RUNNING HEAD: Pig muscle transcriptomics

Application of the microarray technology to the transcriptional analysis of muscle phenotypes in pigs

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Summary

The transcriptome refers to the collection of all transcripts present in a cell. Gene expression has a very dynamic nature: it acts as a bridge between epigenetic marks, DNA sequence and proteins, and changes to accommodate the requirements of the cell at each given time.. Recent technological advances have created new opportunities to study complex phenotypes from a global point of view. From an animal production perspective, muscle transcriptomics have been investigated in relation with muscle growth, carcass fattening and meat quality traits. In this review, we discuss the impact of nutritional, anatomic and genetic factors on muscle gene expression and meat quality of pigs assessed by microarray technologies. Altogether, several common themes have been revealed by the in-depth analysis of the current body of knowledge. For instance, the involvement of genes related to energy balance and substrate turnover in the oxidative/glycolytic phenotype of red/white muscle fibre types and in the storage of intramuscular fat. The review also covers recent advances in the discovery of expression QTL and regulatory RNAs in porcine breeds, as well as technical developments in the field of deep-sequencing technologies that are expected to substantially increase our knowledge about the genetic architecture of meat quality and production traits.

Keywords: muscle, meat, swine, RNA-seq, microarray, gene expression

High-throughput tools used in gene expression studies in pigs

The transcriptome represents a key link between information encoded in DNA and proteins, the functional effectors that shape phenotypes. Gene expression is highly dynamic and responds to many internal and external cues such as hormone levels, energy status, diet composition, and exposure of the animal to stress or to pathogens, all of which contribute to the epigenetic and transcriptional regulation of gene expression. Recent technological advances have created new opportunities to study complex phenotypes from a global point of view using large scale molecular gene expression profiles, gene clusters and networks that are characteristic of a biological process or a specific trait (Ozsolak & Milos 2011). The development of high-throughput techniques such as cDNA and oligo-based arrays or RNA-seq approaches represents valuable tools to study the transcriptome and its regulatory mechanisms (Table 1).

Initial characterisation of the transcriptome of model organisms was performed with sequencing based approaches involving the cloning, sequencing and quantitation of partial to full-length cDNA molecules (expressed sequenced tags (EST) libraries) or of short cDNA tags (serial analysis of gene expression (SAGE)). The first global gene expression experiments recorded in pigs used in-house glass or nylon printed arrays developed with information from tissue-specific EST libraries (Bai *et al.* 2003; da Costa *et al.* 2004; Te Pas *et al.* 2005; Hausman *et al.* 2006; Hausman *et al.* 2007; Li *et al.* 2008; Lobjois *et al.* 2008). These arrays were based on long stretches of cDNA sequences, whose length varied widely from spot to spot. Genome coverage was only partial (in general, less than 5,000 spots) which made the comparison across platforms challenging. Another drawback from these first-generation cDNA arrays was that hybridisation efficiency was very inconsistent from

spot to spot due to the unequal length of the cDNA clones. Moreover, the use of these custom cDNA arrays was restricted to the (few) research groups that could afford to acquire and maintain an automatic spotter. To overcome these limitations, several scientific teams explored the possibility of using human or murine microarrays, with more or less success (Lin & Hsu 2005). The first commercially available pig microarray (Operon *Porcine AROS v1.0*) was released in 2003 and consisted of a set of 10,665 oligo-sets designed from NCBI and TIGR swine expressed sequence tag databases (Zhao *et al.* 2005). This commercial tool overcame the uneven hybridisation problem by designing a set of 70-nucleotide-long oligos of similar thermodynamic properties. Despite the high degree of redundancy of this oligo-set (>30%) (Zhao *et al.* 2005), it had the advantage of allowing each group to customize and print their own arrays or, alternatively, ready-made arrays could be purchased directly from the company. An extended *AROS v1.1* was released in 2006 which increased gene coverage by adding 2,632 extra probes to the oligo-set. Subsequent microarray experiments comparing gene expression profiles in a panel of healthy tissues from humans (Shyamsundar *et al.* 2005) and pigs (Hornshoj *et al.* 2007; Steibel *et al.* 2009) highlighted the importance of not-limiting *a priori* the number of genes per array as most genes are ubiquitously expressed although at a tissue-specific level (*i.e.* expression in many tissues but at different levels).

Thus, next generation pig expression arrays offer a more exhaustive coverage of the transcriptome (Table 1). Three of these oligo-based arrays are commercial (Affymetrix' *Porcine Genome Array*, Illumina's *PigOligoArray* and Agilent's *Porcine Gene Expression Microarray*) while several others have been developed by public research bodies (e.g DIAS (Denmark), INRA (France), USDA (USA), Wageningen University (Netherlands)). These arrays are mostly composed of 40- to 70-mer oligonucleotides spotted on a glass slide, with

the aim of guaranteeing an efficient hybridisation to the target probe and, simultaneously, a low level of cross-hybridisation (Steibel *et al.* 2009). Among them, only the Affymetrix array supports a one-channel hybridisation platform. It is worth to mention that this technology allows each particular sample to be hybridised on an individual array. This non-competitive hybridisation has clear advantages when analysing data from several classes or groups of animals, as it does not require a reference sample to make comparisons, a feature which is of particular importance when analysing large datasets.

Recent advancements, particularly in the last five years, have resulted in the establishment of novel deep-sequencing applications to the field of transcriptomics. Second generation sequencers, such as Solexa (Illumina), 454 (Roche) and SOLiD and Ion Torrent (Life Technologies) have been used to characterise transcripts at a whole genome scale (RNA-seq). The main advantages of these technologies are that they allow gathering sequence (mutations, exon usage, new transcripts) and expression information (at the level of number of copies transcribed) in a single experiment. Next Generation sequencing has also allowed researchers to investigate the expression of long and short non-coding RNAs as well as the evaluation of the consequences of epigenetic marks on gene expression. Moreover, single molecule third-generation sequencers (such as those developed by Helicos Genetic Analysis Platform, Pacific Biosciences and VisiGen Biotechnologies), which do not need a pre-amplification step, are currently available and they will likely offer new perspectives on the RNA landscape of livestock species.

