.

65. Wu X, Jin W, Liu X, Fu H, Gong P, Xu J, Cui G, Ni Y, Ke K, Gao Z, Gao Y: Cyclic AMP response element modulator-1 (CREM-1) involves in neuronal apoptosis after traumatic brain injury. J Mol Neurosci 2012, 47(2):357-367.

66. YT, Wang Y, Solomou EE, Li Y, Mawrin C, Tenbrock K, Kyttaris VC, Tsokos GC: Systemic lupus erythematosus serum IgG increases CREM binding to the IL-2

promoter and suppresses IL-2 production through CaMKIV. J Clin Invest 2005, 115(4):996-1005.

67. Xu WD, Zhang YJ, Wang W, Li R, Pan HF, Ye DQ: Role of CREM in systemic lupus erythematosus. Cell Immunol 2012, 276(1-2):10-15.

68. Nantel F, Monaco L, Foulkes NS, Masquilier D, LeMeur M, Henriksén K, Dierich A, Parvinen M, Sassone-Corsi P, Spermiogenesis deficiency and germ-cell apoptosis in CREM-mutant mice. Nature 1996, 380(6570):159-162.

69. Kosir R, Juvan P, Perse M, Budefeld T, Majdic G, Fink M, Sassone-Corsi P, Rozman D. Novel insights into the downstream pathways and targets controlled by transcription factors CREM in the testis. PLoS One 2012; 7(2):e31798.

70. Lonergan EH, Zhang W, Lonergan SM: Biochemistry of postmortem muscle - Lessons on mechanisms of meat tenderization. Meat Sci 2010, 86(1): 184–195.

71. Xu J, Liao X, Wong C: Downregulations of B-cell lymphoma 2 and myeloid cell leukemia sequence 1 by microRNA 153 induce apoptosis in a glioblastoma cell line DBTRG-05MG. Int J Cancer 2010, 126(4):1029-1035.

72. Xu J, Liao X, Lu N, Liu W, Wong CW: Chromatin-modifying drugs induce miRNA-153 expression to suppress Irs-2 in glioblastoma cell lines. Int J Cancer 2011, 129(10):2527-2531.

73. Agarwal P, Srivastava R, Srivastava AK, Ali S, Datta M: miR-135a targets IRS2 and regulates insulin signaling and glucose uptake in the diabetic gastrocnemius skeletal muscle. Biochim Biophys Acta 2013, 1832(8):1294-303.

74. Kubota T, Kubota N, Kumagai H, Yamaguchi S, Kozono H, Takahashi T, Inoue M, Itoh S, Takamoto I, Sasako T, Kumagai K, Kawai T, Hashimoto S, Kobayashi T, Sato M, Tokuyama K, Nishimura S, Tsunoda M, Ide T, Murakami K, Yamazaki T, Ezaki O, Kawamura K, Masuda H, Moroi M, Sugi K, Oike Y, Shimokawa H, Yanagihara N, Tsutsui M, Terauchi Y, Tobe K, Nagai R, Kamata K, Inoue K, Kodama T, Ueki K, Kadowaki T: Impaired insulin signaling in endothelial cells reduces insulin-induced glucose uptake by skeletal muscle. Cell Metab 2011, 13(3):294-307.

75. Milan D, Jeon JT, Looft C, Amarger V, Robic A, Thelander M, Rogel-Gaillard C, Paul S, Iannuccelli N, Rask L, Ronne H, Lundström K, Reinsch N, Gellin J, Kalm E, Roy PL, Chardon P, Andersson L: A mutation in PRKAG3 associated with excess glycogen content in pig skeletal muscle. Science 2000, 288(5469):1248-51.

76. Andersson L: Identification and characterization of AMPK gamma 3 mutations in the pig. Biochem Soc Trans 2003, 31(Pt 1):232-235.

77. Li P, Jiao J, Gao G: Prabhakar BS. Control of mitochondrial activity by miRNAs. J Cell Biochem 2012, 113(4):1104-1110.

78. Aschrafi A, Schwechter AD, Mameza MG, Natera-Naranjo O, Gioio AE, Kaplan BB: MicroRNA-338 regulates local cytochrome c oxidase IV mRNA levels and oxidative phosphorylation in the axons of sympathetic neurons. J Neurosci 2008, 28(47):12581–12590.

79. Aschrafi A, Kar AN, Natera-Naranjo O, Macgibeny MA, Gioio AE, Kaplan BB: MicroRNA-338 regulates the axonal expression of multiple nuclear-encoded mitochondrial mRNAs encoding subunits of the oxidative phosphorylation machinery. Cell Mol Life Sci 2012, PMID:22773120

80. Nishi H, Ono K, Iwanaga Y, Horie T, Nagao K, Takemura G, Kinoshita M, Kuwabara Y, Mori RT, Hasegawa K, Kita T, Kimura T: MicroRNA-15b modulates cellular ATP levels and degenerates mitochondria via Arl2 in neonatal rat cardiac myocytes. J Biol Chem 2010, 285(7):4920–4930.

81. Das S, Ferlito M, Kent OA, Fox-Talbot K, Wang R, Liu D, Raghavachari N, Yang Y, Wheelan SJ, Murphy E, Steenbergen C: Nuclear miRNA regulates the mitochondrial genome in the heart. Circ Res 2012, 110(12):1596-1603.

82. Gandellini P, Profumo V, Casamichele A, Fenderico N, Borrelli S, Petrovich G, Santilli G, Callari M, Colecchia M, Pozzi S, De Cesare M, Folini M, Valdagni R, Mantovani R, Zaffaroni N: miR-205 regulates basement membrane deposition in human prostate: implications for cancer development. Cell Death Differ 2012, PMID: 22555458.

83. Li Z, Hassan MQ, Jafferji M, Aqeilan RI, Garzon R, Croce CM, van Wijnen AJ, Stein JL, Stein GS, Lian JB: Biological functions of miR-29b contribute to positive regulation of osteoblast differentiation. J Biol Chem 2009, 284(23):15676-1584.

84. Duisters RF, Tijsen AJ, Schroen B, Leenders JJ, Lentink V, van der Made I, Herias V, van Leeuwen RE, Schellings MW, Barenbrug P, Maessen JG, Heymans S, Pinto YM, Creemers EE: miR-133 and miR-30 regulate connective tissue growth factor: implications for a role of microRNAs in myocardial matrix remodeling. Circ Res 2009, 104(2):170-178.

85. Carraro G, El-Hashash A, Guidolin D, Tiozzo C, Turcatel G, Young BM, De Langhe SP, Bellusci S, Shi W, Parnigotto PP, Warburton D: Mir-17 family of microrna controls fgf10-mediated embryonic lung epithelial branching morphogenesis through mapk14 and stat3 regulation of e-cadherin distribution. Dev Biol 2009, 333(2):238-250.

86. Lee YB, Bantounas I, Lee DY, Phylactou L, Caldwell MA, Uney JB: Twist-1 regulates the mir-199a/214 cluster during development. Nucleic Acids Res 2009, 37(1):123–128.

87 Huynh TP, Muráni E, Maak S, Ponsuksili S, Wimmers K: UBE3B and ZRANB1 polymorphisms and transcript abundance are associated with water holding capacity of porcine M. longissimus dorsi. Meat Sci 2013, 95(2):166-172.

88. Nielsen S, Scheele C, Yfanti C, Akerström T, Nielsen AR, Pedersen BK, Laye MJ: Muscle specific microRNAs are regulated by endurance exercise in human skeletal muscle. J Physiol 2010, 588(Pt 20):4029-4037.

89. Zhang P, Chen X, Fan M: Signaling mechanisms involved in disuse muscle atrophy. Med Hypotheses 2007, 69(2):310–321.

90. Jackman RW, Kandarian SC: The molecular basis of skeletal muscle atrophy. Am J Physiol Cell Physiol 2004, 287(4):C834–843.

91. Honikel KO: Wasserbindungsvermögen von Fleisch. Mitteilungsblatt der BAFF. 1986, 6:7150-7154.

92. Naraballobh W, Chomdej S, Murani E, Wimmers K, Ponsuksili S: Annotation and in silico localization of the Affymetrix GeneChip Porcine Genome Array. Arch Tierz 2010, 53: 230-238.

93. Ponsuksili S, Du Y, Murani E, Schwerin M, Wimmers K: Elucidating molecular networks that either affect or respond to plasma cortisol concentration in target tissues of liver and muscle. Genetics 2012, 192(3):1109-22.

94. Storey JD, Tibshirani R. Statistical significance for genome-wide experiments. PNAS 2003,100: 9440-9445.

95. Zhang B, Horvath S: A general framework for weighted gene co-expression network analysis. Stat Appl Genet Mol Biol 2005, 4:17.

96. Huang DW, Sherman BT, Lempicki RA: Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 2009a, 37(1):1-13.

97. Huang DW, Sherman BT, Lempicki RA: Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. Nature Protoc 2009b, 4(1):44-57.

98. Rehmsmeier M, Steffen P, Höchsmann M, Giegerich R: Fast and effective prediction of microRNA/target duplexes. RNA 2004, 10:1507-1517.

99. Lewis BP, Burge CB, Bartel DP: Conserved Seed Pairing, Often Flanked by Adenosines, Indicates that Thousands of Human Genes are MicroRNA Targets. Cell 2005, 120:15-20.

CHAPTER 5

MicroRNAs regulate cellular ATP levels by targeting mitochondrial energy metabolism genes during C2C12 myoblast differentiation

Puntita Siengdee, Nares Trakooljul, Eduard Murani, Manfred Schwerin, Klaus Wimmers, Siriluck Ponsuksili

Manuscript under minor revision (*PLOS ONE*)

MicroRNAs regulate cellular ATP levels by targeting mitochondrial energy metabolism genes during C2C12 myoblast differentiation

Abstract

MicroRNAs (miRNAs) regulate various biological pathways through miRNA-dependent networks. In a previous study, we identified an miRNA regulatory network involved in energy metabolism in porcine muscle. To better understand the involvement of miRNAs in cellular ATP production and energy metabolism, here we used C2C12 myoblasts, in which ATP levels increase during differentiation, to identify miRNAs modulating these processes. Correlation analyses were performed between ATP level and miRNA and mRNA microarray expression profiles during C2C12 differentiation into myotubes. The results suggest 14 miRNAs (miR-423-3p, miR-17, miR-130b, miR-301a/b, miR-345, miR-15a, miR-16a, miR-128, miR-615, miR-1968, miR-1a/b, and miR-194) as cellular ATP regulators targeting genes involved in mitochondrial energy metabolism (*Cox4i2, Cox6a2, Ndufb7, Ndufs4, Ndufs5,* and *Ndufv1*) during C2C12 differentiation. Among these, miR-423-3p showed a high inverse correlation with increased ATP level. Besides, it has been implicated in promoting cell growth and cell cycle progression, yet its function in cellular ATP regulation is unknown. Therefore, miR-423-3p was selected for a functional validation together with 3 of its potential target genes, Cox6a2, Ndufb7, Ndufs5, which also have predicted miRNA-423-3p binding sites on the 3'-UTR. Overexpression of synthetic miR-423-3p mimics in C2C12 myogenic differentiation produced decreased cellular ATP level and decreased expression of *Cox6a2, Ndufb7, Ndufs5* compared to the negative control. These results identify miR-423-3p as a novel regulator of ATP/energy metabolism via its targeting of *Cox6a2, Ndufb7,* and *Ndufs5.*

Keywords: C2C12, myogenesis, microRNA, miRNA-423-3p, ATP

Introduction

MicroRNAs (miRNAs) are evolutionarily conserved, short (~22 nucleotides), single-stranded, non-coding RNA molecules that regulate gene expression often by degrading or repressing translation of target mRNAs. miRNAs contain a 5' "seed sequence" (nucleotide positions 2-8) used to predict binding sites on the 3'-UTR of target genes; however, the entire miRNA sequence can influence its binding affinity and effects. Functional miRNAs undergo biogenesis pathways including transcription, pre-processing, maturation, and, finally, formation of the RNA-induced silencing complex (RISC) [1-4]. Mature miRNAs play important roles in regulating of diverse biological processes including cellular development, differentiation, growth, proliferation, apoptosis, and metabolism [5-8]. Further, accumulating evidence indicates that miRNAs are involved in numerous regulatory networks. For example, estrogen-related receptor γ (ERRγ) establishes a nuclear receptor/miRNA regulatory network dictating the fibre-type composition in muscle [9]. The involvement of miRNAs in many cellular processes implies a likely contribution to energy metabolism. Energy metabolism produces ATP, the energy source vital to cellular functions, through several pathways including oxidative phosphorylation in the mitochondria. We previously identified an miRNA-dependent regulatory network implicated in energy metabolism in porcine muscle [10]. However, knowledge on which miRNAs are involved in ATP production/energy metabolism remains scarce, and functional validation is needed to confirm the contribution of miRNAs to these processes.

Here, we used a myogenesis model to study miRNAs regulating cellular energy metabolism. Myogenesis is a multistep pathway in which myoblasts form from mesodermal precursor cells, withdraw from the cell cycle, express muscle-specific genes, and, eventually, differentiate into myotubes [11, 12]. To identify miRNAs that can modulate expression of genes involved in energy metabolism, we performed correlation analyses between ATP levels and both miRNA and mRNA expression profiles during the C2C12 murine myotube differentiation. The resulting list of ATP/miRNA/mRNA correlation interactions revealed several candidate miRNAs, from which miR-423-3p was selected for functional validation.

Experimental Procedures

Cell culture

Mouse skeletal muscle C2C12 myoblasts passage no. 7–9 (ATCC® CRL1772™) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 1 g/L glucose and supplemented with 10% FBS (PAA). At 80–90% confluence, myogenic differentiation was induced by switching to 2% (horse) serum-supplemented DMEM. Differentiation medium was changed every day during the course of myotube induction (see experimental scheme, Figure 5.1A). All cultures were maintained at 37°C under 5% CO_2, and all media were supplemented with 1% penicillin/streptomycin (Biochrom).

Bioluminescent luciferase assay

Intracellular ATP content was measured during the induction of myogenic differentiation on days 0 (D0) to 8 (D8) using an Adenosine 5'-triphosphate Bioluminescent Assay kit according to the manufacturer's recommendations (Sigma). Briefly, C2C12 myoblasts were grown in 10 mL growth medium in a 75-cm^2 flask at a density of 4×10^5 cells/flask. Cultured cells were harvested at 24-hour intervals from D0 to D8 post-induction for an ATP measurement (Figure 5.1A). Cell suspensions were adjusted to a concentration of 5×10^5 cells/mL by the Trypan Blue solution (Sigma) exclusion test and resuspended in a lysis buffer from the kit. Cell lysate was mixed with ATP assay solution, and bioluminescence was measured immediately with a DTX 880 Multimode Detector (Beckman Coulter). All measurements were performed in triplicate. Cellular ATP content was calculated from an ATP calibration curve generated from ATP standard solutions.

RNA isolation

Total RNA was extracted from cultured C2C12 cells using Tri-Reagent (Sigma-Aldrich, Germany) followed by an on-column DNase treatment. For small RNA isolation and enrichment, an miReasy Mini kit and an RNeasy MinElute Cleanup kit (Qiagen, Germany) were used according to the manufacturer's protocols. RNA quality and quantity were assessed with an Agilent 2100 Bioanalyzer (Agilent) using an Agilent RNA 6000 Nano kit (total RNA) and an Agilent small RNA kit (small RNA).

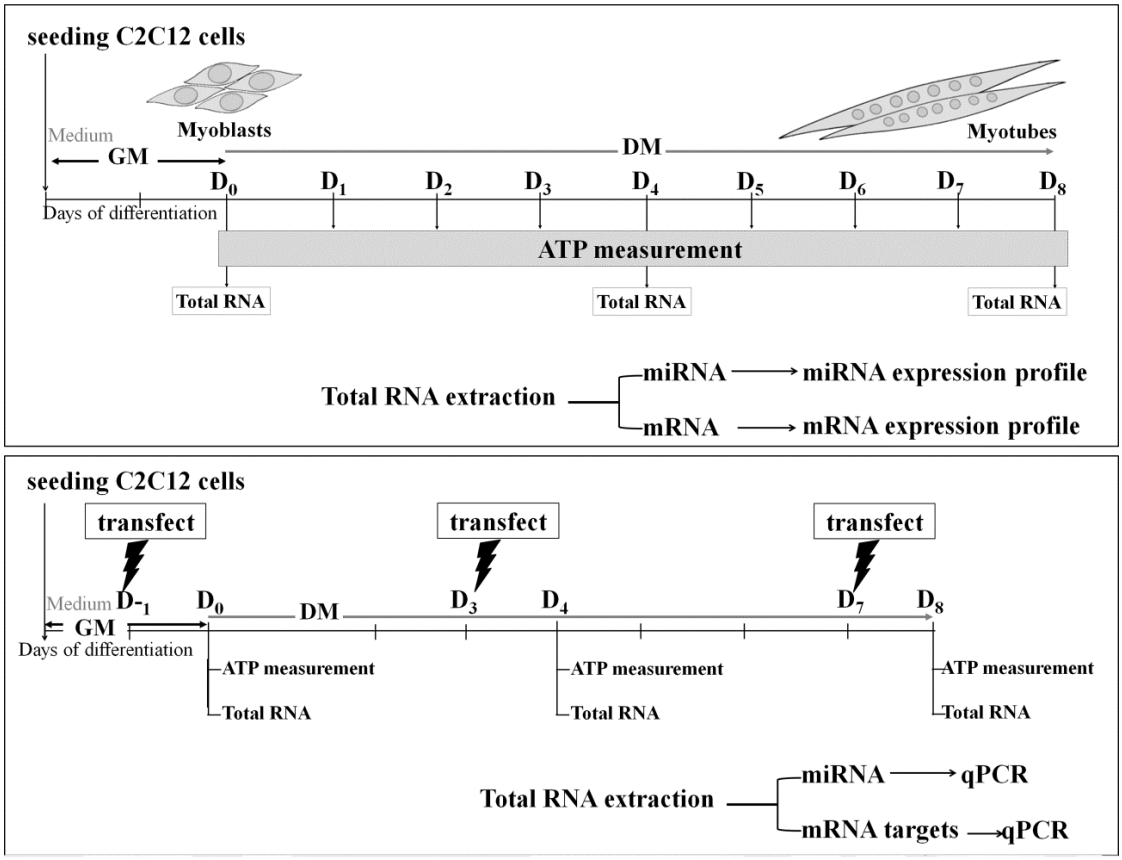

Figure 5.1: Experimental scheme. **(A)** ATP measurement and expression profiling of microRNA and target mRNA. C2C12 myoblasts were maintained in growth medium (GM) for 2 days (80–90% confluence). Myogenic differentiation was then induced from D0 to D8 by switching to differentiated medium (DM). Cells were harvested for (1) intracellular ATP measurement (2) expression profiling of miRNA (Affymetrix GeneChip miRNA 3.0 Array) and target mRNAs (Qiagen RT^2 Profiler PCR Array focusing on mitochondrial energy metabolism genes). **(B)** miR-423-3p overexpression by transfection of synthetic miR-423-3p mimic. C2C12 myoblasts were transfected with miR-423-3p mimic 24 hours prior to myotube induction (D -1), D3 and D7 post-induction and were then collected on D0, D4, and D8, respectively, for miRNA and target gene qPCR as well as ATP measurement.

