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Identification of differentially expressed small RNAs and prediction of target genes in Italian Large White pigs with divergent backfat deposition

Running head: MiRNA-gene interaction in pig backfat

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Summary

The identification of the molecular mechanisms regulating pathways associated to the potential of fat deposition in pigs can lead to the detection of key genes and markers for the genetic improvement of fat traits. MicroRNAs (miRNAs) interactions with target RNAs regulate gene expression and modulate pathway activation in cells and tissues. In pigs, miRNA discovery is well far from saturation and the knowledge of miRNA expression in backfat tissue and particularly of the impact of miRNA variations are still fragmentary. We characterized by RNA-seq the small RNAs (sRNAs) expression profiles in Italian Large White pig backfat tissue. Comparing two groups of pigs divergent for backfat deposition, we detected 31 significant differentially expressed (DE) sRNAs, 14 up-regulated (including ssc-miR-132 ,ssc-miR-146b, ssc-miR-221-5p, ssc-miR-365-5p, and the moRNA ssc-moR-21-5p) and 17 down-regulated (including ssc-miR-136, ssc-miR-195, ssc-miR-199a-5p, and ssc-miR-335). To understand the biological impact of the observed miRNA expression variations, we used the expression correlation of DE miRNA target transcripts expressed in the same samples to define a regulatory network of 193 interactions between DE miRNAs and 40 DE target transcripts showing opposite expression profiles and being involved in specific pathways. Several miRNAs and mRNAs in the network resulted to be expressed from backfat related pig QTLs. These results are informative on the complex mechanisms influencing fat traits, shed light on a new aspect of the genetic regulation of fat deposition in pigs, and facilitate the perspective implementation of innovative strategies of pig genetic improvement based on genomic markers.

Keywords: swine, backfat, microRNA, moRNA, differential expression, regulatory network, genetic improvement

Introduction

Backfat deposition in pigs is an important selection trait closely connected with carcass and meat quality. MicroRNAs (miRNAs) and miRNA-like short RNAs (sRNAs) represent a functionally relevant RNA category modulating the expression of coding messenger RNAs (mRNAs) and noncoding transcripts in almost all tissues. In pigs, only 411 mature miRNAs are included in the current miRBase release 21 (Kozomara and Griffiths-Jones, 2013), being less than 16% of the number of human miRNAs, indicating that miRNA discovery in pigs is far from reaching saturation. To date, only few papers describing miRNAs expression in porcine adipose tissues have been published (Li et al., 2011; Chen et al, 2012; Li et al., 2012; Bai et al., 2014). Recently, the transcription profiles of sRNAs in backfat of two Italian Large White pigs have been described in Gaffo et al. (2014), reporting new miRNAs, isomiRs and moRNAs, and showing the complexity of sRNA population in the porcine backfat tissue. RNA-seq was recently used to study transcriptome variations in backfat tissue comparing two groups of Italian Large White pigs divergent for backfat deposition, getting a deeper knowledge of the molecular processes regulating the potential of fat deposition in pigs (Zambonelli et al., 2016), that is essential to identify key genes and markers for the genetic improvement of fat traits.

59 In this study, we used RNA-seq to describe the transcription profile of sRNAs expressed in
60 porcine backfat tissue and to identify sRNAs differentially expressed between fat and lean pigs.
61 Furthermore, linking miRNAs and target transcripts whose expression vary in relation to backfat
62 deposition we reconstructed a regulatory network that disclose the impact of miRNAs
63 regulatory action on fat traits.
64

65 Materials and Methods

66 Samples collection and sequencing

67 For this analysis we used 18 of the Italian Large White pigs and the backfat samples collected
68 and described in Zambonelli et al. (2016). All animals used in this study were kept according to
69 Italian and European laws for pig production and all the adopted procedures were fully
70 compliant with national and European Union regulations for animal care and slaughtering. We
71 considered short RNA-seq data of backfat tissue in 18 animals, comprising 9 pigs with high
72 backfat thickness (FAT) and 9 pigs with low backfat thickness (LEAN), as described before in
73 Zambonelli et al. (2016). The 18 RNA samples were newly sequenced (series record GSE108829)
74 as detailed below.
75 Backfat samples stored at -80 °C in a deep freezer were used for total RNA extraction using
76 Trizol (Invitrogen) according to the manufacturer's instructions. Extracted RNA was quantified
77 using the Nanodrop ND-1000 spectrophotometer, and the quality of the extracted RNA was
78 assayed using an Agilent 2100 BioAnalyzer (Supplementary Table 1). Eighteen small RNA
79 libraries were prepared from total RNA using the TruSeq Small RNA kit (Illumina) and version 3

80 of the reagents following the manufacturer's suggested protocol. The libraries were individually
81 tagged and run on 9 lanes of an Illumina GAII.
82

83 Computational analysis of small RNA sequencing data

84 The bioinformatics pipeline used is summarized in Supplementary Figure 1, informing also on
85 the study design. RNA-seq data were processed with a combination of the miR&moRe pipeline
86 (Bortoluzzi et al., 2012; Gaffo et al., 2014) and of miRDeep2 (Friedländer et al., 2011), which
87 was exploited to discover new precursor hairpins that express mature miRNAs (NPmiRNAs) in
88 the samples: we run miR&moRe on the known porcine miRNAs plus a high confidence set of
89 predictions, obtained by miRDeep2.
90 MiRDeep2 v0.0.5 was run with default parameters pooling the sample read sets. Input genome
91 and miRNA annotation were the same used for miR&moRe (UCSC database susScr3 genome
92 and miRBase release 21). We filtered miRDeep2 predictions restricting to precursors with
93 miRDeep2 score greater than 1.0, length greater than or equal to 50 nt and predicted
94 probability of being a miRNA greater than 60% (Londin et al., 2015). The novel precursor
95 annotation predicted by miRDeep2 was added to miRBase swine miRNA annotation and given
96 as input to miR&moRe, which was run on the 18 newly sequenced samples.
97 MiR&moRe quantifies small RNAs from RNA-seq experiments, detect as well miRNA isoforms
98 (isomiRs), novel miRNAs expressed in known hairpin precursors with only one miRNA
99 annotated, and identify also miRNA-offset RNAs (moRNAs; Bortoluzzi et al., 2011; Bortoluzzi et
100 al., 2012; Guglielmelli et al., 2015). The miR&moRe pipeline performs a preliminary cleaning
101 and quality preprocessing of the input raw sequences. Reads passing the quality filter are

102 aligned to the reference miRNA precursors and to the reference genome for expression
103 quantification. Identification and expression quantification of isomiRs and moRNAs follow from
104 the alignments and sequence folding predictions. Small RNAs expression levels are measured as
105 read alignment counts in each sample.
106

107 Differential expression assessment and validation

108 Read counts were normalized across all the samples according to the DESeq2 (v1.4.5; Love et
109 al., 2014) approach. Small RNAs represented by less than ten normalized reads considering all
110 samples were excluded from further analysis. Short RNA differential expression comparing FAT
111 and LEAN groups was assessed by DESeq2, considering significant those variations with FDR <
112 0.05 (Benjamini-Hochberg adjusted P-values).
113 Validations of miRNAs differentially expressed according to RNA-seq analysis have been
114 obtained by Quantitative Real Time PCR (qRT-PCR) analysis on the same samples, performed in
115 triplicate. The TaqMan® Micro RNA Assay kit (Applied Biosystems) was used, following the
116 customer protocol, as reported in Gaffo et al. (2014), and using as internal reference the U6
117 snRNA (Chen et al., 2012; Yu et al., 2012). For the relative miRNA quantification, the PCR-
118 derived cycle threshold (Cq) of target miRNAs is compared with that of a stably expressed
119 endogenous miRNA from the same sample. The difference between these values is the ΔCq
120 value (Rai et al., 2012). A t-test was used to assess the significance of the differential expression
121 estimated by qRT-PCR, considering a significance threshold of P-value <0.05.
122

