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Research paper

The sheep miRNAome: Characterization and distribution of miRNAs in 21 tissues

Martin Bilbao-Arribas [1], Aitor Guisasola-Serrano [1], Endika Varela-Martínez, Begoña M. Jugo [*]

Department of Genetics, Physical Anthropology and Animal Physiology, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), Leioa, Spain

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ABSTRACT

MicroRNAs (miRNAs) are evolutionarily conserved small non-coding RNAs that regulate gene expression. In livestock species, miRNAs also show great potential as biomarkers for animal health and product quality. In sheep, few miRNAs have been described in comparison with other livestock species or model organisms. We uniformly analyzed 172 public ovine small RNA sequencing datasets from 21 different tissues in order to predict conserved and novel miRNA precursors and profile their expression patterns. In addition to the 106 annotated sheep miRNAs, 1047 previously unannotated miRNA precursor sequences were detected and 41 % of them were assigned an orthologue from other close species. Considering expression levels, a set of miRNAs with high sequence conservation were detected in all tissues, while 733 mature miRNAs were robustly expressed in at least one tissue. 270 miRNAs showed high tissue specificity index values. Brain, male reproductive tissues and PBMCs showed the most distinct expression patterns. Strikingly, over one hundred precursors from the ruminant specific family of mir-2284/mir-2285 miRNAs were found, which were enriched in immune related tissues. This work supports the known high conservation of many miRNAs, but also highlights the potential of clade-specific innovations in ruminant evolution.

1. Introduction

MicroRNAs (miRNAs) are evolutionarily conserved small non-coding RNAs that regulate gene expression by targeting mRNAs and provoking destabilization or translational repression (Bartel, 2018). Moreover, they have been associated with many diseases and are used as markers for molecular diagnosis in humans (Saliminejad et al., 2019). In livestock species, miRNAs also show great potential as biomarkers for animal health and product quality, or as biomarkers for the selection and improvement of phenotypes of commercial interest in breeding programs (Liu et al., 2010; Miretti et al., 2020).

Livestock genomes remain under-annotated in terms of miRNAs compared with other model organisms such as human or mouse but the information in sheep is scarce. In livestock species such as goat, cattle, horse or pig hundreds of miRNAs have been described, but in sheep there are only 106 miRNA genes in miRBase database (Kozomara et al., 2019), there are 355 miRNA genes annotated in the latest Ensembl (v.104) annotation and sheep is not among the supported species in the latest MirGeneDB (2.1) update (Fromm et al., 2022). The RumimiR database (Bourdon et al., 2019) is the most comprehensive repository. It stores ruminant miRNA sequences from the literature as they were published and is a useful resource to find out which sequences have already been detected. Considering the important regulatory roles miRNAs have, their proper characterization in sheep is of prime importance.

Integrated miRNA expression profiling across tissues has been performed to identify tissue specific miRNAs in other livestock species such as horse or cattle (Pacholewska et al., 2016; Sun et al., 2019). Different sheep tissues, such as ovaries, heart, lungs or intestines have been analyzed by small RNA sequencing in several functional experiments (Hou et al., 2018; Long et al., 2019; Pokharel et al., 2018). Recently, our group has also analyzed the miRNA expression in experiments related to the immune response in infection diseases and vaccination experiments in different tissues such as lungs (Bilbao-Arribas et al., 2019), cerebral

Abbreviations: CPM, Counts per million; miRNA, microRNA; miRNA-seq, microRNA sequencing; PBMC, Peripheral Blood Mononuclear Cells; SRA, Sequence Read Archive; TSI, Tissue specificity index; tSNE, t-distributed stochastic neighbor embedding.

* Corresponding author.
E-mail addresses: martin.bilbao@ehu.eus (M. Bilbao-Arribas), aguisasola013@ikasle.ehu.eus (A. Guisasola-Serrano), endika.varela@ehu.eus (E. Varela-Martínez), begonamarina.jugo@ehu.eus (B.M. Jugo).

[1] Coauthorship.

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cortex (Varela-Martínez et al., 2020), peripheral blood mononuclear cells (PBMCs) (Varela-Martínez et al., 2018) and spleen (Varela-Martínez et al., under revision).

Secondary analysis of genomic data deposited in public databases represents an opportunity for scientific questions that could not be possible with individual datasets (Sielemann et al., 2020). In this work, we collected raw miRNA-seq samples deposited in public databases by multiple projects, comprising a wide range of sheep tissues, and analyzed them in a uniform way. Thus, the main objective of this work was to characterize the sheep miRNA microRNAome by predicting unannotated miRNAs and analyzing their expression profile across different tissues in an integrated manner. We focused on the identification of tissue-specific miRNAs and the dissection of miRNA expression distribution, as dominant miRNAs can constitute a significant fraction of the expressed miRNAome, in clear contrast to protein-coding genes (Olive et al., 2015).

2. Methods

2.1. Sample selection and data preprocessing

Firstly, searches in NCBI PubMed and SRA databases were performed using "mirna" and "sheep" keywords. 226 articles were recovered from PubMed (search performed on 20/07/2021), and all the projects including the two keywords in the SRA database were reviewed. Two conditions were established for dataset selection: high-throughput small RNA sequencing should have been performed in an Illumina platform and the raw sequencing files should have been uploaded to a public repository with clear metadata. Three conditions were set up for sample selection: samples without any experimental treatment (e.g. an infection), with at least two biological replicates in the same study and from animals older than 6 months were selected.

All samples were downloaded with the SRA toolkit version 2.10.8 (SRA Toolkit Development Team). Quality control was performed using Fastqc version 0.11.5. Adapters, low-quality sequences and small sequences (>16 bp) were removed using Trimmomatic version 0.39 (Bolger et al., 2014).

Some tissues were grouped into more general groups: ovary and corpus luteum samples were grouped as "Ovary"; cerebral cortex and hypothalamus samples as "Brain"; colon and intestine samples as "Intestines" and omasum and rumen samples as "Stomach".

2.2. Characterization and quantification of miRNAs

Preprocessed miRNA reads were mapped against the *Ovis aries* reference genome Oar_rambouillet_v1.0 with the *mapper.pl* script from miRDeep2 v.0.1.2 (Friedländer et al., 2012), which internally uses bowtie (Langmead et al., 2009). During mapping, we allowed one mismatch in the seed sequence, defined as the first 18 nucleotides, and removed reads mapped to more than five locations. Then, the miRDeep2 core program was used in order to predict bona fide miRNAs. We used miRNAs from sheep and other species from miRBase release 22.1 (Kozomara et al., 2019) to guide the search for unannotated miRNAs. Predicted sequences with 5 or higher miRDeep2 scores were kept. All the resulting sequences were blasted against the sheep RNA sequences in RNAcentral database with standalone BLAST + v.2.9.0 (Camacho et al., 2009) in order to filter out potentially hairpin-forming RNA classes, such as tRNAs and other small RNAs. A single miRNA was retained when two precursor sequences overlapped>16 base pairs in their location. Known miRBase miRNAs mapping to a different location were treated as unannotated copies.