MicroRNA-microarray analysis

MicroRNA expression profiling was performed using Affymetrix Gene Chip Micro 3.0 Array (Affymetrix, Inc, Santa Clara, CA, USA) containing 16,772 entries representing hairpin precursor (miRBase v17), total probe set 19,724 for detection most of miRNA from 153 species, provides >3-log dynamic range, with >95% reproducibility and 85% transcript detection at 1.0 amol, for a total RNA input of 130-500 ng. A total of 9 enriched small-RNA pools derived from D0, D4, and D8 post-induction of C2C12 myoblasts (three each) were used in the array hybridizations. Each RNA pool was generated from 5 individual RNA samples extracted from independent cultures. 200 ng of small RNA were used in sample preparation with a FlashTag™ Biotin RNA Labeling Kit for Affymetrix GeneChip miRNA arrays (Genisphere). The labeled RNA was then hybridized for 16 hours to an Affymetrix GeneChip miRNA array according to the manufacturer's recommendations (Affymetrix), washed and stained in the Affymetrix Fluidics Station 450, and scanned on the Affymetrix G3000 GeneArray Scanner. The image files were analyzed using the Affymetrix software (Expression Console), Robust Multi-array Average (RMA) background correction, log-2 transformations and quantile normalization methods implemented in JMP Genomics 5.1 were performed for data pre-processing, normalization, and statistical analysis.

RT^2 Profiler PCR Array

Expression levels of 89 genes functionally associated with mitochondrial energy metabolism were determined using Mouse Mitochondrial Energy Metabolism RT^2 Profiler™ PCR Array (Qiagen). The array is a pre-optimized qPCR panel of pathway-focused genes in a 96-well plate format including five standard housekeeping genes. Briefly, the real-time PCR was performed according to manufacturer's recommendations using SYBR Green PCR Master Mix and 20 ng (total RNA) cDNA/reaction well on a LightCycler 480 (Roche). The thermal cycler program was 10 min at 95°C, followed by 45 cycles of 15 sec at 95°C and 1 min at 60°C. Dissociation curve analysis was performed immediately after the last PCR amplification cycle.

mmu-miR-423-3p mimic transfection

Cells were seeded at a density of 1.5×10^5 cells/well on a 6-well plate in 2 mL of the growth medium. The C2C12 myoblasts were transfected with 150 nM mmu-miR-423-3p miScript miRNA mimic (5'-AGCUCGGUCUGAGGCCCCUCAGU -3', Qiagen) as double-stranded RNA oligonucleotides using the HiPerFect transfection reagent (Qiagen) at three different time points: 1 day prior to myotube induction and D3 and D7 post-induction (Fig 5.1B). Transfected myoblasts were harvested 24 hours post-transfection on D0, D4, and D8 post-induction for RNA extraction and qPCR. A random-sequence double-stranded RNA oligonucleotide was used as a negative control in all transfections (Qiagen). Transfection conditions were pre-optimized using the siGLO Green transfection indicator (Thermo Scientific) according to the manufacture's recommendations; resulting transfection efficiencies exceeded 97%.

Validation of microRNA-microarray results

Expression differences for differentially-expressed miRNAs identified from microarray analysis (mmu-miR-423-3p, mmu-miR-128-3p, and mmu-miR-301a-3p) were validated by two-step real-time PCR. First, single-stranded cDNA was synthesized from total RNA using a Megaplex™ RT Primers kit, Rodent Pool Set v3.0 (Life Technologies) containing rodent-specific stem-loop primers of 641 and 373 unique microRNAs for mouse and rat, respectively. Real-time PCR was performed using a standard LightCycler 480 SYBR Green I Master (Roche) on the LightCycler 480 system (Roche). U6 was used as an internal standard and the relative abundance of miRNAs was calculated using a comparative threshold cycle ΔΔCt method [31]. Validation of mmu-miR-423-3p mimic transfection was carried out using the same method.

Bioinformatic and statistical analysis

We predicted targets using the computational software RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/), which detects the most energetically favorable hybridization sites of a small RNA (miRNA) within a large RNA (mRNA) [14, 15]. Here, we tested the miRNA probe sets with the following parameters: energy cutoff (mfe) = −20 kcal/mol, allowing the G:U wobble base-pair, 1 mismatch seed, and ≤5 internal bulging nucleotides in the seed region. Identified targets were located on the 3′-UTR of genes. Statistical analysis for microRNA microarray and

RT^2 PCR array data was performed using a general linear model on JMP Genomics 6 (SAS Institute Inc., http://www.jmp.com). Adjustment for multiple tests across the Type 3 tests for the fixed effects was calculated using the post-hoc Tukey-Kramer test. For controlling false discovery rate, we chose the FDR according to Benjamini and Hochberg, 1995 [32]. Correlation coefficient analysis (r) between miRNA or mitochondria gene expression and ATP level was performed using SAS version 9.3. The expression data are available in the Gene Expression Omnibus public repository with the GEO accession number GSE52410: GSM1265694- GSM1265710

Results

Increased intracellular ATP during C2C12 myotube induction

To identify a critical time point for a functional study of ATP-miRNA regulatory pathways, intracellular ATP content was measured during the myotube induction of C2C12 myoblasts using a luciferase assay. At D0, cells were mononucleated and 80–90% confluent. After 4 days post-induction in low serum medium, they gradually differentiated and, by D8, were predominantly multinucleated myotubes (Figure 5.2A). Induction of differentiation was confirmed by measuring mRNA expression of myogenic markers *Tnnt1*, *Myh1,* and *Myh3,* all of which were up-regulated (Figure 5.2B). Intracellular ATP content was determined in the differentiating cells at an interval of 24 hours during the course of induced differentiation (D0 to D8). ATP level increased gradually from D0–D5, increased sharply from D5–D6, and reached an approximate two-fold increase at D6–D8 compared to D0 (Figure 5.2C).

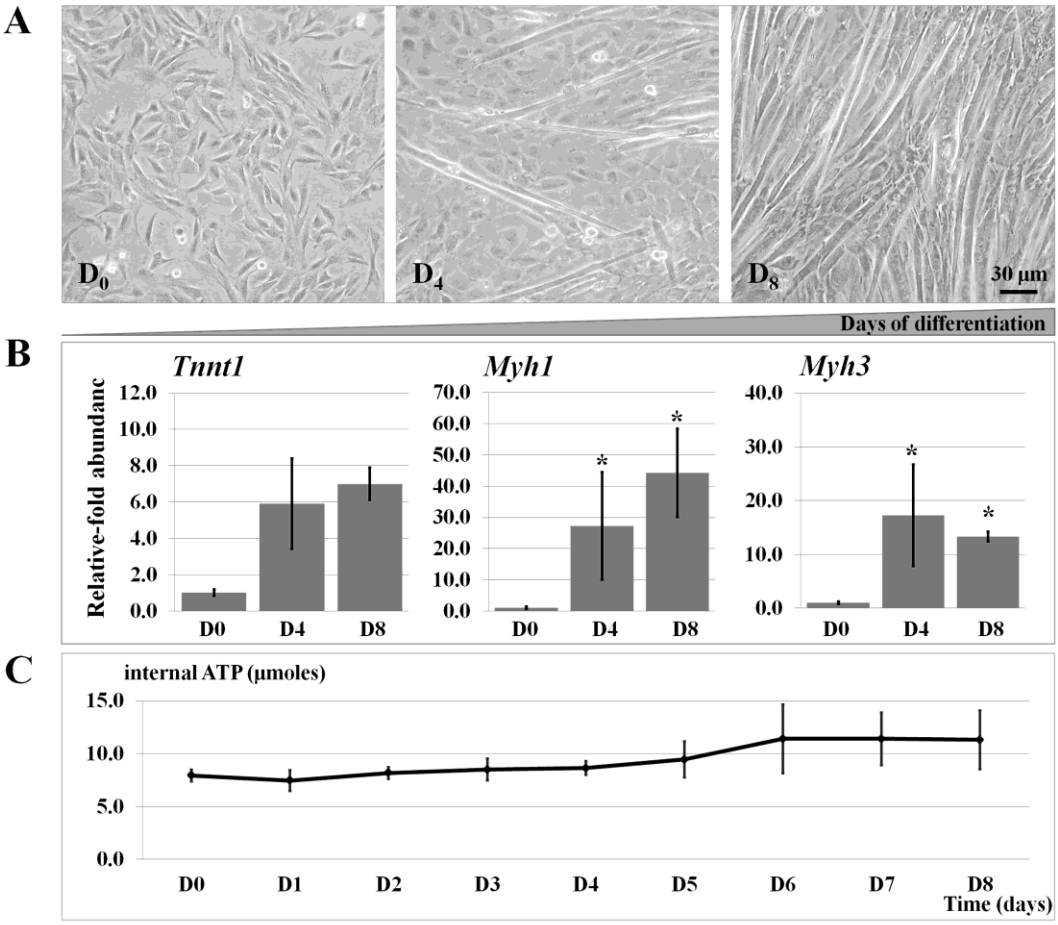

Figure 5.2: Characteristics of myogenic differentiation. **(A)** Morphological change of C2C12 myoblasts differentiating into myotubes at the post-induction D0 (undifferentiated mononucleated cells), D4 (elongated confluent cells), and D8 (long multinucleated myotubes). **(B)** Myogenic differentiation was accompanied by the up-regulation of *Tnnt1, Myh1, and Myh3*. **(C)** An increase in intracellular ATP level (µmoles) during the course of myogenic differentiation. The ATP level is shown as mean ± SEM (n=5).

Altered miRNA expression during C2C12 myogenic differentiation

Expression of miRNA during myogenic differentiation of C2C12 myoblasts was profiled at D0, D4, and D8 post-induction. After data pre-processing, 5,040 probes passed the quality control and filtering criteria and were analyzed further. To identify differentially-expressed miRNAs, the expression level of miRNAs was compared between pre- (D0) and post- (D4, D8) myotube induction. At D4 and D8, 179 and 188 probes, respectively, had significant expression changes from D0 (FDR $\leq$ 0.05). To focus on miRNA-ATP regulatory pathways, a correlation analysis between the

expression of miRNA probes which significantly changes from D0 and ATP level was applied; this filtering resulted in 95 (D4) and 103 (D8) probes of interest. Of these, 46 probes representing 13 unique miRNAs (miR-301, miR-301a/b, miR-423-3p, miR-615-3p, miR-130b, miR-140-3p, miR-17-3p, miR-183, miR-345, miR-15a, miR-16, and miR-16a) were down-regulated (compared to D0) and negatively correlated with ATP at D4 post-induction. In contrast, 49 probes representing 10 miRNAs (miR-296, miR-128, miR-128a/b, miR-1968, miR-206, miR-194, miR-1968 and miR-1a/b) were up-regulated and positively correlated with ATP level at D4. At D8 post-induction, expression of 55 out of 103 probes was lower compared to D0. These corresponded to 15 unique down-regulated miRNAs whose expression level negatively correlated with intracellular ATP content (miR-130b, miR-140, miR-15a, miR-16, miR-16a, miR-17-3p, miR-296-3p, miR-301, miR-301a/b, miR-345-3p, miR-423-3p, miR-542, miR-615-3p, and miR-183). Expression of 48 probes was higher, corresponding to 9 up-regulated miRNAs positively correlated with the ATP level (miR-194, miR-1968, miR-206, miR-128, miR-128a/b and miR-1, miR-1a/b). Notably, among them, well-known muscle miRNAs miR-1 and miR-128 were highly up-regulated (> 10-fold). The majority of differentially-expressed miRNAs were found at both D4 and D8 (Figure 5.3).

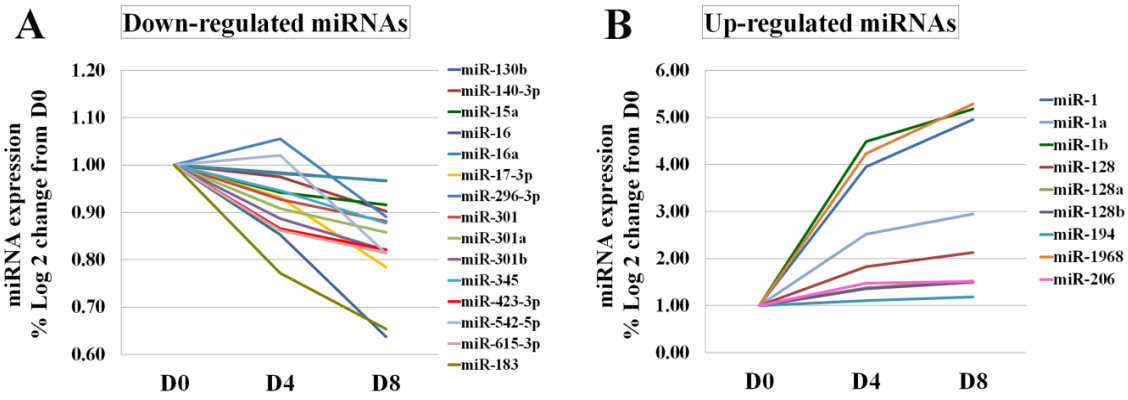

Figure 5.3: Alteration of miRNA expression in C2C12 myoblasts post-myotube induction. **(A)** 15 down-regulated miRNAs that were negatively correlated with the ATP level (correlation coefficient, r = -0.67 to -0.83, P< 0.05). **(B)** 9 up-regulated miRNAs that were positively correlated with the ATP level (r = 0.67 to 0.79, P< 0.05). MiR-423-3p, miR-128-3p and miR-301a-3p were random selected for validation by real-time PCR. A highly correlation coefficient (0.93-0.99) between microarray and real-time PCR results was found (Figure 5.4).

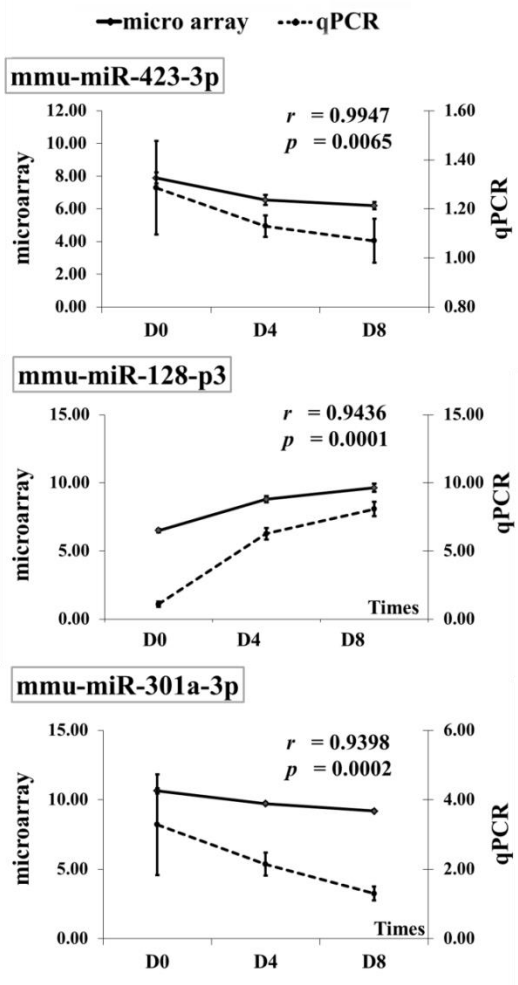

Figure 5.4: Confirmation of microRNA-microarray results by qPCR. miR-423-3p, miR-128-3p, and miR-301a-3p were selected for qPCR validation. Mean ± SEM (n=4) of the log2 transformed microarray result (–•– on primary y-axis) and relative expression ($2^{-\Delta\Delta Ct}$) derived from qPCR (--•--secondary y-axis) are overlaid. A correlation coefficient (*r)* and *p*-value are indicated.

Identification of genes associated with cellular ATP content during C2C12 differentiation

To functionally link miRNAs to cellular ATP production, mRNA targets must be identified. We therefore performed an expression analysis of target genes on the same samples used in miRNA profiling. Here, the RT^2 Profiler PCR Array system containing a panel of 89 mitochondrial energy metabolism-related genes was used. Analysis of this array identified 35 and 49 genes that were differentially expressed at D4 and D8, respectively, compared to D0. Further, a correlation analysis of these differentially-

expressed genes with ATP content, performed as with the miRNA analysis, narrowed down the gene list to 13 genes significantly positively correlated with ATP level (r = 0.63 – 0.92, *p*-value < 0.05) (Table 5.1). Of these, the mRNA abundance of 7 out of 13 genes significantly increased as early as D4 and all 13 genes were ultimately up-regulated at D8. Among them 8 genes showed > 2-fold change at D4 and/or D8 post-myotube induction.

Associations between miRNAs and mitochondrial energy-metabolism genes

A pairwise correlation analysis was carried out between the expression level of miRNAs (5,040 filtered probes) and 89 focused genes derived from the RT^2 PCR array analysis. A Pearson correlation coefficient analysis revealed 4,260 significant correlation interactions (FDR < 0.05). Further filtering criteria were applied to consider only those interactions in which both miRNAs and target mRNAs were significantly associated with differentiation and ATP level (FDR < 0.1) of C2C12 myotube induction. These resulted in 168 positive and 77 negative correlation interactions. Among these, 14 miRNAs (miR-423-3p, miR-17, miR-130b, miR-301a/b, miR-345, miR-15a, miR-16a, miR-128, miR-615, miR-1968, miR-1a/b, and miR-194) and 6 target mRNAs (*Cox4i2*, *Cox6a2, Ndufb7, Ndufs4, Ndufs5,* and *Ndufv1*) were identified. The miRNAs and mRNAs with high correlation interactions included miR-1 and *Ndufv1* (r = 0.988, p = 5.486E-07) and miR-423-3p and *Cox6a2* (r = - 0.971; p = 1.401E-05). Although most miRNAs inhibit their target mRNAs, some, particularly those miRNAs encoded within mRNAs, are positively correlated with expression of their targets [13] However, positive correlations could also indicate a connection via as-yet-undetermined indirect regulations. Therefore, we included both positive and negative correlation interactions in an IPA pathway analysis (Figure 5.5). Additionally, a computational bioinformatic web tool (RNAhybrid) [14, 15] was used to scan significant correlation pairs of miRNA-mRNA to obtain additional evidence for their functional links (Table 5.2). This analysis identified a miR-423-3p as an miRNA of interest for potential interactions with several mRNAs in energy metabolism.

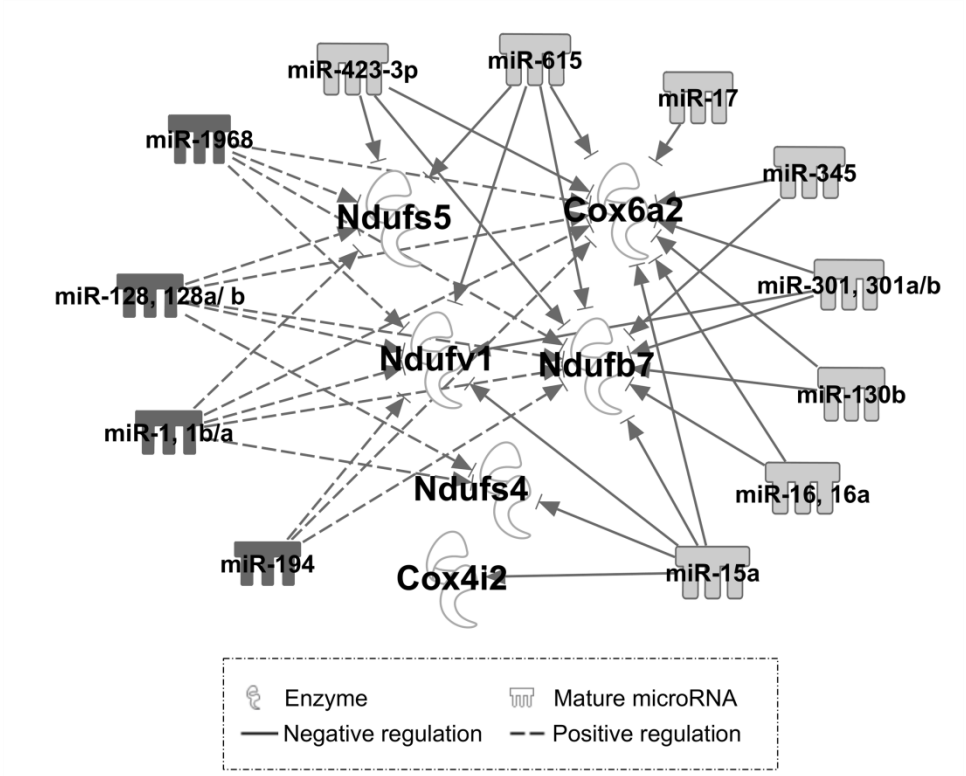

Figure 5.5: A regulatory network of differentially-expressed miRNAs and genes in C2C12 myogenesis, focusing on mitochondrial energy-metabolism pathways. Pearson correlation coefficient analysis identified a number of miRNAs-mRNA correlation interactions. Only those interactions in which both miRNAs and target mRNAs significantly associated with differentiation and ATP level (FDR < 0.1) during C2C12 myotube induction were utilized to model an miRNA-regulatory network (IPA pathway designer tool). White nodes indicate mRNA target; edges indicate regulatory interaction between miRNA and target gene. Solid line label denotes negative regulation with negative correlation interaction; dashed line denotes indirect positive regulation with positive correlation interaction.

miR-423-3p as an ATP-regulated miRNA candidate

Our microarray and correlation analysis indicated that expression of miR-423-3p decreased > 2-fold (FDR < 0.05) and was negatively correlated with the up-regulation of mitochondrial energy metabolism-related genes throughout the course of C2C12 myotube induction. *In silico* investigation of miR-423-3p showed that the miRNA is highly conserved across several species (Figure 5.6A). Moreover, the target genes *Cox6a2*, *Ndufb7*, and *Ndufs5* in our energy metabolism gene list (see also Table 5.1) each possess at least one predicted binding site for miR-423-3p in the 3'-UTR

(RNAhybrid; Figure 5.6B). To our knowledge, miR-423-3p has not yet been associated with ATP metabolism. Therefore, miR-423-3p was selected for functional validation.