As these technologies become increasingly affordable, the in-depth characterization of the transcriptome and its regulatory elements is progressing at a fast rate (see the Sequence Read Archive -SRA- at NCBI for updated information). However, as the number of reports dealing with pig muscle gene expression measured by massive sequencing is still limited,

we have decided to focus the review on the many articles that have used cDNA and oligo microarrays to characterize the porcine transcriptome.

Global gene expression patterns in pig muscle

The availability of microarray technology for most livestock species has provided new opportunities for researchers to characterise global gene expression profiles. In the field of pork production, most studies have focused on the growth and development of skeletal muscle. In this way, microarrays have been used to evaluate the impact of genotype (breed), nutrition and fibre type composition on muscle gene expression (Table 2). In the following pages, we will discuss transcriptomic profiles associated with meat quality attributes such as water-holding capacity, tenderness, fiber type and intramuscular fat content and composition.

Impact of restricted protein diet on muscle gene expression and intramuscular fat accumulation

Da Costa *et al.* (2004) examined the influence of both protein and energy diet restriction on gene expression in skeletal muscle of growing pigs. Dietary restriction (20% less protein and 7% less energy) induced accumulation of intramuscular fat (IMF) in both red and white muscles (*psoas major* and *longissimus dorsi*, respectively) suggesting that changes in gene expression may be of relevance to meat quality and nutrient utilization. The restricted diet increased the expression of genes involved in substrate (protein, glycogen and lipid) turnover, favouring the generation of ATP, mitochondrial function, and

raising the glycolytic and oxidative capacity in both red and white muscles, including fatty acid β-oxidation. This pattern differs from the intramuscular lipid droplet accumulation phenotype associated with pathological states such as type II diabetes mellitus in humans (Schrauwen & Hesselink 2004). Dietary protein restriction also results in reduced growth (Hamill *et al.* 2013) which has been linked with a general transcriptional repression of cell cycle and muscle growth regulation. The accumulation of intramuscular fat in pigs fed with a low protein diet is driven by the enhanced expression of both lipogenic and lipolytic genes (Hamill *et al.* 2013). In agreement with the above, swine receiving a protein restricted diet display a significant increase in the expression and activity of lipogenic stearoyl-coA desaturase (SCD) in muscle but not in subcutaneous adipose tissue (Doran *et al.* 2006). Moreover, SCD protein expression is positively and significantly correlated with total fat content in muscle (Doran *et al.* 2006). It can be inferred from these results that SCD might be an interesting candidate biomarker for IMF accumulation in swine.

Dietary regulation of muscle gene expression starts well before birth. Feeding pregnant sows with either high and low protein diets has short- and long-term consequences on the muscle gene expression profile of their offspring. Indeed, protein-rich diets result in the overexpression of genes related with muscle growth and organisation in 94 dpc foetus and newborn piglets. These differences, however, are not seen in older pigs (Oster *et al.* 2012a). In contrast, most differences in muscle gene expression are evidenced in the long-term when sows are exposed to low-protein diets (Oster *et al.* 2012b). At 188 days of age, offspring from treated sows exhibit higher expression levels of genes involved in the glycolysis and oxidative phosphorylation pathways and lower mRNA levels of cell cycle and growth genes. It is remarkable that this observation agrees with the findings described above for growing pigs fed a low protein diet (da Costa *et al.* 2004; Hamill *et al.* 2013).

Taken together, these results suggest that the transcriptional consequences of dietary protein restriction are similar whether the treatment is applied to piglets or to their mothers.

Gene expression differences between muscle fibre types

Diverse studies have focused on the characterization of expression differences between red and white muscle fibre types (Bai *et al.* 2003; da Costa *et al.* 2004; Li *et al.* 2010). These muscle fibre types differ in the number of glycolytic and oxidative fibres. Red-fibre or highly oxidative muscles are richer in slow-twitch oxidative fibres and have a higher lipid concentration which is often associated with a more tender meat (Chang 2007). Bai *et al.* (2003) compared the transcriptional profile of *psoas* major and *longissimus dorsi* (muscles predominantly composed of red and white fibres) from one 22-week-old Berkshire pig using a muscle-specific cDNA microarray which contained 5,500 probes. More than half of the genes overexpressed in *psoas* were of mitochondrial origin, agreeing with the higher mitochondria content of type-I fibre-rich muscles. Although in a much lower proportion, genes of the gluconeogenesis pathway were also differentially expressed. Conversely, the majority of genes overexpressed in the white-fibre muscle encoded sarcomeric/structural proteins. The other two groups of genes highly expressed in *longissimus dorsi* were involved in glycolysis and in the transcriptional regulation of muscle cell differentiation. Metabolic differences between these two muscle fibre types were also observed after feeding pigs with an energy and protein restricted diet (da Costa *et al.* 2004). On the whole the restricted diet promoted in both muscle fibres the expression of genes involved in ATP-generating processes. However, the oxidative and glycolytic functions were particularly activated in red- and white-fibre muscles, respectively.