Unannotated miRNAs were named based on sequence similarity with other species. Precursor sequences were blasted against individual datasets of miRBase precursor sequences of goat, cattle, horse, pig and human miRNAs with standalone BLAST + v.2.9.0 (Camacho et al., 2009). Alignments with Q value < 0.01 and query coverage > 80 were kept, and the one with highest identity was selected in each species. When a sequence was present in more than one species, we named the unannotated miRNA with the name of the evolutionarily closest species. All miRNA loci were intersected with the Ensembl v.104 miRNA genes with bedtools v.2.26.0 (Quinlan and Hall, 2010). All miRNA loci were grouped into clusters with a minimum genomic distance of 10 kb with bedtools v.2.26.0 (Quinlan and Hall, 2010).

2.3. Analysis of miRNA expression

Expression quantification of known and predicted miRNAs was performed with the *quantifier.pl* script from miRDeep2. We conserved the expression of a single representative miRNA in the case of identical miRNAs located in different genomic loci. For that, we clustered the mature miRNA sequences into groups with complete identity using cd-hit-est from the CD-HIT suite (Fu et al., 2012). miRNAs that were not expressed in at least one tissue with 10 reads on average were removed.

Read counts were normalized to counts per million (CPM), using the cpm function of EdgeR v.3.26.8 R package (Robinson et al., 2010). The expression matrix was log10-transformed after adding 0.1 to all the values and the normalized expression matrix was used for visualization with the t-distributed stochastic neighbor embedding (t-SNE) method of dimension reduction with Rtsne v.0.15 R package. Correlation between samples was analyzed using the Pearson correlation coefficient and was visualized as a heatmap using the pheatmap v.1.0.12 R package. The miRNA expression distribution was analyzed by averaging the CPM values in each tissue. The bioinformatic workflow schema followed in this study is available as a supplementary figure (Fig. S1).

2.4. Analysis of tissue specificity

Two procedures were applied to analyze tissue specific miRNAs. Firstly, it was verified in which tissue combinations were expressed the miRNAs using a minimum of 5 CPM to consider them expressed, and it was visualized with UpSetR v1.4.0 R package (Conway et al., 2017). Secondly, a previously described tissue specificity index (TSI) called tau (τ) was used (Yanai et al., 2005). The range of the TSI values for a miRNA is between 0 and 1, where 1 represents a miRNA expressed in a single tissue and 0 represents a miRNA expressed in all tissues. miRNAs with 0.9 or greater TSI values were considered tissue-specific and those who had a 0.25 or smaller TSI value were considered housekeeping miRNAs.

2.5. Target prediction

The target genes of the novel miRNAs were predicted using the standalone version of TargetScan v.7.0 (Agarwal et al., 2015) and the UTR sequences defined in the ovine Ensembl gene annotation (release 107). Predicted miRNA-gene interactions were filtered by a context ++ score percentile > 95 in order to retain the most confident targets. Gene set enrichment analysis of targets gene sets was done with gprofiler2 v.0.2.1 R package (Raudvere et al., 2019). Benjamini-Hochberg FDR correction was applied to the p-values and the threshold was set to 0.01.

3. Results

3.1. Data retrieval

We selected 20 ovine small RNA sequencing datasets available through NCBI SRA. Among all the datasets, four had been produced in our lab and another one was produced by The Ovine FAANG Project. In total, the selected dataset comprises 172 samples and 21 tissues, with ovary being the tissue with the largest number of samples (Table 1). Information of all samples selected for the study is provided as supplementary data (Supplementary Table S1). From the 21 tissues, 3 corresponded to the female sheep reproductive system (endometrium, ovary

Table 1
Summary of samples and publications retrieved for this work.

BioProject accession	Tissues (number of samples)	References
PRJNA451237	Ovary (6), endometrium (2)	(Yang J et al., 2019)
PRJNA354833	Adipose tissue (2)	(Zhou et al., 2017)
PRJEB22101	Ovary (10)	(Pokharel et al., 2018)
PRJEB32852	Corpus luteum (10)	(Pokharel et al., 2020b)
PRJEB32852	Endometrium (10)	(Pokharel et al., 2020a)
PRJNA392421	Intestine (3)	(Hou et al., 2018)
PRJEB20781	Lymph node (6)	Unpublished[a]
PRJNA505702	Ovary (6)	(Gu et al., 2019)
PRJNA474913	Lung (5)	(Bilbao-Arribas et al., 2019)
PRJNA532808	Hypothalamus (12)	(Zhang et al., 2020)
PRJNA511987	Heart (6), Muscle (6), Lung (6), Kidney (4), Liver (5), Spleen (6)	(Long et al., 2019)
PRJNA414087	Gallbladder (2), Heart (2), Skin (2), Muscle (2), Lymph node (2), Colon (2), Omasum (2), Rumen (2), Oviduct (2), Ureter (2)	(Salavati et al., 2020)[b]
PRJNA454385	PBMC (6)	(Varela-Martínez et al., 2018)
PRJNA528259	Cerebral cortex (5)	(Varela-Martínez et al., 2020)
PRJNA748757	Spleen (4)	Varela-Martínez et al.[c]
PRJNA638028	Ovary (3)	(Shabbir et al., 2021)
PRJNA613135	Testis (8)	(Li et al., 2021)
PRJNA608075	Mammary gland (6)	(Hao et al., 2021)
PRJNA694531	Epididymis (9)	(Wu et al., 2021)
PRJNA607580	Mammary gland (6)	(Wang J et al., 2021)

[a] Permission from the author. [b]The Ovine FAANG Project. [c]Under review.

and oviduct), 2 to the male sheep reproductive system (testis and epididymis), 4 to the digestive system (stomach, intestine, liver and gallbladder), 3 to the immune system (spleen, PBMC and lymph node) and 2 to the renal system (kidney and ureter). Four of the samples were obtained from unpublished spleen miRNA-seq experiments that were produced by our group.