Overexpression of miRNA-423-3p mimic negatively regulated ATP level in C2C12 differentiation

To determine whether miR-423-3p regulates ATP metabolism, synthetic miRNA-423-3p mimics were transfected into C2C12 cells one day prior to differentiation induction and on D3 and D7 post-induction. A random-sequence double-stranded RNA oligonucleotide was used as a negative control and baseline at each time point. Gene expression analysis by real-time PCR for miR-423-3p, *Cox6a2*, *Ndufb7*, and *Ndufs5* and ATP measurements (see experimental scheme, Figure 1B) were performed 24 hours after transfection. miR-423-3p expression was detected in cells after transfection, and endogenous miR-423-3p expression was significantly lower in the negative control (Figure 7A). ATP levels tended to decrease in miR-423-3p mimic-treated cells compared to the negative control at D0 and D4 and were significantly lower at D8 (Figure 5.7B). Additionally, expression of *Cox6a2*, *Ndufb7*, and *Ndufs5* was significantly down-regulated at D4 and D8 (Figure 5.7C). These results suggest that miR-423-3p modulates the ATP level partly by regulating the expression of *Cox6a2*, *Ndufb7* and *Ndufs5*. Additionally, mRNA expression of myogenic marker *Tnnt1* was significantly down-regulated at D8, while *Myh1* was significantly down-regulated at D4 and D8 in miR-423-3p mimic-treated cells (Figure 5.7D).

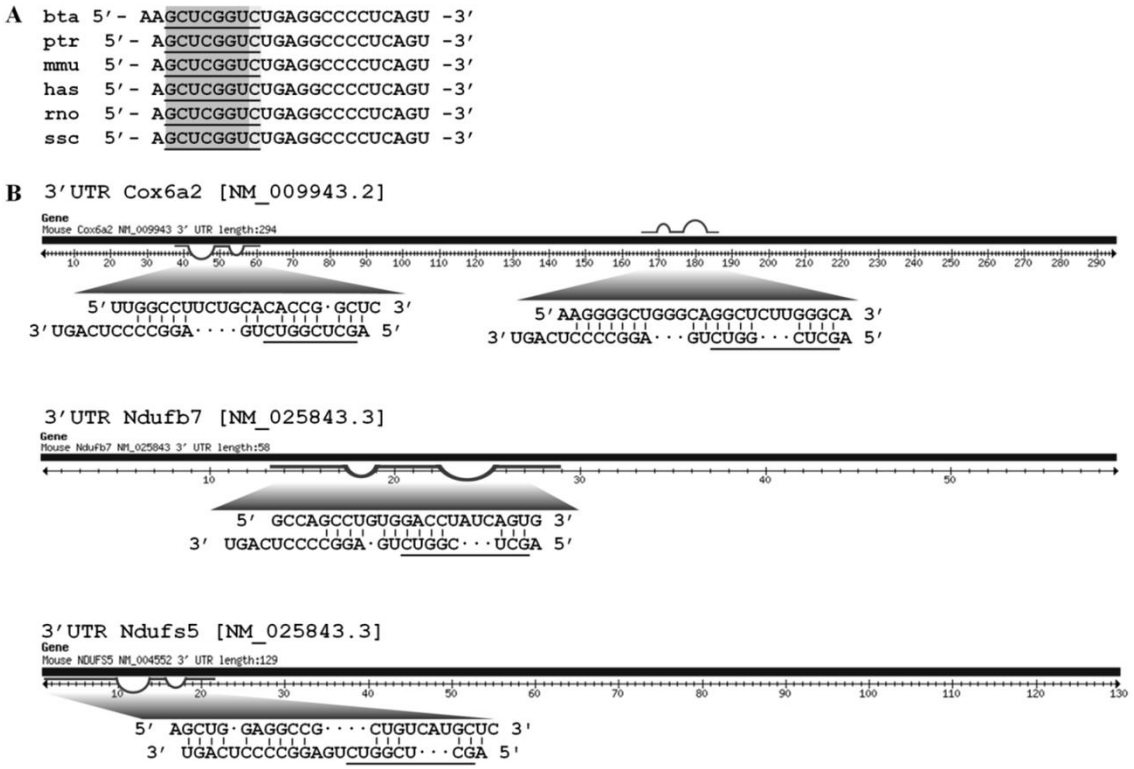

Figure 5.6: **miR-423-3p as a candidate miRNA for functional validation. (A)** Conservation of miRNA-423-3p across species; Bos taurus (bta), Pan troglodytes (ptr), Mus musculus (mmu), Homo sapiens (hsa), Rattus norvegicus (rno), and Sus scrofa (ssc). The seed region (2-8 nt) is highlighted in a gray box and underlined. **(B)** Predicted targets of mmu-miR-423-3p in the 3′-UTR of Cox6a2, Ndufb7, and Ndufs5 (RNAhybrid). The prediction criteria include free energy ≥ - 20 kcal/mol and allowing the G:U wobble base-pair and bulging nucleotides in the seed region. The seed match is underlined.

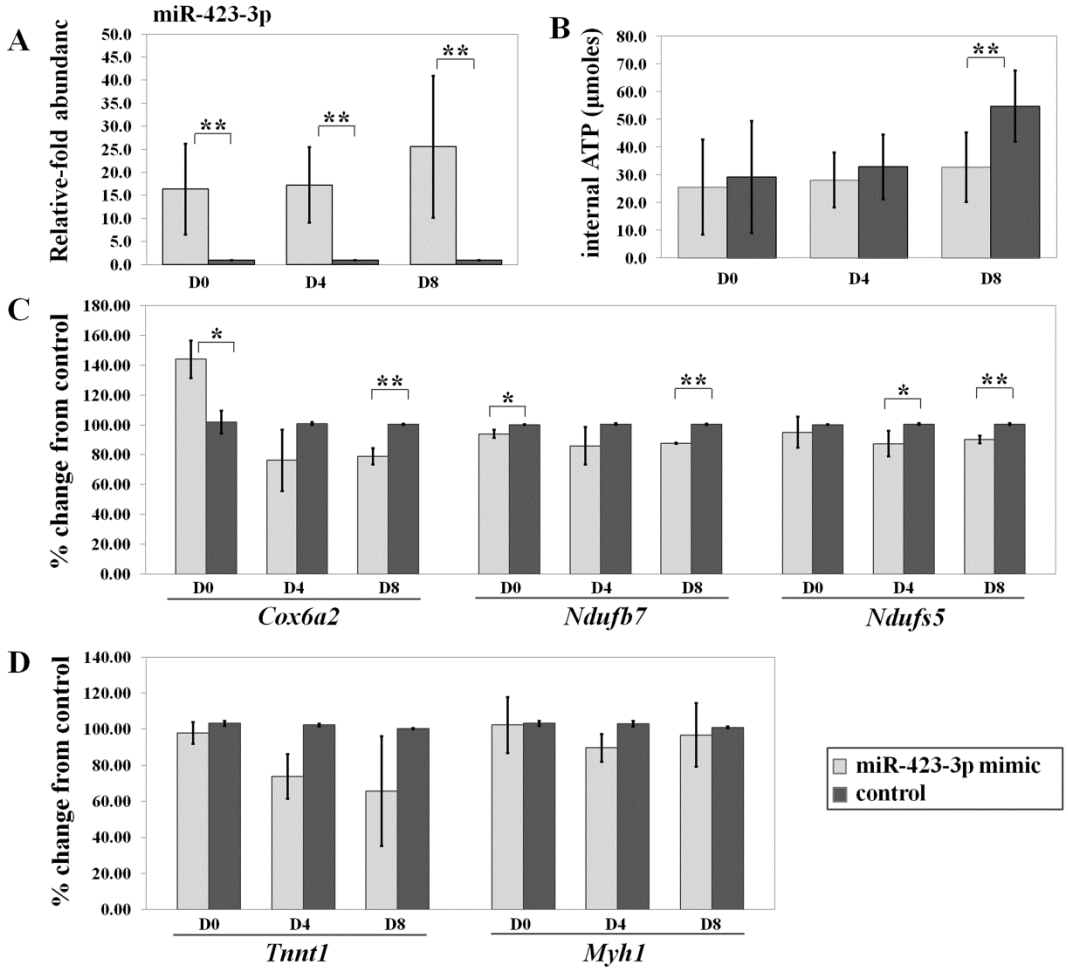

Figure 5.7: Transfection of synthetic miR-423-3p mimic negatively regulates Cox6a2, Ndufb7, and Ndufs5 as well as ATP level during C2C12 myotube induction. (A) Relative abundance of miRNA-423-3p by qPCR post miRNA-423-3p mimic transfection (n=3). **(B)** 24 hours post-transfection, the cellular ATP level was lower (not significant) in the miR-423-3p mimic-transfected cells compared to the negative control at D0 and D4 and was significantly lower at D8. **(C)** Significant down-regulation of *Cox6a2*, *Ndufb7*, and *Ndufs5* by qPCR 24 hours post-transfection. **(D)** Myogenic differentiation marker genes. * and ** denotes p-values ≤ 0.05 and ≤ 0.01, respectively. All values are presented as mean ± SEM (n = 3).

Discussion

Using differentiating C2C12 myoblasts, we identified correlations between miRNAs, target mRNAs, and cellular ATP levels. Importantly, C2C12 myoblasts exhibited changing ATP levels during differentiation, gradually increasing at first (days 0-5), then rapidly (days 6-8) reaching 200% of the baseline level, demonstrating the increased production of ATP during induced differentiation. Previous work on ATP level and myogenic differentiation showed that extracellular ATP has an inhibitory effect on cell proliferation while simultaneously promoting myogenic differentiation of satellite cells [16]. The changing ATP level during myoblast differentiation provided a good model to search for potential regulators of ATP production.

In the correlation analyses of miRNA expression changes and ATP level, we first considered down-regulated miRNAs that were negatively correlated with the increased ATP level. Of the miRNAs identified in this group, miR-15a shares its seed sequence with that of miR-15b, which has been reported to modulate cellular ATP levels and degenerate mitochondria by targeting the down-regulation of *Arl2* in rat myocytes [17]. MiR-301 and miR-17-3p function in mitochondrial metabolism by regulating ATPase and translocase [18]. Consistent with previous findings [19], the expression of miR-140, a chondrocyte-specific marker [20], decreased during myogenic differentiation. On the other hand, the miRNAs those were up-regulated and positively correlated with ATP level during C2C12 myogenic differentiation have not been well-described. Of these, miR-206 and miR-1 are muscle-specific miRNAs with important functions in muscle development, as regulators of cell proliferation and differentiation during myogenesis [21-23].

Because mitochondria produce ATP through oxidative phosphorylation to provide energy for cellular activities, like growth and differentiation, our microarray expression analysis focused on mitochondrial energy-metabolism genes. A number of genes were differentially expressed during C2C12 myogenic differentiation; however, only *Cox6a2, Ndufb7, Ndufs5, Ndufv1, Bcs1l, Ndufs4*, and *Cox4i2* were significantly up-regulated and simultaneously correlated with increased ATP throughout D0-D8.

Cox4i2 and *Cox6a2* are nuclear genes localized in the inner mitochondrial membrane and specifically expressed in heart and muscle tissues. The COX complex functions in electron transfer in oxidative phosphorylation, which is responsible for 90% of ATP

synthesis for muscle energy [24]. The COX4I2 protein encoded by *Cox4i2* is up-regulated in a limited-oxygen environment to increase ATP levels and enhance the efficiency of cellular respiration [25]. *Cox6a2*, expressed in heart and skeletal muscle, encodes one of thirteen subunits of the respiratory chain complex IV protein, the COX6A subunit. It has been implicated in stimulating enzymatic activity of the functional complex IV and directly affects ROS production in skeletal muscle [24]. *Cox6a2* null mice have severely reduced skeletal muscle complex IV activity, decreased ATP levels, enhanced respiratory uncoupling, and increased thermogenesis, energy expenditure, mitochondrial biogenesis, and muscle-fibre type switching. These changes explain the unique phenotype of the null mice, which includes leanness and resistance to diet-induced obesity [24].

Ndufb7, *Ndufs5*, *Ndufv1*, and *Ndufs4* are members of the NADH dehydrogenase (ubiquinone) family of mitochondrial respiratory chain complex I, which represents the largest and first complex mediating electron transfer through the electron transfer chain. These enzymes transfer electrons from NADH to ubiquinone and through the respiratory chain, resulting in the generation of ATP, which is important for energy metabolism [26, 27]. *Ndufs4* and *Ndufs5* are classified in the "iron–sulfur group" with a phosphorylation function, while *Ndufb7* is classified in the "hydrophobic group". *Ndufv1* is classified in the "flavoprotein group" with NADH-binding and oxidizing properties [27, 28].

A correlation analysis (considering both positive and negative relationships) between the expression of miRNAs and mitochondria-related mRNAs during myogenesis was used as the first line of evidence to build an miRNA-mRNA network. A negative correlation interaction between an miRNA and an mRNA could imply direct regulation; in contrast, a positive correlation interaction could suggest indirect regulation via (unknown) intermediate pathways. Additionally, positive correlation interactions can indicate direct positive regulation of mRNA transcript abundance by miRNAs [13]. The finding of significant negative correlations of miR-15a and miR-16a with *Cox4i2*, *Cox6a2*, *Ndufb7*, *Ndufv1*, and *Ndufs4* is supported by previous work identifying miR-15 and miR-16 as ATP modulators affecting oxygen consumption [17] as well as direct regulators of *Bcl2* to induce apoptosis through the regulation of mitochondrial function [17, 29].

Analyzing each miRNA-mRNA correlation interaction to identify mRNAs targets with 3'-UTRs containing potential binding sites for the corresponding correlated miRNAs revealed three mitochondria-related genes (*Ndufb7*, *Ndufs5*, and *Cox6a2*) sharing at least one binding site on the 3'-UTR for the same miRNA: miR-423-3p. We therefore concluded that these genes compose a potential miRNA-mRNA network for cellular ATP production and energy metabolism. miR-423-3p has been previously shown to involve in promoting cell growth and cell cycle progression [30]. This supports our hypothesis that down-regulation of miR-423-3p is required for C2C12 myogenic differentiation. Overexpression of miR-423-3p during C2C12 myogenic differentiation resulted in dramatically lower ATP levels and significantly lowers expression of Cox6a2, Ndufb7, and Ndufs5. Similarly, two myogenic markers were down-regulated following miRNA-423-3p overexpression. These results suggest the first time that miR-423-3p not only modulates ATP production by targeting Cox6a2, Ndufb7, and Ndufs5 but also regulates myogenesis.

Acknowledgements

The authors thank Joana Bittner and Annette Jugert for an excellent technical assistance. This research was supported by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG; PO 753/7-1 and WI 1754/10-14). The authors declare no conflict of interest.

Table 5.1 List of differentially-expressed genes affecting ATP level during myogenic differentiation in C2C12 cells

Gene symbol	Ref. sequence	Description	ATP correlation coefficient	P-value of correlation coefficient	Fold-change (D0)-(D4)	p-value (D0)-(D4)	FDR (D0)-(D4)	Fold-change (D0)-(D8)	p-value (D0)-(D8)	FDR (D0)-(D8)
Cox4i2	**NM_053091**	Cytochrome c oxidase subunit IV isoform 2	0.9234	0.0004	1.7275	0.0297	0.0890	2.2130	0.0050	0.0350
Ndufs7	**NM_029272**	NADH dehydrogenase (ubiquinone) Fe-S protein 7	0.8551	0.0033	1.2272	0.0644	0.1504	1.3591	0.0150	0.0626
Ndufb7	**NM_025843**	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7	0.8314	0.0055	1.4096	0.0033	0.0279	1.6771	0.0005	0.0104
Cox6a2	**NM_009943**	Cytochrome c oxidase, subunit VI a, polypeptide 2	0.7727	0.0147	37.5828	0.0011	0.0142	57.3876	0.0002	0.0060
Ndufs4	**NM_010887**	NADH dehydrogenase (ubiquinone) Fe-S protein 4	0.7673	0.0158	1.7275	0.0257	0.0860	2.0411	0.0092	0.0478
Ndufb3	**NM_025597**	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 3	0.7624	0.0169	1.1345	0.2691	0.4037	1.3560	0.0288	0.0873
Ndufv1	**NM_133666**	NADH dehydrogenase (ubiquinone) flavoprotein 1	0.7415	0.0222	1.5569	0.0052	0.0350	1.6888	0.0024	0.0217
Bcs1l	**NM_025784**	BCS1-like (yeast)	0.7417	0.0222	1.1163	0.0237	0.0828	1.1914	0.0047	0.0343
Lhpp	**NM_029609**	Phospholysine phosphohistidine inorganic pyrophosphate phosphatase	0.7161	0.0300	1.1991	0.2450	0.3874	1.5080	0.0265	0.0864
Ndufs3	**NM_026688**	NADH dehydrogenase (ubiquinone) Fe-S protein 3	0.7122	0.0313	1.3031	0.0526	0.1360	1.3941	0.0261	0.0862
Ndufs5	**NM_001030274**	NADH dehydrogenase (ubiquinone) Fe-S protein 5	0.7089	0.0325	1.4526	0.0051	0.0350	1.5977	0.0018	0.0180
Ndufa2	**NM_010885**	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2	0.7021	0.0350	1.1060	0.1315	0.2486	1.2449	0.0114	0.0538
Atp6v0d2	**NM_175406**	ATPase, H+ transporting, lysosomal V0 subunit D2	0.6921	0.0388	3.6774	0.2224	0.3647	18.2860	0.0059	0.0383

Data are shown as fold regulation levels compared to control group **(**D0**)** and normalized to mean of housekeeping genes (*Actb*, *B2m*, *Gapdh*, *Gusb*, and *Hsp90ab1*), then assessed for correlation with ATP levels.