123 Small RNA target prediction on backfat long RNA data

124 The transcription profiles of long RNAs in the same pig backfat samples (GSE68007) already
125 analyzed in Zambonelli et al. (2016) were used for integrative analyses with miRNA expression
126 profiles, considering matched miRNA and transcript expression data in backfat of FAT and LEAN
127 animals. MiRanda v3.3a (Enright et al., 2003) was applied to predict target sites on the 63,418
128 transcript sequences reported by Zambonelli et al. (2016). For each differentially expressed
129 miRNA (DEM), the prediction was computed using the most abundant isomiR sequences
130 (detected as reported in Gaffo et al., 2014), keeping only isomiRs contributing each at least 10%
131 of the miRNA expression.
132 Next, focusing on differentially expressed small RNAs, we calculated Spearman pairwise
133 correlations between sRNA and DEM expression profiles, and tested the significance of the
134 correlations. According to largely prevalent repressive role of miRNAs on target transcripts, we
135 focused on the negative correlations, whose association P-values were corrected for multiple
136 tests (Benjamini-Hochberg). Only negative correlations with FDR at most 10% were considered
137 significant to identify miRNA-transcript relations that are supported by expression data analysis.
138 Significant miRNA-transcript relations with correlation <-0.6 were selected to draw an
139 interaction network, using Cytoscape version 3.3. Pathways enriched in genes included in the
140 network have been calculated with EnrichR (http://amp.pharm.mssm.edu/Enrichr/; Kuleshov et
141 al., 2016), selecting KEGG and Reactome databases and considering significant those pathways
142 with adjusted P-value at most 0.1 and including at least two genes.
143

144 Quantitative Trait Loci enrichment on target genomic regions

145 For each pig QTL annotated in the PigQTL database (http://www.animalgenome.org/cgi-
146 bin/QTLdb/SS/download?file=gbpSS_10.2), we counted the number of differentially expressed
147 sRNAs and their predicted target genes considering only those with differentially expressed
148 transcripts with FDR at most 30% according to Zambonelli et al. (2016) mapping in the genomic
149 region. QTL enrichment in miRNA and genes was tested using the upper-tailed hypergeometric
150 test for over-representation, with one side mid p-values as defined in Rivals et al. (2007). The
151 analysis used as background the pig genome annotation (v 10.2.80) merged with the new genes
152 discovered in Zambonelli et al. (2016), resulting in 34,617 genes, and the new sRNAs detected
153 in this study. We considered as significantly enriched only those QTLs showing adjusted P-
154 values <0.1.
155

156 **Results**

157 Small RNA identification and quantification from RNA-seq data

158 RNA-seq analysis produced in 97 million reads per sample on average, that after read trimming
159 and subsequent filtering steps (Supplementary Table 2) were reduced to 37 million high quality
160 reads per sample, in average, that were further processed for the sRNAs characterization. We
161 applied stringent filtering criteria in the processing of the raw data to obtain high quality read
162 sets deprived of sequencing artifacts, at the cost of high number of discarded sequences. This
163 approach and the number of biological replicates contributed to the reliability of downstream
164 predictions, in particular regarding novel elements like moRNAs and isomiRs.

165 MiRDeep2 analysis of RNA-seq data predicted 1,340 new miRNA precursors characteristic of pig
166 genome, expressing 2,680 putative novel mature miRNAs, whose sequences do not overlap
167 each other and with different sequence from pig miRNA precursors reported in miRBase. Of
168 these, 103 NPmiRNAs passed the filtering criteria and were considered for following analyses.
169 Overall, 426 sRNAs resulted expressed in the new dataset, including 231 known miRNAs, 69
170 new miRNAs from known precursors, 103 new miRNAs from new precursors, and 23 moRNAs
171 (Supplementary Table 3).
172 As observed in Gaffo et al. (2014), the expression distribution was very skewed: only 24 known
173 miRNAs (5.6% of the expressed sRNAs) accounted for the 90% of the total expression, and only
174 21.1% of expressed sRNAs accounted the 99% of the total expression (Supplementary Figure 2).
175 Ssc-miR-10b, ssc-miR-143-3p and ssc-148a-3p resulted very abundant and accounted together
176 for about the 52% of the total expression, with the first two accounting for respectively about
177 33% and 13% of the expression.
178

179 Differentially expressed sRNAs

180 Among the 426 sRNAs expressed in the considered porcine backfat samples, 31 resulted
181 differentially expressed comparing LEAN and FAT animals (Table 1, Supplementary Table 4): 18
182 known miRNAs, 6 new sister miRNAs, 6 miRNAs from new precursors and ssc-moR-21-5p,
183 whose existence was previously validated by qRT-PCR (Gaffo et al., 2014). Figure 1 shows the
184 unsupervised clustering and heatmap of expression profiles of the 31 DEM in the 18 samples
185 and two animal groups considered. SRNAs up- and down-regulated in FAT animals were 14 and

186 17, respectively. Up-regulated sRNAs include six known miRNAs (ssc-miR-146b, ssc-miR-365-5p,
187 ssc-miR-221-5p, ssc-miR-222, ssc-miR-132 and ssc-miR-1306-5p) the moRNA ssc-moR-21-5p, a
188 new pig sister miRNA (ssc-miR-128-1-5p) and six mature miRNAs from newly predicted
189 precursors. All down-regulated sRNAs are miRNAs from already annotated precursors, including
190 12 known miRNAs (ssc-let-7c, ssc-miR-130a, ssc-miR-181c, ssc-miR-199a-5p, ssc-miR-199a-3p,
191 ssc-miR-199b-3p, ssc-miR-195, ssc-miR-193a-3p, ssc-miR-335, ssc-miR-133a-3p, ssc-miR-545-3p,
192 ssc-miR-136) and 5 new sister miRNAs (ssc-miR-214-5p, ssc-miR-99a-3p, ssc-miR-101-2-5p, ssc-
193 miR-136-3p, ssc-miR-362-3p, ssc-miR-128-1-5p).
194 We selected 9 DEMs, 8 miRNAs and ssc-moR-21-5p, for the qRT-PCR validation of differential
195 expression. A good agreement between RNA-seq and qRT-PCR expression estimates was
196 observed (R^2=0.78, Figure 2A) supporting the robustness of the data reported for all the small
197 RNAs detected in this study. Indeed, all the comparisons performed show the same trend
198 between the two analyses, and 5 out of 9 DE sRNAs resulted significantly differentially
199 expressed also according to RT-PCR estimations (Figure 2B).
200

201 ## IsomiR composition of differentially expressed sRNAs

202 IsomiRs were investigated for the DEM showing more than one variant (18 known and 6 novel-
203 precursor miRNAs out of the 31 DEM). Fifty-nine distinct isomiRs were detected, specifically 58
204 in LEAN and 55 in FAT of which 54 were in common. Four isomiRs are specific for LEAN samples
205 and one is specific for FAT samples (Supplementary Table 5).
206 The number of isomiRs contributing at least 10% of each DEM expression ranged from one to
207 four, a maximum value detected in three cases (chr5_31322-3p, JH118494-1_44794-3p,

208 chr9_40038-5p). Three miRNAs (chr7_37486-3p, ssc-miR-136 and ssc-miR-193a-3p) are
209 represented by only one major isoform accounting for more than half of the miRNA expression
210 (Supplementary Figure 3A). Most isoforms, according to miR&moRe classification, are
211 variations of the length: isomiRs in the "shorter_or_longer" category are 32 out of 58 in LEAN
212 and 29 out of 55 in FAT; the variants perfectly matching the reference sequence ("exact"
213 variants, or "canonical" isomiRs) are 18 in both groups; and "one-mismatch" variation isomiRs
214 are eight. Less than half of DEMs (10 cases in LEAN and 11 in FAT) express the canonical isomiR
215 as the major form. Conversely, "shorter or longer" isoforms compose the largest part of the
216 expression in 12 LEAN and 11 FAT cases. Notably, in 6 DEMs the exact isomiR contributed less
217 than 10% to the miRNA expression (Supplementary Figure 3B). These findings are consistent
218 with our previous results (Gaffo et al., 2014) showing a similar distribution of isomiRs and
219 indicating that in pig backfat the canonical miRNA isoform is not always the most expressed for
220 the miRNA.
221

222 Network of short and long differentially expressed RNA interactions in pig backfat

223 The target prediction considered 66 sRNA sequences, including 59 abundant isomiRs from the
224 18 known miRNAs and the 6 NPmiRNAs, plus 6 new sister-miRNAs and ssc-moR-21-5p. The
225 predicted relations with significant strong negative correlations accounted for 56,683 sRNA-
226 transcript interactions, involving 22,362 transcripts from 12,373 unique genes. All the 66
227 isomiRs potentially targeted at least one transcript each. Among the known miRNAs, ssc-miR-
228 365-5p presented the largest number of targets (3,095 different transcripts targeted,
229 corresponding to 2,624 genes), while in absolute chr3_26283-5p has the largest number of