Similar results were obtained in a recent report (Li *et al.* 2010), where the expression profiles of red-fibre (*soleus*) and white-fibre (*longissimus dorsi*) muscles of Chinese Meishan pigs were compared using a second generation array with a more exhaustive coverage of the transcriptome (Affymetrix GeneChip array). Among the structural proteins, gene expression of components of the contractile cytoskeleton was consistent with the fibre composition of these two muscles. Thus, myosin heavy chain *MyHCI* (oxidative fibre) and *MyHCIIa* (intermediate fibre) were significantly overexpressed in *soleus*, in contrast to *MyHCIIb* (glycolytic fibre) expression which was significantly higher in *longissimus dorsi*. Additionally, expression of several collagen and extracellular matrix proteins differed between red- and white- fibre muscles. Red-fibre muscle expressed, in addition to genes from lipogenesis and oxidative processes, higher levels of cathepsins B, H and Z, whose role in the process of muscle tenderization is still controversial (Kemp *et al.* 2010). Moreover, Li *et al.* (2010) highlighted that certain transcription factors (including GATA6, TGFB1, TGFB3, MEF2C, EGF and HMOX1) seem to act in a muscle fibre-dependent manner. Most of them are overexpressed in red- *vs* white- fibre muscle. Consequently, these transcription factors are important candidates for transcriptional regulation of the distinct metabolic and contractile features of these two types of muscle fibres. As a whole, transcriptomic analyses agree with descriptive studies on mechanical, structural and metabolic differences between red and white fibre types at both mRNA and protein level, in rats (Okumura *et al.* 2005). Importantly, they also indicate that these differences are regulated, to a significant extent, at the transcriptional level.

Gene expression differences between pigs of distinct genetic lines and breeds

Global gene expression studies are also a worthy approach to study differences between muscle phenotypes across breeds. It is estimated that genetic factors explain around 30% of the variation in meat quality traits (Olsson & Pickova 2005). Thus, many studies have focused on the comparison of pigs of different genotypes (breeds) which represent distinct muscle phenotypes (Table 2). For instance, Lin and Hsu (2005) compared the patterns of gene expression in the *longissimus dorsi* muscle of adult Duroc and Taoyuan pigs, which differ in their postnatal muscle growth rate. Consistent with the heavier muscling and leaner phenotypes observed in Duroc pigs, a group of genes related to glycolytic metabolism and fast twitch-related myosin heavy chains are overexpressed. This result suggests that leaner phenotypes induce a shift towards a more glycolytic and less oxidative fibre type, thus favouring carbohydrates, rather than lipids, as energy substrates (Lefaucheur *et al.* 2004). Pre-natal differentiation processes determine not only muscle mass but also itsphysiological properties, such as total muscle fibre number and, likely, the amount of IMF. Early expression of fatty acid metabolism genes has been shown to be an important factor in relation to IMF content at slaughter (Cagnazzo *et al.* 2006). When compared to Duroc pigs of the same age, the heavier muscled and leaner Piétrain foetuses exhibit a delayed pattern of lipogenesis, muscle differentiation and structural gene activation, both during the primary and secondary wave of myogenesis.. The Piétrain developmental program leads to an increase in the number of muscle fibres, thus enhancing muscle post-natal hypertrophy. A similar delay in the gene expression pattern associated with muscle development has been reported in other lean breeds when compared to fatter breeds (D'Andrea *et al.* 2011; Sollero *et al.* 2011). A longitudinal analysis of embryo and adult muscle development in Piétrain and Landrace pigs identified a network of MyoD functional modulators, including

two fast twitch-specific modulators of myoblast differentiation (TNNC2 and AKT1), and IGF2, as major determinants of embryo differences, while the family of TGF-β factors were differentially expressed in adult Piétrain and Landrace myotubes probably because these molecules are involved in the enhancement of myofibroblast differentiation (Siengdee *et al.* 2013).

Due to its central role in the modulation of body energy balance, liver metabolism is one of the main determinants of body lean/fat phenotype and, consequently, of IMF deposition. The liver is a key organ regulating whole-body metabolism. It can be regarded as the central link between the supply and utilization of fuel by the tissues, the direction and flux of which is mediated by the endocrine system. Skeletal muscle constitutes about 45% of body weight and therefore represents an important peripheral target for dietary energy. Muscle and liver essentially interact through pathways related with protein and lipid metabolism (e.g. VLDL lipoproteins released from the liver are uptaken by the muscle). Gene expression changes that alter hepatic metabolism often have indirect consequences on the energy supply to muscle, with potential effects on growth and fat deposition. In this context, Ponsuksili *et al.* (2007) described the time-course transcriptional activation of liver genes in lean Piétrain and fat German Landrace pigs. These authors described breed-specific liver transactivation events that initiated during early prenatal development. The most prominent differences took place at peripubertal age with (i) an up-regulation of key genes integrated in lipid metabolism pathways (*FASN*, *ACSL2*, *ACACA*) in German Landrace pigs, and (ii) an up-regulation of genes related with cell growth, proliferation and protein synthesis (*PPARD*, *POU1F1*, *IGF2R*) in Piétrain.

Comparison of transcriptomic levels between pigs from the same population but with divergent muscle phenotypes has also been used to study IMF deposition in the *longissimus*

dorsi (Liu *et al.* 2009; Hamill *et al.* 2012) and muscle lipid content and composition in the *gluteus medius* (Canovas *et al.* 2010) and *longissimus dorsi* (Pena *et al.* 2013). These three reports highlighted the prominent role of glycolytic enzymes on intramuscular fat deposition and revealed a general trend towards promoting lipogenesis at the expense of lipolysis in fatter pigs. These differences in glycolytic enzyme content were also confirmed at the protein level by Liu and co-workers (2009). The glycolytic pathway is important in the first steps of glucose conversion into lipids, and *de novo* lipogenesis is directly involved in IMF deposition in pig muscles (Mourot & Kouba 1999). Lipid deposition in muscle adipocytes is regulated by controlling the ratio of lipogenesis to lipolysis rather than enhancing only one of these pathways (Gardan *et al.* 2006). This seems to be the case in pig muscle, as fatter animals have higher mRNA levels for both lipogenic and lipolytic enzymes (Liu *et al.* 2009; Canovas *et al.* 2010; Pena *et al.* 2013). Another important group of genes differentially expressed in pigs with divergent fatness phenotypes are those involved in the regulation of cell energy balance through the insulin, *PPAR* and adipokines signalling pathways (Canovas *et al.* 2010).