Table 5.2 Predicted binding sites for miRNAs in the 3'-UTRs of their correlated mRNAs (RNAhybrid)

Regulatory interaction	N/a me	Sequence	3'-UTR site No.* [mfe (kcal/mol)]					
			Cox6a2	***Ndufb7***	***Ndufs5***	***Ndufv1***	***Ndufs4***	***Cox4i2***
negative	miR-423-3p	AGCUCGGUCUGAGGCCCCUCAGU	3 (-33.1to-22.7)	1 (-20.2)	1 (-22.8)		4	
	miR-17-3p	ACUGCAGUGAGGGCACUUGUAG	1 (-22.7)				4	1
	miR-301a-3p	CAGUGCAAUAGUAUUGUCAAAGC	n/a **	n/a		n/a	1	
	miR-345-3p	CCUGAACUAGGGGUCUGGAG	2 (-26.1to-22.1)	1 (-20.9)		n/a		
	miR-345-3p	CCUGAACUAGGGGUCUGGAGAC	2 (-27.2to-25.4)	1 (-20.9)		1	4	
	miR-15a-5p	UAGCAGCACAUAAUGGUUUGUG	1 (-18.7)	n/a		1 (-18.3)	3 (-20.8to-18.7)	1 (-19.4)
	miR-16-5p	UAGCAGCACGUAAAUAUUGGCG	n/a	n/a			2	
	miR-615-3p	UCCGAGCCUGGGUCUCCCUCUU	3 (-28.0to-19.8)	1 (-19.1)	n/a	n/a	1	
	miR-130b	CAGUGCAAUAAUGAAAGGGCAU	1 (-19.2)	n/a	2		1	
positive	miR-128-3p	UCACAGUGAACCGGUCUCUUU	1 (-22.6)	n/a	1 (-22.6)	n/a	1 (-20.6)	
	miR-1b	UGGAAUGUAAAGAAGUAUGGGU	n/a	n/a	n/a	n/a	5 (-23.1to-18.0)	
	miR-1a	UGGAAUGUAAAGAAGUAUGUA	n/a	n/a	n/a	n/a	1 (-19.1)	
	miR-1-3p	UGGAAUGUAAAGAAGUAUGGAG	n/a	n/a	n/a	n/a	2 (-20.5to-18.1)	
	miR-194-5p	UGUAACAGCAACUCCAUGUGGA	n/a	n/a	1	n/a	4	
	miR-1968-5p	UGCAGCUGUUAAGGAUGGUGGACU	2 (-23.5to-20.0)	n/a	1 (-21.2)	1 (-22.7)	4	2

The gray zone highlight the paired miRNA-mRNA that were significantly correlated by Pearson correlation coefficient analysis.

* The number of binding sites at the 3´-UTR of genes and the free energy hybridizations of miRNA and target (in parentheses) .

** n/a, no binding site available at 3´-UTR of gene.

References of chapter 5

1. Winter, J., Jung, S., Keller, S., Gregory, R. I. & Diederichs, S. (2009) Many roads to maturity: microRNA biogenesis pathways and their regulation, Nat Cell Biol. 11, 228 - 234.

2. Pasquinelli, A. E. (2012) MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship, Nat Rev Genet. 13, 271-82.

3. Filipowicz, W., Bhattacharyya, S. N. & Sonenberg, N. (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?, Nat Rev Genet. 9, 102-114.

4. Grimson, A., Farh, K., Johnston, W., Garrett-Engele, P., Lim, L. & Barte, l. D. (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing., Mol Cell. 27, 91-105.

5. Ambros, V. (2004) The functions of animal microRNAs, Nature. 16, 350-355.

6. Calin, G. A. & Croce, C. M. (2006) MicroRNA-cancer connection: the beginning of a new tale., Cancer Res. 66, 7390-7394.

7. Cuellar, T. L. & McManus, M. T. (2005) MicroRNAs and endocrine biology, J Endocrinol. 187, 327-332.

8. Boehm, M. & Slack, F. (2005) A developmental timing microRNA and its target regulate life span in C. elegans., Science 23, 1954-1957.

9. Gan, Z., Rumsey, J., Hazen, B., Lai, L., Leone, T., Vega, R., Xie, H., Conley, K., Auwerx, J., Smith, S., Olson, E., Kralli, A. & Kelly, D. (2013) Nuclear receptor/microRNA circuitry links muscle fiber type to energy metabolism., J Clin Invest 123, 2564-75.

10. Ponsuksili, S., Du, Y., Hadlich, F., Siengdee, P., Murani, E., Schwerin, M. & Wimmers, K. (2013) Correlated mRNAs and miRNAs from co-expression and regulatory networks affect porcine muscle and finally meat properties, BMC Genomics. 14, 533.

11. Sartorelli, V. & Caretti, G. (2005) Mechanisms underlying the transcriptional regulation of skeletal myogenesis, Curr Opin Genet Dev. 15, 528-535.

12. Sartorelli, V., Puri, P. L., Hamamori, Y., Ogryzko, V., Chung, G., Nakatani, Y., Wang, J. Y. J. & Kedes, L. (1999) Acetylation of MyoD directed by PCAF is necessary for the execution of the muscle program., Molecular Cell. 4, 725-734.

13. Wang, Y. P. & Li, K. B. (2009) Correlation of expression profiles between microRNAs and mRNA targets using NCI-60 data., BMC Genomics. 10.

14. Krüger, J. & Rehmsmeier, M. (2006) RNAhybrid: microRNA target prediction easy, fast and flexible, Nucleic Acids Res. 1, W451-4.

15. Rehmsmeier, M., Steffen, P., Hochsmann, M. & Giegerich, R. (2004) Fast and effective prediction of microRNA/target duplexes., RNA, 1507-17.

16. Ryten, M., Dunn, P., Neary, J. & Burnstock, G. (2002) ATP regulates the differentiation of mammalian skeletal muscle by activation of a P2X5 receptor on satellite cells, J Cell Biol 158, 345–355.

17. Nishi, H., Ono, K., Iwanaga, Y., Horie, T., Nagao, K., Takemura, G., Kinoshita, M., Kuwabara, Y., Mori, R., Hasegawa, K., Kita, T. & Kimura, T. (2010) MicroRNA-15b Modulates Cellular ATP Levels and Degenerates Mitochondria via Arl2 in Neonatal Rat Cardiac Myocytes, J Biol Chem 285, 4920-30.

18. Demongeot, J., Cohen, O., Doncescu, A. & Henrion-Caude, A. (2013). MitomiRs and Energetic Regulation. Paper presented at the 27th International Conference on Advanced Information Networking and Applications Workshops, Barcelona, Spain

19. Dong, S., Yang, B., Guo, H. & Kang, F. (2012) MicroRNAs regulate osteogenesis and chondrogenesis., Biochem Biophys Res Commun 418, 587-91.

20. Tuddenham, L., Wheeler, G., Ntounia-Fousara, S., Waters, J., Hajihosseini, M., Clark, I. & Dalmay, T. (2006) The cartilage specific microRNA-140 targets histone deacetylase 4 in mouse cells., FEBS Lett. 580, 4214-7.

21. Chen, J.-F., Mandel, E. M., Thomson, J. M., Wu, Q., Callis, T. E., Hammond, S. M., Conlon, F. L. & Wang, D.-Z. (2005) The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation, Nature Genetics 38, 228 - 233

22. Goljanek-Whysall, K., Pais, H., Rathjen, T., Sweetman, D., Dalmay, T. & Münsterberg, A. (2012) Regulation of multiple target genes by miR-1 and miR-206 is pivotal for C2C12 myoblast differentiation., J Cell Sci. 125, 3590-600.

23. Kim, H. K., Lee, Y. S., Sivaprasad, U., Malhotra, A. & Dutta, A. (2006) Muscle-specific microRNA miR-206 promotes muscle differentiation, J Cell Biol. 174, 677-687.

24. Quintens, R., Singh, S., Lemaire, K., De Bock, K., Granvik, M., Schraenen, A., Vroegrijk, I., Costa, V., Van Noten, P., Lambrechts, D., Lehnert, S., Van Lommel, L., Thorrez, L., De Faudeur, G., Romijn, J., Shelton, J., Scorrano, L., Lijnen, H., Voshol,

P., Carmeliet, P., Mammen, P. & Schuit, F. (2013) Mice deficient in the respiratory chain gene Cox6a2 are protected against high-fat diet-induced obesity and insulin resistance., PLoS One. 8.

25. Fukuda, R., Zhang, H., Kim, J., Shimoda, L., Dang, C. & Semenza, G. (2007) HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells., Cell 129, 111-22.

26. Triepels, R., Van Den Heuvel, L., Trijbels, J. & Smeitink, J. (2001) Respiratory chain complex I deficiency., Am J Med Genet. 106, 37-45.

27. Mimaki, M., Wang, X., McKenzie, M., Thorburn, D. & Ryan, M. (2012) Understanding mitochondrial complex I assembly in health and disease., Biochim Biophys Acta 1817, 851-62.

28. Koene, S., Willems, P., Roestenberg, P., Koopman, W. & Smeitink, J. (2011) Mouse models for nuclear DNA-encoded mitochondrial complex I deficiency., J Inherit Metab Dis. 34, 293-307.

29. Cimmino, A., Calin, G., Fabbri, M., Iorio, M., Ferracin, M., Shimizu, M., Wojcik, S., Aqeilan, R., Zupo, S., Dono, M., Rassenti, L., Alder, H., Volinia, S., Liu, C., Kipps, T., Negrini, M. & Croce, C. (2005) miR-15 and miR-16 induce apoptosis by targeting BCL2., Proc Natl Acad Sci U S A 102, 13944-9.

30. Lin, J., Huang, S., Wu, S., Ding, J., Zhao, Y., Liang, L., Tian, Q., Zha, R., Zhan, R. & He, X. (2011) MicroRNA-423 promotes cell growth and regulates G(1)/S transition by targeting p21Cip1/Waf1 in hepatocellular carcinoma., Carcinogenesis. 32, 1641-7.

31. Livaka, K. J. & Schmittgen, T. D. (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method, Methods. 25, 402-408

32. Benjamin, Y. & Hochberg, Y. (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing, J R Stat Soc Ser B. 57, 289-300.

CHAPTER 6

General discussions

General discussions

Skeletal muscle is the most abundant tissue which is a primary product of animal breeding, known as “meat”. Improving the quality of meat is always in the interest of scientific research these days. Rational approaches for enhancing the quality of the meat production depended implicitly on the fundamental knowledge of the skeletal muscle tissue growth and development controlling mechanisms. Skeletal muscle growth and development are very complicate processes and involve with many factors. These are included their genetic control, environmental factors, muscle structure and metabolism as well as slaughtering procedures,which impact on muscle development and meat quality (Sellier 1994; Rehfeldt *et al.* 2011). Of these factors influence and/or play a role in the meat production control from early prenatal development to postnatal growth. After the discovery of miRNAs and its function, numerous studies have demonstrated that miRNAs regulate the expression of target genes in diverse cellular processes. Hence, miRNAs play important roles in an array of physiological processes including muscle development and growth control (Chen *et al.* 2006; Chen *et al.* 2009; Chen *et al.* 2010; Braun & Gautel 2011) as well as metabolic control (Esau *et al.* 2006; Granjon *et al.* 2009). This significantly increases our understanding in the regulatory mechanism and gene-regulatory networks complexity of miRNAs in muscle. However, factors that govern miRNA synthesis and expression in response to changes in the cellular environment are still largely unknown and inherent challenges. Therefore, the involvement of miRNAs in the complex of skeletal muscle growth and development processes warrants further investigation. Thus, this thesis explaining the potential miRNAs and their target genes regulation networks that involve in skeletal muscle by understanding their biological roles and regulations under different physiological conditions of muscle. That starts from skeletal muscle development period until fully developed skeletal muscle tissue.

In this discussion section, it is firstly focused on the determination of interesting miRNAs and their mRNA target relationships or their genes regulation networks and their biological function. Then the results of the study are discussed and finally the future prospects are mentioned.

Interesting miRNA-mRNA relationships and their biological function associated with different stages of skeletal muscle development in two pig breeds (DL) Vs. (Pi)

In the **chapter 2**, we first used custom miRNA array to identify the miRNAs expression profile that are involved in porcine muscle development and therefore may contribute to the emergence of phenotypic differences in muscularity. This part performed comparative miRNA expression profiling of skeletal muscle across three developmental stages in two different breeds, DL and Pi. Muscle samples (*Musculus longissimus dorsi*) collected at 63 and 91 dpc, during secondary muscle fibre development which is critical for predetermines muscle characteristics in adult pigs (Rehfeldt *et al.* 1999; Bérard *et al.* 2011) as well as adult age of 180 days was chosen to represent the ultimate muscle phenotype (2 breeds×3 stages×3 biological replicates). Our custom miRNA array was designed from 100% homology search of multi-species miRNA to the porcine genome sequence and known porcine miRNA (miRBase version 14.0) as well as those that are public literature studies reported (Huang *et al.* 2008; Reddy *et al.* 2009).

In this result, most of our differentially expressed miRNAs between two different muscle breeds (i.e. let-7, miR-1, -17, -19, -23, -25, -27, -106, -133, -181, -199 and -214) were generally concurred with the previous findings (Chen *et al.* 2006; Flynt *et al.* 2007; Huang *et al.* 2008; Chen *et al.* 2008 ; Chen *et al.* 2009; Nielsen *et al.* 2009; McDaneld *et al.* 2009 ; Zhou *et al.* 2010). Confirmed with numerous of previous studies, these miRNAs may have expression abundance in muscles and are likely conserved across species and play an important role in muscle. Information of the Ingenuity Knowledge Base was used in order to assign biological functions of the miRNAs found to be differentially expressed. Many miRNA regulations were observed to be associated with breed type. Such as let-7, miR-30, -15, -199 and -17 families (miR-17, -18a/b, -20 and -106) were consistently higher expressed in Pi pigs compared to DL pigs. All of these are involve in porcine skeletal muscle growth and development and play a role in proliferation of cells (Huang *et al.* 2008; Chen *et al.* 2008 ; Duisters *et al.* 2009; Zhou *et al.* 2010). Some miRNAs such as miR-143, miR-30 and miR-103 were also higher expressed in Pi at 63 dpc and adult stage. These miRNAs are important for adipocyte differentiation and enhanced adipogenesis in human rat as well as porcine adipose tissue (Xie *et al.* 2009; Keller *et al.* 2011; Li *et al.* 2011; Soh *et al.* 2013). MiRNAs up-regulated in DL pigs at 63-dpc and/or the adult stage include miR-

122, -133, -181, and -214. Of these miR-133 is well known as a muscle-specific miRNA (Chen *et al.* 2006) that also controls brown adipose tissue regulation (Yin *et al.* 2013). MiR-181and miR-214 likely influence muscle development (Naguibneva *et al.* 2006; Flynt *et al.* 2007; Li *et al.* 2012a). Interestingly, MiR-122, which plays a crucial role in cholesterol and lipid metabolism (Esau *et al.* 2006; Li *et al.* 2011; Chen *et al.* 2011) showed high abundance in DL at both prenatal stages.

The results reflected the expression pattern of miRNAs in muscle which were enriched by genes belonging to cellular growth and development as well as cell proliferation and the cell cycle. Thus, revealing that multiple miRNAs have similar biological functions to their targets. Manner several miRNAs were associated with breed type and expressed in a developmental-specific. This is of interest because DL and Pi pigs are considerably different in numerous traits including growth rate, body composition, muscularity and fat content: Pi pigs are leaner and have a lower ration of fat/meat content compared to DL pigs (Rehfeldt *et al.* 2000; Ponsuksili *et al.* 2007).

An integration of miRNA and mRNA expression profiling data (**in chapter 3**) derived from the same samples is useful for systems-level studies of gene regulation to establish miRNA-mRNA co-expression networks. The second part of this study, a total of 24 Affymetrix GeneChip porcine Genome Arrays was used. Differentially expressed genes compared between two ontogenetic stages within each breed showed the same shift of abundance along ontogenetic stages in both breeds. The highest number of significantly different probes was found at the adult stage, 35 dpc, 63 dpc and 91 dpc, respectively. The bio-functions of differentially expressed genes from IPA analysis represented groups of gene which related to muscle growth and development including “cellular growth and proliferation”, “cellular function and maintenance”, “cell death”, “cell cycle”, “cell morphology”, “skeletal and muscular system development and function” and “lipid metabolism”. These were overrepresented with different significant levels associated with breed and stage. Moreover, the number of differential expressed genes involved in lipid-metabolism were perpetually overrepresented in DL breed type at almost all stages of muscle development compared to Pi concordant with the trait characteristics (Rehfeldt *et al.* 2000; Ponsuksili *et al.* 2007). IPA microRNA Target Filter analysis revealed miRNA-dependent regulatory networks sorted by negative-correlations between miRNA-mRNA and focused on cellular growth and proliferation and lipid-metabolism functions.

The miRNA-mRNA interactions are known to play important roles in many biological processes of skeletal muscle including skeletal muscle development; *TGFBR2* (Inamoto *et al.* 2010), *JARID2* (Walters et al. 2013) and *BCL2* (Biral et al. 2002), muscle hypertrophy; *CALM1* (Frey *et al.* 2000), muscle mass; *ACVR2B* (Walsh *et al.* 2007), and muscle energy metabolism; *ACSL4* (Mercadé *et al.* 2006; Ruść *et al.* 2011). These results emerged a complex miRNA-dependent regulatory network during muscle development and further added a comprehensive knowledge on the regulation of transcriptome during muscle development in pigs.

Co-expression and regulatory networks of mRNA-miRNA pairs of mature skeletal muscle of pig affecting on meat properties

This part of study aimed to expose a complex network between miRNA and gene interactions in skeletal muscle that associated with meat quality and carcass composition of meat production. Previous studies introduced methods for identification of miRNA-mRNA regulatory networks, by predicting putative target genes of miRNAs (Peng *et al.* 2009; Liu *et al.* 2010). In order to achieve our aim of constructing miRNA-mRNA co-expression networks, weighted gene co-expression network analysis WGCNA (Langfelder & Horvath 2008) was applied for clustering genes/ miRNA into modules based on their co-expression across a set of samples and finally to relate these modules to the traits. The gene in the module which negatively correlated and targets of miRNAs were used to construct miRNA-mRNA networks.

In **chapter 4**, total of 7 carcass traits and 13 meat quality traits were determind from 207 performance-tested crossbred pig [PI×(DL×DE)] samples. WGCNA grouped the genes into 22 modules based on patterns of co-expression. Of these only 5 modules (dark-turquoise, orange, red, black, and tan) were significantly correlated with meat quality. The functional annotation of the genes in modules was performed by using the database annotation from GO enrichment analysis and DAVID (Huang *et al.* 2009). Modules dark-turquoise and orange that positively correlated to pH traits and negatively to drip loss were highly enriched for genes belonging to the cluster “glucose metabolic process” and “response to wounding”, respectively. Whereas, modules red, black, and tan which negatively correlated to pH traits and positively to drip loss were enriched for genes functional annotation clusters of “mitochondrial ribosome”, “mitochondrion or oxidative phosphorylation”, and “extracellular matrix”, respectively.

All processes largely impact on postnatal development and growth and also on improvement of meat quality by playing a significant role in the cellular energy production of muscle cell (Rosenvold & Andersen 2003; Figueiredo *et al.* 2008; Romanello *et al.* 2010; Gan *et al.* 2013).

For the microRNA part, WGCNA revealed only 2 modules that were associated with meat quality. Module purple (consisted of 8 miRNA families: miR-17, -30, -221, -185, -324, -362, -500, and -542) was positively correlated to LF24MLD and negatively to pH24MLD and IMP24MLD. Module blue (consisted of 29 miRNA families: let-7, miR-15, -17, -31, -95, -103, -105, -122, -124, -130, -138, -154, -184, -185, -197, -202, -204, -212, -214, -320, -326, -335, -346, -383, -467, -491, -744, -1224, and -1296) was positively correlated to pH45MLD and IMP24MLD. Pairwise correlation coefficient analysis was performed and integrated to module-trait expression set and further confirm the miRNA targets by TargetScan (Friedman *et al.* 2009; Garcia *et al.* 2011) and RNAhybrid (Rehmsmeier *et al.* 2004) prediction tools. Final results identified major groups of co-expression gene (mRNA) - miRNA networks that are correlated with organismal traits and related to carcass and meat quality. According to the fundamental function of miRNA, an inverse expression relationship between miRNA and their target mRNAs can be expected in most cases (Huang *et al.* 2007; Pasquinelli 2012). Therefore, only the negative correlation between mRNA-miRNA pair relationship was concerned. In module dark-turquoise, we found that, the important apoptosis promoters *CREM* (Kosir et al. 2012) was highly negatively correlated with miR-153. Many miRNAs were found to negatively correlate to their target genes in modules red and black. These modules were enriched for genes related to mitochondrial pathways. Some important miRNA were energy metabolism modulator such as miR-338 (Aschrafi *et al.* 2008; Aschrafi *et al.* 2012), ATP production modulators like miR-15b, -16, -195, and -424 (Nishi *et al.* 2010) and electron transport modulator; miR-181c (Das *et al.* 2012). In module tan, one of the major fibrillar collagen family *Col6a1* (Gelse et al. 2003) was found as a target for many miRNAs including miR-132, -205, -320, -330, -375, -423, and -425. Also, skeletal muscle specific miRNAs including miR-1, miR-133, and miR-181 (Chen *et al.* 2006; Naguibneva *et al.* 2006; McCarthy & Esser 2007) were found in many modules. Altogether, this part of study enabled us to interpret the differentially-regulated genes from a systems perspective, yielding new

insight into several biological pathways underlying carcass and meat quality traits differences.