230 target transcripts (3,383; 2,878 genes). Ssc-miR-136-3p has the smallest number of targets (524;
231 465 genes), while among the new miRNAs chr7_37486-3p has the minimum (1,011; 862 genes).
232 Considering the very large number of predicted interactions we focused particularly on the
233 predicted sRNA targets included among the 86 DETs reported in Zambonelli et al. (2016),
234 resulting in 193 putative regulatory interactions between 30 DEMs and 40 target DETs
235 (Supplementary Table 6) that may contribute to regulate the metabolic activity of porcine
236 backfat. The network in Figure 3A-B show the interactions supported by strong correlation.
237 KEGG and Reactome pathways significantly enriched in target DETs in the network are shown in
238 Figure 3C. The down regulated genes *HSPA1A, HSPA1B*, and *DNAJB1* are linked to cellular stress
239 responses, to the regulation of stress-induced transcription by *HSF1*, and its modulation by
240 attenuation that occurs during continuous exposure to intermediate heat shock conditions or
241 upon recovery from stress. The same three genes and *MRC1* and *ATP6V0D2* participate in
242 several pathways linked to immunity. Furthermore, five genes (*ADSSL1*, *AKAP5*, *BCAT1*, *HMOX1*,
243 and *PLIN2*) are linked to metabolic pathways.
244 The involvement of several genes in the network in specific pathways is informative on the
245 putative regulatory activity of selected miRNAs beyond single targets, suggesting their impact
246 on pathway regulation by coordinated activity on several functionally connected genes. Four of
247 the metabolism-linked genes are overexpressed in FAT animals and resulted under putative
248 control of common underexpressed miRNAs, with both *BCAT1* and *AKAP5* possibly repressed by
249 the ssc-let-7c, and *HMOX1* and *BCAT1* by ssc-miR-195. The underexpressed stress responses
250 genes are under putative control of specific miRNAs, as *HSPA1A, HSPA1B*, and *DNAJB1* can be

251 coordinately repressed by the upregulated and validated ssc-miR-146, and the triplet *HSPA1B*,
252 *DNAJB1* and *HSP70* by ssc-miR-365.
253

254 ## QTL enrichment of differentially expressed sRNAs and mRNAs loci

255 As miRNAs and genes with transcripts differentially expressed in LEAN and FAT animals and
256 their interactions could be implicated in genetic modulation of backfat deposition, we looked
257 for the overlap between their loci and pig QTL, and we observed 56 significantly enriched QTL
258 (Supplementary Table 7). Notably, a significant enrichment in miRNAs and their target DE genes
259 was observed for four backfat-specific carcass quality QTLs (Supplementary Table 7). Two
260 largely overlapping QTLs (QTL 21222 "Average backfat thickness" chr4:74,725,606-82,527,275;
261 QTL 21228 "Backfat at first rib" chr4: 76,741,953-83,693,913) covering approximately 9 Mb
262 contains four DE transcripts from *PENK* gene and from a predicted gene. Two enriched
263 "Average backfat thickness" QTLs were identified in chromosome 8 (QTL 29568, chr8:
264 33,033,004-33,985,796, XLOC_032101 / *UCHL1*) and 12 (QTL 5990, chr12: 23,672,762-
265 60,040,247). The most interesting enriched QTL is located in chromosome 12 and contains the
266 genes expressing eight DETs, and notably five DE miRNAs (the up-regulated ssc-miR-193a-3p
267 and ssc-miR-195 and the down-regulated ssc-miR-132, ssc-miR-365 and ssc-moR-21-5p)
268 strengthening the indication of a possible role of these sRNAs in the backfat phenotype
269 variation.
270

271 # Discussion

272 We reported the landscape of sRNA expression in swine backfat, detecting and quantifying 426
273 sRNAs expressed, including 403 miRNAs and 23 moRNAs, confirming previous data (Gaffo et al.,
274 2014) and extending them, by increasing the number of sRNAs detected in pig backfat.
275 The FAT and LEAN animals expression profile comparison disclosed 31 differentially expressed
276 sRNAs whose abundance is related to backfat level, including 30 miRNAs and a moRNA, ssc-
277 moR-21-5p, that resulted downregulated in FAT animals. These results are backed up by
278 quantitative real-time PCR validations conducted on a sizeable subset of the DE sRNAs detected
279 by RNA-seq (Figure 2), including known miRNAs, new mature miRNAs of known precursors, and
280 a moRNA, with different expression levels and variable variations strengths in the comparison
281 between FAT and LEAN animals.
282 Thirthy DE miRNAs possibly targeted 40 out of 86 backfat DETs, while no putative targets were
283 detected among the same backfat DETs for ssc-moR-21-5p. This study confirmed a possible link
284 between ssc-moR-21 and adipocyte physiology (Gaffo et al., 2014) that deserves further
285 investigation.
286 Altogether, almost two thousands putative miRNA-target interactions emerged considering the
287 correlation between the DE miRNAs and the DE mRNAs and represented as a functional
288 network (Figure 3).
289 The majority of the transcripts included in the network are coding (green squares) while there
290 are four putative noncoding (grey and orange squares). *BCAT1 (*branched chain amino acid
291 transaminase 1) and *GPNMB (*glycoprotein Nmb) are represented by both coding and one
292 noncoding isoforms.

293 The less expressed ssc-miR-199a-5p potentially regulate several transcripts, including coding
294 transcripts of different genes, different isoforms derived from the same gene, and unannotated
295 transcripts found overexpressed in FAT vs LEAN pigs. As already described in Zambonelli et al.
296 (2016) some of them are involved in lipid metabolism (perilipin 2, *PLIN2*) or in the regulation of
297 food intake (*GPNMB*). Ssc-miR-199a-5p was found under-expressed in the FAT pigs, in
298 agreement with previous data on the underexpression of this miRNA in attenuating cell
299 proliferation and promoting lipid deposition in porcine pre-adipocytes (Shi X-E et al., 2014).
300 Ssc-miR136, ssc-miR-99a-3p, and ssc-miR-214-5p were down-regulated in FAT vs LEAN pigs. In
301 lambs, miR-136 was differentially expressed in subcutaneous adipose tissue compared with
302 perirenal fat (Meale et al., 2014). Guo et al. (2012) linked miR-99a to preadipocyte
303 differentiation both in porcine intramuscular and subcutaneous vascular stem cells. The same
304 miRNA was twofold more expressed in adult subcutaneous adipose tissue than in juvenile
305 adipose tissue in pigs by Li et al. (2011). miR-214 was previously related to adipose-derived
306 stem cells differentiation in rats) and resulted up-regulated in rat stem cells isolated from
307 subcutaneous fat compared with omentum (Hu et al., 2017).
308 The importance of ssc-miR-335 in the regulation of biological and molecular processes
309 responsible of different fat deposition between FAT and LEAN animals is supported by previous
310 data linking this miRNA to the control of fat traits (Nakanishi et al., 2009; Oger et al., 2014; and
311 Zhu et al., 2014). In our network, the down-regulation of miR-335 is linked to *BCAT1*
312 overexpression. Under-expressed putative targets of overexpressed miRNAs include one
313 protein coding gene transcripts (*DNAJB1*) and noncoding transcripts, mostly belonging to heat
314 shock protein genes (*HSP70*, *HSPA1A*, *HSPA1B*) that deserve further investigation.