The relationship between muscle transcriptome and meat quality traits

In the context of other meat quality-related traits, a regression analysis between expression data and Warner–Bratzler shear force values was used to identify genes related with cooked meat tenderness in commercial pigs (Lobjois *et al.* 2008). The 63 genes that were associated with this attribute happened to be involved in cell cycle regulation, energy metabolism, and muscle development and organization. Similarly, comparing transcriptomic profiles of hybrid gilts with divergent Warner–Bratzler shear force values in the *longissimus dorsi* muscle allowed the detection of 151 differentially expressed genes

over-represented in processes related to growth and development, myofibrillar and proteolytic genes (Hamill *et al.* 2012). Taken together, these results suggest that meat tenderness is associated with a transition from fast, glycolytic to slow, oxidative fibre type with an increased lipid oxidation capacity, thus confirming the positive relationship between slow fibre abundance and tenderness and/or juiciness (Maltin *et al.* 2003). Another muscle attribute investigated at the global transcriptomic level is water-holding capacity (or drip loss), an important meat quality trait for the pork industry (Ponsuksili *et al.* 2008b). Pigs with higher drip losses exhibit lower expression of genes involved in the oxidative metabolism of skeletal muscle and in response to cellular stressors. Pigs with lower water-holding capacity also have reduced expression of lipid metabolism genes, in agreement with the negative phenotypic correlation that exists between fatness traits and drip loss (Ponsuksili *et al.* 2008b).

Gene expression characterization of intramuscular adipocytes

Intramuscular adipocytes are morphologically and functionally different to adipocytes of other fat depots. Recent studies in growing pigs indicate that not only are they smaller and hold reduced lipid vesicles, but they also exhibit a more immature metabolic phenotype compared to subcutaneous and perirenal adipocytes. This metabolic profile characteristic of IMF adipocytes is associated with lower mRNA levels and/or activities of enzymes involved in lipogenesis, lipolysis and transcriptional regulation of lipid metabolism (Gardan *et al.* 2006; Gondret *et al.* 2008; Zhou *et al.* 2010b). Moreover, secretion of adipocytokines (leptin, adiponectin), IGF1 and hormone-sensitive lipase is also reduced. Only *IGF2* expression is higher in intramuscular adipocytes than in other adipocytes. Intramuscular

adipocytes also exhibit lower levels for insulin, IGF and growth hormone receptors. The same pattern was observed in an *in vitro* differentiation assay of subcutaneous and intramuscular pig pre-adipocytes (Zhou *et al.* 2010b). In addition, subcutaneous pre-adipocytes showed an enhanced proliferation, in term of cell cycle regulators measured at the mRNA and protein levels, when compared to their intramuscular counterparts. These depot-specific differences indicate that intramuscular adipocytes are not just an ectopic extension of other fat locations but display specific biological and metabolic features. Therefore, it should be feasible to identify genetic markers with specific effects on intramuscular adipocyte physiology.

Genomic regulation of muscle gene expression

A limited number of studies have used genetical genomic approaches to study the regulation of gene expression in pig skeletal muscle. This strategy involves the performance of a genome-wide scan for expression data with the aim to identify genomic regions affecting gene expression levels (*i.e.* expression quantitative trait loci or eQTL). Transcriptional regulation of a given gene can be affected by *cis*-acting (located within the gene or in a flanking region) and *trans*-acting (located elsewhere) factors. Although most eQTL have not yet been characterised in full, *cis*-acting eQTL are produced by changes in the regulatory sequences of genes (proximal and distal promoters, enhancers, etc) with effects on their expression while *trans*-eQTL are likely to involve mutations of genes encoding transcription factors or other intermediate players regulating gene expression networks. The relative importance of *cis*- *vs trans*-acting factors is currently unknown and

estimates vary substantially among studies because of differences in experimental design, number of replicas and overall statistical power.

Certain genomic regions are responsible for the transcriptional regulation of an important number of genes. These genomic regions are designed as eQTL hotspots (Kang *et al.* 2008) and represent master regulators of expression, several of which are tissue-dependent. In a recent experiment, Liaubet *et al.* (2011) identified 335 eQTL affecting the expression of 272 transcripts in the muscle. A significant proportion of these eQTL were related with proteins involved in muscle development and metabolism, cell morphology, assembly and organization and also in stress response and apoptosis. Expression QTL hotspots were detected on pig chromosomes 1, 2, 10, 13, 16, and 18. Similarly, Canovas *et al.* (2012) identified eleven *trans*-regulatory eQTL hotspots, affecting the expression levels of four to 16 genes in the *gluteus medius* muscle, on pig chromosomes 1, 2, 3, 5, 6, 7, 12 and 18.

A suitable experimental design pre-selecting animals that diverge for a given trait can increase the power to detect regulatory regions that are directly involved in modulating gene expression. For instance, Ponsuksili and co-workers identified eQTL based on the statistical comparison of all genotype combinations for a major drip loss QTL in pigs with divergent phenotypes for this trait (Ponsuksili *et al.* 2008a) and other technological attributes of pork quality such as pH, conductivity, colour and shear force (Ponsuksili *et al.* 2010; Wimmers *et al.* 2010). Other groups have investigated the genomic trans-regulation of lipid muscle content and composition (Canovas *et al.* 2012) and back fat thickness/loin muscle area (Steibel *et al.* 2011). Undoubtedly, genetical genomics represents a key source of information in the search of functional candidate genes responsible for muscle and meat phenotypes. Studies carried out so far have just reported the genomic location of eQTL but

not the underlying causal mutations and their mechanisms of action, an issue that remains largely unexplored.