MiRNA-mRNA correlations in mitochondrial energy metabolism function during C2C12 myoblast differentiation

Related to the previous part, to precede the presumption that an mRNA-miRNA regulatory network involved in the muscle energy and metabolism, mouse C2C12 myoblast cells were used as a model tool for muscle differentiation. C2C12 is a well characterized C2C12 myogenic cell that reflects a concerted and controlled activation of transcription starting from mononucleated myoblasts until their fusing to form multinucleated myotubes (Burattini *et al.* 2004). This part of the study intended to identify miRNAs that can modulate the expression of genes involved in energy metabolism. The correlation between miRNA, mitochondrial energy metabolism genes and cellular ATP levels was analyses. In **chapter 5**, the ATP levels were increased during myogenic differentiating of C2C12 myoblasts. The relationship between ATP levels and myogenic differentiation has been previously analysed (Higham *et al.* 1993; Martinello *et al.* 2011), suggesting that ATP has an inhibitory effect on cell proliferation while simultaneously promoting myogenic differentiation of satellite cells (Ryten *et al.* 2002). Thus, changing ATP level during myoblast differentiation provided a good model to search for potential regulators of ATP production. Correlation coefficient analysis of miRNAs, mitochondria-related mRNAs, and cellular ATP levels at D0, D4, and D8 post-differentiation induction was used to build an miRNA-mRNA network. Both negative and positive correlation relationships were considered. The final results suggest 14 potential miRNAs (miR-423, -17, -130b, -301, -345, -15a, -16a, -128, -615, -1968, -1a/b, and -194) as cellular ATP regulators targeting genes interacting with 6 mitochondrial energy metabolism genes (*Cox4i2, Cox6a2, Ndufb7, Ndufs4, Ndufs5,* and *Ndufv1*). *Cox4i2* and *Cox6a2* are specifically expressed in heart and muscle tissues and functions in electron transfer in oxidative phosphorylation, which is responsible for 90% of ATP synthesis for muscle energy (Quintens *et al.* 2013). *Ndufb7, Ndufs5, Ndufv1,* and *Ndufs4* are members of the NADH dehydrogenase (ubiquinone) family of mitochondrial respiratory chain complex I, which represents the largest complex mediating electron transfer through the respiratory chain (Triepels *et al.* 2001; Mimaki *et al.* 2012). Therefore, all of them are important for ATP generation and energy

metabolism. By considering the down-regulated miRNAs which were negatively correlated with the increase ATP level, a group of miRNA was identified including miR-423, -17, -130b, -301, -345, -15a, -16a, and -615. Of this miR-15 and miR-16 have been reported to modulate cellular ATP levels by down regulation of *Arl2* target (Nishi *et al.* 2010) and directly targeting *BCL2* which leads to apoptosis induction through the regulation of mitochondrial function (Cimmino *et al.* 2005). MiR-301 and miR-17 also function on mitochondrial metabolism by regulating ATPase and translocase (Demongeot *et al.* 2013). The other group of miRNAs which was up-regulated and hence positively correlated with the ATP level during C2C12 myogenic differentiation includes miR-1a/b, -128, -194, and -1968. MiR-1 is well known as skeletal muscle-specific miRNA which play important functions on muscle development by regulating cell proliferation and differentiation during myogenesis (Chen *et al.* 2006; Goljanek-Whysall *et al.* 2012). MiR-128 also regulates muscle cell proliferation and differentiation by repressing one of the myogenic gene *Pax3*, in muscle side population cells (Motohashi et al. 2012). Among these potential miRNAs, miR-423-3p was selected for further functional validation with 3 of its potential target genes, *Cox6a2*, *Ndufb7*, *Ndufs5,* due to the reason that miR-423-3p showed a high inverse correlation with increased ATP level. Besides, it has been implicated in promoting cell growth and cell cycle progression (Lin *et al.* 2011), yet its function in cellular ATP regulation is still unknown. The RNAhybrid tool (Rehmsmeier *et al.* 2004) was used under the default criteria, and revealed at least one binding site on 3'-UTR of target mRNAs for the miR-423 (miR-423-3p in mouse). As a functional validation, miR-423-3p was overexpressed during C2C12 myogenic differentiation using mouse synthetic miR-423-3p mimic transfection. After 24 hours post-transfection, miR-423-3p mimic produced decreased cellular ATP level and decreased the expression of *Cox6a2, Ndufb7,* and *Ndufs5* and increased the expression of two myogenic markers, *Tnnt1* and *Myh1* compared to the negative control. In conclusion of this section, the results suggested that miR-423-3p function as an ATP/energy metabolism modulator by targeting *Cox6a2, Ndufb7,* and *Ndufs5* and may be required for C2C12 myogenic differentiation.

The multistate study was designed to meet the need for new, fundamental information about the molecular and cellular processes that regulate the development of skeletal muscle. Each chapter of this thesis could be considered as a stand-alone study, but the common purpose of each was to gain and identify the potential miRNAs and their

candidate target in muscle. Taken together, this thesis has identified the potential miRNAs and their candidate target genes in regulatory networks that provide insight into the mechanism underlying of skeletal muscle development, growth and metabolism which affect meat quality. Many miRNA-mRNA correlations were identified that need to be further validated in functional studies of these miRNAs in skeletal muscle.

Future perspectives

In future efforts, we will continue to clarify the the relationship of particular miRNAs and mRNAs on an experimental level in biological functional analyses for a concrete and consistent validation of current results. More studies are required to fully comprehend how miRNAs impact on skeletal muscle development, growth and metabolism. Future experiment will be including the *in vitro* functional validation of transient over-expression or knockdown of miRNAs using a design shRNA vector and also the determination of their target at the protein levels.

At present, numerous reports show that the expression profiling of miRNAs is a useful diagnostic and prognostic screening tool. MiRNAs may serve as novel biomarkers for some physiological and pathological conditions (Chen *et al.* 2008; Bader *et al.* 2011; Mo *et al.* 2012). Circulating miRNA biomarkers are commonly used. A remarkably stable form of miRNAs can be readily detected in serum (Chen *et al.* 2008; Fang *et al.* 2012), plasma or whole blood (Heneghan *et al.* 2010). Some of muscle specific miRNA: miR-1, -133 and -208 have been reported as circulating markers for muscular disorders such as cardiomyocyte death in myocardial infarction (Wang *et al.* 2010; Kuwabara *et al.* 2011; Wang *et al.* 2013) and other myopathies (Eisenberg *et al.* 2008; Cacchiarelli *et al.* 2011). The up-regulated miRNA in peripheral circulation is mainly originated from different types of injuries of body compartments (Schöler *et al.* 2010). These intracellular actions of miRNAs are suggesting that they may have roles distant from the cell from which they originate. Moreover, one recent observation from Zhang *et al.* (2012) provided a groundbreaking new possibility on the environmental dietary effects across broad phylogenies. Intake of exogenous plant miRNA by food can pass through gastrointestinal track and enter in the bloodstream, then resulting in cross-kingdomly modulated target gene regulation in animals (Zhang *et al.* 2012b). Consistent with all of these evident discoveries, in future work, it would be a challenge to further investigate and discover new generations of miRNA biomarkers of some

specific phenotype correlated with the meat quality traits. This could allow extending the area of investigation of the direct oral RNA delivery to improve the meat characteristics in domestic animals. In conclusion, we expect that our further validation results will help to develop further new miRNAs biomarkers to aid in the prevention or selection of meat quality traits or diseases in farm animals in the future.

CHAPTER 7

Summary

Summary

In domestic animals, skeletal muscle is a tissue of major economic importance for meat production. Maintaining skeletal muscle function throughout the lifespan is necessary for good health and living, which finally influence in meat production. For the improvement of the muscle function at optimal levels, the efficient regulation of complex processes that control muscle development, growth and metabolism are required.

At present, molecular genetic methods are used to identify genes that control variation in phenotypes like meat quality. Recently, a new regulatory factor which is able to regulate gene expression at the post-transcriptional level has been discovered. MiRNAs are a set of small, non-protein-coding RNAs. Mature miRNAs comprise single-stranded ~22 nucleotides in length. The regulation of miRNA expression can occur both at the transcriptional and at the post-transcriptional level, by binding to the 3'-UTR of the target genes and either repression of protein translation or induction of mRNA degradation. Due to general target recognition capability of miRNA, several miRNAs consent to target the same 3'-UTR and a single miRNA can directly inhibit the expression of numbers of mRNAs/genes, thus creating a complex regulatory network. Therefore, the potential miRNAs and their target gene regulation networks may enable providing new directions in molecular animal breeding. It will extend the fundamental knowledge of the factors controlling skeletal muscle function including muscle development, growth and metabolism and resulting in improved efficiency of meat production.

The aims of the thesis have been achieved; the potential miRNAs and their target genes regulation networks which are involved in skeletal muscle have been identified and the understanding of their biological roles and regulations under different physiological conditions of muscle has been extended. In **chapter 2,** custom miRNA array were used for comparative miRNA expression profiling of skeletal muscle across three muscle fibre developmental stages and adult pigs in two different breeds. German Landrace (DL) and Pietrain (Pi) pigs are considerably different in numerous traits including growth rate, body composition, muscularity and fat content. Differentially expressed miRNAs were identified between the two breeds. Several miRNAs were observed to be associated with breed type and expressed in a developmental stages-specific manner.

Such as, Let-7, miR-103, -143, -15, -199, -17 families and miR-30 were consistently higher expressed in Pi pigs compared to DL pigs, whereas, miR-122, -133, -181, and -214 were up-regulated in DL pigs compared to Pi. Related to the previous study, in **chapter 3,** Affymetrix GeneChip porcine Genome Arrays were used to perform the profiling of differential gene expression upon each developmental stage. A combination of mRNA and miRNA expression profiling data derived from the same samples (in **chapter 2**) was achieved by Ingenuity Pathway Analysis. This method has shown to be effective in identifying the most prominent interactions from the databases of putative targets as well as validated information. The analysis established a complex miRNA–dependent regulatory network and revealed insights into their role during skeletal muscle development and growth. Interestingly, a number of miRNA-mRNA interactions was associated to cellular growth and proliferation and lipid-metabolism functions. During prenatal stage, the differential expression genes overrepresented in these bio-functions tend to be higher expressed in DL than in Pi. But at adult stage, they are overrepresented in Pi breed. **Chapter 4** addresses *post mortem* pig traits obtained in a crossbred [PI×(DL×DE)] population (n = 207). To identify miRNA-mRNA co-expression networks correlated with muscle phenotypes, both custom miRNA arrays and Affymetrix GeneChip porcine Genome Arrays were used. This study integrated miRNA and mRNA expression results as well as network analysis by using WGCNA, pairwise correlation coefficient analysis and miRNA target prediction tools. The final results identified groups of negative miRNA-mRNA co-expression networks enriched in several biological pathways underlying the difference of carcass and meat quality traits for example glucose metabolism, mitochondrial processes and oxidative phosphorylation. Moreover, some of these established miRNA-mRNA co-expression interactions have confirmed miRNA binding sites according to TargetScan and RNAhybrid. The functional study of such mRNAs-miRNAs regulatory networks involved in the muscle energy metabolism was further demonstrated. In **chapter 5,** mouse C2C12 cells were used as a model tool for muscle differentiation starting from myoblasts to form multinucleated myotubes. Previous publications revealed the relationship between ATP levels and myogenic differentiation. Therefore, changing ATP level during myoblast differentiation provided a good model to search for potential regulators of ATP production. This chapter identify miRNAs that can modulate expression of genes involved in energy metabolism and revealed a link between

miRNA, mitochondrial energy metabolism genes and cellular ATP levels by correlation coefficient analysis. Both negative and positive miRNA-mRNA correlation relationships were considered. The final results suggest 14 miRNAs (miR-423, -17, -130b, -301, -345, -15a, -16a, -128, -615, -1968, -1a/b, and -194) as cellular ATP regulators targeting genes including 6 genes of mitochondrial energy metabolism genes (*Cox4i2, Cox6a2, Ndufb7, Ndufs4, Ndufs5,* and *Ndufv1*) Of these, miR-423-3p was selected for further functional validation with 3 of its potential target genes, *Cox6a2, Ndufb7, Ndufs5*. Overexpression of miR-423-3p during C2C12 myogenic differentiation decreased cellular ATP level and decreased the expression of all target genes as well as down-regulated the expression of two myogenic markers, *Tnnt1* and *Myh1* compared to the negative control. In conclusion, the results suggest that miR-423-3p functions as an ATP/energy metabolism modulator by targeting Cox6a2, Ndufb7, and Ndufs5 and may be required for C2C12 myogenic differentiation.

In summary, many miRNA involved in regulation of biological processes of muscle development, growth and metabolism and finally meat quality or quantity were identify. This thesis was performed, mainly by *in vivo* studies that relate miRNAs abundance in muscle development at different stages, and abundance in mature muscle to phenotypic carcass and meat quality traits. The *in vitro* study, identified potential miRNAs, which are involved in the process of muscle differentiation and mitochondria energy metabolism. Finally, this thesis brought together the potential miRNAs and their candidate target genes and established regulatory networks that provide insight into the mechanism underlying skeletal muscle growth and development and muscle metabolism. Many miRNA-mRNA correlations were identified and need to be further validated in functional studies of these miRNAs in skeletal muscle.

Zusammenfassung

Bei Nutztieren ist die Skelettmuskulatur von entscheidender ökonomischer Bedeutung für die Fleischproduktion. Die Aufrechterhaltung der Funktion der Skelettmuskulatur über die gesamte Lebensdauer ist zudem essentiell für die Gesundheit und das Wohlbefinden der Tiere und beeinflusst die Fleischqualität. Die effiziente Regulation von komplexen Prozessen, die der Steuerung von Muskelentwicklung, -wachstum und -stoffwechsel dienen, ist notwendig, um die Muskelfunktionen nachhaltig zu verbessern.
Mit den erst kürzlich entdeckten miRNAs steht ein großes Repertoire an Regulatoren zur Verfügung, welche die Genexpression und damit die Merkmalsausprägung beeinflussen können. Reife miRNAs bestehen aus 22 Nukleotiden langen Einzelsträngen, die nicht für Proteine kodierenden. MiRNAs können die Genexpression sowohl auf transkriptioneller als auch auf post-transkriptioneller Ebene regulieren. Auf post-transkriptioneller Ebene binden miRNAs an das 3'UTR von regulierten Zieltranskripten und können dadurch die Translation des Proteins hemmen oder den Abbau der mRNA induzieren. Aufgrund der Interaktion von miRNAs mit Zieltranskripten über kurze Erkennungssequenzen können mehrere miRNAs das gleiche 3'UTR binden und dementsprechend auch einzelne miRNAs zahlreiche mRNAs bzw. Gene direkt inhibieren. Dadurch entstehen komplexe regulatorische Netzwerke die neue Möglichkeiten für die molekulare Tierzucht darstellen. Die Analyse derartiger Netzwerke wird wesentlich zum Wissensstand über Faktoren beitragen, die an der Regulation der Muskelfunktion, insbesondere der Muskelentwicklung, des Muskelwachstums und des Muskelstoffwechsely, beiteiligt sind und mit einer effizienten Fleischproduktion einhergehen.
Die gesteckten Ziele dieser Arbeit konnten erreicht werden; potentielle miRNAs und von diesen regulierte Gennetzwerke, die für die Skelettmuskulatur relvant sind, konnten identifiziert werden und haben darüber hinaus wesentlich zum Wissensstand über die biologische Funktion von miRNAs und ihrer Regulation im Kontext unterschiedlicher physiologischer Zustände des Muskels beigetragen.
In Kapitel 2, konnten unter Verwendung von spezifisch gefertigten miRNA-Arrays vergleichende miRNA-Expressionsprofile der Skelettmuskulatur über drei Entwicklungsstadien der Muskelfaser sowie in erwachsenen Schweinen von zwei verschiedenen Rassen erzeugt werden. Die Schweine der ausgewählten Rassen

Deutsche Landrasse (DL) und Pietrain (Pi) unterscheiden sich deutlich in zahlreichen relevanten Merkmalen, einschließlich dem Wachstum, der Körperzusammensetzung, dem Muskelansatz und dem Fettgehalt. Auf dieser Grundlage konnten differenziell exprimierte miRNAs zwischen beiden Fleischrassen aufgezeigt werden. Weiterhin konnte in Bezug auf die Regulation einiger miRNAs nachgewiesen werden, dass diese sowohl rassenspezifische, als auch entwicklungsspezifische Expressionsmuster haben. Die miRNAs der Let-7, miR-103, -143, -15, -199 und -17 Familien und miR-30 waren in Pi konsistent höher exprimiert als in DL. Im Gegensatz dazu waren miR-122, -133, -181, und -214 in DL hochreguliert. Im Zusammenhang mit der vorausgehenden Studie zeigt Kapitel 3 die unter Verwendung von porcinen Genommikroarrays (Affymetrix GeneChip) erzeugten Profile der differentiell exprimierten Transkripte in jedem Entwicklungsstadium. Auf dieser Grundlage erfolgte die Integration der Analysen mit den miRNA-Expressionsdaten der gleichen Proben (Kapitel 2) mittels Ingenuity Pathway Analysis. Dieses Verfahren hat sich als effektiv erwiesen, um die wichtigsten Interaktionen aus den Datenbanken der Zieltranskripte zu identifizieren und die Informationen zu validieren. Aus den Analysen geht ein komplexes miRNA-abhängiges regulatorisches Netzwerk hervor, welches Einblicke in die Rolle der miRNAs während der Entwicklung und dem Wachstum der Skelettmuskulatur ermöglicht. Interessanterweise sind zahlreiche Interaktion von miRNAs und mRNAs mit Funktionen des Zellwachstums, der Proliferation und des Lipidstoffwechsels assoziiert. In der pränatalen Phase sind die meisten der differentiell exprimierten Gene der genannten biologischen Funktionen in DL tendenziell stärker reguliert als in Pi, während sich diese Verhältnisse im adulten Stadium umgekehrt darstellen. Kapitel 4 beinhaltet Untersuchungen zur Ausprägung von *post mortem* Merkmalen erfasst in einer Population von Tieren der Dreirassenkreuzung PI×(DL×DE) (n = 207). Um Ko-Expressionsnetzwerke von miRNA und mRNA zu identifizieren, die mit bestimmten phänotypischen Ausprägungen der Muskulatur korrelieren, wurden sowohl spezifisch gefertigte miRNA-Arrays als auch porcine mRNA Mikroarrays verwendet. Die Ergebnisse der miRNA- und mRNA-Expressionsanalysen wurden im Weiteren mittels gewichteten Netzwerkanalysen (WGCNA), paarweisen Analysen von Korrelationskoeffizenten und Programmen zur Vorhersage von miRNA-*targets* integriert. Die Ergebnisse zeigen, dass negative Ko-Expressionsnetzwerke in Signalwegen angereichert sind, die für die phänotypischen Unterschiede der

Schlachtkörper- und Fleischqualitätsmerkmale ursächlich sind. Dazu gehören beispielsweise Prozesse des Glucosestoffwechsels, des mitochondrialen Stoffwechsels und die oxidativen Phosphorylierung. Zudem konnten für einige der nachgewiesenen Interaktionen zwischen miRNA und mRNA entsprechende miRNA-Bindungsstellen in den Transkripten mittels Targetscan und RNAhybrid identifizierten werden. Die funktionelle Untersuchung solcher mRNA-miRNA regulatorischen Netzwerke mit Anteil am Energie- und Muskelstoffwechsel wurden weiter fokussiert. In Kapitel 5 sind die Untersuchungen am murinen C2C12-Zellensystem dargestellt, welches als *in vitro* Modell für die myogene Differenzierung ausgehend vom Myoblasten bis hin zum vielkernigen Myotuben dient. Aufgrund des nachgewiesenen Zusammenhangs zwischen dem ATP-Spiegel und der myogenen Differenzierung eignen sich die veränderlichen ATP-Spiegel während der Differenzierung der Myoblasten als gutes Modell, um potentielle Regulatoren der ATP-Produktion zu identifizieren. Dabei wurden miRNAs identifiziert, welche in der Lage sind, die Expression von Genen des Energiehaushalts zu modulieren. Zudem konnten durch die Analyse der Korrelationskoeffizienten Zusammenhänge zwischen miRNAs, Genen des mitochondrialen Energiemetabolismus und zellulären ATP-Gehalten aufgezeigt werden. Sowohl negative als auch positive miRNA-mRNA-Korrelationen wurden dabei berücksichtigt. Die endgültigen Ergebnisse legen nahe, dass 14 potentielle miRNAs (miR-423, -17, -130b, -301, -345, -15a, -16a, -128, -615, -1968, -1a/b, und -194) als zelluläre ATP-Regulatoren fungieren und mit Genen interagieren, einschließlich 6 Genen (Cox4i2, Cox6a2, Ndufb7, Ndufs4, Ndufs5 und NDUFV1) des mitochondrialen Energiestoffwechsels. Davon wurden miR-423-3p und die potentiellen Zielgene Cox6a2, Ndufb7 und Ndufs5 zur weiteren funktionellen Validierung ausgewählt. Die Überexpression von miR-423-3p während der C2C12-Differenzierung führte zu reduzierten zellulären ATP-Konzentrationen, zur verringerten Expression aller Zielgene sowie der verringerten Expression der myogenen Marker Tnnt1 und MYH1 im Vergleich zur Negativkontrolle. Die Ergebnisse deuten darauf hin, dass miR-423-3p durch die Interaktion mit Cox6a2, Ndufb7 und Ndufs5 als Modulator des ATP- bzw. Energiemetabolismus fungiert und somit für die myogene Differenzierung von C2C12-Zellen notwendig ist.

