Biochemical and Biophysical Research Communications 375 (2008) 484–489

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Concatameric cloning of porcine microRNA molecules after assembly PCR

Soroush Sharbati-Tehrani *, Barbara Kutz-Lohroff, Jutta Scholven, Ralf Einspanier

*Institute of Veterinary-Biochemistry, Freie Universität Berlin, Oertzenweg 19b, 14163 Berlin, Germany*

ARTICLE INFO

*Article history:*
Received 8 August 2008
Available online 21 August 2008

*Keywords:*
microRNA
RNA interference
Cloning
Assembly PCR
miR-Q

ABSTRACT

While the number of human or murine microRNAs (miRNAs) increases continuously, there are limited data available from other species. We report a novel identification method of small RNAs such as miRNAs, which allows simultaneous cloning of five RNA molecules within the same insert. First, RNA molecules <40 nt were polyadenylated and five concatamerising 5′ DNA adaptors were ligated to the molecules in independent reactions. Reverse transcription was carried out using oligo $d(T)_{18}$ primers with concatamerising 5′ overhangs. The introduced complementary termini in the different reactions enabled the subsequent coupling of five purified antisense strands to one molecule by means of an assembly PCR. After cloning, small RNAs were identified by DNA sequencing. By means of this cloning approach, we identified 10 novel and one known porcine miRNAs. Furthermore, the endogenous expression of the cloned miRNAs was quantified in various tissues using a qRT-PCR approach.

© 2008 Elsevier Inc. All rights reserved.

Due to numerous genome sequencing projects, expression of various eukaryotic protein coding genes, their role in development or disease, and their regulation become more and more deciphered. Since phylogenetically distant animals share the majority of protein-coding gene families, morphological complexity in animal evolution implies a sophisticated level of gene regulation [1]. Recently, miRNAs are recognised as major regulators of eukaryotic gene expression. This class of small non-coding RNAs is encoded in the genome of most eukaryotes and it is suggested that up to 90% of human genes may be negatively controlled by miRNAs [2]. Animal miRNAs direct gene regulation by binding to a target site in the 3′ untranslated region (3′ UTR) of mRNAs with incomplete complementarity [3]. The resulting regulatory effect is mainly based on the reduction of translation efficiency, rather than on enhanced mRNA degradation [4]. They are involved in essential developmental processes such as timing, embryogenesis, organogenesis, growth control, and programmed cell death [3]; but they also play a role in human disease such as viral infections [5] and cancer.

Besides their enormous importance in ontogeny or disease recent studies indicate that miRNAs are responsible for morphological complexity during animal evolution. The gain of new miRNA families seems to be directly connected to phylogenetic development of metazoa. For example the let-7 family has been reported to be evolutionary conserved among diverse bilateria but apparently absent in basic metazoan phyla like cnidarians or poriferans [6]. Niwa & Slack [1] have related the acquisition of several miRNA families to the increasing complexity in animal phylogeny. They suggested that the successive gain of different miRNA families has led to the branches of vertebrates and eutheria. Finally, primates share a considerable number of specific miRNAs. These data support the hypothesis that the acquisition of new miRNAs during vertebrate evolution seems to be associated with more sophisticated body-plans and phenotypic variation within related species.

The high level of conservation of miRNAs, conventional sequencing of small RNA libraries and the introduction of new "deep sequencing" methods have led to the identification of miRNAs in selected species. The miRNA database miRBase Release 11.0 [7] comprises miRNA sequences from 21 mammalian species. While 678 human and 472 murine miRNA sequences are known, there are less than 100 identified sequences for more than 60% of the listed species. "Deep sequencing" technologies have been introduced to provide fast miRNA sequence reads [8–10]. Although these technologies are rapidly evolving they are still costly and hence unaffordable for numerous research groups worldwide. Thus, there is still a need for cost effective, convenient and fast cloning methods to obtain sequence data of unknown mature miRNAs from different species.

The aim of this study was to introduce a new cloning approach for small RNA molecules, which allows the simultaneous sequencing of up to five small RNAs. The method was used to create a porcine miRNA library.

## Materials and methods

*Isolation of small RNA*. Total RNA was isolated from different porcine tissues using the mirVana miRNA Isolation Kit (Ambion, Darmstadt, Germany), according to the manufacturers protocol. The porcine samples included the jejunum, ileum, spleen and

* Corresponding author. Fax: +49 3083862584.
*E-mail address:* sharbati@zedat.fu-berlin.de (S. Sharbati-Tehrani).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.08.048

kidney of ten 31-day-old piglets (EUROC x Pietrain). The RNA quality and quantity of all samples were proven using the Agilent 2100 Bioanalyzer and the RNA Nano Chips (Agilent, Waldbronn, Germany) and the Nanodrop 1000 Spectrophotometer (Thermo Scientific, MA, USA). Afterwards, small RNAs below 40 nucleotides were isolated from 10 μg total RNA using the flashPAGE fractionator and pre-cast gels (Ambion) following the provided protocol.