3.2. Characterization of known and unannotated miRNAs

Before data preprocessing, samples had an average of 15.37 ± 0.53 million reads and 14.07 ± 0.5 million reads remained after quality filtering and adapter trimming. Samples with bad quality, very low sequencing depth or read length distribution not centered around 20–22 base pairs were discarded from the analysis. An average of 82.85 ± 0.8 % of the filtered reads were unambiguously mapped against the sheep genome.

1047 sequences were selected as bona fide miRNAs, corresponding to the sequences with a probability of being a true miRNA of 0.83 ± 0.01 according to miRDeep2, and after removing other RNA classes and overlapping predictions. Despite some miRNAs being already annotated in miRBase, eleven of them did not reach the minimum established miRDeep2 score to be considered a true miRNA. Comparing with the Ensembl miRNA gene set, we detected 284 out of the 355 miRNA precursors (80 %), of which 97 were annotated in miRBase and 187 were not. Sequences and coordinates of all detected miRNA loci are provided as supplementary data (Supplementary Table S2).

3.3. Sequence conservation and miRNA clusters

Regarding the 1047 unannotated miRNAs, they showed differing levels of conservation. 455 (43 %) were found at least in another species using a stringent approach. Due to some miRNAs being located at more than a single genomic loci, they were homologs of 428 miRNAs in other species and most unannotated miRNAs were named based on a goat or cow homolog (Fig. 1). 161 miRNAs (35 % of the conserved miRNAs) were found in all five species and 432 miRNAs (95 %) had homologs in cattle. The reason for finding so many cattle homologs is the higher number of annotated cattle miRNAs, comparable to that of humans, and especially the high number of ruminant specific miRNAs. Strikingly, we found 146 precursors of the ruminant specific family of mir-2284 and mir-2285 miRNAs, which have 206 annotated precursors in cattle and 5 precursors in goat, but none in sheep.

We identified 95 miRNA clusters, miRNA groups closely located in the genome, five of them with at least 5 miRNAs. The biggest one was the known mammal miR-379/miR-656 cluster located in chromosome 18. It harbors 48 miRNAs included in this study, mainly from miRBase, but three novel conserved loci were also found in this location. Interestingly, we found two novel clusters located on chromosome X, with 8 members each, exclusively made up of unannotated conserved miRNAs. One of them contains miRNAs homologs to the cattle bta-mir-6526 and horse eca-mir-8908 families, while the other contains miRNAs homologs to the cattle and goat miRNA families mir-424, mir-450, mir-503 and mir-542. Many of the small clusters (<5 miRNAs) were comprised of multicopy miRNAs, very similar precursors that produce the same mature sequence.

3.4. Exploratory analysis of miRNA expression

In total, 1014 miRNAs were quantified, 98 of which were included in miRBase and 916 were previously unannotated miRNAs. These miRNAs represent 1985 unique mature miRNAs as different pre-miRNAs can produce the same mature miRNA product. Removing lowly and inconsistently expressed miRNAs, a dataset of 1082 mature miRNAs for the analysis of expression was obtained (Supplementary Table S3). Fourteen % (147) of the expressed mature miRNAs were miRBase miRNAs, 53 % (574) were miRNAs with homologs in other species, and 33 % (361) were unannotated novel miRNAs. MiRBase miRNAs consistently showed higher expression levels than unannotated miRNAs, regardless of homology (Fig. 2A). The miRNAs included in miRBase had a mean expression of 8369.21 CPM, while conserved miRNAs and novel miRNAs had a mean expression of 525.66 CPM and 6.36 CPM, respectively. Each tissue expressed nearly 500 miRNAs on average (468.7 ± 25.6 miRNAs), with the highest number in the brain (695) and the lowest number in adipose tissue (173).

The first exploratory analysis showed that tissues were generally well grouped according with their tissue type (Fig. 2B). Samples of the same tissue but from different works often did not group together, which highlights how other variables can affect miRNA expression. Brain samples were clustered into two closely related groups, based on their cerebral cortex and hypothalamus origin. We can observe a similar pattern in the sample correlation matrix (Fig. 2C). Brain, male reproductive tissues and PBMCs showed the most distinct expression patterns.

The distribution of miRNA expression was skewed towards a handful of highly expressed molecules (Fig. 3A). In some tissues, a single miRNA took more than half of the total expression (adipose tissue, intestine, stomach and ureter). In all tissues, the expression levels decline sharply from the eighth more expressed miRNA. The miRNAs oar-mir-143, oar-mir-26a, oar-mir-10a and oar-mir-10b were among the most expressed in most of the tissues, and in many tissues (intestine, gallbladder, lymph node, stomach, oviduct, ureter, lung, spleen and epididymis), miR-143 was the predominant miRNA. Most of the predominantly expressed miRNAs were annotated in miRBase.

3.5. Tissue specificity analysis

To get a picture of the miRNAs expressed in each tissue, we

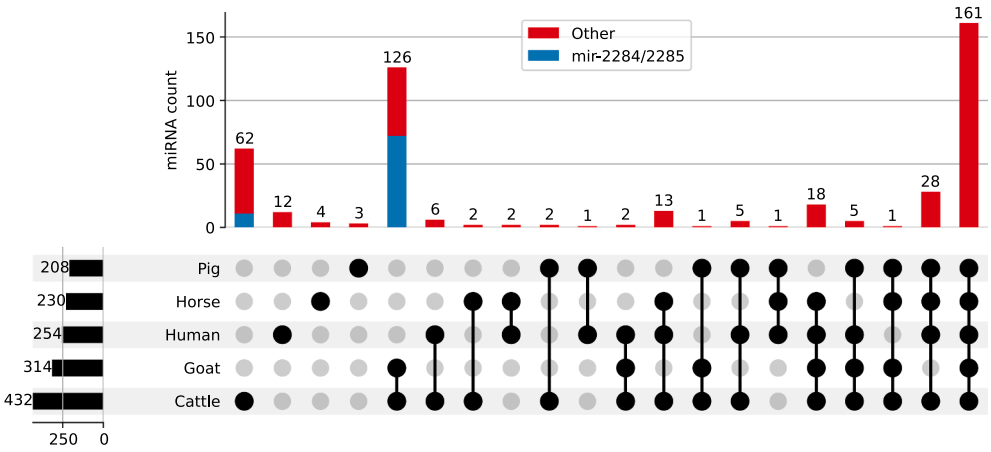

Fig. 1. Comparison between species of miRNA precursor sequences. All the novel precursors found conserved between sheep and at least another species were visualized as an upset plot. Horizontal bars represent the number of sequences with a match in each species. Vertical bars represent the number of sequences common to each intersection. The family of mir-2284/mir-2285 miRNAs was given a different color in the intersection bars to highlight the ruminant specificity.

considered all miRNAs expressed above a threshold of 5 CPM as strongly expressed in that tissue. 733 miRNAs were expressed above this threshold in half of the samples of at least one tissue, (Fig. 3B). A set of miRNAs, containing 89 miRNAs, was expressed in all tissues. However, there were several miRNA strongly expressed exclusively in one tissue. In this analysis, 43 cerebral specific miRNAs, 42 PBMCs specific miRNAs and 37 testis specific miRNAs were detected. Other tissues with many strongly expressed exclusive miRNAs were epididymis (14), mammary gland (10) and gallbladder (9). The aforementioned tissues correspond to those that showed a lower correlation with other tissues.