315 Ssc-miR-146b could control the expression of three genes down-regulated all coding for heat
316 shock proteins (*DNAJB1*, *HSPA1A*, *HSPA1B*), whereas ssc-miR-365-5p was linked to *DNAJB1*,
317 *HSPA1A* and *HSP70*, indicating that the two overexpressed *miRNAs* could contribute to
318 regulation pattern of common targets possibly with a synergistic effect. The action of miR-146b
319 on tissue inflammation was described in human obesity by different Authors (Hulsmans et al.,
320 2012; Shi C et al., 2014) who reported that, despite the obese condition, an attenuation of
321 cytokine signaling reduced the effect of the inflammation on the tissues. In visceral and
322 subcutaneous adipose tissue of over weighted and obese human subjects, miR-146b
323 overexpression promoted preadipocyte differentiation, not impacting proliferation (Ahn et al.,
324 2013; Chen et al., 2014). These evidences suggest a likely explanation of the lower level of
325 expression on backfat tissue in FAT pigs of heat shock protein DETs as response to a chronic
326 inflammation (Zambonelli et al., 2016) providing as well a link with miRNA expression and
327 regulatory activity.
328 Of miRNAs overexpressed in FAT animals, ssc-miR-132 was upregulated in the hypothalamus of
329 rats fed with a high fat diet (Sangiao-Alvarellos et al., 2014), and in omental fat and plasma of
330 obese humans (Heneghan et al., 2011), whereas the overexpression of miR-221 was associated
331 to an increase in adipocitokines expression in mice (Parra et al., 2010) and with body mass
332 index increase in subcutaneous and abdominal adipose tissue in human Pima Indians (Meerson
333 et al., 2013) where, intriguingly, the increase of body mass index was associated also with the
334 under-expression of miR-199a-3p, fully in accordance with our observations.
335 Several elements of the network, are expressed by genomic regions harboring QTL associated
336 with backfat thickness, supporting the presence and contributing to the identification of

337 positional and functional candidate genes for fat traits. The SSC12 QTL region arbors both the
338 overexpressed ssc-mir-132 and the under-expressed ssc-mir-195. Within a QTL it is possible to
339 found an epistatic effect of different genetic elements acting on opposite direction in the
340 phenotype determination (Cordell, 2002). However, the action on the phenotype of these two
341 miRNAs could be concordant or even synergistic if they repress the expression of genes whose
342 products have opposite roles in biological processes or signaling pathways.
343 Collectively, our data and the above discussed literature informed on the involvement of
344 miRNAs in the regulation of genes active in adipose tissue, suggesting that the identified DE
345 miRNAs can modulate several functions related to the adipose tissue development and growth,
346 in particular adipocyte differentiation, adipogenesis, lipid deposition, and obesity in humans.
347 Specific miRNAs might modulate as well heat shock transcript expression, supporting the idea
348 that pigs with an increased potential for fat deposition present some aspects similar to those
349 described in human obesity in which a mild inflammation blocks an excessive harmful effect on
350 a high level of inflammation when a chronic increase of fat tissue as in obesity occurs.
351 We found new genes, mRNAs and miRNAs that, according to sequence and expression data,
352 could be involved in the complex network of regulation of backfat deposition. Our results
353 explain part of the complex mechanisms influencing fat traits, shedding light on new aspect of
354 the genetic regulation of fat deposition in pigs, and facilitate the perspective implementation of
355 innovative strategies of pig genetic improvement based on genomic markers.
356

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362

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479
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481 ## Supporting information

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

483 **Supplementary Tables are included in the file: DEM-DET-**

484 **Target_SupplementaryTables_rev.xlsx**

485

486 **Table S1 (ST1).** Samples RIN numbers.

487

488 **Table S2 (ST2).** Sample raw reads filtering.

489

490 **Table S3 (ST3).** Small RNAs expressed.

491

492 **Table S4 (ST4).** List of all DE tests obtained.

493

494 **Table S5 (ST5).** IsomiRs specific for FAT or LEAN samples.

495

496 **Table S6 (ST6).** Differentially expressed small RNA targets among differentially expressed

497 transcripts.

498

499 **Table S7(ST7).** QTL enrichment for differentially expressed sRNAs and target genes.

500

501

502 **Figure S1.** Outline of the computational analysis workflow. Long and short RNA-seq data in the
503 same backfat samples of 10 LEAN and 10 FAT animals have been considered; after detection,
504 discovery and quantification of miRNA and miRNA-like small RNAs, integrative analyses of
505 target prediction and of miRNA and transcript expression profiles were used for miRNA-
506 transcript regulatory network inference.
507 **File: FigureSF1_rev.pdf**
508
509 **Figure S2.** Cumulative expression of gene expression measures show a similar pattern in the
510 FAT and LEAN groups. The small panel inside the figure corresponds to the region shaded in
511 light blue in the main panel.
512 **File: FigureSF2_rev.pdf**
513
514 **Figure S3.** IsomiR composition of known and newly predicted miRNAs resulting differentially
515 expressed in backfat tissue of LEAN and FAT animals. Colors and labels are relative to each
516 single bar-chart. The same color may refer to different labels. Portion of the bars not reaching
517 100% represents isoforms composing each less than 10% of the overall miR expression.
518 **File: FigureSF3_rev.pdf**
519
520

521 # Figure captions

522 **Figure 1.** Heatmap of expression profiles of miRNAs differentially expressed (DEMs) comparing
523 FAT vs LEAN animals.
524 Rows of the heatmap represent the 31 DEMs, columns correspond to samples. The heatmap
525 cells are colored according to the deviance of the sRNA expression in the sample from the
526 average expression of the sRNA, thus red and blue cells represent respectively expression
527 values higher or lower than mean expression across all samples (white) with color intensity
528 proportional to the difference from the mean, in the regularized logarithmic scale.
529 **File: Figure1_rev.pdf**
530

531 **Figure 2.** Validation by qRT-PCR of eight DE miRNAs and of the DE moRNA detected by RNA-seq.
532 A) Correlation between fold changes calculated according to RNA-seq (horizontal axis) and qRT-
533 PCR (vertical axis) estimations. Dashed line indicates linear smoothing of the points. R_s =
534 Spearman's rank correlation coefficient. B) Significance of differential expression tests
535 according to qRT-PCR data. T-test p-value: ns = not significant; * = <0.05, ** <0.01; *** =
536 <0.001. ΔCq = difference between U6 and the target miRNA PCR-derived cycle thresholds.
537 **File: Figure2_rev.pdf**
538

539 **Figure 3.** Differentially expressed miRNAs and their putative predicted target transcripts
540 differentially expressed. The network depicts the predicted interactions between: A) miRNAs
541 downregulated in FAT animals and putative target transcripts upregulated in FAT animals; B)