*Generation of concatamerising termini*. The purified small RNA was polyadenylated in a total volume of 40 μl using 2 U of the *Escherichia coli* Poly (A) Polymerase (Ambion), 5× E-PAP buffer, 2.5 mM $MnCl_2$ and 1 mM ATP. The reaction was performed for 2 h at 37 °C. Poly (A) tailed small RNA was recovered by phenol/chloroform extraction followed by ethanol precipitation and the RNA was dissolved in water. The recovered RNA was used for ligation to five different concatamerising 5′ DNA adaptors. For this purpose, 5 μl of poly (A) tailed small RNA was used in five parallel reactions each containing 25 pmol of the concatamerising 5′ DNA adaptors (for combinations see Table 1), 10 U $T_4$ RNA Ligase (New England Biolabs, Frankfurt am Main, Germany), 2 μg BSA, 1 mM ATP and 10× $T_4$ RNA Ligase buffer in a 20 μl final volume. The reaction was carried out at 37 °C for 2 h followed by an inactivation step at 70 °C for 10 min. The chimeric DNA–RNA molecules were recovered by phenol/chloroform extraction followed by ethanol precipitation and dissolved in 8 μl water. Each of the five recovered chimera pools was reverse transcribed using 50 pmol of five different anchored oligo $d(T)_{18}$ primers with concatamerising 5′ termini (Table 1), the RevertAid™ M-MuLV Reverse Transcriptase (Fermentas GmbH, St. Leon-Roth, Germany), 1 mM dNTPs and 5× RT buffer in 20 μl final volume. The reaction was carried out at 42 °C for 1 h followed by an inactivation step at 70 °C for 10 min. Six microliters of the cDNA was employed as template for initial independently PCR amplifications using the related biotinylated concatamerising 5′ DNA adaptors as well as the anchored oligo $d(T)_{18}$ primers with concatamerising 5′ termini (Table 1). The reactions were performed in 50 μl final volume using 400 nM of each reaction specific oligonucleotide, 1.25 U Accusure DNA Polymerase (Bioline, Luckenwalde, Germany), 1 mM $MgCl_2$, 0.2 mM dNTPs and 10× buffer. The amplification was started at 95 °C for 8 min, followed by 20 cycles with 30 s at 95 °C, 15 s at 57 °C, and 30 s at 68 °C followed by 2 min at 68 °C.

*Strand selection, assembly PCR, cloning and sequencing*. For the final assembly PCR, antisense strands of the five resulting amplicons were collected using streptavidin coated magnetic beads (Dynabeads M-270 Streptavidin; Invitrogen GmbH, Karlsruhe, Germany). For this purpose, 125 μl dynabeads were washed twice in 2× B&W buffer (10 mM Tris–HCl, pH 7.5; 1 mM EDTA; 2.0 M NaCl), resuspended in 250 μl 2× B&W buffer and aliquoted in 50 μl volumes. Fifty microliters of each of the five initial PCRs was added to one aliquot of the beads and incubated at room temperature for 15 min with gentle shaking. Beads were washed twice with 1× B&W buffer and were resuspended in 40 μl of water. Bead associated amplicons were denatured at 95 °C for 4 min with gentle agitation and were immediately placed on ice. The biotinylated sense strands were captured using a magnetic stand while the supernatants containing the liberated antisense single-strands were recovered for further processing.

The terminal concatamerisation of the isolated antisense strands was performed by two successive steps of an assembly PCR. Therefore, about 20 ng of each of the five collected antisense strands were introduced to the assembly PCR using 1.25 U Accusure DNA Polymerase, 1 mM $MgCl_2$, 0.2 mM dNTPs and 10× buffer in 25 μl final volume. The amplification was started at 95 °C for 8 min, followed by 10 cycles with 30 s at 95 °C, 15 s at 57 °C, and 30 s at 68 °C followed by 2 min at 68 °C. Then, the volume was brought up to 50 μl using 20 pmol of each terminal cloning primer (Cloning fw & Cloning rev, Table 1), 1.5 U Accusure DNA Polymerase, 1 mM $MgCl_2$, 0.2 mM dNTPs and 10× buffer. The amplification was initiated by a first step at 95 °C for 8 min, followed by 25 cycles with 30 s at 95 °C, 15 s at 60 °C, and 30 s at 68 °C followed by 2 min at 68 °C.

The resulting PCR product was separated by 2% agarose gel electrophoresis. The amplicon with the expected size (about 300 bp) was cut out and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Subsequently, the product was cloned using the CloneJET™ PCR Cloning Kit (Fermentas) following the recommendations of the supplier. *Escherichia coli* DH5α were transformed as described earlier [11] and positive clones were picked and grown overnight. Recombinant plasmids were isolated using the NucleoSpin Plasmid (Macherey-Nagel, Düren, Germany) and screened for the right insert by restriction analysis using the enzyme HindIII (Fermentas). Recombinant plasmids containing inserts of about 300 bp were chosen for further analysis. Both strands of the inserts were sequenced using the primers "adv pJET1 fw" and adv "pJET1 rev"