To identify tissue specific and housekeeping miRNAs in a quantitative manner, a tissue specificity index (TSI) was calculated (Supplementary Table S4). 270 miRNAs could be considered tissue specific with a TSI value higher than 0.9 (Fig. 4A). Of these, 18 were known miRBase sheep miRNAs and 92 miRNAs were conserved in humans, pigs, goats, cows or horses (Fig. 4B). 25 miRNAs were exclusively expressed by a single tissue, most of them novel miRNAs.

Nearly all tissue specific miRNAs showed their highest expression in one of the following tissues: brain (94), testis (54), epididymis (44) and PBMCs (37). It should be noted that there is a set of 46 miRNAs with their highest expression in the brain that are also highly expressed in testis or epididymis, or vice versa. These miRNAs include miRBase miRNAs such as oar-mir-433, oar-mir-1193, oar-mir-758, an orthologue of human hsa-mir-2113 or an ortholog of goat chi-mir-873. Samples were better grouped into tissues by the tissue specific miRNA heatmap (Fig. 4A). Immune system-related tissues (PBMCs, spleen, and lymph nodes) were grouped together, even if there were almost no miRNAs specific to spleen or lymph nodes. This indicates that there is a common miRNA expression profile in relation to immunity.

Housekeeping miRNAs, defined here as miRNAs with a TSI < 0.25, were generally highly expressed and there was not any novel molecule among them. The 47 housekeeping miRNAs include oar-mir-143, oar-mir-26a and oar-mir-10b, present among the top 5 most expressed miRNAs in many tissues, and many members of the let-7 family. One of the only exceptions among the predominant miRNAs was chi-mir-122, which was specifically expressed in liver. Its most expressed mature arm, chi-mir-122-5p, had a TSI of 0.83, and the other arm, chi-mir-122-3p had a TSI of 0.97.

3.6. Ruminant specific mir-2284/mir-2285 family

In this work, we detected 146 miRNA loci expressing precursors belonging to the family of mir-2284/mir-2285 miRNAs. They were identified mainly based on sequence similarity with the over 200 cattle miRBase miRNAs from this family. Due to the high similarity between these miRNAs at precursor and mature miRNA level, exact one-to-one homologies were not given to the novel miRNAs. Instead, they were sequentially named (Supplementary Table S2). Regarding precursor sequences, there were 137 unique mir-2284/2285 family miRNAs, and regarding mature sequences, defined as the most expressed mature product from the same hairpin, there were 108 unique sequences belonging to this family. Thus, several copies of identical or very similar miRNAs are located through the sheep genome.

There were 156 mature miRNAs from the mir-2284/mir-2285 family expressed above the expression threshold. In general, their expression was lower than annotated miRNAs and other conserved miRNAs, but higher than novel miRNAs. In some tissues, their expression was significantly higher than in all tissues (Fig. 5A). This was true for immune related tissues such as PBMCs (Mann-Whitney *U* test, P = 4x10-19) and lymph nodes (Mann-Whitney *U* test, P = 7x10-7), but not for spleen. The difference was also significant in testis (Mann-Whitney *U* test, P = 6x10-8). Those tissues with high mir-2284/mir-2285 expression coincide with some of the most transcriptionally distinct tissues, but, interestingly, in the brain, the most different tissue and a tissue with the most tissue specific miRNAs, the expression of mir-2284/mir-2285 family miRNAs was significantly lower (Mann-Whitney *U* test, P = 1x10-12). Overall, immune-related tissues and testis expressed the highest number of these miRNAs (Fig. 5B). There were 16 tissue-specific miRNAs from this miRNA family, representing the 10 % of expressed mir-2284/mir-2285 miRNAs, but this is lower than the fraction of tissue specific miRNAs from the whole dataset (25 %) (Fig. 5C). Those miRNAs were mainly specific of male reproductive tissues or PBMCs.

Because lowly expressed miRNAs with similar seeds can have an additive effect on target gene repression, the 98 genes that were predicted to be targeted by>10 mature miRNAs of the mir-2284/mir-2285 family were selected as their putative targets. AP3S1 was predicted to be targeted by 34 different miRNAs from this family, much more than any other gene. The GO overrepresentation test revealed that the set of 98 target genes was enriched in processes related to hormonal regulation, regulation of female sex organs and response to external stimuli (Fig. 5D). The most significant term was *regulation of hormone levels* (GO:0010817, FDR = 3.9x10-4), with genes such as AFP, FSHB, VAMP7 or ESR1. Other significant GO terms include *ovulation cycle process* (GO:0022602, FDR = 2.4x10-3), with genes such as AFP, LHCGR or ESR1; and *regulation of response to external stimulus* (GO: 0032101, FDR = 9.7x10-3), with genes such as IFNG, CXCL8 or CD200R1.