The role of micro RNA in muscle gene expression regulation

In addition to the transcriptional control of gene expression, another source of regulation of mRNA levels is represented by a population of small non-coding RNAs (sncRNAs) known as microRNAs (miR). MicroRNAs are ≈ 22-nucleotides-long and either inhibit translation or promote mRNA degradation by annealing to complementary sequences mainly in the 3' untranslated regions of specific target mRNAs (Williams *et al.* 2009). MicroRNAs derive from the transcriptionally active genome, and the precursor genes from which they are transcribed can be contained in exonic and intronic regions of both coding and non-coding genes. The number of miRNAs in mammals is estimated to be around 800-1,000, and in general their sequences are well-conserved between species. MicroRNAs have been reported to play very relevant roles in the development and physiology of embryonic and adult tissues by fine-tuning gene expression patterns, although they can also act as on-off switches of gene expression.

MicroRNAs are known to have important regulatory functions in muscle. Thus, during muscle cell proliferation and differentiation, several feedback loops fine-tune a transcriptional network involving the muscle-specific miR-1, miR-206 and miR-133 as well as the serum response factor (SRF) and the myogenic basic helix-loop-helix transcription factors encoded by *MyoD*, *Myf5*, *myogenin* and *MRF4* (Williams *et al.* 2009). As an example of their involvement in determining muscle phenotype, muscle-specific miRs have been reported to regulate the expression of the myostatin gene of heavily muscled Belgian

Texel sheep, resulting in a decreased translation of the myostatin protein and a consequent increase in muscle mass (Clop *et al.* 2006). A number of recent studies have assessed the role of miR in regulating pig muscle development and function using several approaches including sequencing of sncRNA muscle libraries (McDaneld *et al.* 2009; Cho *et al.* 2010; Xie *et al.* 2010), miR microarrays (Huang *et al.* 2008; Zhou *et al.* 2010a) and, more recently, RNA-seq (Nielsen *et al.* 2010; Guo *et al.* 2012; McDaneld *et al.* 2012; Liu *et al.* 2013). These studies offer an in-depth characterization of miR species and potential targets in adult and foetal pig muscle. At present 220-250 miR species have been identified as expressed in adult porcine skeletal muscle. Four or the five most abundant miRs are muscle-specific and include miR-1 (87.1% of all sequence reads), miR-206 (5.6%) and miR-133 (0.05%) (Nielsen *et al.* 2010). The ubiquitously expressed let-7 miR also ranked amongst the five highest expressed miRs in pig muscle (1.7% of all reads). Several time-course analyses have described developmental changes of miR abundance between the two embryonic waves of myogenesis as well as newborn and adult pig muscles (McDaneld *et al.* 2009; Nielsen *et al.* 2010; Zhou *et al.* 2010a) . These studies have shown that the expression patterns of each physiological stage are unique. For instance, during development miR-1 promotes myogenesis by targeting histone deacetylase 4 (HDAC4), a signal dependent chromatin regulator that represses the expression of the myogenic factor MEF2. In contrast, miR-133 enhances myoblast proliferation by repressing SRF, an essential regulator for muscle proliferation and differentiation In adult cells, miR-1 and miR-206 facilitate satellite cell differentiation by restricting satellite cell proliferative potential through the regulation of *Pax7* (*paired box 7*), an essential stem cell maintenance gene in satellite cells and one of their main targets of miR-1 and miR-206 (Chen *et al.* 2010).

The role of miRs in defining the oxidative and glycolytic potential of red- and white-fibre muscles has also been studied. For instance, using deep sequencing of the small RNA fraction, Liu and co-workers (2013) described differences in miR concentrations between oxidative (predominantly red fibre) and glycolytic (predominantly white fibre) muscles. A total of 80 and 256 miRs were specifically expressed in the white- and red-fibre muscles, respectively, although these fibre-specific miRs accounted for less than 0.02% of total sequence counts. Muscle-specific miR-1 and miR-133, which are transcriptionally regulated by myogenic differentiation factors, showed expression differences between these two muscle fibre types. White-fibre muscle also contains higher levels of miR-23, a regulator of *PPARGC1A* mRNA expression. Intramuscular and subcutaneous adipocytes and pre-adipocytes also show differences in miR species and concentrations, which mostly affect the less abundant miRs (Guo *et al.* 2012).

Limitations of gene expression studies and future opportunities

Microarray technology, like all experimental approaches, has important limitations that must be acknowledged and kept in mind when experiments are designed and interpreted. Of particular importance, regarding studies on skeletal muscle, is the fact that muscle tissue is not a homogeneous cell population but a mixture of muscle, adipose, connective, nervous and vascular cells together with their respective precursors. Differences in the proportions of these cell types may alter gene expression profiles. In this regard, the number and size of intramuscular adipocytes are the main determinants of total lipid content variability in muscles. This must be taken into account when comparing expression profiles from pigs with extreme intramuscular adipocyte content. Other physiological parameters that

influence muscle gene expression patterns are sex and age (Cagnazzo *et al.* 2006; Ferraz *et al.* 2008; D'Andrea *et al.* 2011), which need to be properly considered in the analysis models.