**Table 1**
DNA adaptors and oligo $d(T)_{18}$ molecules used in this work

| | Sequence (5′ to 3′) |
|---|---|
| *Pool #1* | |
| Concatamerising 5′ adaptor: Ad1/3′ | 5′-Biotin-CGA TCT GAT AGC CCA ACT CG-3′ |
| Concatamerising anchored oligo $d(T)_{18}$: Ad1/5′ | 5′-CAC AAC GGT GTG TTC CCT CTT TTT TTT TTT TTT TTT TTN-3′ |
| *Pool #2* | |
| Concatamerising 5′ adaptor: Ad2/3′ | 5′-Biotin-ACT GAG CCT GGT TGA TGG TC-3′ |
| Concatamerising anchored oligo $d(T)_{18}$: Ad2/5′ | 5′-GGT GTA TCT CAT GCG GGT CTT TTT TTT TTT TTT TTT TTN-3′ |
| *Pool #3* | |
| Concatamerising 5′ adaptor: Ad3/3′ | 5′-Biotin-CTG ATA GCC CAA CTC CAA GG-3′ |
| Concatamerising anchored oligo $d(T)_{18}$: Ad3/5′ | 5′-GGA GGT GGT GTA GGA GAA GTT TTT TTT TTT TTT TTT TTN-3′ |
| *Pool #4* | |
| Concatamerising 5′ adaptor: rcAd1/3′ | 5′-Biotin-CGA GTT GGG CTA TCA GAT CG-3′ |
| Concatamerising anchored oligo $d(T)_{18}$: rcAd2/5′ | 5′-AGA CCC GCA TGA GAT ACA CCT TTT TTT TTT TTT TTT TTN-3′ |
| *Pool #5* | |
| Concatamerising 5′ adaptor: rcAd2/3′ | 5′-Biotin-GAC CAT CAA CCA GGC TCA GT-3′ |
| Concatamerising anchored oligo $d(T)_{18}$: rcAd3/5′ | 5′-ACT TCT CCT ACA CCA CCT CCT TTT TTT TTT TTT TTT TTN-3′ |
| *Cloning primers* | |
| Cloning fw | 5′-GTA CAA GCT TCA CAA CGG TGT GTT CCC TCT-3′ |
| Cloning rev | 5′-GTA CAA GCT TCT GAT AGC CCA ACT CCA AGG-3′ |
| *Sequencing primers* | |
| adv pJET1 fw | 5′-TCA ACT GCT TTA ACA CTT GTG C-3′ |
| adv pJET1 rev | 5′-CAG CCT GAA AAT CTT GAG AGA A-3′ |

**Table 2**
Oligonucleotides for the relating miR-Q assays

| | Sequence (5′ to 3′) |
|---|---|
| *miR-Q miR-21* | |
| RT6-miR-21 | 5′-TGT CAG GCA ACC GTA TTC ACC GTG AGT GGT TCA ACA-3′ |
| short-miR-21-rev | 5′-CGT CAG ATG TCC GAG TAG AGG GGG AAC GGC GTA GCT TAT CAG ACT GA-3′ |
| *miR-Q miR-24* | |
| RT6-miR-24 | 5′-TGT CAG GCA ACC GTA TTC ACC GTG AGT GGT CTG TTC-3′ |
| short-miR-24-rev | 5′-CGT CAG ATG TCC GAG TAG AGG GGG AAC GGC GTG GCT CAG TTC AGC AG-3′ |
| *miR-Q miR-143* | |
| RT6-miR-143 | 5′-TGT CAG GCA ACC GTA TTC ACC GTG AGT GGT TGA GCT-3′ |
| short-miR-143-rev | 5′-CGT CAG ATG TCC GAG TAG AGG GGG AAC GGC GTG AGA TGA AGC ACT GT-3′ |
| *miR-Q ssc-miR-181a* | |
| RT6-ssc-miR-181a | 5′-TGT CAG GCA ACC GTA TTC ACC GTG AGT GGT CAC CGA-3′ |
| short-ssc-miR-181a-rev | 5′-CGT CAG ATG TCC GAG TAG AGG GGG AAC GGC GAA CAT TCA ACG CTG-3′ |
| *miR-Q miR-326* | |
| RT6-miR-326 | 5′-TGT CAG GCA ACC GTA TTC ACC GTG AGT GGT CTG GAG-3′ |
| short-miR-326-rev | 5′-CGT CAG ATG TCC GAG TAG AGG GGG AAC GGC GCC TCT GGG CCC TTC-3′ |
| *miR-Q ssc-miR-423* | |
| RT6-ssc-miR-423 | 5′-TGT CAG GCA ACC GTA TTC ACC GTG AGT GGT TGA GGG-3′ |
| short-ssc-miR-423-rev | 5′-CGT CAG ATG TCC GAG TAG AGG GGG AAC GGC GAG CTC GGT CTG AGG C-3′ |
| *miR-Q miR-451* | |
| RT6-miR-451 | 5′-TGT CAG GCA ACC GTA TTC ACC GTG AGT GGT AAA CTC-3′ |
| adv-short-miR-451-rev | 5′-CGT CAG ATG TCC GAG TAG AGG GGG AAC GGC GAA ACC GTT ACC ATT ACT GAG-3′ |
| *miR-Q ssc-miR-484* | |
| RT6-ssc-miR-484 | 5′-TGT CAG GCA ACC GTA TTC ACC GTG AGT GGT ATC GGG-3′ |
| short-ssc-miR-484-rev | 5′-CGT CAG ATG TCC GAG TAG AGG GGG AAC GGC GTC AGG CTC AGT CCC CT-3′ |

(Table 1) with the Prism Big Dye™ FS Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Darmstadt, Germany).