Insgesamt konnten zahlreiche miRNAs identifiziert werden, die an der Regulation von biologischen Prozessen der Entwicklung, des Wachstum und des Stoffwechsel der

Muskulatur beteiligt sind und im Endeffekt für die Fleischqualität und -quantität von Bedeutung sind. Insbesondere aus den durgeführten *in vivo* Studien geht hervor, dass miRNAs in verschiedenen Stadien der Skelettmuskelentwicklung präsent sind und auch im ausdifferenzierten Muskeln mit Merkmalen des Schlachtkörpers und der Fleischqualität im Zusammenhang stehen. Die *in vitro* Studien führten zur Identifizierung potentieller miRNAs, die an Prozessen der Muskeldifferenzierung und dem mitochondiralem Energiestoffwechsel beteiligt sind. Durch die Studien im Rahmen der vorliegenden Dissertation konnten die miRNAs und ihre potentiellen Zielgene zu komplexen regulatorischen Netzwerken verknüpft werden und ein Einblick in die zugrundeliegenden Mechanismen des Wachstum, der Entwicklung und des Metabolismus der Skelettmuskulatur ermöglicht werden. Ein Kernpunkt zukünftiger Studien wird die experimentelle Validierung der identifizierten Zusammenhänge zwischen miRNAs und mRNAs durch funktionelle Studien der miRNAs in der Skelettmuskulatur sein.

CHAPTER 8

References

References

Ambros V. (2004) The functions of animal microRNAs. *Nature* **16**, 350-5.

Aschrafi A., Kar A., Natera-Naranjo O., Macgibeny M., Gioio A. & Kaplan B. (2012) MicroRNA-338 regulates the axonal expression of multiple nuclear-encoded mitochondrial mRNAs encoding subunits of the oxidative phosphorylation machinery. *Cell Mol Life Sci.* **69**, 4017-27.

Aschrafi A., Schwechter A., Mameza M., Natera-Naranjo O., Gioio A. & Kaplan B. (2008) MicroRNA-338 regulates local cytochrome c oxidase IV mRNA levels and oxidative phosphorylation in the axons of sympathetic neurons. *J Neurosci.* **28**, 12581-90.

Ashmore C.R., Addis P.B. & Doerr L. (1973) Development of Muscle Fibers in the Fetal Pig. *J ANIM SCI* **36**, 1088-93.

Bader A., Brown D., Stoudemire J. & Lammers P. (2011) Developing therapeutic microRNAs for cancer. *Gene Ther.* **18**, 1121-6.

Baek D., Villén J., Shin C., Camargo F., Gygi S. & Bartel D. (2008) The impact of microRNAs on protein output. *Nature.* **455**, 64-71.

Baker J., McCormick M. & Robergs R. (2010) Interaction among Skeletal Muscle Metabolic Energy Systems during Intense Exercise. In: *J Nutr Metab.*

Bartel D. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* **116**, 281-97.

Bartel D. (2009) MicroRNAs: target recognition and regulatory functions. *Cell.* **136**, 215-33.

Benjamin Y. & Hochberg Y. (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J R Stat Soc Ser B* **57**, 289-300.

Bentzinger C., Wang Y. & Rudnicki M. (2012) Building muscle: molecular regulation of myogenesis. *Cold Spring Harb Perspect Biol.* **4**, pii: a008342.

Bérard J., Kalbe C., Lösel D., Tuchscherer A. & Rehfeldt C. (2011) Potential sources of early-postnatal increase in myofibre number in pig skeletal muscle. *Histochem Cell Biol* **136**, 217-25.

Betel D., Koppal A., Agius P., Sander C. & Leslie C. (2010) Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. *Genome Biol.* **11**, R90.

Betel D., Wilson M., Gabow A., Marks D. & Sander C. (2008) The microRNA.org resource: targets and expression. *Nucleic Acids Res.* **36**, D149-53.

Biral D., Jakubiec-Puka A. & Betto R. (2002) Expression of Bcl-2 Family Proteins in Recovering and Regenerating Muscles. *Basic and Applied Myology* **12**, 43-6.

Bracken C., Szubert J., Mercer T., Dinger M., Thomson D., Mattick J., Michael M. & Goodall G. (2011) Global analysis of the mammalian RNA degradome reveals widespread miRNA-dependent and miRNA-independent endonucleolytic cleavage. *Nucleic Acids Res.* **39**, 5658-68.

Braun T. & Gautel M. (2011) Transcriptional mechanisms regulating skeletal muscle differentiation, growth and homeostasis. *Nat Rev Mol Cell Biol.* **12**, 349-61.

Brennecke J., Stark A., Russell R. & Cohen S. (2005) Principles of microRNA-target recognition. *PLoS Biol.* **3**, e85.

Brent A. & Tabin C. (2002) Developmental regulation of somite derivatives: muscle, cartilage and tendon. *Curr Opin Genet Dev.* **12**, 548-57.

Bryson-Richardson R. & Currie P. (2008) The genetics of vertebrate myogenesis. *Nat Rev Genet.* **9**, 632-46.

Burattini S., Ferri P., Battistelli M., Curci R., Luchetti F. & Falcieri E. (2004) C2C12 murine myoblasts as a model of skeletal muscle development: morpho-functional characterization. *Eur J Histochem.* **48**, 223-33.

Burnstock G., Knight G. & Greig A. (2012) Purinergic signaling in healthy and diseased skin. *J Invest Dermatol.* **132**, 526-46.

Cacchiarelli D., Legnini I., Martone J., Cazzella V., A. D.A., E. B. & I. B. (2011) miRNAs as serum biomarkers for Duchenne muscular dystrophy. *EMBO Mol Med.* **3**, 258-65.

Callis T., Pandya K., Seok H., Tang R., Tatsuguchi M., Huang Z., Chen J., Deng Z., Gunn B., Shumate J., Willis M., Selzman C. & Wang D. (2009) MicroRNA-208a is a regulator of cardiac hypertrophy and conduction in mice. *J Clin Invest.* **119**, 2772-86.

Cardinali B., Castellani L., Fasanaro P., Basso A., Alemà S., Martelli F. & Falcone G. (2009) Microrna-221 and Microrna-222 Modulate Differentiation and Maturation of Skeletal Muscle Cells. *PLoS ONE* **4**, e7607.

Carraro G., El-Hashash A., Guidolin D., Tiozzo C., Turcatel G., Young B.M., De Langhe S.P., Bellusci S., Shi W., Parnigotto P.P. & Warburton D. (2009) miR-

17 family of microRNAs controls FGF10-mediated embryonic lung epithelial branching morphogenesis through MAPK14 and STAT3 regulation of E-Cadherin distribution. *Dev. Biol.* **333** 238-50.

Chang J., Nicolas E., Marks D., Sander C., Lerro A., Buendia M., Xu C., Mason W., Moloshok T., Bort R., Zaret K. & Taylor J. (2004) miR-122, a mammalian liver-specific microRNA, is processed from hcr mRNA and may downregulate the high affinity cationic amino acid transporter CAT-1. *RNA Biol.*, 106-13.

Chargé S. & Rudnicki M. (2004) Cellular and Molecular Regulation of Muscle Regeneration. *Physiol Rev.* **84**, 209-38.

Chen C., Ai H., Ren J., Li W., Li P., Qiao R., Ouyang J., Yang M., Ma J. & Huang L. (2011) A global view of porcine transcriptome in three tissues from a full-sib pair with extreme phenotypes in growth and fat deposition by paired-end RNA sequencing. *BMC Genomics* **12**.

Chen J.-F., Callis T.E. & Wang D.-Z. (2009) microRNAs and muscle disorders. *Journal of Cell Science* **122**, 13-20.

Chen J.-F., Mandel E.M., Thomson J.M., Wu Q., Callis T.E., Hammond S.M., Conlon F.L. & Wang D.-Z. (2006) The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nature Genetics* **38**, 228 - 33.

Chen J.-F., Tao Y., Li J., Deng Z., Yan Z., Xiao X. & Wang D. (2010) microRNA-1 and microRNA-206 regulate skeletal muscle satellite cell proliferation and differentiation by repressing Pax7. *J Cell Biol.* **190**, 867-79.

Chen J.-H., Wei W.-J., Xiao X., Zhu M.-J., Fan B. & Zhao S.-H. (2008) Expression analysis of miRNAs in porcine fetal skeletal muscle on days 65 and 90 of gestation. *Asian-Aust. J. Anim. Sci* **21**, 954-60.

Chen K. & Rajewsky N. (2006) Natural selection on human microRNA binding sites inferred from SNP data. *Nat Genet.* **38**, 1452-6.

Chen X., Ba Y., Ma L., Cai X., Yin Y., Wang K., Guo J., Zhang Y., Chen J., Guo X., Li Q., Li X., Wang W., Zhang Y., Wang J., Jiang X., Xiang Y., Xu C., Zheng P., Zhang J., Li R., Zhang H., Shang X., Gong T., Ning G., Wang J., Zen K., Zhang J. & Zhang C. (2008) Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res.* **18**, 997-1006.

Cimmino A., Calin G., Fabbri M., Iorio M., Ferracin M., Shimizu M., Wojcik S., Aqeilan R., Zupo S., Dono M., Rassenti L., Alder H., Volinia S., Liu C., Kipps

T., Negrini M. & Croce C. (2005) miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A.* **102**, 13944-9.

Clop A., Marcq F., Takeda H., Pirottin D., Tordoir X., Bibé B., Bouix J., Caiment F., Elsen J., Eychenne F., Larzul C., Laville E., Meish F., Milenkovic D., Tobin J., Charlier C. & Georges M. (2006) A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. *Nat Genet.* **38**, 813-8.

Crist C., Montarras D., Pallafacchina G., Rocancourt D., Cumano A., Conway S. & Buckingham M. (2009) Muscle stem cell behavior is modified by microRNA-27 regulation of Pax3 expression. *Proc Natl Acad Sci U S A.* **106**, 13383-7.

da Costa Martins P., Salic K., Gladka M., Armand A., Leptidis S., el Azzouzi H., Hansen A., Coenen-de Roo C., Bierhuizen M., van der Nagel R., van Kuik J., de Weger R., de Bruin A., Condorelli G., Arbones M., Eschenhagen T. & De Windt L. (2010) MicroRNA-199b targets the nuclear kinase Dyrk1a in an auto-amplification loop promoting calcineurin/NFAT signalling. *Nat Cell Biol.* **12**, 1220-7.

Das S., Ferlito M., Kent O., Fox-Talbot K., Wang R., Liu D., Raghavachari N., Yang Y., Wheelan S., Murphy E. & Steenbergen C. (2012) Nuclear miRNA regulates the mitochondrial genome in the heart. *Circ Res.* **110**, 1596-603.

Demongeot J., Cohen O., Doncescu A. & Henrion-Caude A. (2013) MitomiRs and Energetic Regulation. In: *27th International Conference on Advanced Information Networking and Applications Workshops*, pp. 1501-8. IEEE Computer Society Washington, DC, USA ©2013, Barcelona, Spain

Dey B., Gagan J. & Dutta A. (2011) miR-206 and -486 induce myoblast differentiation by downregulating Pax7. *Mol Cell Biol.* **31**, 1329.

Doench J. & Sharp P. (2004) Specificity of microRNA target selection in translational repression. *Genes Dev.* **18**, 504-11.

Dong S., Yang B., Guo H. & Kang F. (2012) MicroRNAs regulate osteogenesis and chondrogenesis. *Biochem Biophys Res Commun.* **418**, 587-91.

Duisters R.F., Tijsen A.J., Schroen B., Leenders J.J., Lentink V., van der Made I., Herias V., van Leeuwen R.E., Schellings M.W., Barenbrug P., Maessen J.G., Heymans S., Pinto Y.M. & Creemers E.E. (2009) miR-133 and miR-30

Regulate Connective Tissue Growth Factor Implications for a Role of MicroRNAs in Myocardial Matrix Remodeling. *Circulation Res.* **104**, 170-8.

Dwyer C., Stickland N. & Fletcher J. (1994) The influence of maternal nutrition on muscle fiber number development in the porcine fetus and on subsequent postnatal growth. *J ANIM SCI* **72**, 911-7.

Eisenberg I., Eran A., Nishino I., Moggio M., Lamperti C., Amato A., Lidov H., Kang P., North K., Mitrani-Rosenbaum S., Flanigan K., Neely L., Whitney D., Beggs A., Kohane I. & Kunkel L. (2008) Distinctive patterns of microRNA expression in primary muscular disorders. *Proc Natl Acad Sci U S A.* **105**, 399.

Elia L., Contu R., Quintavalle M., Varrone F., Chimenti C., Russo M., Cimino V., De Marinis L., Frustaci A., Catalucci D. & Condorelli G. (2009) Reciprocal regulation of microRNA-1 and insulin-like growth factor-1 signal transduction cascade in cardiac and skeletal muscle in physiological and pathological conditions. *Circulation.* **120**, 2377-85.

Esau C., Davis S., Murray S.F., Xian Yu X., Pandey S.K., Pear M., Watts L., Booten S.L., Graham M., McKay R., Subramaniam A., Propp S., Lollo B.A., Freier S., Bennett C.F., Bhanot S. & Monia B.P. (2006) miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab.* **3**, 87-98.

Fabian M., Sonenberg N. & Filipowicz W. (2010) Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem.* **79**, 351-79.

Fang C., Zhu D., Dong H., Zhou Z., Wang Y., Liu L., Fan L., Miao K., Liu P., Xu W. & Li J. (2012) Serum microRNAs are promising novel biomarkers for diffuse large B cell lymphoma. *Ann Hematol.* **91**, 553-9.

Favier F., Benoit H. & Freyssenet D. (2008) Cellular and molecular events controlling skeletal muscle mass in response to altered use. *Pflügers Archiv - European Journal of Physiology* **456**, 587-600.

Figueiredo P., Mota M., Appell H. & Duarte J. (2008) The role of mitochondria in aging of skeletal muscle. *Biogerontology.* **9**, 67-84.

Filipowicz W., Bhattacharyya S.N. & Sonenberg N. (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat. Rev. Genet.* **9**, 102-14.

Flynt A.S., Li N., Thatcher E.J., Solnica-Krezel L. & Patton J.G. (2007) Zebrafish miR-214 modulates Hedgehog signaling to specify muscle cell fate. *Nature Genetics* **39**, 259 - 63.

Foxcroft G., Dixon W., Novak S., Putman C., Town S. & Vinsky M. (2006) The biological basis for prenatal programming of postnatal performance in pigs. *J Anim Sci.* **84**, E105-E12.

Frey N., Richardson J. & Olson E. (2000) Calsarcins, a novel family of sarcomeric calcineurin-binding proteins. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 14632-7.

Friedman R.C., Farh K.K.-H., Burge C.B. & Bartel D.P. (2009) Most mammalian mRNAs are conserved targets of microRNAs. *Genome. Res.* **19**, 92-105.

Gambardella S., Rinaldi F., Lepore S., Viola A., Loro E., Angelini C., Vergani L., Novelli G. & Botta A. (2010) Overexpression of microRNA-206 in the skeletal muscle from myotonic dystrophy type 1 patients. *J Transl Med.* **8**, 48.

Gan Z., Rumsey J., Hazen B., Lai L., Leone T., Vega R., Xie H., Conley K., Auwerx J., Smith S., Olson E., Kralli A. & Kelly D. (2013) Nuclear receptor/microRNA circuitry links muscle fiber type to energy metabolism. *J Clin Invest.* **123**, 2564-75.

Garcia D., Baek D., Shin C., Bell G., Grimson A. & Bartel D. (2011) Weak seed-pairing stability and high target-site abundance decrease the proficiency of lsy-6 and other microRNAs. *Nat Struct Mol Biol.* **18**, 1139-46.

Gelse K., Pöschl E. & Aigner T. (2003) Collagens--structure, function, and biosynthesis. *Adv Drug Deliv Rev.* **55**, 1531-46.

Gilbert S. (2000) *Developmental Biology*. Sinauer Associates, Sunderland

Goljanek-Whysall K., Pais H., Rathjen T., Sweetman D., Dalmay T. & Münsterberg A. (2012) Regulation of multiple target genes by miR-1 and miR-206 is pivotal for C2C12 myoblast differentiation. *J Cell Sci* **125**, 3590-600.

Goljanek-Whysall K., Sweetman D, Abu-Elmagd M., Chapnik E., Dalmay T., Hornstein E. & Münsterberg A. (2011) MicroRNA regulation of the paired-box transcription factor Pax3 confers robustness to developmental timing of myogenesis. *Proc Natl Acad Sci U S A.* **108**, 11936-41.

Granjon A., Gustin M., Rieusset J., Lefai E., Meugnier E., Güller I., Cerutti C., Paultre C., Disse E., Rabasa-Lhoret R., Laville M., Vidal H. & Rome S. (2009) The

microRNA signature in response to insulin reveals its implication in the transcriptional action of insulin in human skeletal muscle and the role of a sterol regulatory element-binding protein-1c/myocyte enhancer factor 2C pathway. *Diabetes.* **58**, 2555-64.

Grimson A., Farh K., Johnston W., Garrett-Engele P., Lim L. & Bartel D. (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell.* **27**, 91-105.