One important drawback of microarray experiments is the large number of comparisons required to minimize the number of false positive results. This is particularly critical for two-channel platforms, since comparison of large numbers of samples require complex looping systems where dye-swap controls must be also taken into consideration. At the same time, whole genome arrays should ideally give a complete coverage of the transcriptome over a range of tissues and conditions. However, not all platforms available for pigs are equally comprehensive. Steibel and co-workers (2009) conducted a comparison study and integration of data from three commercial platforms (PigOligoArray, Operon/QIAgen and Affymetrix) within the context of gene expression analysis in pigs. Each platform used distinct probes to interrogate porcine genes, a circumstance which made the comparison among platforms quite challenging because transcripts may have alternative structures that can be recognized with a differential efficiency depending on the probe. Regarding genome coverage, Operon/QIAgen was the least comprehensive one. Besides, the quality of annotation information was very different among the three platforms, being the one from Affymetrix the poorest one. Thus, based on the available gene annotation, substantially more oligonucleotides were identified for the PigOligoarray than for the Affymetrix or Operon/Qiagen arrays.

All of the above makes comparisons between experiments a very complex issue. Interpretation of microarray results is not straightforward and must be made with caution. Besides errors and/or lack of data in the annotation files, technical issues such as cross-hybridisation between members of the same gene family cannot be disregarded. Moreover,

results should be considered as provisional until they can be confirmed by an independent study, either via another microarray tool or through other assays such as quantitative PCR or Northern blot analysis.

Most of these issues are overcome by next generation sequencing techniques for global gene expression profiling based on direct massively parallel cDNA sequencing (RNA-seq). This approach has considerable advantages for examining the transcriptome. First, it delivers greater sensitivity and accuracy compared to microarray measurements, resulting in a more comprehensive characterization of RNA expression profiles. The advantages of RNA-seq include the direct access to sequence information; therefore, junctions between exons can be assayed without prior knowledge of gene structure. Moreover, RNA editing and alternative splicing events can be detected. Quantification of individual transcript isoforms and identification of novel or known polymorphisms can provide direct measurements of allele-specific expression profiles and can be used even in species for which a whole-genome sequence is not available (Malone & Oliver 2011). On the other hand, the high economic cost of this technique limits the number of biological replicates. Of particular relevance is the depth of sequencing required to effectively sample the transcriptome, which needs to be determined for each species/tissue combination. Moreover, as with most novel techniques, there are not validated and generally-accepted protocols for data analysis and interpretation, yet. There are contrasting reports about the agreement between expression data obtained from microarray and RNA-seq platforms. Studies in human and mice indicate an overall good agreement between both data sets, although RNA-seq agrees much better with quantitative PCR data, confirming that microarray experiments often generate less accurate results due to the saturation of large signals from highly expressed genes and large errors in the measurement of low signals

(Malone & Oliver 2011). In contrast, a comparative study of microarray and RNA-seq approaches aimed to measure gene expression in pig heart and skeletal muscle demonstrated high reproducibility within each assay, but scarce agreement across both technologies (Hornshoj *et al.* 2009). This outcome might be due to the less homogeneous hybridisation conditions obtained with cDNA arrays compared to the oligo arrays used by Malone and Oliver (2011).

Future advances in high-throughput transcriptome analysis will mostly rely on novel developments in the next generation sequencing technologies. The epigenetic control of gene expression is particularly gathering much interest. So far, adaptation of chromatin-immunoprecipitation protocols to the next generation sequencing analysis (ChIP-seq) has been used in humans and model organisms, in the framework of the ENCODE and modENCODE projects, to analyse histone and DNA epigenetic marks. The cross-analysis of ChIP-seq and RNA-seq data will be particularly informative in describing non-genetic contributions to gene expression. Undoubtedly, this approach will be extended to livestock species as these techniques become more affordable. As a first example, Li and co-workers (2012) have used ChIP-seq to compare the methylome of pig muscle and subcutaneous fat cells. The large datasets gathered by microarray and RNA-seq techniques will give impetus to the implementation of novel computational approaches. New avenues that should be further explored are the effective integration of nucleotide variation and gene expression data, the minimisation of experimental biases, and the comparison of gene expression patterns in livestock and model organisms through meta-analysis approaches.

Conclusions

Despite several technical limitations, microarrays represent a first attempt to characterise and functionally describe global transcriptomic profiles. In the context of muscle physiology, data gathered during the last decade allow to distinguish overall two main patterns of muscle gene expression that are closely associated with fibre type (Figure 1). Metabolic and biochemical characteristics, such as oxidative and glycolytic capacities, fibre size, colour, and glycogen and lipid contents, have been found to vary between MyHC fibre types (Chang 2007). Slow MyHC-I fibres, those with a high oxidative capacity, are characterised by containing slow isoform contractile proteins, high levels of myoglobin and lipids and an increased mitochondrial volume. Important meat traits such as colour and tenderness have been found to closely associate with an increased abundance of red muscle fibres. By contrast, fast MyHC-IIb fibres are the major contributors of hypertrophic growth, and are characterised by fast isoform contractile proteins, low amounts of myoglobin and mitochondria, high glycolytic capacity and low lipid contents.

Fibre type composition varies between muscles according to their functional adaptation. Muscles with predominant red fibres are under continual (postural) use and comprise a high proportion of oxidative fibres. White fibre-rich muscles (used for intensive activities) possess large numbers of fast fibres. Thus fibre population in muscle is a continuum of pure and mixed fibres that can be altered in the fast-to-slow or slow-to-fast direction under appropriate stimulatory conditions (Chang 2007). Thus, pigs fed with a protein restricted diet or displaying a fat phenotype (different breeds or within lines) tend to express a transcriptomic profile typical of slow MyHC-I fibres (Figure 1). In response to environmental stimuli, the dynamics of the muscle transcriptome seems to follow the muscle metabolic adaptation in terms of fibre type content. In the future, these data should

instruct us on how to manage environmental cues in order to modulate gene expression towards improving meat quality.

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Figure Legends

Figure 1.