*Sequence analysis*. Sequence data was analysed using the Vector NTI 10.3.0 package (Invitrogen). Elimination of cloned RNA species other than miRNAs and degradation products was performed by blastn analysis (http://blast.ncbi.nlm.nih.gov/). Small RNA sequences ⩾18 nt molecules were subjected to blastn analysis against known miRNAs using the miRBase [7] search option (http://microrna.sanger.ac.uk/sequences/search.shtml).

*Quantification of miRNA expression by means of miR-Q.* The expression profiling of the cloned miRNAs was carried out by means of a miRNA quantitative reverse transcription PCR (qRT-PCR) called miR-Q [12]. Absolute quantification was performed as described earlier [12] using the oligonucleotides in Table 2. miRNA expression profiling was performed by triplicate measurements for each sample, compared with a calibration curve established by reverse transcription of serially diluted amounts of the particular synthetic miRNA in the presence of 50 ng bacterial total RNA. The RT reaction of non-spiked bacterial total RNA samples and no template controls were used as negative controls. All reactions were run in the StepOnePlus™ Real-Time PCR System (Applied Biosystems).

## Results and discussion

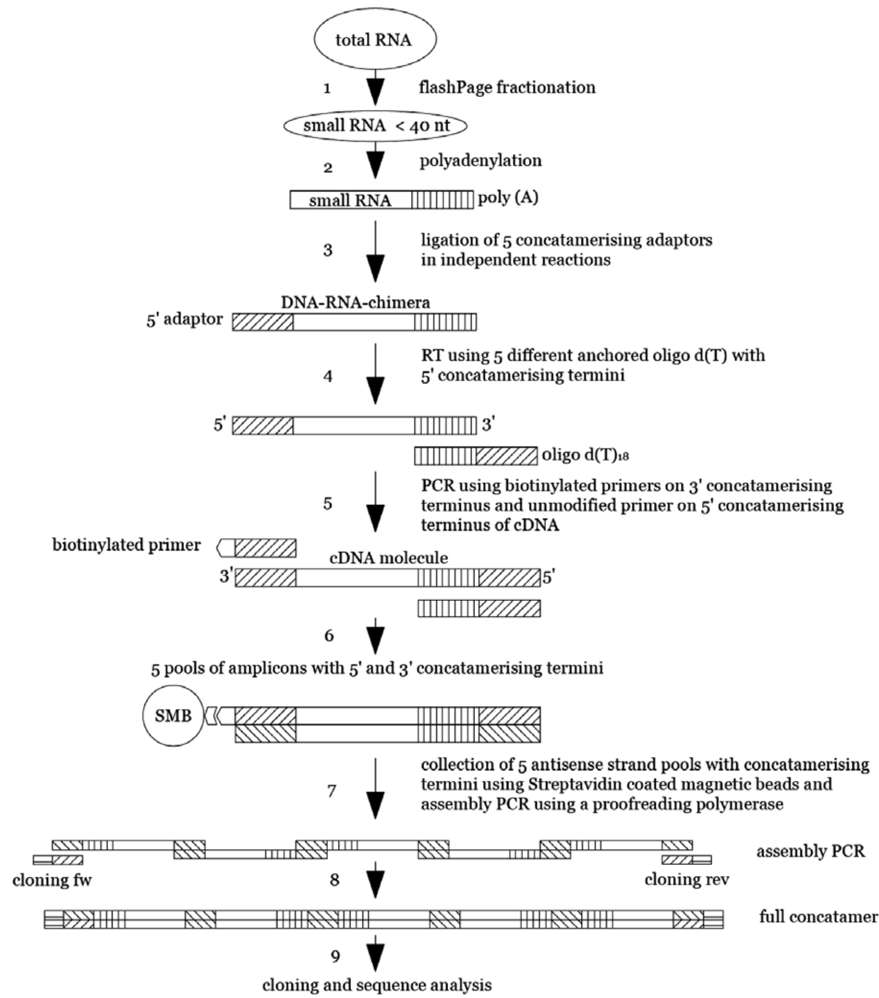
In recent years thousands of regulating miRNAs from selected species were identified but many need to be discovered e.g. in further species used as animal models or in farm animals. Although the miRBase miRNA sequence database Release 11 [7] comprises 6396 entries, the distribution of known miRNAs among different species is highly divergent. In contrast to 700 verified human miRNAs, only 55 porcine miRNAs have been deposited in the miRBase and 19 more have been recently reported elsewhere [13].

