III-Materials and methods

III-I Animals sampled

Wild-type mice on FVBN genetic background have been used. All animals were housed and handled according to the approved protocol established by the Institutional Animal Care and Use Committee and NIH guidelines.

For the mammary gland expression analyses of the miRNA the fifth pair of mammary gland of 2 mice was taken out after taking away the lymph node. The tissue was frozen using liquid nitrogen and conserved at –80° C or immediately used for RNA extraction.

The stages analyzed were: Virgin 4 and 8 weeks, Gestation 2, 4-6-9-12-18-days, Lactation 1, 3 days, Involution 1-3-6 days. The involution was provoked taking away the offspring after three days of lactation.

For the organ-expression analyses of the miRNAs 2 mice were sacrificed and the brain, heart, liver, lung, muscle, kidney, ovaries, spleen and thyme were taken out, frozen and conserved like for the mammary gland.

In the 'clear fat pad experiment' the mice, after an anesthesia, were operated at one of the two mammary glands of the fifth pair : in the stage of early virgin, when the mice weight less than 18 grams, the rudimentary tree of the epithelial tissue is taken away. 2 mice were sacrificed for each of the stages considered in this experiment: virgin 18 weeks, gestation 12 and 18 days, lactation 1 day.

The growing epithelial tissue taken away from these mice was spread on glass slides, fixed, colored and observed at the microscope to verify its shape and the occurrence of the complete removal of it.

The epithelial tissue on the glass slide is fixed for 2 to 4 hours at room temperature in Carnoy's fixative composed of 6 parts of 100% ethanol, 3 parts of chloroform, 1 part of glacial acetic acid. Later it is washed sequentially in solution containing decreasing concentration of ethanol: 15 minutes in a solution of 50% of ethanol, 15 minutes in another at 30%, 5 minutes in water. Later it is stained in Carmin Aluminium Staining overnight and the following day it is washed 15 minutes in 3 solutions with increasing concentration of ethanol: 70, 95 and 100%. After that it is mounted on glass slides with Permount (Sigma).

III-II RNA extraction and Northern Blot analyses

The total RNA has been isolated with the reagent RNA NOW^{TM} (Biogentex).

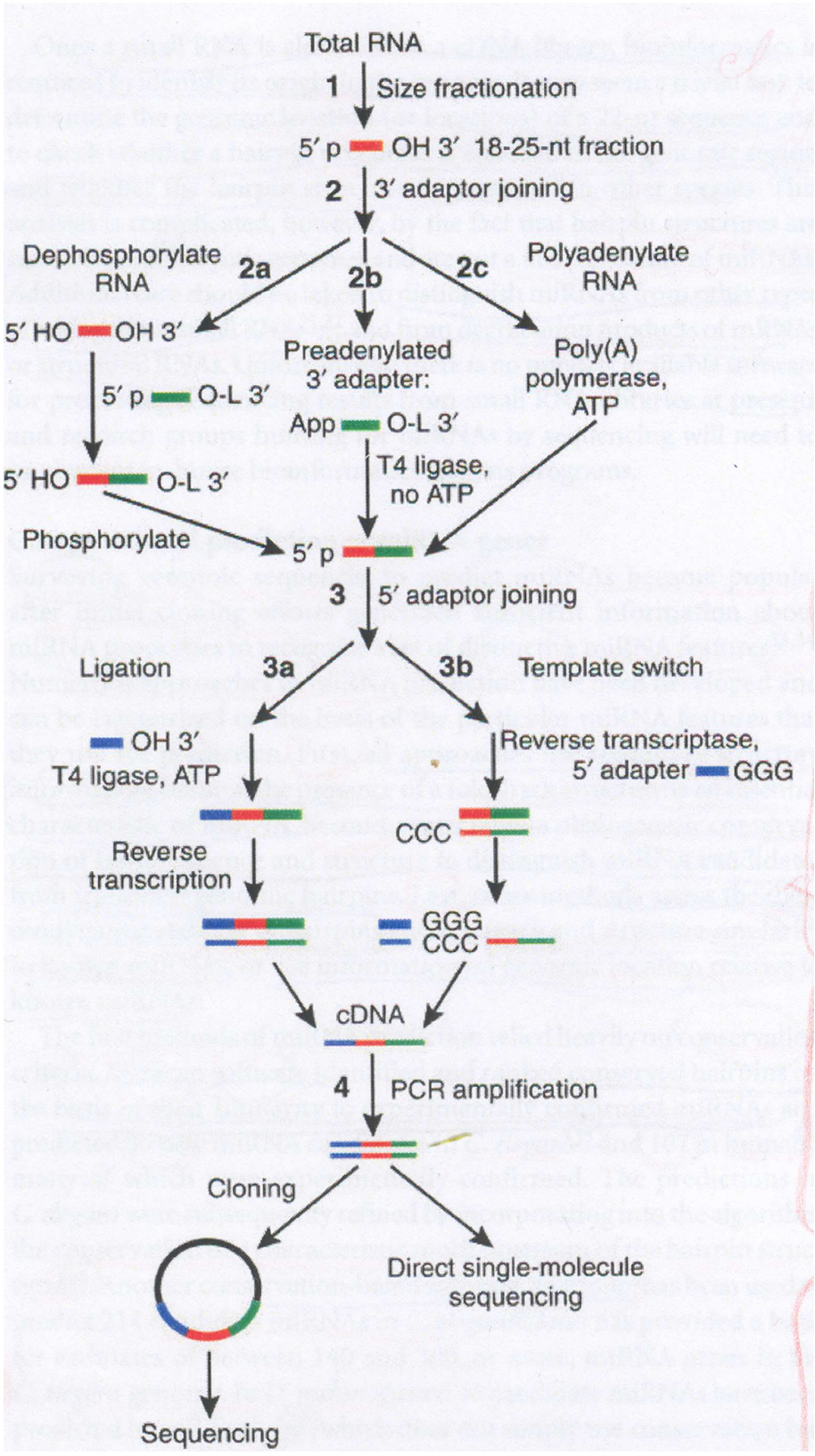
The reagent includes a cocktail of chaotropic agents, such as guanidinium salt derivative compound, which works synergistically to effectively alter the secondary and tertiary structures of proteins and polysaccharides and permits the extraction of RNA from other organic components.