542 miRNAs upregulated in FAT animals and putative target transcripts downregulated in FAT
543 animals; miRNA-target relations with expression profiles correlation < -0.6 are reported,
544 involving 26 miRNA (blue circle nodes) and 28 transcripts (square nodes, coloured green if
545 coding, lilac if non-coding, and orange if reliably non-coding) for a total of 92 interactions
546 (edges). Edge thickness is inversely proportional to the correlation intensity (the smaller the
547 correlation, the thicker the line). Nodes borders are coloured in red if representing a newly
548 predicted transcript or miRNA precursor, orange for new sister miRNAs, and green for known
549 miRNAs. C) KEGG and Reactome pathways significantly enriched in genes included in the
550 network of panel A-B.
551 **File: Figure3_rev.pdf**
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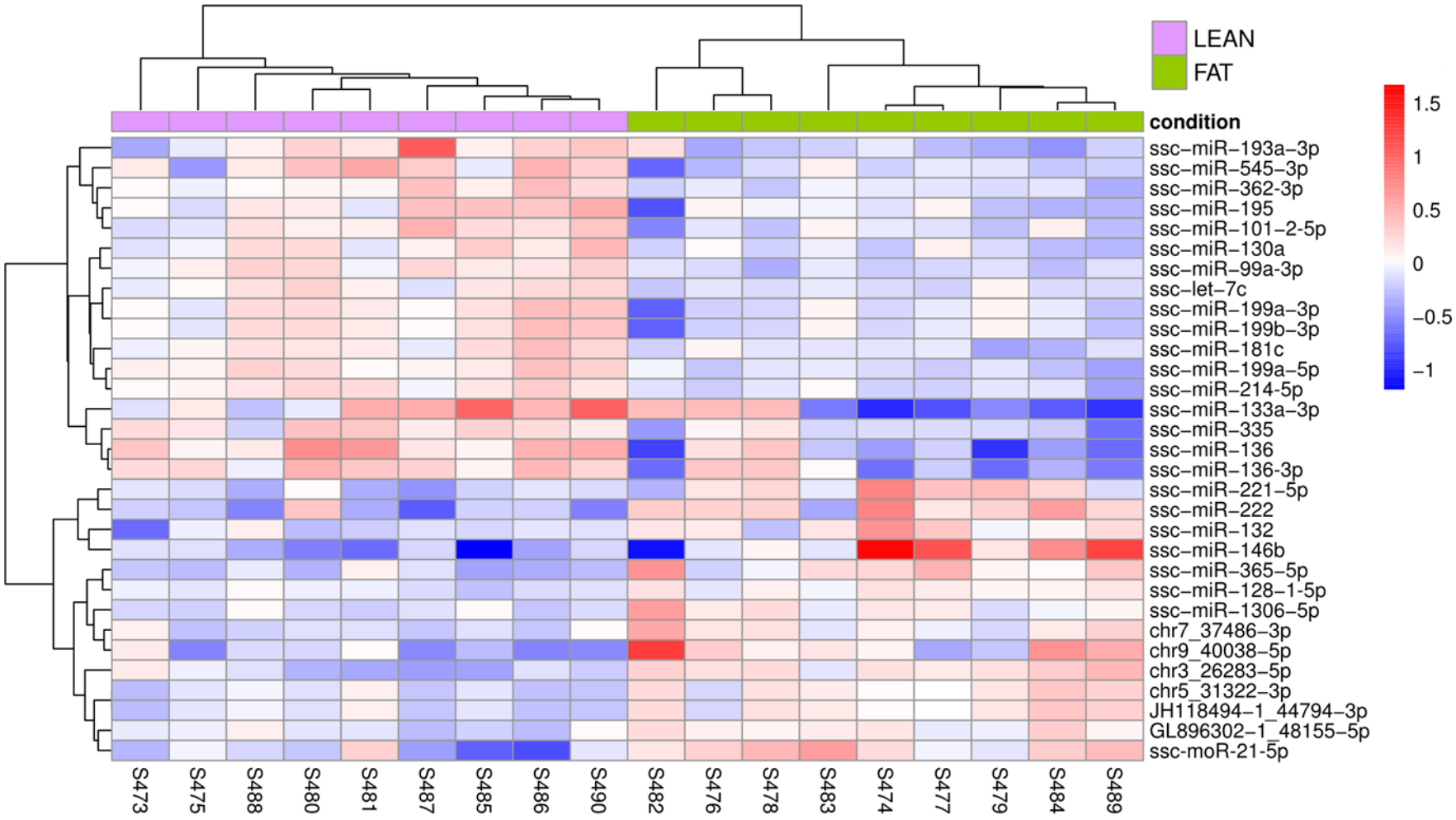
554 # Tables

555 **Table 1.** Short RNAs resulting differentially expressed in backfat of FAT vs LEAN animals

556 according to RNA-seq data.

Name	Type	Mature sequence	Average expression (RPM)*	% of expressed sRNAs	Log_2 Fold Change	Adjusted p-value
ssc-miR-146b	known	ugagaacugaauuccauaggc	20131.80	2.13E-02	0.97	0.01
ssc-miR-365-5p	known	gagggacuuucaggggcagcugu	799.48	1.15E-03	0.89	0.00
chr9_40038-5p	new	cuccuggcuggcucgcca	21.81	8.53E-03	0.83	0.01
ssc-miR-221-5p	known	accuggcauacaauguagauuucug	2916.44	1.20E-03	0.77	0.01
ssc-moR-21-5p	moRNA	ctccatggctgtaccaccttgtcgg	9.63	3.01E-03	0.76	0.01
ssc-miR-222	known	agcuacaucuggcuacugggucuc	2863.37	2.35E-02	0.75	0.01
chr5_31322-3p	new	ucugagaugugaccugggcau	27.77	6.09E-04	0.72	0.01
JH118494-1_44794-	new	ucugagaugugaccugggcau	11.40	6.09E-04	0.72	0.01
chr7_37486-3p	new	aagucccaucugggucgcc	24.49	6.79E-04	0.69	0.02
chr3_26283-5p	new	uuggcucugcgaggucggcuca	30.91	2.56E-03	0.69	0.00
ssc-miR-128-1-5p	new sister	cggggccgtagcactgtctgag	138.40	5.09E-04	0.53	0.02
ssc-miR-132	known	uaacagucuacagccauggucg	55877.75	4.04E-03	0.50	0.03
ssc-miR-1306-5p	known	ccaccuccccugcaaacgucca	70505.29	2.34E-03	0.47	0.02
GL896302-1_48155-	new	ucucugggccugugucuuaggcu	12.42	2.09E-03	0.39	0.02
ssc-let-7c	known	ugagguaguagguuguaugguu	2435309.00	5.98E-01	-0.32	0.01
ssc-miR-130a	known	cagugcaauguuaaaagggcau	56716.38	4.26E-02	-0.38	0.02
ssc-miR-181c	known	aacauucaaccugucggugagu	9716.73	1.15E-01	-0.39	0.01
ssc-miR-214-5p	new sister	tgcctgtctacacttgctgtgc	93.15	8.13E-03	-0.42	0.00
ssc-miR-199a-5p	known	cccaguguucagacuaccuguuc	5221.81	1.71E-01	-0.44	0.00
ssc-miR-199a-3p	known	acaguagucugcacauugguua	5352.48	1.34E+00	-0.45	0.01
ssc-miR-199b-3p	known	uacaguagucugcacauugguu	5118.61	6.70E-01	-0.45	0.01
ssc-miR-99a-3p	new sister	caagctcgcttctatgggtctg	56.14	1.81E-02	-0.47	0.00
ssc-miR-195	known	uagcagcacagaaauauuggc	6255.99	1.60E-01	-0.50	0.02
ssc-miR-101-2-5p	new sister	tcagttatcacagtgctgatgct	152.31	1.45E-03	-0.61	0.02
ssc-miR-193a-3p	known	aacuggccuacaaagucccagu	6485.75	2.93E-02	-0.62	0.02
ssc-miR-136-3p	new sister	catcatcgtctcaaatgagtct	133.91	8.57E-03	-0.63	0.02
ssc-miR-335	known	ucaagagcaauaacgaaaaaug	1118.26	8.38E-04	-0.75	0.02
ssc-miR-133a-3p	known	uuggucccuucaaccagcug	52301.03	1.44E-02	-0.79	0.03
ssc-miR-545-3p	known	aucaacaaacauuuauugugug	381.19	7.45E-04	-0.81	0.04
ssc-miR-136	known	acuccauuuguuuugaugaugga	32337.35	2.42E-03	-0.94	0.01
ssc-miR-362-3p	new sister	aacacacctattcaaggattc	76.01	2.91E-04	-0.97	0.01