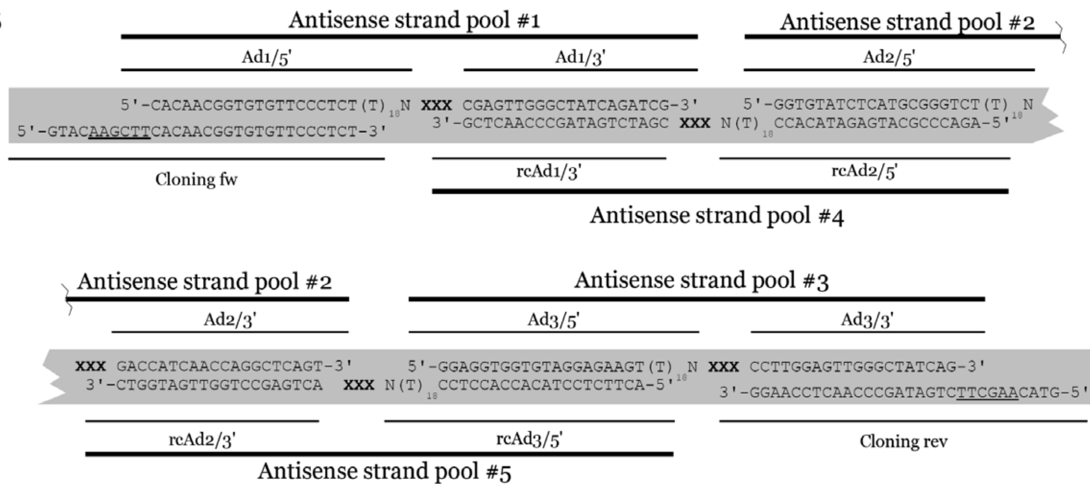
Therefore, a novel strategy for cloning of small sized RNA molecules below 40 nt from distinct sources has been developed here. This method was applied to clone porcine miRNAs from different tissues contributing to a significantly improved porcine miRNA library. The proposed cloning procedure presents an advanced approach based on polyadenylation, 5′ adaptor ligation and reverse transcription [14]. The use of introduced complementary termini allows the concatamerisation of five molecules each of which harbouring a small RNA molecule. The novel combination of adaptor ligation, reverse transcription followed by the assembly PCR allows the cloning of up to five different small RNA molecules within the same insert. A detailed step-by-step scheme of the strategy is given in Fig. 1A. In brief, RNA molecules smaller than 40 nt were isolated and subsequently polyadenylated. After purification, five different 5′ DNA adaptors were ligated to the molecules in independent reactions enabling the following concatamerisation. Subsequently, RT reactions were carried out using anchored oligo $d(T)_{18}$ primers with concatamerising 5′ termini. Amplification of cDNA pools was performed using a proofreading polymerase and biotinylated primers (Table 1) in independent PCRs. Afterwards, the antisense strands were released and recovered using streptavidin coated

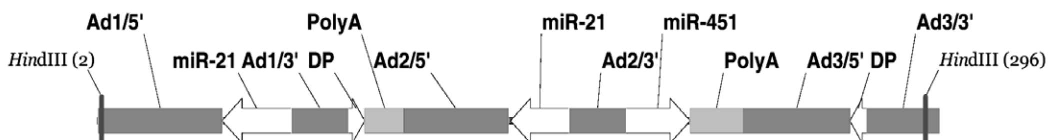
→

**Fig. 1.** Step-by-step schematic description of the assembly PCR and cloning strategy. (A) Small RNAs were polyadenylated (vertically hatched bars) and five different 5′ DNA adaptors (diagonally hatched bars) were ligated to the molecules in independent reactions. After RT reactions using oligo $d(T)_{18}$ primers with 5′ termini (diagonally hatched bars) and subsequent independent PCRs, the antisense strands were purified and collected using Streptavidin coated beads. The introduced complementary 5′ DNA adaptors and 5′ termini of the oligo $d(T)_{18}$ primers in the different reactions enabled the subsequent concatamerisation of the five purified antisense strands to one molecule in the following assembly PCR. Horizontally hatched bars indicate the introduced HindIII sites. It has to be considered that the steps 3–7 are shown only for one pool of molecules as an example but effectively five independent reactions are performed in parallel. Abbreviations: nt; nucleotide, RT; reverse transcription, SMB; streptavidin coated magnetic beads. (B) Configuration of the purified antisense strands within the assembly PCR. The complementary termini are indicated by thin bars, while generated and recovered antisense strands are indicated by bold bars. Each of the antisense molecules harbours one small RNA molecule, which is indicated by "**XXX**". The terminal primers Cloning fw and rev were applied for amplification of the fully assembled product and to introduce HindIII restriction sites (underlined letters). (C) Sequenced insert of the clone pMPg12 with a size of 300 bp. The exemplified full concatameric insert harbours ssc-miR-21 twice and ssc-miR-451. The other two small RNAs were below 18 nt and were regarded as degradation products (DP).

magnetic beads. The generated antisense strand pools #1–#5 (Fig. 1B) comprised complementary termini that were introduced by the 5′ DNA adaptors and the 5′ overhangs of the oligo $d(T)_{18}$ primers. These concatamerising termini enabled the consequent assembly of the five purified antisense strands to one molecule in the following assembly PCR without using a ligase (Fig. 1B). This