A piece of tissue of approximately 0,5 cm of diameter was disrupted in 2 ml of reagent by a mechanical homogenizer. The RNA extraction occurs after the addition of 0,5 volume of chloroform during a centrifugation of 10 minutes at 15000 rpm at 4° C. The RNA is recovered in the aqueous phase. The precipitation of RNA occurs adding one volume of isopropanol and leaving the RNA at -20° C overnight.
The day later the RNA is precipitated and washed one time with 70% ethanol, the pellet is dried and resuspended at 65° C for 5 minutes in 50 µl of distilled water.
The quality of extraction of RNA has been evaluated by testing samples by electrophoresis on 1% agarose gel with Ethidium Bromide. The concentration has been measured using a spectrometer and a range of 0,5-5 µg/µl was obtained. The less concentrated samples have been precipitated overnight at -20° C in a solution of ethanol and NaCl 0,3 M before Northern Blot analyses.

20 µg of total RNA of each sample has been fractionated using a 15 % denaturing poyacrylamid gel. 75 µl of ammonium persulfate (10% wight/volume) and 12 µl of temed (from a solution of 99% of concentration, Sigma) are added after melting 12 ml of gel, to favor the polymerization. The RNA contained within the gel has been transferred overnight to nitrocellulose membrane (Hybond-N+ , Amersham Bionsciences) by capillarity. The RNA has been fixed to the membrane under UV radiation for 3 minutes.
15 pmol of 20-22 nucleotides probes have been labeled with γ-^{32}P dATP (Perkin Elmer) in a final volume reaction of 50 µl using 20 units of T4 polynucleotide kinase (Roche Diagnostices) and 5 µl of 10X reaction buffer for 30 minutes at 37° C. Pre-hybridizations and hybridizations have been carried on at 55° C for half an hour and overnight, respectively, in a phosphate buffer solution (0,5 M pH 7,2) added with sodium dodecyl phosphate (SDS) (7%). The membranes have been washed 5 minutes two times at 55° C in a pre-warmed aqueous solution of SSC 2X (SSC 1X : sodium chloride 150 mM; sodium citrate 15 mM, pH 7).
The revelation has been effected at the red light developing an auto-radiographic film (Hyperfilm, Amersham) in contact with the membrane in a closed cassette or using Phosphor Screen and the StormScan software, that produces an image on the computer starting from the data of the Phosphoimager, a scanner able to count the radioactivity of the Phosphor Screen.
The hybridized membranes have been deshybridized in contact with a boiling aqueous solution of SSC 0,1% and SDS 0,1% and reused for 3-4 subsequent hybridizations.