557 * Read Per Million


LEAN
FAT
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0.5
0
−0.5
−1
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ssc−miR−545−3p
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ssc−miR−101−2-5p
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ssc−miR−181c
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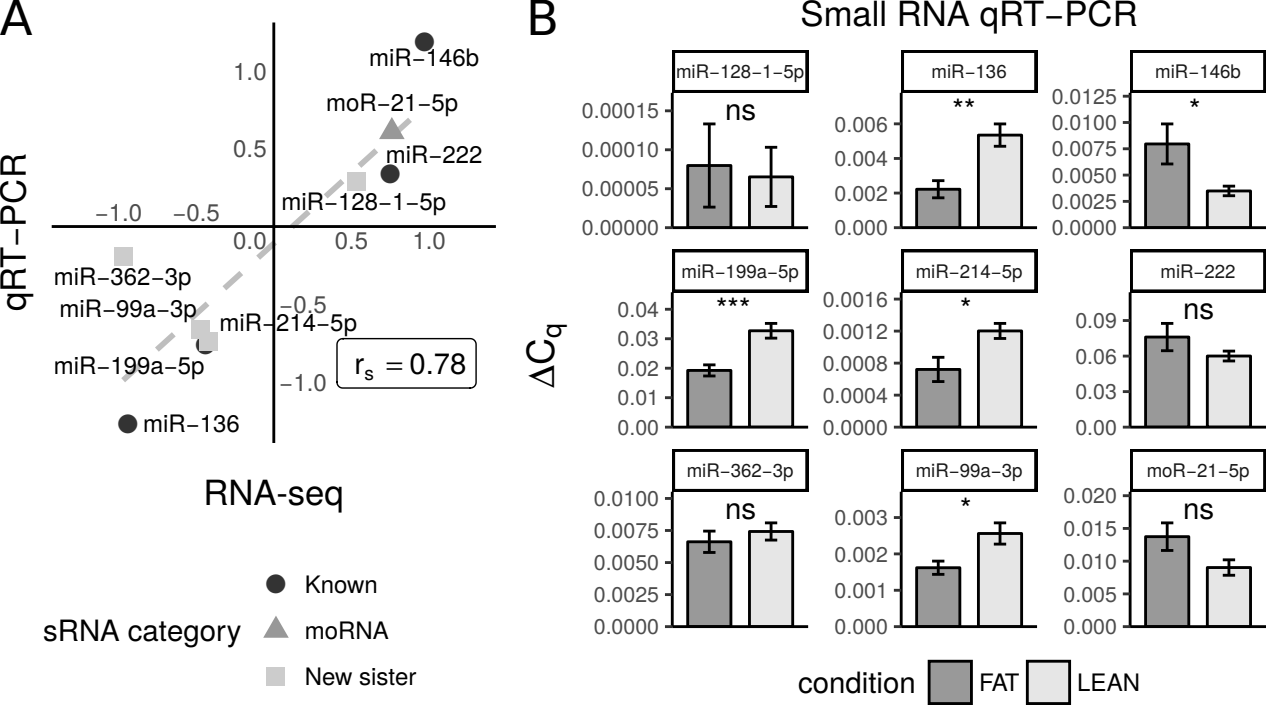
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Small RNA qRT−PCR
qRT−PCR
RNA-seq
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miR−222
miR−128−1−5p
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miR−99a−3p
miR−214−5p
miR−199a−5p
miR−136
$r_s = 0.78$
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0.5
0.0
−0.5
−1.0
sRNA category
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moRNA
New sister
ΔC_q
miR−128−1−5p
miR−136
miR−146b
miR−199a−5p
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moR−21−5p
ns
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*
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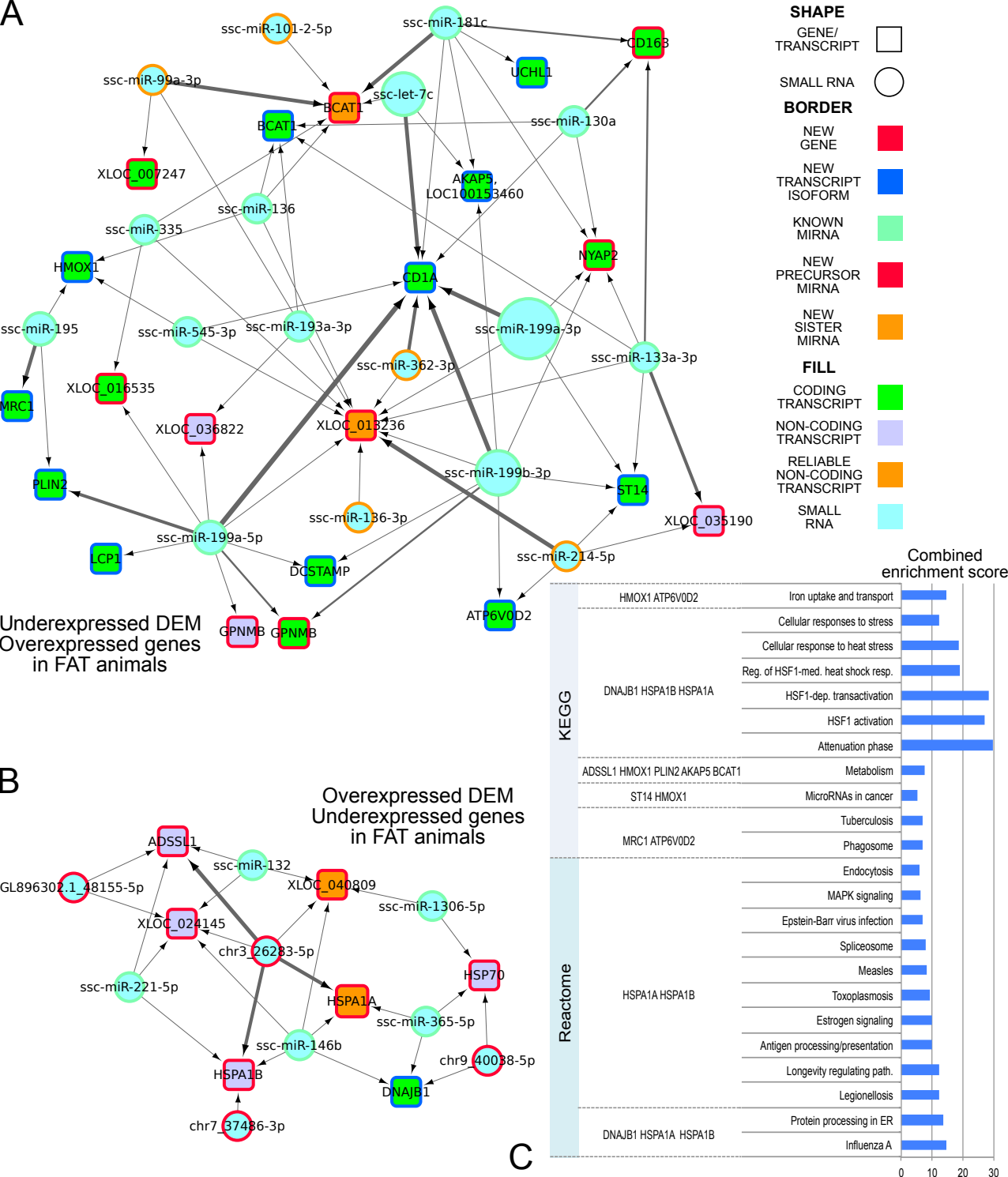
FAT
LEAN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LCP1