| miRNA | Sequence | Tissue and accession no. |
| --- | --- | --- |
| | 1 … 22 | |
| hsa-let-7f | UGAGGUAGUAGAUUGUAUAGUU | |
| mmu-let-7f | UGAGGUAGUAGAUUGUAUAGU- | kidney |
| cloned ssc-let-7f | UGAGGUAGUAGAUUGUAUAGUC | AM920653 |
| | 1 … 22 | |
| hsa-miR-21 | UAGCUUAUCAGACUGAUGUUGA | |
| mmu-miR-21 | UAGCUUAUCAGACUGAUGUUGA | |
| ssc-miR-21 | UAGCUUAUCAGACUGAUGUUGA | spleen |
| cloned ssc-miR-21 | UAGCUUAUCAGACUGAUGUUG- | AM777928 |
| | 1 … 22 | |
| hsa-miR-24 | UGGCUCAGUUCAGCAGGAACAG | |
| mmu-miR-24 | UGGCUCAGUUCAGCAGGAACAG | kidney, spleen |
| cloned ssc-miR-24 | -GGCUCAGUUCAGCAGGAACAG | AM920655 |
| | 1 … 22 | |
| hsa-miR-143 | UGAGAUGAAGCACUGUAGCUCA | |
| mmu-miR-143 | UGAGAUGAAGCACUGUAGCUCA | spleen |
| cloned ssc-miR-143 | UGAGAUGAAGCACUGUAGCUCU | AM920654 |
| | 1 … 23 | |
| hsa-miR-181a | AACAUUCAACGCUGUCGGUGAGU | |
| mmu-miR-181a | AACAUUCAACGCUGUCGGUGAGU | kidney |
| cloned ssc-miR-181a | AACAUUCAACGCUGUCGGUGA-- | AM777927 |
| | 1 … 25 | |
| mmu-miR-292-3p | --AAGUGCCGCCAGGUUUUGAGUGU | |
| rno-miR-292-3p | --AAGUGCCGCCAGGUUUUGAGUGU | spleen |
| cloned ssc-miR-292-3p | GGUGUUUCCGCCCGGUUUCGA---- | AM777929 |
| | 1 … 21 | |
| hsa-miR-326 | CCUCUGGGCCCUUCCUCCAG- | |
| mmu-miR-326 | CCUCUGGGCCCUUCCUCCAGU | jejunum |
| cloned ssc-miR-326 | CCUCUGGGCCCUUCCUCCAG- | AM777930 |
| | 1 … 23 | |
| hsa-miR-423-3p | AGCUCGGUCUGAGGCCCCUCAGU | |
| mmu-miR-423-3p | AGCUCGGUCUGAGGCCCCUCAGU | jejunum, kidney, spleen |
| cloned ssc-miR-423-3p | AGCUCGGUCUGAGGCCCCUCA-- | AM777932 |
| | 1 … 23 | |
| hsa-miR-451 | AAACCGUUACCAUUACUGAGUU- | |
| mmu-miR-451 | AAACCGUUACCAUUACUGAGUU- | kidney, spleen |
| cloned ssc-miR-451 | AAACCGUUACCAUUACUGAGUUU | AM777934 |
| | 1 … 22 | |
| hsa-miR-484 | UCAGGCUCAGUCCCCUCCCGAU | |
| mmu-miR-484 | UCAGGCUCAGUCCCCUCCCGAU | jejunum, ileum |
| cloned ssc-miR-484 | -CAGGCUCAGUCCCCUCCCGAU | AM920656 |
| | 1 … 27 | |
| hsa-miR-1826 | AUUGAUCAUCGACACUUCGAACGCAAU | spleen |
| clonedssc-miR-1826 | AUUGAUCAUCGACACUUCGAACGCACU | FM163471 |

**Fig. 2.** Alignment of novel porcine miRNAs with known orthologues. The cloned porcine miRNAs were aligned with their human or murine orthologues, showing high similarity. The GenBank accession numbers and the employed tissues for cloning are indicated in the right columns.

approach was deduced from a previously published method [15] for synthesis of long DNA sequences without the use of a DNA ligase. While the reported approach was based on using synthetic oligonucleotides, the advancement of our proposed method relies on generating chimera harbouring unknown sequences derived from the ligated and reverse transcribed small RNA molecules. As shown in Fig. 1A and B, the complementary nature of the introduced termini enabled the mutual priming of the obtained five single stranded molecules. This in turn led to successive assembly and amplification of the single strands to one amplicon possessing a size of about 300 bp finally used for cloning. The obtained product not only entails the advantage over existing methods [14] to harbour several small RNA molecules below 40 nt (Fig. 1C) but also provides a convenient size for cloning. Furthermore, the initial size extraction leads to a higher cloning efficiency and preferential cloning of mature small RNA molecules. The entire PCRs were performed using a proofreading polymerase possessing high fidelity and avoiding mismatches. The application of the terminal cloning primers (Table 1) not only promoted the selected amplification of full concatamers but also provided two HindIII sites (Fig. 1B and C) for restriction analysis of clones.