4. Discussion

In recent years, thanks to the advances in sequencing technologies,

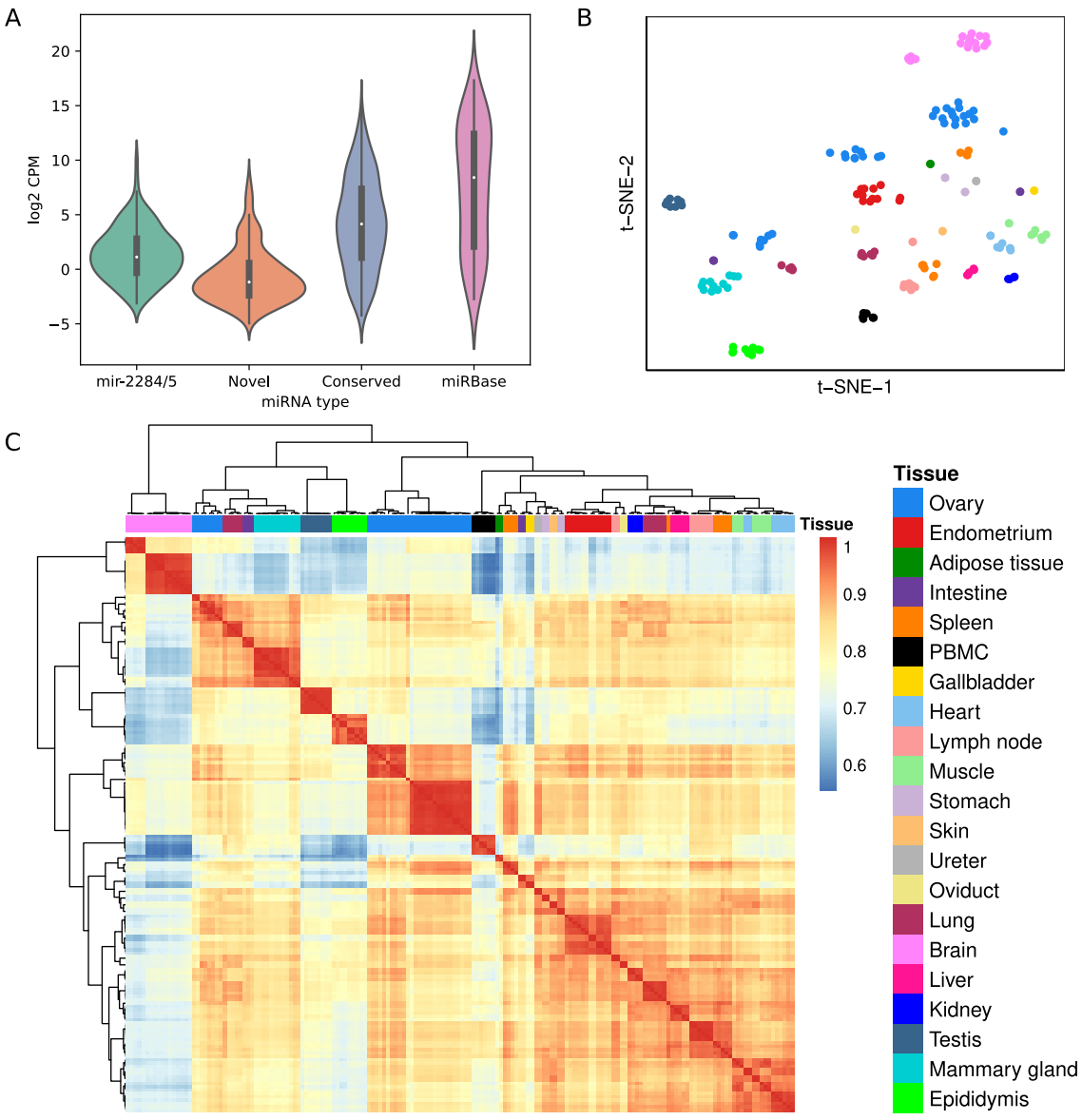

Fig. 2. Exploratory analysis of miRNA expression. (A) Expression levels of all the miRNAs separated in categories based on sequence conservation. Mir-2284/5 family has been represented separately to see its specific pattern. (B) t-SNE plot of all the samples colored by tissue. (C) Correlation heat-map and clustering of all the samples using Pearson correlation. Color legend shared by (B) and (C) subfigures.

many species have been extensively annotated for miRNAs. Among livestock species, nearly 1000 miRNAs have been annotated in cattle, whereas around 300 have been characterized in pig and goat. However, the current state of sheep miRNA annotation in the reference sources lies behind human and other livestock species. While there are 106 precursors in miRBase v.22 database (Kozomara et al., 2019) and 355 in the Ensembl v.104 annotation, we identify 1047 unannotated miRNA gene loci significantly expressed in any of the 172 samples analyzed. Moreover, most of the annotated miRNAs from both sources were detected in this study. 455 of these miRNAs were found to be conserved in another livestock species or in humans, while the remaining were classified as novel. Some of them could be orthologues of human miRNAs that were not defined as such due to the sequence divergence between species.

One of the advantages of this work is that it harmonizes the naming of all reanalyzed studies for an easier comparison between tissues. Other useful resources like the RumimiR database (Bourdon et al., 2019) contain an exhaustive in-depth description of the miRNAs from the literature, but we go further by reanalyzing all the raw data in an uniform way using the latest sheep genome (Oar_rambouillet_v1.0) in Ensembl (release 104). It should be noted that, due to the data being produced by different projects, there is some unavoidable variability, which could be caused by the experimental procedure for sample and RNA extraction, breed, diet, sex or age of the animals (Benítez et al., 2019; Long et al., 2019; Meder et al., 2014; Sielemann et al., 2020). Besides, the data for the tissues with many samples is more reliable than the tissues with few samples, more affected by variability, but, in general, the data seems representative for most tissues. We used the CPM normalization method, which compared to other methods appears to yield better reproducibility between individuals while keeping the distinction between cell types (De Rie et al., 2017).

miRNAs are frequently clustered in the genome, 25 % of human miRNAs are located in clusters and multicopy miRNAs tend to be in the same cluster (Guo et al., 2014). Some interesting clusters were detected in this work. One of them, the miR-379/miR-656 cluster has already been reported in sheep, is conserved across placental mammals and is located in an imprinted region (Pokharel et al., 2020a). Interestingly, two novel big clusters were detected in this study on chromosome X, and one of them is potentially specific to the ruminant or ungulate clades,