DCSTAMP
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ATP6V0D2
GPNMB
GPNMB
Underexpressed DEM
Overexpressed genes
in FAT animals
SHAPE
GENE/ TRANSCRIPT
SMALL RNA
BORDER
NEW GENE
NEW TRANSCRIPT ISOFORM
KNOWN MIRNA
NEW PRECURSOR MIRNA
NEW SISTER MIRNA
FILL
CODING TRANSCRIPT
NON-CODING TRANSCRIPT
RELIABLE NON-CODING TRANSCRIPT
SMALL RNA
B
Overexpressed DEM
Underexpressed genes
in FAT animals
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Combined enrichment score
KEGG
Reactome
HMOX1 ATP6V0D2
Iron uptake and transport
Cellular responses to stress
Cellular response to heat stress
Reg. of HSF1-med. heat shock resp.
DNAJB1 HSPA1B HSPA1A
HSF1-dep. transactivation
HSF1 activation
Attenuation phase
ADSSL1 HMOX1 PLIN2 AKAP5 BCAT1
Metabolism
ST14 HMOX1
MicroRNAs in cancer
Tuberculosis
MRC1 ATP6V0D2
Phagosome
Endocytosis
MAPK signaling
Epstein-Barr virus infection
Spliceosome
Measles
HSPA1A HSPA1B
Toxoplasmosis
Estrogen signaling
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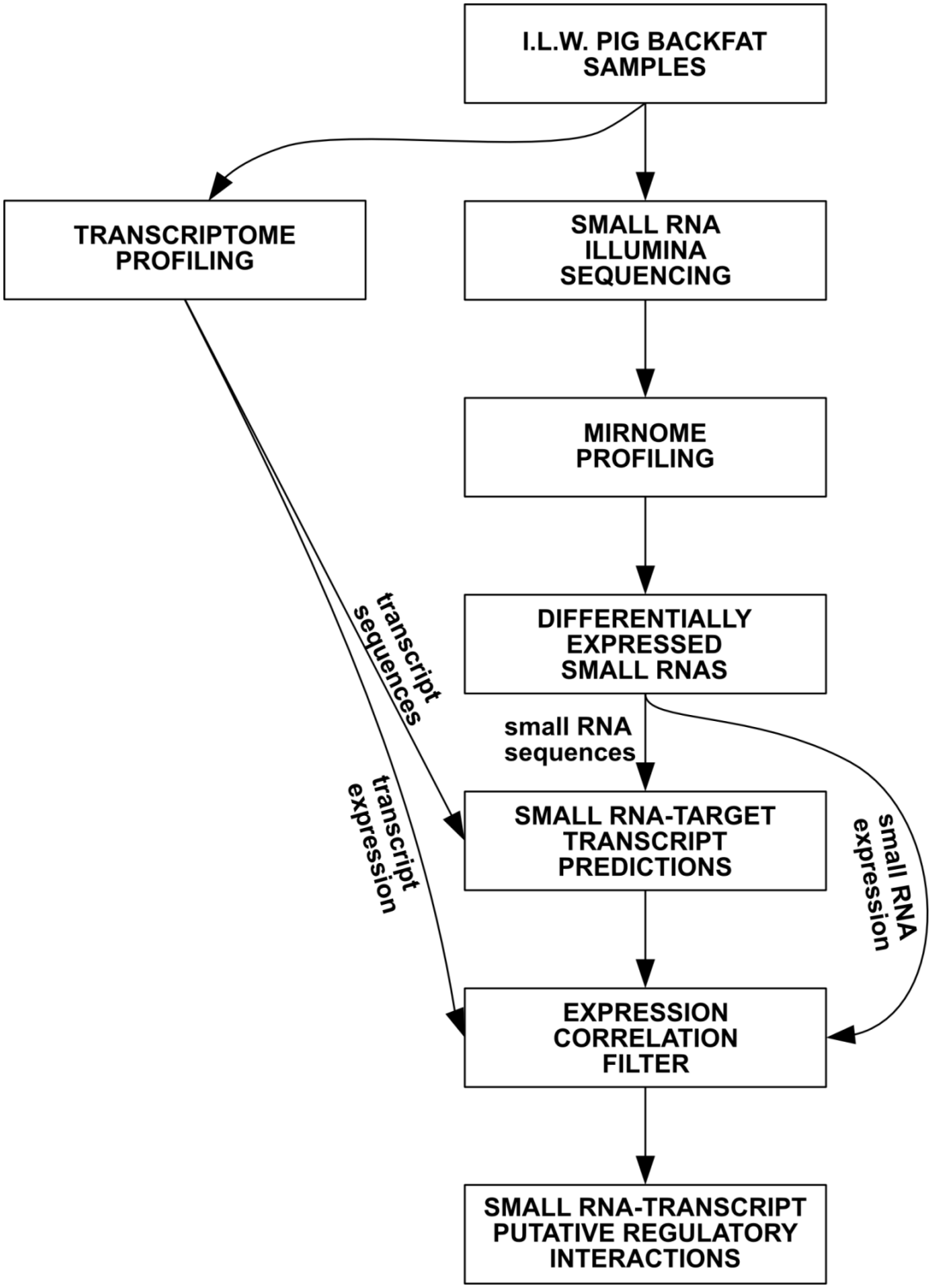
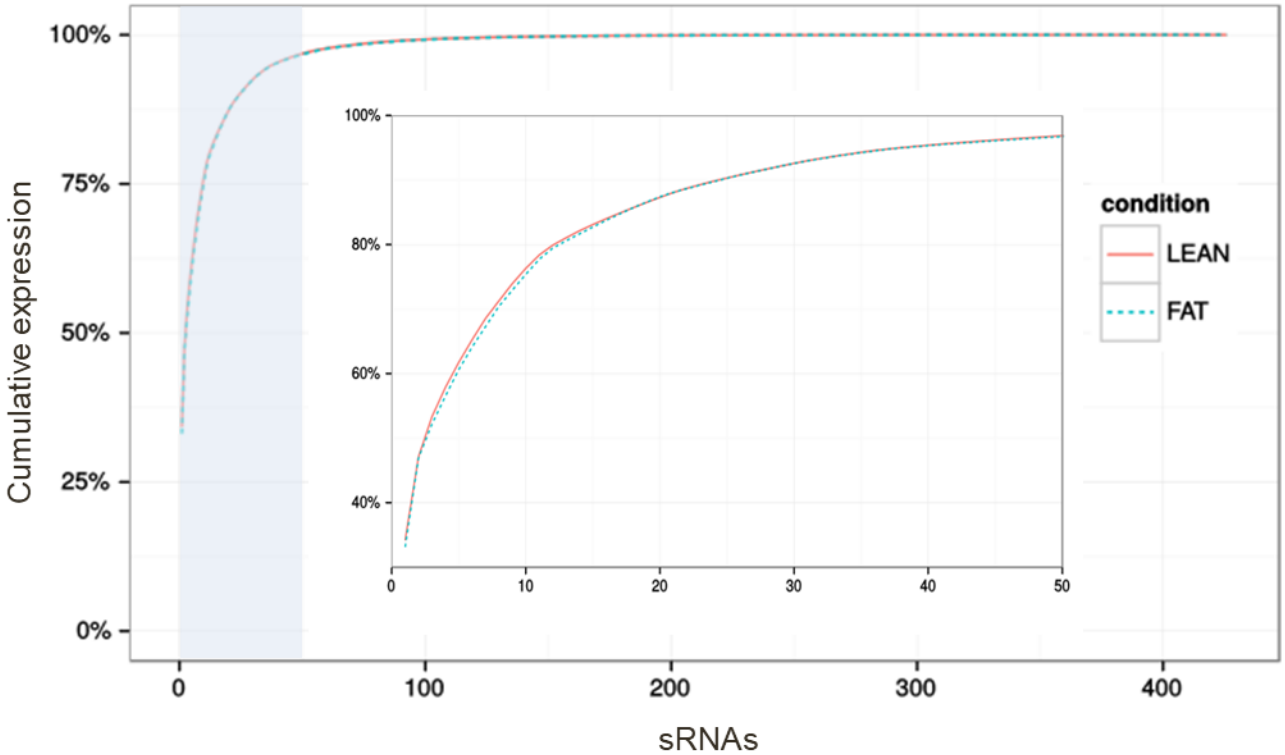
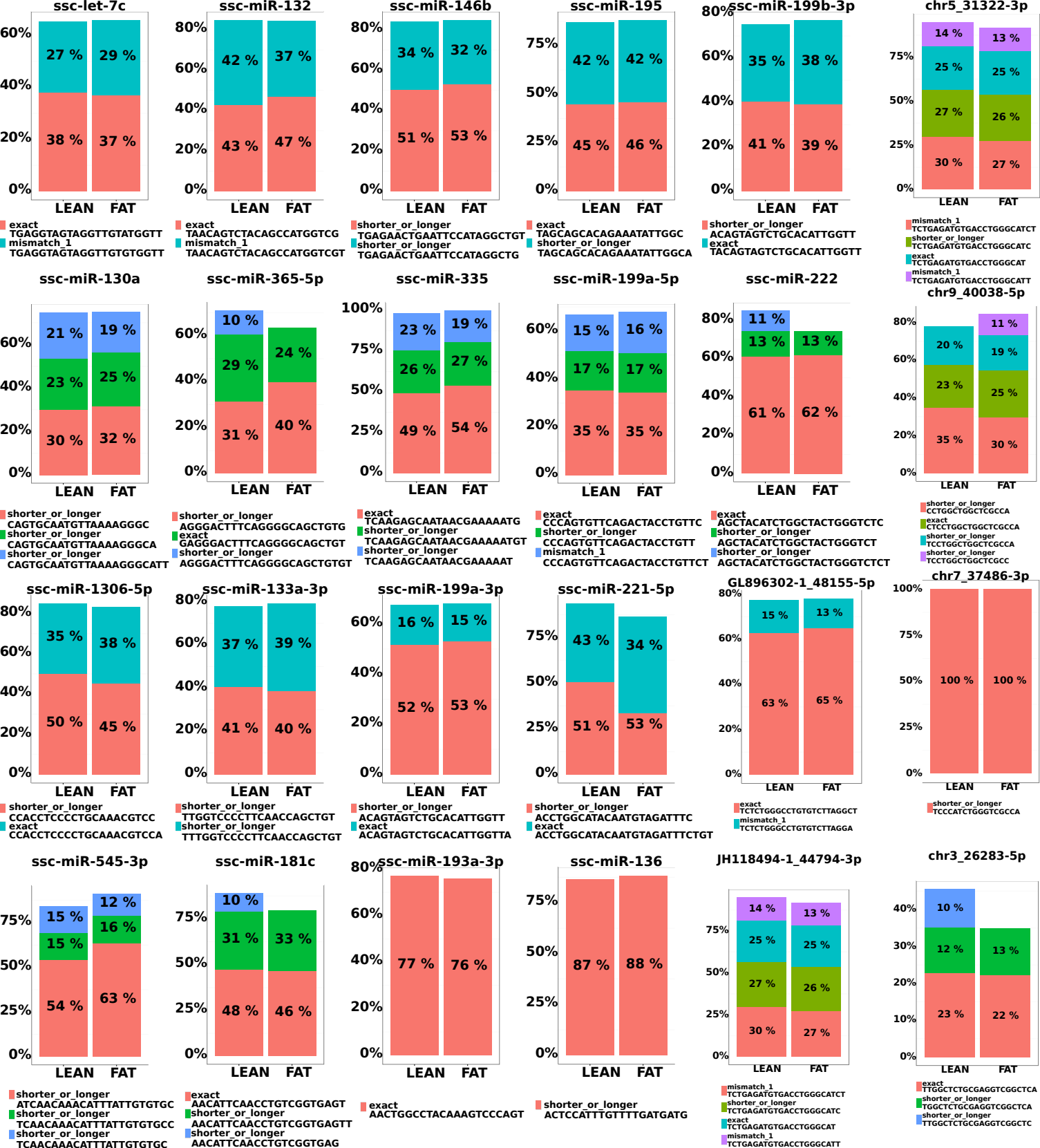