By means of this cloning approach a library for small RNAs was constructed using RNA samples from porcine intestinal tissues, kidney and spleen. After restriction analysis of 57 clones, candidates that showed the expected insert size were selected for sequencing. Sequencing revealed 30 clones (corresponding to 52.6%) with fully assembled concatamers including porcine small RNA sequences. Cloned RNA molecules below 18 nt were regarded as degradation products and excluded from any further analysis. The remaining sequences were subjected to blastn analysis at http://blast.ncbi.nlm.nih.gov/. After eliminating mRNA, rRNA, snoRNA or tRNA sequences, the remaining RNAs were subjected to a blastn analysis against known miRNAs using the miRBase search option. This analysis revealed 11 miRNA sequences, which were highly similar to sequences from human or mouse orthologues. Only one of those (ssc-miR-21) was reported earlier for the pig, hence the remaining 10 were regarded as novel porcine miRNAs. Consequently, these new miRNAs were named following the established human or mouse nomenclature (Fig. 2). Ten out of 11 of the new porcine miRNAs (ssc-let-7f, ssc-miR-21, ssc-miR-24, ssc-miR-143, ssc-miR-181a, ssc-miR-326, ssc-miR-423-3p, ssc-miR-451, ssc-miR-484 and ssc-miR-1826) differed from human or murine sequences in one or two nucleotides at the 5′- or 3′ termini (Fig. 2). The remaining core sequences corresponded 100% with the human and murine orthologues with only one exception (ssc-miR-292-3p), which showed 61.9% identity to the relating murine orthologue. All of the nucleotide sequences were submitted to the EMBL nucleotide database and the accession numbers are indicated in Fig. 2. Some of the identified miRNAs were frequently found in different tissues. The total number of cloned verified miRNA molecules amounted to 26. After this analysis 12 additional sequences of sizes ranging from 18 to 24 nt showed no similarity to any known molecule within the miRBase. Blasting of these sequences against the NCBI porcine HTGS database revealed all molecules to be of porcine origin and chromosomal loci could be identified (data not shown).

MiRNAs show diverse expression patterns, some show time- or tissue-dependent expression and others are expressed in various tissues. To verify the expression of the novel porcine miRNAs, their expression was studied in all employed tissues by means of the miRNA qRT-PCR approach miR-Q [12]. Using this method, expression of eight novel porcine miRNAs was confirmed and quantified among different tissues. While ssc-miR-21, ssc-miR-24, ssc-miR-181a, ssc-miR-326, ssc-miR-423-3p and ssc-miR-484 were expressed throughout all analysed tissues, ssc-miR-143 was only expressed in the intestine and spleen and ssc-miR-451 was only detected in spleen and kidney, respectively. Expression levels of miRNAs ranged from approximately 20 fM to more than 40,000 fM depending on the miRNA and the studied tissue (Fig. 3). Interestingly, there was a direct correlation between cloning frequency of miRNAs and their expression levels. For example ssc-miR-24, ssc-miR-484 and ssc-miR-451 showed high expression levels in kidney and spleen and were predominantly cloned from those tissues. The expression of ssc-miR-484 in intestinal tissues could also be connected to the cloning frequency. The expression analysis of ssc-let-7f, ssc-miR-292-3p and ssc-miR-1826 failed probably due to the detection limit of the corresponding miR-Q assays.