Graphical summary of the main gene expression patterns generated with microarrays and associated with pig muscle fibre type, growth and fat deposition. Genes activated in the red slow twitch fibre-rich muscles promote a more rapid substrate turnover that results in the accumulation of intramuscular fat (IMF). Protein-restricted diets promote a shift in the muscle transcriptome towards a red muscle fibre phenotype. This profile is also displayed by fat pigs with a more tender meat. Conversely, white fast twitch fibre-rich muscles overexpress structural proteins and myogenic factors that lead to a leaner and hypertrophic phenotype. Three muscle-specific microRNAs, which regulate myogenic signalling in embryonic and satellite muscle cells, are overexpressed in white fibres. Pigs with leaner phenotypes or producing meat with increased drip losses show a shift in their transcriptomic pattern that recalls that of white muscle fibres.

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Table 1 High-throughput tools used in the global characterization of gene expression in pigs

Tool name	Technology	Taxonomy	Spots	Contact	Date
Commercial					
Affymetrix Porcine Snowball Array	25-mer oligos	*Sus scrofa*	47845	Affymetrix	2013
Agilent Porcine Gene Expression Microarray	60-mer oligos	*Sus scrofa*	43603	Agilent Technologies	2009
PigOligoArray	70-mer oligos	*Sus scrofa*	20736	Illumina	2008
Affymetrix Porcine Genome Array	25-mer oligos	*Sus scrofa*	24123	Affymetrix	2006
Operon Porcine AROS v1.1	70-mer oligos	*Sus scrofa*	13297	Operon	2006
Operon Porcine AROS v1.0	70-mer oligos	*Sus scrofa*	10665	Operon	2003
Custom/Custom-commercial					
INRA FH Sus scrofa 15K muscle array	60-mer oligos	*Sus scrofa*	15744	INRA	2012
EmbryoGene Porcine Array v1	60-mer oligos	*Sus scrofa*	45220	Univ Alberta	2012
INRA Sus scrofa 15K Adipose Tissue	60-mer oligos	*Sus scrofa*	15744	INRA	2011
SLA/NRSP8 Pig 70 mers (3.8K + 13.3K) v1	70-mer oligos	*Sus scrofa*	19200	INRA/Operon	2009
Pig Pre-implantation Embryo 40K oligo array	60-mer oligos	*Sus scrofa*	45220	USDA-ARS/Agilent	2009
Porcine oligo microarray version 3	75-mer oligos	*Sus scrofa*	2160	DTU	2008
Porcine oligo microarray version 4	60/70-mer oligos	*Sus scrofa*	366	DTU	2008
Pork Quality Operon 70-mer oligo array	70-mer oligos	*Sus scrofa*	656	pigebv/Operon	2008
ASG Porcine jejunum spleen cDNA array	spotted DNA/cDNA	*Sus scrofa*	26496	Wageningen UR	2008
SLA_PrV porcine DNA/cDNA microarray	spotted DNA/cDNA	*Sus scrofa*	2304	INRA	2007
Porcine testis cDNA microarray	spotted DNA/cDNA	*Sus scrofa*	10080	ATIT	2007
NLI_SSC_11.5K_cDNA_V1	spotted DNA/cDNA	*Sus scrofa*	11520	CAU	2007
Sus scrofa 1.2K mono array (ovary)	spotted DNA/cDNA	*Sus scrofa*	1152	INRA	2006
Spotting_muscle_21OCT03	spotted DNA/cDNA (Nylon)	*Sus scrofa*	4608	INRA	2006
PigGeneric2_9216 (ovary)	spotted DNA/cDNA	*Sus scrofa*	9216	INRA	2006
DIAS_PIG_27K2_v2	mixed spotted oligos/cDNA	*Sus scrofa*	27648	DIAS/NimbleGen	2006
DIAS_PIG_55K2_v1	spotted DNA/cDNA	*Sus scrofa*	55488	DIAS/NimbleGen	2006