He A., Zhu L., Gupta N., Chang Y. & Fang F. (2007) Overexpression of micro ribonucleic acid 29, highly up-regulated in diabetic rats, leads to insulin resistance in 3T3-L1 adipocytes. *Mol Endocrinol.* **21**, 2785-94.

Heneghan H., Miller N., Lowery A., Sweeney K., Newell J. & Kerin M. (2010) Circulating microRNAs as novel minimally invasive biomarkers for breast cancer. *Ann Surg.* **251**, 499-505.

Higham S., Melikian J., Karin N., Ismail-Beigi F. & Pressley T. (1993) Na,K-ATPase expression in C2C12 cells during myogenesis: minimal contribution of alpha 2 isoform to Na,K transport. *J Membr Biol.* **131**, 129-36.

Hirai H., Verma M., Watanabe S., Tastad C., Asakura Y. & Asakura A. (2010) MyoD regulates apoptosis of myoblasts through microRNA-mediated down-regulation of Pax3. *J Cell Biol.* **191**, 347-65.

Hocquettea J.F., Ortigues-Martya I., Pethickb D., Herpinc P. & . F.X. (1998) Nutritional and hormonal regulation of energy metabolism in skeletal muscles of meat-producing animals. *Livestock Production Science* **56**, 115-43.

Huang D., Sherman B. & Lempicki R. (2009) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* **37**, 1-13.

Huang J., Babak T., Corson T., Chua G., Khan S., Gallie B., Hughes T., Blencowe B., Frey B. & Morris Q. (2007) Using expression profiling data to identify human microRNA targets. *Nat Methods.* **4**, 1045-9.

Huang T.-H., Zhu M.-J., Li X.-Y. & Zhao S.-H. (2008) Discovery of Porcine microRNAs and Profiling from Skeletal Muscle Tissues during Development. *PLoS ONE* **3**, 3225.

Huang Z., Chen X., Yu B., He J. & Chen D. (2012) MicroRNA-27a promotes myoblast proliferation by targeting myostatin. *Biochem Biophys Res Commun.* **423**, 265-9. .

Huntzinger E. & Izaurralde E. (2011) Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet.* **12**, 99-110.

Inamoto S., Kwartler C.S., Lafont A.L., Liang Y.Y., Fadulu V.T., Duraisamy S., Willing M., Estrera A., Safi H., Hannibal M.C., Carey J., Wiktorowicz J., Tan F.K., Feng X.-H., Pannu H. & Milewicz D.M. (2010) TGFBR2 mutations alter smooth muscle cell phenotype and predispose to thoracic aortic aneurysms and dissections. *Cardiovascular Research* **88**, 520-9.

Keller P., Gburcik V., Petrovic N., Gallagher I.J., Nedergaard J., Cannon B. & Timmons J.A. (2011) Gene-chip studies of adipogenesis-regulated microRNAs in mouse primary adipocytes and human obesity. *Endocrine Disorders* **11**.

Kertesz M., Iovino N., Unnerstall U., Gaul U. & Segal E. (2007) The role of site accessibility in microRNA target recognition. *Nat Genet.* **39**, 1278 - 84

Khvorova A., Reynolds A. & Jayasena S. (2003) Functional siRNAs and miRNAs exhibit strand bias. *Cell.* **115**, 209-16.

Kim H.K., Lee Y.S., Sivaprasad U., Malhotra A. & Dutta A. (2006) Muscle-specifi c microRNA miR-206 promotes muscle differentiation. *J. Cell. Biol.* **174**, 677-87.

Kim V. (2005) Small RNAs: Classification, Biogenesis, and Function. *Mol Cells* **19**, 1-15.

Kim V. & Nam J. (2006) Genomics of microRNA. *Trends in Genetics* **22**, 165-73.

Knight J. & Kothary R. (2011) The myogenic kinome: protein kinases critical to mammalian skeletal myogenesis. *Skelet Muscle.* **29**.

Kosir R., Juvan P., Perse M., Budefeld T., Majdic G., Fink M., Sassone-Corsi P. & Rozman D. (2012) Novel insights into the downstream pathways and targets controlled by transcription factors CREM in the testis. *PLoS One.* **7**, e31798.

Krol J., Loedige I. & Filipowicz W. (2010) The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet.*, 597-610.

Kuhn A.R., Schlauch K., Lao R., Halayko A.J., Gerthoffer W.T. & Singer C.A. (2010) MicroRNA Expression in Human Airway Smooth Muscle Cells: Role of miR-25 in Regulation of Airway Smooth Muscle Phenotype. *Am J Respir Cell Mol Biol.* **42**, 506-13.

Kuwabara Y., Ono K., Horie T., Nishi H., Nagao K., Kinoshita M., Watanabe S., Baba O., Kojima Y., Shizuta S., Imai M., Tamura T., Kita T. & Kimura T. (2011) Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate myocardial damage. *Circ Cardiovasc Genet.* **4**, 446-54.

Langfelder P. & Horvath S. (2008) WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics.*, 559.

Lee S. (2004) Regulation of muscle mass by myostatin. *Annu Rev Cell Dev Biol.* **20**, 61-86.

Lefaucheur L., Edom F., Ecolan P. & Butler-Browne G.S. (1995) Pattern of muscle fiber type formation in the pig. *Dev. Dyn.* **203**, 27-41.

Li G., Li Y., Li X., Ning X., Li M. & Yang G. (2011) MicroRNA identity and abundance in developing swine adipose tissue as determined by solexa sequencing. *J. Cell. Biochem.* **112**, 1318-28.

Li H., Xi Q., Xiong Y., Liu X., Cheng X., Shu G., Wang S., Wang L., Gao P., Zhu X., Jiang Q., Yuan L. & Zhang Y. (2012a) Identification and comparison of microRNAs from skeletal muscle and adipose tissues from two porcine breeds. *Animal Genetics*, 1-10.

Li L., Shi J., Zhu G. & Shi B. (2012b) MiR-17-92 Cluster Regulates Cell Proliferation and Collagen Synthesis by Targeting TGFB Pathway in Mouse Palatal Mesenchymal Cells. *Journal of Cellular Biochemistry* **113**, 1235-44.

Liao Y. & Lönnerdal B. (2010) Beta-catenin/TCF4 transactivates miR-30e during intestinal cell differentiation. *Cellular and Molecular Life Sciences* **67**, 2969–78.

Lin J., Huang S., Wu S., Ding J., Zhao Y., Liang L., Tian Q., Zha R., Zhan R. & He X. (2011) MicroRNA-423 promotes cell growth and regulates G(1)/S transition by targeting p21Cip1/Waf1 in hepatocellular carcinoma. *Carcinogenesis.* **32**, 1641-7.

Liu H., Brannon A., Reddy A., Alexe G., Seiler M., Arreola A., Oza J., Yao M., Juan D., Liou L., Ganesan S., Levine A., Rathmell W. & Bhanot G. (2010) Identifying mRNA targets of microRNA dysregulated in cancer: with application to clear cell Renal Cell Carcinoma. *BMC Syst Biol.*, 51-68.

Liu N., Williams A., Kim Y., McAnally J., Bezprozvannaya S., Sutherland L., Richardson J., Bassel-Duby R. & Olson E. (2007) An intragenic MEF2-

dependent enhancer directs muscle-specific expression of microRNAs 1 and 133. *Proc Natl Acad Sci U S A.* **104**, 20844-9.

Lu H., Buchan R. & Cook S. (2010) MicroRNA-223 regulates Glut4 expression and cardiomyocyte glucose metabolism. *Cardiovasc Res.* **86**, 410-20.

Lytle J., Yario T. & Steitz J. (2007) Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR.

. *Proc Natl Acad Sci U S A.* **104**, 9667-72.

Manni I., Artuso S., Careccia S., Rizzo M., Baserga R., Piaggio G. & Sacchi A. (2009) The microRNA miR-92 increases proliferation of myeloid cells and by targeting p63 modulates the abundance of its isoforms. *FASEB J* **23**, 3957-66. .

Maroto M., Reshef R., Münsterberg A., Koester S., Goulding M. & Lassar A. (1997) Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. *Cell.* **89**, 139-48.

Martinello T., Baldoin M., Morbiato L., Paganin M., Tarricone E., Schiavo G., Bianchini E., Sandonà D. & Betto R. (2011) Extracellular ATP signaling during differentiation of C2C12 skeletal muscle cells: role in proliferation. *Mol Cell Biochem.* **351**, 183-96.

Mathonnet G., Fabian M., Svitkin Y., Parsyan A., Huck L., Murata T., Biffo S., Merrick W., Darzynkiewicz E., Pillai R., Filipowicz W., Duchaine T. & Sonenberg N. (2007) MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. *Science* **317**, 1764-7. .

McCarthy J. & Esser K. (2007) MicroRNA-1 and microRNA-133a expression are decreased during skeletal muscle hypertrophy. *J Appl Physiol (1985)* **102**, 306-13.

McCarthy J., Mula J., Miyazaki M., Erfani R., Garrison K., Farooqui A., Srikuea R., Lawson B., Grimes B., Keller C., Van Zant G., Campbell K., Esser K., Dupont-Versteegden E. & Peterson C. (2011) Effective fiber hypertrophy in satellite cell-depleted skeletal muscle. *Development* **138**, 3657-66.

McDaneld T.G., Smith T.P., Doumit M.E., Miles J.R., Coutinho L.L., Sonstegard T.S., Matukumalli L.K., Nonneman D.J. & Wiedmann R.T. (2009) MicroRNA transcriptome profiles during swine skeletal muscle development. *BMC Genomics* **10**, 77.

Meister G. (2007) miRNAs Get an Early Start on Translational Silencing. *Cell.* **131**, 25-8.

Mercadé A., Estellé J., Pérez-Enciso M., Varona L., Silió L., Noguera J.L., Sánchez A. & Folch J.M. (2006) Characterization of the porcine acyl-CoA synthetase long-chain 4 gene and its association with growth and meat quality traits. *Animal Genetics* **37**, 219-24.

Mimaki M., Wang X., McKenzie M., Thorburn D. & Ryan M. (2012) Understanding mitochondrial complex I assembly in health and disease. *Biochim Biophys Acta.* **1817**, 851-62.

Miretti S., Martignani E., Accornero P. & Baratta M. (2013) Functional effect of mir-27b on myostatin expression: a relationship in Piedmontese cattle with double-muscled phenotype. *BMC Genomics.* **14**.

Mishra P., Tyagi N., Kundu S. & Tyagi S. (2009) MicroRNAs are involved in homocysteine-induced cardiac remodeling. *Cell Biochem Biophys* **55**, 153-62.

Mo M., Chen L., Fu Y., Wang W. & Fu S. (2012) Cell-free Circulating miRNA Biomarkers in Cancer. *J Cancer.* **3**, 432-48.

Motohashi N., Alexander M., Casar J. & Kunkel L. (2012) Identification of a novel microRNA that regulates the proliferation and differentiation in muscle side population cells. *Stem Cells Dev.* **21**, 3031-43. .

Muráni E., Murániová M., Ponsuksili S., Schellander K. & Wimmers K. (2007) Identification of genes differentially expressed during prenatal development of skeletal muscle in two pig breeds differing in muscularity. *BMC Dev Biol* **7**, 109.

Naguibneva I., Ameyar-Zazoua M., Polesskaya A., Ait-Si-Ali S., Groisman R., Souidi M., Cuvellier S. & Harel-Bellan A. (2006) The microRNA miR-181 targets the homeobox protein Hox-A11 during mammalian myoblast differentiation. *Nat. Cell. Biol.* **8**, 278 - 84

Nielsen M., Hansen J.H., Hedegaard J., Nielsen R.O., Panitz F., Bendixen C. & Thomsen B. (2009) MicroRNA identity and abundance in porcine skeletal muscles determined by deep sequencing. *Animal Genetics* **41**, 159-68.

Nishi H., Ono K., Iwanaga Y., Horie T., Nagao K., Takemura G., Kinoshita M., Kuwabara Y., Mori R., Hasegawa K., Kita T. & Kimura T. (2010) MicroRNA-

15b Modulates Cellular ATP Levels and Degenerates Mitochondria via Arl2 in Neonatal Rat Cardiac Myocytes. *J Biol Chem.* **285**, 4920-30.

Noji H. & Yoshida M. (2001) The rotary machine in the cell, ATP synthase. *J Biol Chem.* **276**, 1665-8.

O'Donnell K., Wentzel E., Zeller K., Dang C. & Mendell J. (2005) c-Myc-regulated microRNAs modulate E2F1 expression. *Nature.* **435**, 839-43.

Pasquinelli A.E. (2012) MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. *Nat Rev Genet.* **13**, 271-82.

Peng X., Li Y., Walters K., Rosenzweig E., Lederer S., Aicher L., Proll S. & Katze M. (2009) Computational identification of hepatitis C virus associated microRNA-mRNA regulatory modules in human livers. *BMC Genomics.*, 373.

Perry R. & Rudnicki M. (2000) Molecular mechanisms regulating myogenic determination and differentiation. *Frontiers in Bioscience* **5**, 750-67.

Petersen C.P., Bordeleau M.E., Pelletier J. & Sharp P.A. (2006) Short RNAs repress translation after initiation in mammalian cells. *Mol. Cell* **21**, 533–42.

Picard B., Lefaucheur L., Berri C. & Duclos M. (2002) Muscle fibre ontogenesis in farm animal species. *Reprod Nutr Dev.* **42**, 415–31.

Pillai R., Bhattacharyya S. & Filipowicz W. (2007) Repression of protein synthesis by miRNAs: how many mechanisms? *Trends Cell Biol.* **17**, 118-26.

Pillai R.S., Bhattacharyya S.N., Artus C.G., Zoller T., Cougot N., Basyuk E., Bertrand E. & Filipowicz W. (2005) Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science* **309**, 1573-6.

Ponsuksili S., Murani E., Walz C., chwerin M. & Wimmers K. (2007) Pre- and postnatal hepatic gene expression profiles of two pig breeds differing in body composition: insight into pathways of metabolic regulation. *Physiol. Genomics.* **29**, 267-79.

Pullamsetti S., Doebele C., Fischer A., Savai R., Kojonazarov B., Dahal B., Ghofrani H., Weissmann N., Grimminger F., Bonauer A., Seeger W., Zeiher A., Dimmeler S. & Schermuly R. (2012) Inhibition of microRNA-17 improves lung and heart function in experimental pulmonary hypertension. *Am J Respir Crit Care Med* **185**, 409-19.

Pullen T., da Silva Xavier G., Kelsey G. & Rutter G. (2011) miR-29a and miR-29b contribute to pancreatic beta-cell-specific silencing of monocarboxylate transporter 1 (Mct1). *Mol Cell Biol.* **31**, 3182-94.

Quintens R., Singh S., Lemaire K., De Bock K., Granvik M., Schraenen A., Vroegrijk I., Costa V., Van Noten P., Lambrechts D., Lehnert S., Van Lommel L., Thorrez L., De Faudeur G., Romijn J., Shelton J., Scorrano L., Lijnen H., Voshol P., Carmeliet P., Mammen P. & Schuit F. (2013) Mice deficient in the respiratory chain gene Cox6a2 are protected against high-fat diet-induced obesity and insulin resistance. *PLoS One.* **8**.

Reddy A.M., Zheng Y., Jagadeeswaran G., Macmil S.L., Graham W.B., Roe B.A., Desilva U., Zhang W. & Sunkar R. (2009) Cloning, characterization and expression analysis of porcine microRNAs. *BMC Genomics* **10**, 65.

Rehfeldt C., Fiedler I., Dietl G. & Ender K. (2000) Myogenesis and postnatal skeletal muscle cell growth as influenced by selection. *Livestock Production Science* **66**, 177-88.

Rehfeldt C., Stickland N.C., Fiedler I. & Wegner J. (1999) Environmental and genetic factors as sources of variation in skeletal muscle fibre number. *Basic and Applied Myology* **9**, 235-53.

Rehfeldt C., Te Pas M.F.W., Wimmers K., Brameld J.M., Nissen P.M., Berri C., Valente L.M.P., Power D.M., Picard B., Stickland N.C. & Oksbjerg N. (2011) Advances in research on the prenatal development of skeletal muscle in animals in relation to the quality of muscle-based food.I. Regulation of myogenesis and environmental impact. *Animal* **5**, 703-17.

Rehmsmeier M., Steffen P., Hochsmann M. & Giegerich R. (2004) Fast and effective prediction of microRNA/target duplexes. *RNA.*, 1507-17.

Romanello V., Guadagnin E., Gomes L., Roder I., Sandri C., Petersen Y., Milan G., Masiero E., Del Piccolo P., Foretz M., Scorrano L., Rudolf R. & Sandri M. (2010) Mitochondrial fission and remodelling contributes to muscle atrophy. *EMBO J.* **29**, 1774-85.

Rosenvold K. & Andersen H. (2003) Factors of significance for pork quality-a review. *Meat Sci.* **64**, 219-37.

Ruść A., Sieczkowska H., Krzęcio E., Antosik K., Zybert A., Koćwin-Podsiadła M. & Kamiński S. (2011) The association between acyl-CoA synthetase (ACSL4)

polymorphism and intramuscular fat content in (Landrace × Yorkshire) × Duroc pigs. *Meat Science* **89** 440-3.

Ryten M., Dunn P., Neary J. & Burnstock G. (2002) ATP regulates the differentiation of mammalian skeletal muscle by activation of a P2X5 receptor on satellite cells. *J Cell Biol.* **158**, 345–55.

Ryten M., Hoebertz A. & Burnstock G. (2001) Sequential expression of three receptor subtypes for extracellular ATP in developing rat skeletal muscle. *Dev Dyn.* **221**, 331-41.

Ryu Y.C. & Kim B.C. (2005) The relationship between muscle fiber characteristics, postmortem metabolic rate, and meat quality of pig longissimus dorsi muscle. *Meat Sci.* **71**, 351-7.

Schiaffino S. & Mammucari C. (2011) Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models. *Skelet Muscle.* **1**, 4.

Schöler N., Langer C., Döhner H., Buske C. & Kuchenbauer F. (2010) Serum microRNAs as a novel class of biomarkers: a comprehensive review of the literature. *Exp Hematol.* **38**, 1126-30. .

Sellier P. (1994) The future role of molecular genetics in the control of meat production and meat quality. *Meat Sci.* **36**, 29-44.

Siomi H. & Siomi M. (2010) Posttranscriptional regulation of microRNA biogenesis in animals. *Mol Cell.* **38**, 323-32.

Smalla E.M., O'Rourkea J.R., Moresia V., Sutherlanda L.B., McAnallya J., Gerarda R.D., Richardsona J.A. & Olsona E.N. (2010) Regulation of PI3-kinase/Akt signaling by muscleenriched microRNA-486. *PNAS* **107**, 4218-23.

Soh J., Iqbal J., Queiroz J., Fernandez-Hernando C. & Hussain M. (2013) MicroRNA-30c reduces hyperlipidemia and atherosclerosis in mice by decreasing lipid synthesis and lipoprotein secretion. *Nat Med.* **19**, 892-900.

Takahashi Y., Forrest A., Maeno E., Hashimoto T., Daub C. & Yasuda J. (2009) MiR-107 and MiR-185 can induce cell cycle arrest in human non small cell lung cancer cell lines. *PLoS ONE* **4**, e6677.

Tan Gana N., Victoriano A. & Okamoto T. (2012) Evaluation of online miRNA resources for biomedical applications. *Genes Cells.* **17**, 11-27.

Tang H., Lee M., Sharpe O., Salamone L., Noonan E., Hoang C., Levine S., Robinson W. & Shrager J. (2012) Oxidative stress-responsive microRNA-320 regulates glycolysis in diverse biological systems. *FASEB J.* **26**, 4710-21.

Tao J., Wu D., Li P., Xu B., Lu Q. & Zhang W. (2012) microRNA-18a, a member of the oncogenic miR-17-92 cluster, targets Dicer and suppresses cell proliferation in bladder cancer T24 cells. *Mol Med Report* **5**, 167-72.