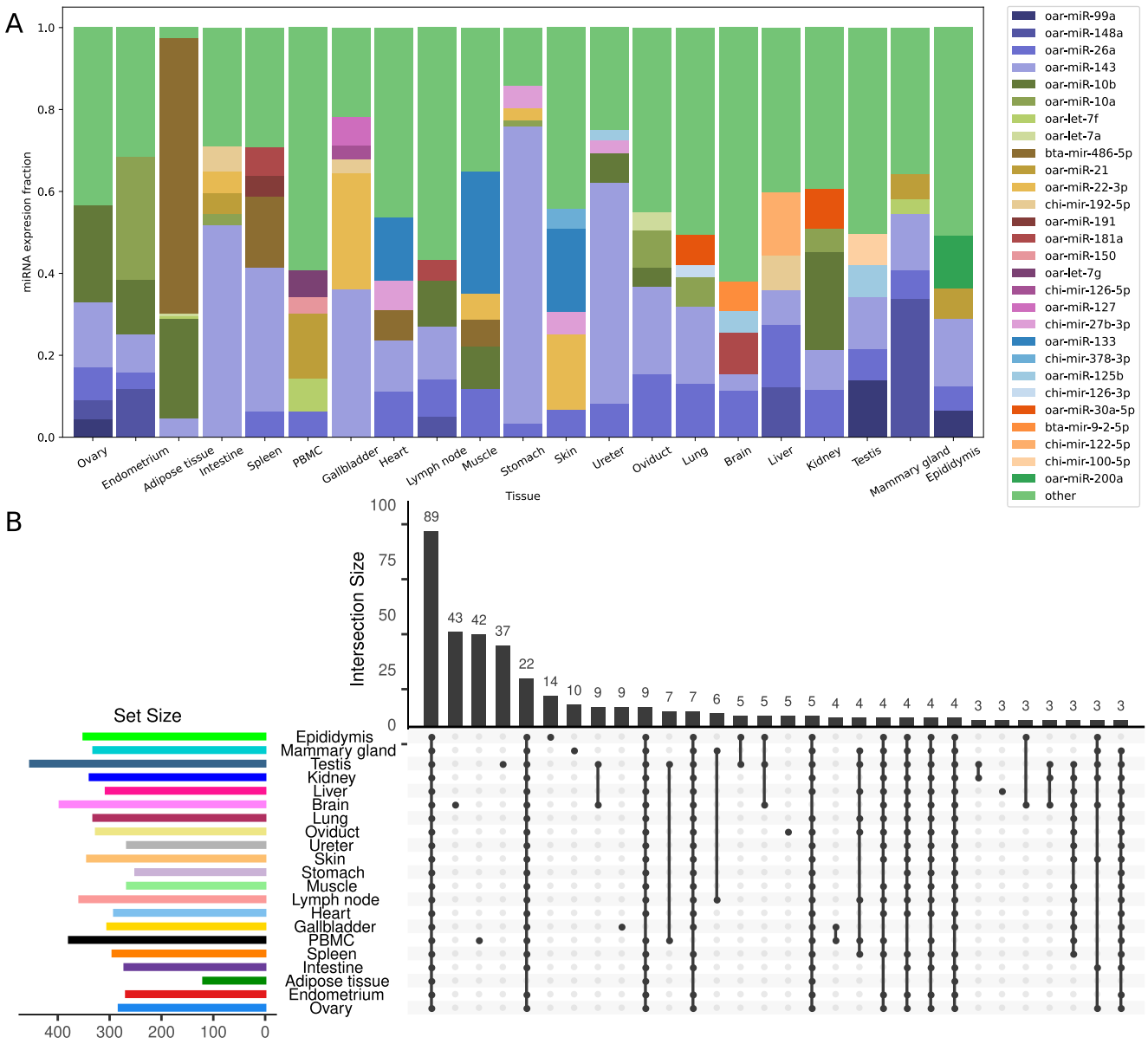

Fig. 3. Distribution of miRNA expression across tissues. (A) Proportion of the five most expressed miRNAs in each tissue as a fraction of total expression, using the mean of all samples in each tissue. (B) Upset plot with the intersection of all the expressed miRNAs above 5 CPM in each tissue. Horizontal bars represent the number of expressed miRNAs in each tissue above the threshold. Intersections with at least three miRNAs are visualized in the vertical bars.

since its members have not been found elsewhere. This redundancy created by polycistronic loci and paralogous loci grants functional robustness to the mammalian miRNAome (Olive et al., 2015). In addition, predominant expression of a miRNA in a tissue, observed here and in the FANTOM5 miRNA atlas (De Rie et al., 2017), also plays a significant role in this functional robustness (Olive et al., 2015). Most tissue-dominant miRNAs such as mir-143, mir-10b, mir-10a or mir-21 have been related to basic functions of cell homeostasis and division, and consequently, to cancer (Peng and Croce, 2016). Still, there also are predominantly expressed miRNAs with roles related to the specific tissue or cell they are expressed in. The clearest example of this is mir-122, specifically expressed and predominant in mammal liver (Bandiera et al., 2015).

In this work, we have identified tissue-specific expression of miRNAs across 21 tissues. Other studies have previously generated miRNA atlases in various mammal species and have identified tissue-specific miRNAs with differing numbers of tissues. For instance, there are works in humans (De Rie et al., 2017; Ludwig et al., 2016), cattle (Sun et al., 2019), giant panda (Wang C et al., 2021), horse (Pacholewska et al., 2016), mice (De Rie et al., 2017; Isakova et al., 2020), rat (Smith et al., 2016) and dog (Koenig et al., 2016). When comparing our dataset to those studies, we found many matching tissue-specific miRNAs, reinforcing the idea that miRNA gene expression of evolutionarily conserved miRNAs is also conserved (Landgraf et al., 2007).

The brain was the tissue that expressed most tissue-specific miRNAs and about half of the conserved sheep brain-specific miRNAs were also found to be specific in other works. Seven of them appeared in, at least, three other mentioned works: mir-129, mir-480, mir-551b, mir-137, mir-383, mir-380 and mir-487b (De Rie et al., 2017; Isakova et al., 2020; Koenig et al., 2016; Ludwig et al., 2016; Pacholewska et al., 2016; Smith et al., 2016; Sun et al., 2019; Wang C et al., 2021). It has been proposed that brain miRNAs are closely related to the mental and behavioral variation during vertebrate evolution, by regulating the complex brain networks (Chen and Qin, 2015). A reproductive tissue such as testis is known to be very transcriptionally complex, with a high number of expressed genes and specific genes, probably due to a more permissive