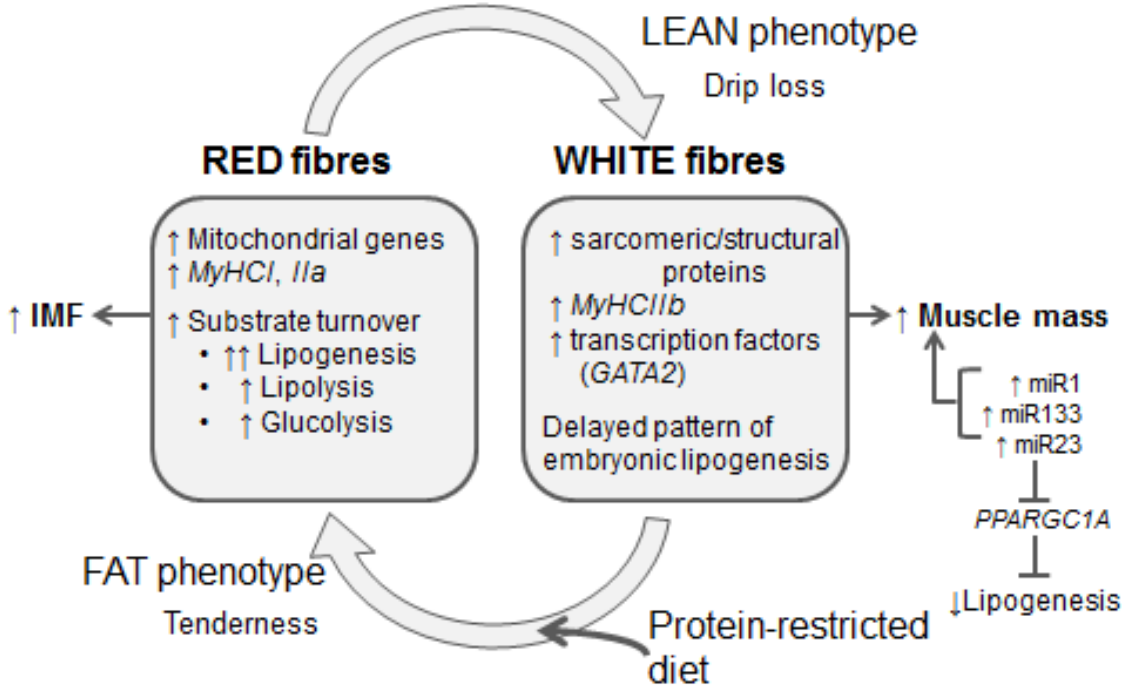
Porcine 1000 embryo gene array	spotted DNA/cDNA	*Sus scrofa*	1015	ISU	2004
PorkChip 2,600 cDNA array	spotted DNA/cDNA	*Sus scrofa*	2600	UMN	2004
UIUC Porcine muscle plus	spotted DNA/cDNA	*Sus scrofa*	2880	UIUC	2003
Porcine Brain Library array	spotted DNA/cDNA	*Sus scrofa*	3888	MSU	2003
Tiling arrays					
MMGG Pig X-tiling path 785 BACs v1	Tiling array	*Sus scrofa*	870	Sanger	2012
NimbleGen_Sus scrofa_135K array	Tiling array	*Sus scrofa*	23806	NimbleGen	2012
NimbleGen agrsci porcine 2.1M v1	Tiling array	*Sus scrofa*	44532	DIAS/NimbleGen	2010
NimbleGen 385K pig array CGH	Tiling array	*Sus scrofa*	392778	DIAS/NimbleGen	2008
miRNA detection					
LC Sciences Pig miRNA array	µParaflo microfluidic chip	*Sus scrofa*	284	LC Sciences	2013
LC sciences pig microRNA 236 V16.0	µParaflo microfluidic chip	*Sus scrofa*	336	LC Sciences	2012
miRCURY LNA microRNA Array	oligo array	mixed	421	Exiqon	2012
Mammalia miRNA 3K Array	oligo array	mixed	3968	INSERM/LC Sciences	2011
Febit Sus Scrofa miRNA Custom 0.8K	oligo array	*Sus scrofa*	798	Febit	2010
Febit Homo Sapiens and Sus Scrofa 1.1K	oligo array	mixed	1101	Febit	2010
FHCRC miRNA Array v1.8.1	oligo array	mixed	3052	FHCRC	2008
RNA-seq					
Illumina HiSeq 2000	deep sequencing	*Sus scrofa*		Illumina	2011
Illumina Genome Analyzer I & II	deep sequencing	*Sus scrofa*		Illumina	2010

Source GEO: http://www.ncbi.nlm.nih.gov/geo (accessed 03-May-2013)

Table 2 Published microarray experiments interrogating diverse pig muscle phenotypes

Trait	N. Animals	Array	Provider	Features	Reference
Protein and energy dietary restriction	4	pig muscle cDNA array	in-house	5,500	da Costa *et al.* 2004
	48	porcine GeneChip array	Affymetrix	23,937	Oster *et al.* 2012a
	11	porcine GeneChip array	Affymetrix	23,937	Hamill *et al.* 2013
High-protein diet	48	porcine GeneChip array	Affymetrix	23,937	Oster *et al.* 2012b
White *vs* Red muscle fibre physiology	1	pig muscle cDNA array	in-house	5,500	Bai *et al.* 2003
	4	pig muscle cDNA array	in-house	5,500	da Costa *et al.* 2004
	3	porcine GeneChip array	Affymetrix	23,937	Li *et al.* 2010
Lean/Fat phenotypes(different breeds)	6	human uniGEM V2	Incyte	9,182	Lin and Hsu 2005
	28	pig muscle cDNA array	in-house	818	Cagnazzo *et al.* 2006
	6	porcine GeneChip array	Affymetrix	23,937	Gao *et al*. 2011
	30	Operon Porcine AROS v1.1	QIAgen	13,297	D'Andrea *et al.* 2011
	42 (14 pools)	PigOligoArray	Illumina	20,736	Sollero *et al.* 2011
	40	Genmascq Chip	In-house	15,198	Damon *et al.* 2012
	36 (12 pools)	porcine GeneChip array	Affymetrix	23,937	Siengdee *et al.* 2013
Intramuscular fat content and composition	16	human/mouse oligo array	in-house	6,681	Liu *et al.* 2009
	70	porcine GeneChip array	Affymetrix	23,937	Canovas *et al.* 2010
	7	cDNA array	in-house	5,400	Hamill *et al.* 2012
	110	porcine GeneChip array	Affymetrix	23,937	Pena *et al.* 2013
Meat tenderness	17	pig muscle cDNA array	in-house	3,456	Lobjois *et al.* 2008
	8	cDNA array	in-house	5,400	Hamill *et al.* 2012
Water-holding capacity	12	porcine GeneChip array	Affymetrix	23,937	Ponsuksili *et al.* 2008a,b

Figure 1


LEAN phenotype
Drip loss
RED fibres
WHITE fibres
↑ Mitochondrial genes
↑ MyHCI, IIa
↑ Substrate turnover
• ↑↑ Lipogenesis
• ↑ Lipolysis
• ↑ Glucolysis
↑ sarcomeric/structural proteins
↑ MyHCIIb
↑ transcription factors (GATA2)
Delayed pattern of embryonic lipogenesis
↑ IMF
↑ Muscle mass
↑ miR1
↑ miR133
↑ miR23
PPARGC1A
↓Lipogenesis
FAT phenotype
Tenderness
Protein-restricted diet