Te Pas M.F.W., Cagnazzo M., De Wit A.A.C., Priem J., Pool M. & Davoli R. (2005) Muscle transcriptomes of Duroc and Pietrain pig breeds during prenatal formation of skeletal muscle tissue using microarray technology *Archiv Tierzucht / Archives Animal Breeding* **48**, 141-7.

Thomas M., Lieberman J. & Lal A. (2010) Desperately seeking microRNA targets. *Nat Struct Mol Biol.* **10**, 1164-74.

Trajkovski M., Hausser J., Soutschek J., Bhat B., Akin A., Zavolan M., Heim M. & Stoffel M. (2011) MicroRNAs 103 and 107 regulate insulin sensitivity. *Nature* **474**, 649-53.

Triepels R., Van Den Heuvel L., Trijbels J. & Smeitink J. (2001) Respiratory chain complex I deficiency. *Am J Med Genet.* **106**, 37-45.

van Rooij E., Quiat D., Johnson B., Sutherland L., Qi X., Richardson J., Kelm RJ J. & Olson E. (2009) A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance. *Dev Cell.* **17**, 662-73. .

van Rooij E., Sutherland L., Qi X., Richardson J., Hill J. & Olson E. (2007) Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science.* **316**, 575-9.

Walsh S., Metter E., Ferrucci L. & Roth S. (2007) Activin-type II receptor B (ACVR2B) and follistatin haplotype associations with muscle mass and strength in humans. *J Appl Physiol (1985)* **102**, 2142-8.

Walters Z., Villarejo-Balcells B., Olmos D., Buist T., Missiaglia E., Allen R., Al-Lazikani B., Garrett M., Blagg J. & Shipley J. (2013) JARID2 is a direct target of the PAX3-FOXO1 fusion protein and inhibits myogenic differentiation of rhabdomyosarcoma cells. *Oncogene.*

Wang F., Long G., Zhao C., Li H., Chaugai S., Wang Y., Chen C. & Wang D. (2013) Plasma microRNA-133a is a new marker for both acute myocardial infarction and underlying coronary artery stenosis. *J Transl Med.*, 222.

Wang G., Zhu J., Zhang J., Li Q., Li Y., He J., Qin Y. & Jing Q. (2010) Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans. *Eur Heart J.* **31**, 659-66.

Wang X., Qian R., Zhang W., Chen S., Jin H. & Hu R. (2009) MicroRNA-320 expression in myocardial microvascular endothelial cells and its relationship with insulin-like growth factor-1 in type 2 diabetic rats. *Clin Exp Pharmacol Physiol* **36**, 181-8.

Wienholds E. & Plasterk R. (2005) MicroRNA function in animal development. *FEBS Lett.* **579**, 5911-22.

Wigmore P. & Evans D. (2002) Molecular and cellular mechanisms involved in the generation of fiber diversity during myogenesis. *International Review of Cytology* **216**, 175-232.

Wigmore P.M.C. & Stickland N.C. (1983) Muscle development in large and small pig fetuses. *Journal of Anatomy* **137**, 235-45.

Williams A.H., Liu N., Rooij E.v. & Olson E.N. (2009) MicroRNA control of muscle development and disease. *Current Opinion in Cell Biology* **21**, 461-9.

Xie H., Lim B. & Lodish H.F. (2009) MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity. *Diabetes* **58**, 1050-7.

Xu H., He J., Xiao Z., Zhang Q., Chen Y., Zhou H. & Qu L. (2010) Liver-enriched transcription factors regulate microRNA-122 that targets CUTL1 during liver development. *Hepatology* **52**, 1431-42.

Yan X., Zhu M.-J., Dodson M.V. & Du M. (2012) Developmental Programming of Fetal Skeletal Muscle and Adipose Tissue Development. *Journal of Genomics* **1**, 29-38.

Yang J. & Lai E. (2011) Alternative miRNA Biogenesis Pathways and the Interpretation of Core miRNA Pathway Mutants. *Mol Cell.* **43**, 892-903.

Yin H., Pasut A., Soleimani V., Bentzinger C., Antoun G., Thorn S., Seale P., Fernando P., van Ijcken W., Grosveld F., Dekemp R., Boushel R., Harper M. & Rudnicki M. (2013) MicroRNA-133 controls brown adipose determination in skeletal muscle satellite cells by targeting Prdm16. *Cell Metab.* **17**, 210-24.

Zhang B., Pan X., Wang Q., Cobb G. & Anderson T. (2006) Computational identification of microRNAs and their targets. *Comput Biol Chem.* **30**, 395-407. .

Zhang J., Ying Z., Tang Z., Long L. & Li K. (2012a) MicroRNA-148a promotes myogenic differentiation by targeting the ROCK1 gene. *J Biol Chem.* **287**, 21093-101.

Zhang L., Hou D., Chen X., Li D., Zhu L., Zhang Y., Li J., Bian Z., Liang X., Cai X., Yin Y., Wang C., Zhang T., Zhu D., Zhang D., Xu J., Chen Q., Ba Y., Liu J., Wang Q., Chen J., Wang J., Wang M., Zhang Q., Zhang J., Zen K. & Zhang C. (2012b) Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. *Cell Res.* **22**, 273-4. .

Zhao S.H., Nettleton D., Liu W., Fitzsimmons C., Ernst C.W., Raney N.E. & Tuggle C.K. (2003) Complementary DNA macroarray analyses of differential gene expression in porcine fetal and postnatal muscle. *J. Anim. Sci.* **81**, 2179-88.

Zhou B., Liu H.L., Shi F.X. & Wang J.Y. (2010) MicroRNA expression profiles of porcine skeletal muscle. *Anim. Genet.* **41**, 499-508.

A Enclosure

A. List of abbreviations

acetyl-CoA	Acetyl-coenzyme A
ACTA1	Actin, Alpha 1, Skeletal Muscle
ADIPOQ	Adiponectin, C1Q And Collagen Domain Containin
Ago	Argonaute
Akt	V-akt murine thymoma viral oncogene cellular homologue
APOB	Apolipoprotein B
ATP	Adenosine triphosphate
BMP	Bone morphogenetic protein
BMP4	Bone morphogenetic protein 4
C/EBP	CCAAT/enhancer-binding protein
CAV1	Caveolin 1
CAV3	Caveolin 3
CBX5	Chromobox Homolog 5
CCNE1	G1/S-specific cyclin-E1
CCNG1	Cyclin G1
CDKN1A	Cyclin-Dependent Kinase Inhibitor 1A (P21, Cip1)
CDKN1B	Cyclin-Dependent Kinase Inhibitor 1B (P27, Kip1)
CDKN1C	Cyclin-Dependent Kinase Inhibitor 1C (P57, Kip2)
c-MET	Hepatocyte growth factor receptor
c-MYC	V-myc myelocytomatosis viral oncogene homolog (avian)
CNN	Smooth muscle calponin
CREB1	CAMP responsive element binding protein 1
CSE	Cystathionine gamma-lyase
CTGF	Connective tissue growth factor
CTGF	Connective tissue growth factor
CUTL1	Cut-like homeobox 1
CYP	Cytochrome P450
DE	German Large White
DGCR8	DiGeorge syndrome chromosomal region 8
DL	German Landrace
DM	Differentiated medium
DMPK	Dystrophia Myotonica Protein Kinase
dpc	Days Post Conception
DYRK1A	Nuclear NFAT kinase dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1a
E2F1	E2F transcription factor 1
E2F2	E2F transcription factor 2
eIFs	Eukaryotic initiation factors (cap-binding protein)
ESRRG	Estrogen-related receptor gamma
FABP4	Fatty acid binding protein 4, adipocyte
FADH2	flavin adenine dinucleotide
FDR	False Discovery Rate
FFAs	Free fatty acids
FMR1	Fragile X mental retardation protein 1

FOXO	Forkhead Box
FOXO3	Forkhead Box O3
GLM	General Linear Model
GM	Growth medium
GW182	Glycine-tryptophan (GW) repeat-containing protein of 182 kDa
HDAC4	Histone Deacetylase 4
HGF	Hepatocyte growth factor
HLH	Helix-loop-helix
HNF	Hepatocyte nuclear factor
HOXA11	Homeobox A11
IGF-1	Insulin-like growth factor 1
IPA	Ingenuity Pathways Analysis
ITGA5	Alpha 5 (Fibronectin Receptor, Alpha Polypeptide)
KIT	V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog
KLF4	Kruppel-like factor 4
LAMB2	Laminin gamma 1
LD	Longissimus dorsi
LETFs	Liver-enriched transcription factors (including C/EBPa HNF1a HNF3b and HNF4a)
LIF	Leukemia inhibitor factor
m7G	7-methylguanosine (m7GpppN cap)
MAP2K	Mitogen-activated protein kinase kinase
MAP3K12	Mitogen-activated protein kinase kinase kinase 12
MAP4K3	Mitogen-activated protein kinase 3
MED13	Mediator Complex Subunit 13
MEF2	Myocyte Enhancer Factor 2
Meox2	Mesenchyme homeobox 2
MHC	Myosin heavy chain
miRNA	MicroRNA
mld	Musculus longissimus dorsi
MMP	Matrix metallopeptidase
MRF4	Myogenic regulatory factor 4
MRFs	Myogenic regulatory factors
MSTN	Myostatin
mTOR	Asmammalian target of rapamycin
MYC	V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
Myf5	Myogenic factor 5
MYH11	Myosin heavy chain 11
MyoD	Myogenic differentiation
NADH	Nicotinamide adenine dinucleotide
ncRNAs	Non-coding RNAs
NFATC	Nuclear Factor Of Activated T-Cells, Cytoplasmic, Calcineurin-Dependent
NOX-4	NADPH oxidase 4
nt	Nucleotides

ORF	Open reading frame
PABPC	Poly(A)-binding protein complex
Pax3	Paired box 3
Pax7	Paired box 7
P-bodies	Cytoplasmic processing bodies
PFKM	Phosphofructokinase, Muscle
Pi	Pietrain
PI3-kinase	Phosphatidylinositide 3-kinases
Pol II	RNA polymerase II
PPARG	Peroxisome Proliferator-Activated Receptor Gamma
PRDM16	PR-Domain Zinc Finger Protein 16
pre-microRNA	Precursor miRNA
pri-miRNA/ pri-microRNA	Primary miRNA
PROX1	Prospero homeobox1
PTCH1	Patched 1
PURB	Purine-Rich Element Binding Protein B
qPCR/ qRT-PCR	Quantitative real time RT-PCR
r	Correlation coefficient
RB1	Retinoblastoma 1
RISC	RNA-induced silencing complexes
RNAi	RNA interference
ROCK1	Rho-associated coiled-coil containing protein kinase 1
Shh	Sonic hedgehog
siRNA	Small interfering RNA
SLC2A4	Solute Carrier Family 2 (Facilitated Glucose Transporter), Member 4
SM22α	Transgelin 2 -alpha homolog
SMA	Smooth muscle actin.
SMAD2	SMAD family member 2
SMAD3	SMAD family member 3
SMAD4	SMAD family member 4
Smarcb1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 1
Smarcd2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 2
SOX6	SRY (sex determining region Y)-box 6
SP1	Sp1 Transcription Factor
SP3	Sp3 Transcription Factor
SRF	Serum response factor (c-fos serum response element-binding transcription factor)
TAGLN2	Transgelin 2
TCA cycle	Tricarboxylic acid cycle
TGF	Transforming growth factor
TGFB	Transforming growth factor beta 1
TGFBR2	Transforming growth factor beta receptor II
Thrap1	The thyroid hormone receptor associated protein-1

TIMP	Tissue inhibitors of metalloproteinases
TNF	Total number of muscle fibers
Wnt	Wingless and Int
β-MHC	Myosin heavy chain beta

B Enclosure

B.1 Acknowledgements

The work presented in this thesis has been carried out at the Department of Molegular Biology, Institute for genomic at Leibniz-Institut für Nutztierbiologie (FBN), Dummerstorf from 2010 to 2014. On this place, I wish to thank all persons involved in this project for the great help and support in various ways during my PhD thesis.

Firstly, I would like to express my deepest gratitude to my supervisor PD. Dr. Siriluck Ponsuksili and Prof. Dr. Klaus Wimmers for giving me the opportunity to work on scientific research at Leibniz-Institut für Nutztierbiologie (FBN), Dummerstorf. I deeply appreciate your guidance, encouragement, support, excellent supervision and the great thought-provoking impulses.during the four years of my project.

I am also very grateful to Prof. Dr. Karl Schellander, the Institute of Animal Science, Animal Breeding and Husbandry Group, University of Bonn and thank for his willingness to be a co-supervisor in this thesis.

I would like to thank to the German Research Foundation, Deutsche Forschungsgemeinschaft (DFG; FOR 753), as well as FBN for the financial support of this work.

I am grateful to Dr. Nares Trakooljul for his helpful guidance and assistance during my study as well as linguistic aid and proof reading throughout the manuscript.

Many thanks go to all technical assistants in Department of Molegular Biology, especially Mrs. Annette Jugert and Mrs.Joana Bittner for their excellent technical help, supporting lab assistance whenever I need and providing good working environment.

A big thank to Henry, Mathias, Andreas G., Philipp, Andreas K. and all of great colleagues for a great social activities in Germany and provide me with friendly atmosphere to study. I also would like to mention all of my Asian friends in Rostock and my fellow doctoral colleagues, Wuttigrai, Loan, Kunsuda, Noppawan,

Watcharapong, Du Yang, Sofia and Kwangjun. Thank you for your friendships and all memorable moment here in Germany.

My deepest and genuine gratitude is extended to my parents, Mr. Prasert and Mrs. Chansom Siengdee and all members of my family for raising me to be what I am today and for their support and encouragement through the ongoing process of growing-up. I am very proud to be a part of a wonderful family.

Finally, I would like to express my gratitude to all those who gave me the possibility to complete this thesis in one way or the other but are not mentioned here.

B.2 Curriculum vitae

1. Personal information:

Name	Miss Puntita Siengdee
Date of Birth	April 24th, 1984
Address	70-71 Taladratana Rd. Sobtui
City	Muang
Province	Lampang
Zip code	52100
Country	Thailand
Email address	siengdee@fbn-dummerstorf.de siengdee.p@gmail.com

2. Education:

2002-2006

University	Chiang Mai University, Faculty of science
Qualifications gained	Bachelor of Science (biology)

2006-2009

University	Chiang Mai University, Faculty of science
Qualifications gained	Master of Science (biology)

2010 – present

(FBN), Dummerstorf, Germany	PhD student at Leibniz-Institut für Nutztierbiologie

3. Certificate/training experience:

2004; December 8-10th work shop in stem cell technology, Chiang Mai University, Chiang Mai, Thailand

2006; April 26th – May 14th workshop in molecular and cellular biology Laboratory, Kasetsart University, Bangkok, Thailand

4. Presentation:

Effect of bromelain on canine articular chondrocyte morphology and efficiency of some biomarkers synthesis. Presented at *The 2008 Annual Conference of the Faculty of Veterinary Medicine*, Chiang Mai University, August 8th 2008, Faculty of Veterinary Medicine Hall, Chiang Mai, Thailand (Oral presentations).

Puntita Siengdee, Weerah Wongkham, Hattaya Kawewong and Boongate Fongkaew. **Effect of mitomycin C on proliferation and viability of 3T3 cell line** Presented at National Science and Technology week, August 16-18, 2007 Chiang Mai, Thailand (Poster)

Puntita Siengdee and Weerach Wongkham. **Mitomycin C and the in vitro effect on cell division**. Presented at *47th Annual Meeting The American society for cell biology*, December 1-5, 2007. Washington convention center, Washington DC, England (Poster)

Puntita Siengdee , Korakot Nganvongpanit, Siriporn Okonogi and Siriwan Ong-chai. **Effect of bromelain on canine articular chondrocyte**. Presented at *Veterinary Practitioner association of Thailand 2008 (VPAT)*, April 27-30, 2008. Queen Sirikit National Convention Center, Bangkok, Thailand (Poster)

5. Proceedings and publications

1. **Puntita Siengdee** , Korakot Nganvongpanit, Siriporn Okonogi and Siriwan Ong-chai. Effect of bromelain on canine articular chondrocyte. *Congress proceeding the Veterinary Practitioner association of Thailand*, 548-550, 2008.

2. Korakot Nganvongpanit, **Puntita Siengdee**, Siriwadee Chomdej, Siriporn Okonogi and Siriwan Ongchai. Effect of bromelain on canine articular chondrocyte morphology and efficiency of some biomarkers synthesis. *Chiang Mai Veterinary Journal***,** 6**:** 67-71, 2007**.**

3. Korakot Nganvongpanit, Patama Chaochird**, Puntita Siengdee**, Siriwadee Chomdej and Prachya Kongtaweelert. The effect of matrix metalloproteinase-3 gene suppression to in vitro chondrocyte morphology and glycosaminoglycan production. *Chiang Mai Veterinary Journal*, 6: 85-88, 2007.

4**.** Korakot Nganvongpanit**,** Patama Chaochird**, Puntita Siengdee,** Peraphan Pothacharoen**,** Kasisin Klunklin**,** Siriwadee Chomdej, Supamit Mekchay and Prachya Kongtaweelert. *In vitro* suppression of the *MMP-3* gene in normal and cytokine-treated human chondrosarcoma using small interfering RNA. *Journal of Orthopaedic Surgery and Research,* 4:45, 2009.

5. **P. Siengdee**, K. Nganvongpanit, P. Pothacharoen, S. Chomdej, S. Mekchay, S. Ong-Chai. Effects of bromelain on cellular characteristics and expression of selected genes in canine in vitro chondrocyte culture. *Veterinarni Medicina*, 55(11) 551–560, 2010.

6. Waranee Pradit, Korakot Nganvongpanit, **Puntita Siengdee,** Kittisak Buddhachat, Maslin Osathanunkul, and Siriwadee Chomdej. In vitro Effects of Polysaccharide Gel Extracted from Durian Rinds (Durio zibethinus L.) on the Enzymatic Activities of MMP-2, MMP-3 and MMP-9 in Canine Chondrocyte Culture. *International Journal of Bioscience, Biochemistry and Bioinformatics*, 2(3): 151-154, 2012.

7. **Puntita Siengdee,** Eduard Murani, Manfred Schwerin, Klaus Wimmers, Siriluck Ponsuksili. Pre- and postnatal microRNA expression profiles of two pig breeds differing in muscularity. *Day of the Doctoral Student*, 24th May 2012, FBN Dummerstorf, Germany.

8. **Siengdee P,** Trakooljul N, Murani E, Schwerin M, Wimmers K, Ponsuksili S. Transcriptional profiling and miRNA-dependent regulatory network analysis of longissimus dorsi muscle during prenatal and adult stages in two distinct pig breeds. *Anim Genet.* 44(4):398-407, 2013. doi: 10.1111/age.12032.

9. Siriluck Ponsuksili, Yang Du, Frieder Hadlich, **Puntita Siengdee**, Eduard Murani, Manfred Schwerin, and Klaus Wimmers. Correlated mRNAs and miRNAs from co-expression and regulatory networks affect porcine muscle and finally meat properties. *BMC Genomics.* 14:533, 2013. doi: 10.1186/1471-2164-14-533.

10. **Puntita Siengdee**, Nares Trakooljul, Eduard Murani, Manfred Schwerin, Klaus Wimmers, Siriluck Ponsuksili. Pre- and postnatal muscle microRNA expression profiles of two pig breeds differing in muscularity. Gene. 561(2):190–198, 2015. doi: 10.1016/j.gene.2015.02.035.

11. Puntita Siengdee, Nares Trakooljul, Eduard Murani, Manfred Schwerin, Klaus Wimmers, Siriluck Ponsuksili. (2014). MicroRNAs regulate cellular ATP levels by targeting mitochondrial energy metabolism genes during C2C12 myoblast differentiation. *PLOS ONE* (Submitted).