I.L.W. PIG BACKFAT SAMPLES
TRANSCRIPTOME PROFILING
SMALL RNA ILLUMINA SEQUENCING
MIRNOME PROFILING
DIFFERENTIALLY EXPRESSED SMALL RNAS
transcript sequences
small RNA sequences
transcript expression
SMALL RNA-TARGET TRANSCRIPT PREDICTIONS
small RNA expression
EXPRESSION CORRELATION FILTER
SMALL RNA-TRANSCRIPT PUTATIVE REGULATORY INTERACTIONS


Cumulative expression
100%
75%
50%
25%
0%
0
100
200
300
400
sRNAs
100%
80%
60%
40%
0
10
20
30
40
50
condition
LEAN
FAT


ssc-let-7c
27 % 29 %
38 % 37 %
LEAN FAT
exact
TGAGGTAGTAGGTTGTATGGTT
mismatch_1
TGAGGTAGTAGGTTGTGTGGTT
ssc-miR-132
42 % 37 %
43 % 47 %
LEAN FAT
exact
TAACAGTCTACAGCCATGGTCG
mismatch_1
TAACAGTCTACAGCCATGGTCGT
ssc-miR-146b
34 % 32 %
51 % 53 %
LEAN FAT
shorter_or_longer
TGAGAACTGAATTCCATAGGCTGT
shorter_or_longer
TGAGAACTGAATTCCATAGGCTG
ssc-miR-195
42 % 42 %
45 % 46 %
LEAN FAT
exact
TAGCAGCACAGAAATATTGGC
shorter_or_longer
TAGCAGCACAGAAATATTGGCA
ssc-miR-199b-3p
35 % 38 %
41 % 39 %
LEAN FAT
shorter_or_longer
ACAGTAGTCTGCACATTGGTT
exact
TACAGTAGTCTGCACATTGGTT
chr5_31322-3p
14 % 13 %
25 % 25 %
27 % 26 %
30 % 27 %
LEAN FAT
mismatch_1
TCTGAGATGTGACCTGGGCATCT
shorter_or_longer
TCTGAGATGTGACCTGGGCATC
exact
TCTGAGATGTGACCTGGGCAT
mismatch_1
TCTGAGATGTGACCTGGGCATT
ssc-miR-130a
21 % 19 %
23 % 25 %
30 % 32 %
LEAN FAT
shorter_or_longer
CAGTGCAATGTTAAAAGGGC
shorter_or_longer
CAGTGCAATGTTAAAAGGGCA
shorter_or_longer
CAGTGCAATGTTAAAAGGGCATT
ssc-miR-365-5p
10 % 24 %
29 % 40 %
31 %
LEAN FAT
shorter_or_longer
AGGGACTTTCAGGGGCAGCTGTG
exact
GAGGGACTTTCAGGGGCAGCTGT
shorter_or_longer
AGGGACTTTCAGGGGCAGCTGTGT
ssc-miR-335
23 % 19 %
26 % 27 %
49 % 54 %
LEAN FAT
exact
TCAAGAGCAATAACGAAAAATG
shorter_or_longer
TCAAGAGCAATAACGAAAAATGT
shorter_or_longer
TCAAGAGCAATAACGAAAAAT
ssc-miR-199a-5p
15 % 16 %
17 % 17 %
35 % 35 %
LEAN FAT
exact
CCCAGTGTTCAGACTACCTGTTC
shorter_or_longer
CCCAGTGTTCAGACTACCTGTT
mismatch_1
CCCAGTGTTCAGACTACCTGTTCT
ssc-miR-222
11 %
13 % 13 %
61 % 62 %
LEAN FAT
exact
AGCTACATCTGGCTACTGGGTCTC
shorter_or_longer
AGCTACATCTGGCTACTGGGTCT
shorter_or_longer
AGCTACATCTGGCTACTGGGTCTCT
chr9_40038-5p
11 %
20 % 19 %
23 % 25 %
35 % 30 %
LEAN FAT
shorter_or_longer
CCTGGCTGGCTCGCCA
exact
CTCCTGGCTGGCTCGCCA
shorter_or_longer
TCCTGGCTGGCTCGCCA
shorter_or_longer
TCCTGGCTGGCTCGCC
ssc-miR-1306-5p
35 % 38 %
50 % 45 %
LEAN FAT
shorter_or_longer
CCACCTCCCCTGCAAACGTCC
exact
CCACCTCCCCTGCAAACGTCCA
ssc-miR-133a-3p
37 % 39 %
41 % 40 %
LEAN FAT
shorter_or_longer
TTGGTCCCCTTCAACCAGCTGT
shorter_or_longer
TTTGGTCCCCTTCAACCAGCTGT
ssc-miR-199a-3p
16 % 15 %
52 % 53 %
LEAN FAT
shorter_or_longer
ACAGTAGTCTGCACATTGGTT
exact
ACAGTAGTCTGCACATTGGTTA
ssc-miR-221-5p
43 % 34 %
51 % 53 %
LEAN FAT
shorter_or_longer
ACCTGGCATACAATGTAGATTTC
exact
ACCTGGCATACAATGTAGATTTCTGT
GL896302-1_48155-5p
15 % 13 %
63 % 65 %
LEAN FAT
exact
TCTCTGGGCCTGTGTCTTAGGCT
mismatch_1
TCTCTGGGCCTGTGTCTTAGGA
chr7_37486-3p
100 % 100 %
LEAN FAT
shorter_or_longer
TCCCATCTGGGTCGCCA
ssc-miR-545-3p
15 % 12 %
15 % 16 %
54 % 63 %
LEAN FAT
shorter_or_longer
ATCAACAAACATTTATTGTGTGC
shorter_or_longer
TCAACAAACATTTATTGTGTGCC
shorter_or_longer
TCAACAAACATTTATTGTGTGC
ssc-miR-181c
10 %
31 % 33 %
48 % 46 %
LEAN FAT
exact
AACATTCAACCTGTCGGTGAGT
shorter_or_longer
AACATTCAACCTGTCGGTGAGTT
shorter_or_longer
AACATTCAACCTGTCGGTGAG
ssc-miR-193a-3p
77 % 76 %
LEAN FAT
exact
AACTGGCCTACAAAGTCCCAGT
ssc-miR-136
87 % 88 %
LEAN FAT
shorter_or_longer
ACTCCATTTGTTTTGATGATG
JH118494-1_44794-3p
14 % 13 %
25 % 25 %
27 % 26 %
30 % 27 %
LEAN FAT
mismatch_1
TCTGAGATGTGACCTGGGCATCT
shorter_or_longer
TCTGAGATGTGACCTGGGCATC
exact
TCTGAGATGTGACCTGGGCAT
mismatch_1
TCTGAGATGTGACCTGGGCATT
chr3_26283-5p
10 %
12 % 13 %
23 % 22 %
LEAN FAT
exact
TTGGCTCTGCGAGGTCGGCTCA
shorter_or_longer
TGGCTCTGCGAGGTCGGCTCA
shorter_or_longer
TTGGCTCTGCGAGGTCGGCTC