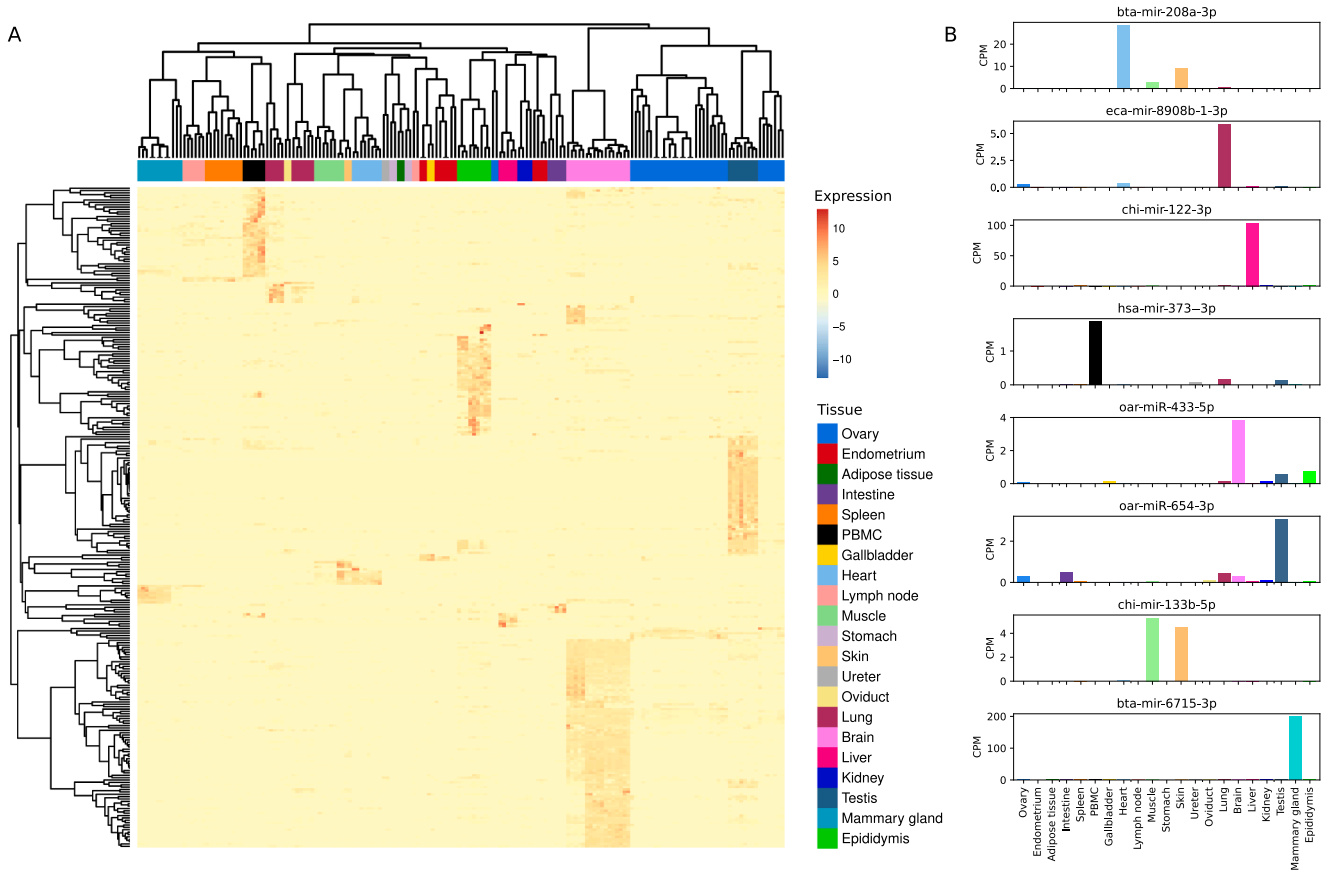

Fig. 4. Tissue-specific miRNA expression. (A) Expression heatmap and sample clustering using all miRNAs with a TSI > 0.9. (B) Expression levels of selected tissue-specific miRNAs in different tissues identified in this study, using the mean of all samples in each tissue.

chromatin (Soumillon et al., 2013). In this dataset, it is the tissue with the highest amount of expressed miRNAs and 54 tissue-specific miRNAs show the highest expression in testis. Out of the 12 known or conserved tissue-specific miRNAs with highest expression in testis, 10 were also supported by other works, including mir-202 and mir-449a (Isakova et al., 2020; Koenig et al., 2016; Ludwig et al., 2016; Smith et al., 2016). miR202 mediates the proliferation, apoptosis, and synthesis function of human Sertoli cells (Yang C et al., 2019) and the mir-449 cluster is essential for spermatogenesis (Wu et al., 2014). Other tissue-specific miRNAs with extensively studied functions and supported by most of the tissue atlases include, for instance, mir-122, mir-133b and mir-208a/mir-208b. mir-122, specifically expressed in liver, is known to be involved in lipid and glucose metabolism (Lynn, 2009). mir-133b, expressed specifically in muscle, has an important role in the differentiation and proliferation of myoblasts (Horak et al., 2016). The family of mir-208 miRNAs are exclusively expressed in cardiac muscle and are encoded in two myosin genes, being responsible for the control myosin content (Horak et al., 2016).

There is a set of highly conserved miRNAs (Bartel, 2018), as well as a great correlation between the miRNA expression profiles of different mammal species (Ludwig et al., 2016). The list of conserved miRNAs has been mostly completed, but there are also clade-specific miRNA families, usually lowly expressed, that could contribute to the phenotypic differences between livestock species (Bartel, 2018; Penso-Dolfin et al., 2018). One of those families is the extensive mir-2284/mir-2285 miRNA family, which was thought to be specific to cattle. This family seems to have evolved by seed shifting and point mutation, has expanded very rapidly and might be related to insulin resistance in ruminants (Bao et al., 2013). Homologs of the mir-2284/mir-2285 family have been previously described by other sheep studies (Bilbao-Arribas et al., 2019; Laganà et al., 2015), but here we report a vast number of members in sheep, comparable to the amount in cattle, thus confirming that the expansion of this miRNA family is, at least, ruminant specific. Functionally, this expansion could have had two outcomes: a progressive subfunctionalization depending on the tissue (Bell et al., 2019), or an additive dosage effect on a restricted number of target genes (Bao et al., 2013). The data from this study suggest the latter, since they are less tissue-specific than other families and many predicted target genes are shared among the paralogues.