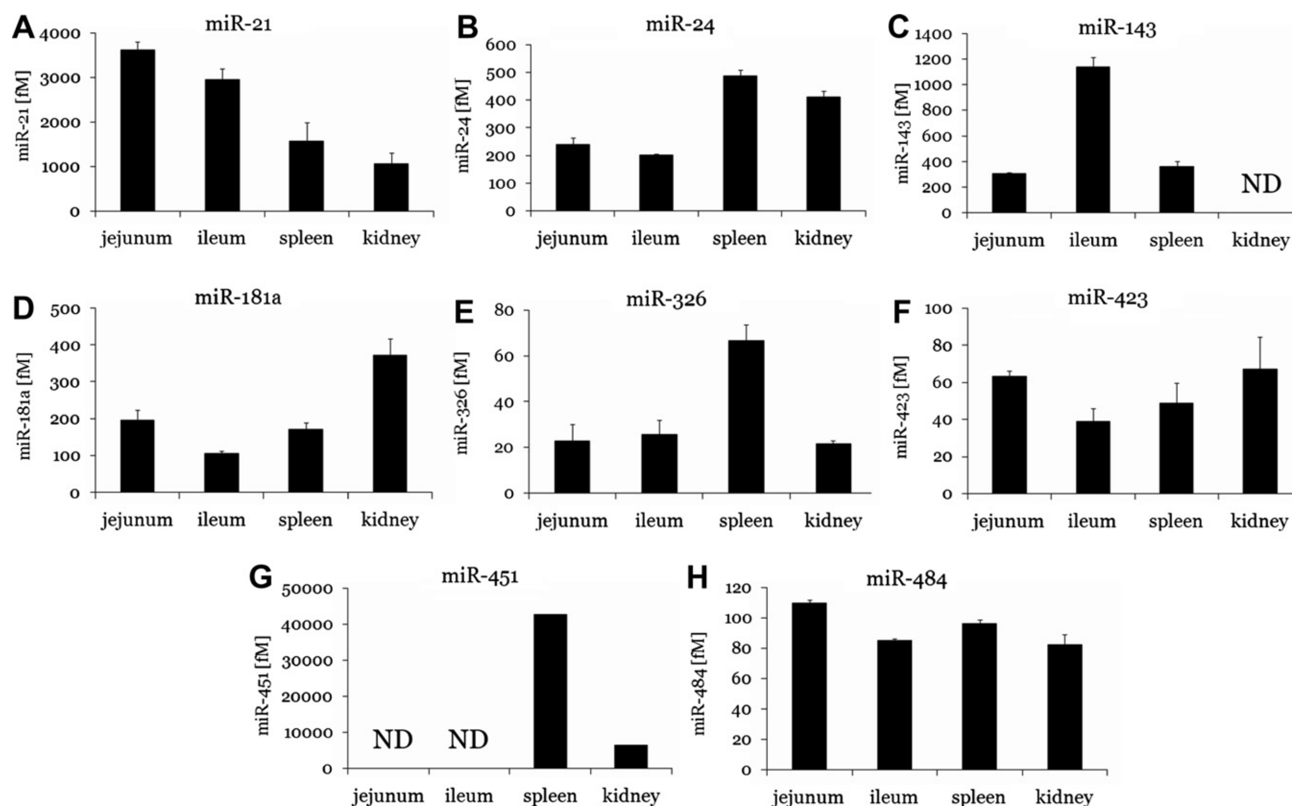



**Fig. 3.** Expression profiling of the novel porcine miRNAs by means of the miR-Q approach. The miRNAs ssc-miR-21 (A), ssc-miR-24 (B), ssc-miR-181a (D), ssc-miR-326 (E), ssc-miR-423-3p (F), and ssc-miR-484 (H) were expressed throughout all analysed tissues, while ssc-miR-143 (C) was only expressed in the intestine and spleen and ssc-miR-451 (G) was only detected in the spleen and kidney samples, respectively. Each column represents the mean (±SD) of three measurements. Abbreviations: ND: not detectable.

In summary, 26 verified miRNA molecules were cloned from different porcine tissues. It can be concluded that 17.3% of all cloned RNA molecules within the 30 analysed clones with fully assembled concatamers represented miRNAs. The calculated percentage might be underestimated taking into account that some of the 12 cloned molecules without similarity to any known miRNAs would represent novel miRNAs. The reported cloning approach represents a convenient and affordable tool in addition to high throughput methods for identification of small RNA molecules. Beside the proven power of this method to discover novel porcine miRNAs it may contribute to identification of any single stranded small RNA such as siRNAs or piRNAs.

## Acknowledgments

The study was funded by the ‘‘Forschungskommission” of the Freie Universität Berlin (Fonds 02235). Tissue Samples were donated by project DFG-Ei296/12-2.

## References

[1] R. Niwa, F.J. Slack, The evolution of animal microRNA function, Curr. Opin. Genet. Dev. 17 (2007) 145–150.
[2] M.P. Perron, P. Provost, Protein interactions and complexes in human microRNA biogenesis and function, Front. Biosci. 13 (2008) 2537–2547.
[3] W.P. Kloosterman, R.H. Plasterk, The diverse functions of microRNAs in animal development and disease, Dev. Cell 11 (2006) 441–450.
[4] R.J. Jackson, N. Standart, How do microRNAs regulate gene expression?, Sci STKE 2007 (2007) re1.
[5] V. Scaria, M. Hariharan, B. Pillai, S. Maiti, S.K. Brahmachari, Host-virus genome interactions: macro roles for microRNAs, Cell. Microbiol. 9 (2007) 2784–2794.
[6] A.E. Pasquinelli, A. McCoy, E. Jimenez, E. Salo, G. Ruvkun, M.Q. Martindale, J. Baguna, Expression of the 22 nucleotide let-7 heterochronic RNA throughout the Metazoa: a role in life history evolution?, Evol Dev. 5 (2003) 372–378.
[7] S. Griffiths-Jones, H.K. Saini, S. van Dongen, A.J. Enright, miRBase: tools for microRNA genomics, Nucleic Acids Res. 36 (2008) D154–D158.
[8] J. Burnside, M. Ouyang, A. Anderson, E. Bernberg, C. Lu, B.C. Meyers, P.J. Green, M. Markis, G.K. Isaacs, E. Huang, R.W. Morgan, Deep Sequencing of Chicken microRNAs, BMC Genomics 9 (2008) 185.
[9] M.R. Friedlander, W. Chen, C. Adamidi, J. Maaskola, R. Einspanier, S. Knespel, N. Rajewsky, Discovering microRNAs from deep sequencing data using miRDeep, Nat. Biotechnol. 26 (2008) 407–415.
[10] J. Lu, Y. Shen, Q. Wu, S. Kumar, B. He, S. Shi, R.W. Carthew, S.M. Wang, C.I. Wu, The birth and death of microRNA genes in *Drosophila*, Nat. Genet. 40 (2008) 351–355.
[11] S. Sharbati-Tehrani, B. Meister, B. Appel, A. Lewin, The porin MspA from Mycobacterium smegmatis improves growth of Mycobacterium bovis BCG, Int. J. Med. Microbiol. 294 (2004) 235–245.
[12] S. Sharbati-Tehrani, B. Kutz-Lohroff, R. Bergbauer, J. Scholven, R. Einspanier, miR-Q: a novel quantitative RT-PCR approach for the expression profiling of small RNA molecules such as miRNAs in a complex sample, BMC Mol. Biol. 9 (2008) 34.
[13] J. Kim, I.S. Cho, J.S. Hong, Y.K. Choi, H. Kim, Y.S. Lee, Identification and characterization of new microRNAs from pig, Mamm. Genome (2008), doi:10.1007/S00335-008-9111-3.
[14] H. Fu, Y. Tie, C. Xu, Z. Zhang, J. Zhu, Y. Shi, H. Jiang, Z. Sun, X. Zheng, Identification of human fetal liver miRNAs by a novel method, FEBS Lett. 579 (2005) 3849–3854.
[15] W.P. Stemmer, A. Crameri, K.D. Ha, T.M. Brennan, H.L. Heyneker, Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides, Gene 164 (1995) 49–53.