As for the biological role of the mir-2284/mir-2285 family miRNAs, in cattle, they are expressed in immune-relevant tissues (Lawless et al., 2014), and show their highest expression in lymph nodes (Bell et al., 2019). Our sheep dataset also follows this trend, as they are highly expressed in PBMCs and lymph nodes. Nevertheless, besides the predicted targeting of immune response and inflammation associated genes, their predicted target genes were also related to hormone regulation and female sex cycle. The main target of this miRNA family was predicted to be AP3S1, which encodes a subunit of the AP3 adaptor complex, involved in intracellular vesicle trafficking. Not only AP3 is involved in the inflammatory response (Mantegazza et al., 2017, 2012; Petnicki-Ocwieja et al., 2015), but also, mice lacking AP3 show dysregulated insulin and other hormone secretion (Sirkis et al., 2013). The specific subunit encoded by AP3S1 seems to play a role in the insulin receptor signaling (VanRenterghem et al., 1998) and variants within AP3S1 have been associated with type 2 diabetes in a Chinese population (Zhou et al., 2010). Considering these results, because vesicle trafficking is important for both, immune cell function and hormone regulation and evolutionary innovations have been important for the development of the unique ruminant digestive system and metabolism (Chen et al., 2019; Clauss et al., 2010; Sasaki, 2002), mir-2284/mir-2285 family miRNAs could have evolved as an adaptation to regulate these processes. The exact phylogenetic origin and functional roles of

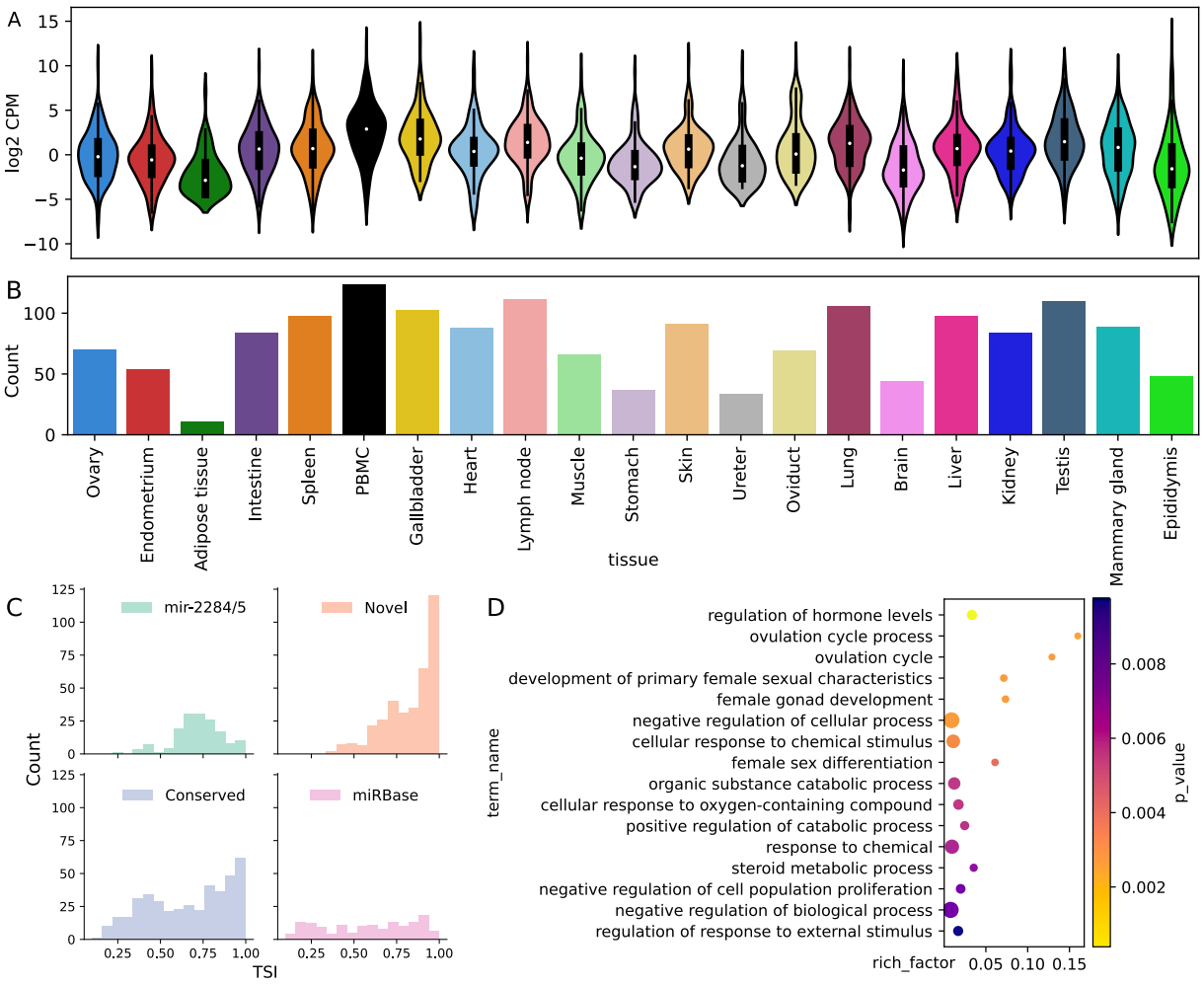

Fig. 5. Expression analysis of miRNAs from the mir-2284/mir-2285 family. (A) Expression levels of all miRNAs from the mir-2284/mir-2285 family in each tissue. (B) Number of expressed miRNAs from the mir-2284/mir-2285 family with mean expression > 1 CPM. Color legend shared by (A) and (B) subfigures. (C) Distribution of TSI values in four miRNA categories based on sequence conservation. Mir-2284/5 family has been represented separately to see its specific pattern. (D) Gene Ontology enrichment results for the set of 98 target genes of the mir-2284/mir-2285 family. P values were corrected with Benjamini-Hochberg FDR.

this family remain to be studied.

5. Conclusions

In this work, we have created an expression atlas of sheep miRNAs by the integration of several small RNA sequencing experiments, including hundreds of previously unannotated and uncharacterized miRNAs. Our analyses support the high conservation of many miRNAs, but also highlight the potential of clade-specific innovations for ruminant evolution, such as the ruminant-specific family of mir-2284/mir-2285. The dataset itself and the analyses regarding expression distribution and specificity of miRNAs should be useful for the field of sheep genomic and veterinary research, as it provides sheep-specific information about the expression of any miRNA in 21 tissues.

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CRediT authorship contribution statement

Martin Bilbao-Arribas: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Aitor Guisasola-Serrano:** Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Endika Varela-Martínez:** Formal analysis, Writing – review & editing. **Begoña M. Jugo:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The code used in this manuscript is available at https://github.com/bilbaom/sheep-miRNAome. Sequences, coordinates and expression of all miRNAs are available as Supplementary tables. The datasets supporting the conclusions of this article have been made publicly available by the original authors and are available in the NCBI SRA repository under the following BioProject accessions: PRJNA451237,

PRJNA354833, PRJEB22101, PRJEB32852, PRJEB32852, PRJNA392421, PRJEB20781, PRJNA505702, PRJNA474913, PRJNA532808, PRJNA511987, PRJNA414087, PRJNA454385, PRJNA528259, PRJNA638028, PRJNA613135, PRJNA608075, PRJNA694531 and PRJNA607580.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2022.146998.

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