III-III Construction of miRNA libraries

There are different protocol and variations of them to built miRNA libraries. In this case it was followed the way 2a and 3a.


Total RNA
1
Size fractionation
5′ p
OH 3′ 18-25-nt fraction
2
3′ adaptor joining
Dephosphorylate RNA
2a
2b
2c
Polyadenylate RNA
5′ HO
OH 3′
Preadenylated 3′ adapter:
Poly(A) polymerase, ATP
5′ p
O-L 3′
App
O-L 3′
T4 ligase, no ATP
5′ HO
O-L 3′
Phosphorylate
5′ p
3
5′ adaptor joining
Ligation
3a
3b
Template switch
OH 3′
T4 ligase, ATP
Reverse transcriptase, 5′ adapter
GGG
CCC
Reverse transcription
GGG
CCC
cDNA
4
PCR amplification
Cloning
Direct single-molecule sequencing
Sequencing

III-III-a Clonage of low-molecular weight RNAs

Low-molecular weight RNA, <200 nucleotides, has been prepared from 100-200 mg of mammary gland tissue using the *miR*Vana^{TM} miRNA Isolation kit (Ambion). The quality and concentration of extractions has been evaluated in 15% denaturing polyacrylamid gel and 40-50 µg of low-molecular weight RNA has been used to isolate 19-25 nucleotides RNA following the instructions of the Lagos-Quintana 2003 cloning protocol.

The RNA has been separated in 15% denaturing polyacrylamid gel and RNA of 19- to 25-nt size has been recovered with a scalpel from the gel by the aid of an RNA size marker γ^{32}P ATP previously labeled using the kit Decade (Ambion). The gel containing small RNA has been eluted overnight in 600 µl of an aqueous solution of NaCl 0,3 M at 4° C, the RNA precipitated with 3 volumes of ethanol 100% and glycogen at the final concentration of 40 µg/ml at -20° C for 1 hour. The pellet was redissolved in distilled water. Following a dephosphorilation at the 3' extremities of the RNA (30 minutes at 50° C in a buffer solution of final volume of 30 µl with 10 units of alkaline phosphates, Roche) a first 5' phosphorylated 3'adapter (table 1), previously labeled radioactively with γ^{32}P ATP, has been ligated to the RNA (after an heat-shock of 30 seconds at 90° C without the ligase, the reaction is carried on for 1 hour at 37° C in a finale volume of 20 µl with 2 µl of 10X reaction buffer, 25 units of T4 RNA ligase, Amersham-Pharmacia, 100 pmol of 3' labeled adapter, 1 nmol of 3' adapter, 15% of DMSO). The reaction has been stopped adding 20 µl of stop solution.

After a second separation of the oligonucleotide RNA-3'adapter by electrophoresis the band of 37-42 nucleotides is recovered, eluted, precipitated like before. A 5' phosphorylation of the RNA-3' adapter oligonucleotides has been carried on (30 minutes at 37° C in a final volume reaction of 20 µl with 2 µl of 10X reaction buffer, 2 mM ATP and 5 units of T4 polynucleotide kinase, NEB; the reaction has been stopped adding 40 µl of a 0,5M NaCl solution). After a purification using the Wizard purification kit (Promega), and the precipitation of the RNA oligonucleotides, the ligation of the 5'adapter (table 1) to the RNA-3' adapter was effected in the same conditions such as for the 3' adapter using 1 nmol of a 5' adapter not radioactively labeled.

The RNA oligonucleotides have been separated by electrophoresis and the band of 55-60 nucleotides was recovered from the gel, eluted, precipitated and resuspended in distilled water like before.

Table 1. Names and composition of the two adapters used in the cloning protocol (UUU= RNA, bold characters= DNA)

Name	Composition
3' adapter	5' phosphateUUU**AACCGCGAATTCCAG** 3'
5' adapter	5' **ACGGAATTCCTCACT**AAA 3'

III-III-b Reverse-transcription and amplification

The 5'adapter-RNA-3'adapter has been retro-transcripted in cDNA using a 30 nucleotides primer (table 2) that contains a Ban I site of restriction.

The RNA has been incubated with 1 µl of RT 5'primer (100 µM) for 30 seconds at 90° C, then with the RT mix (0,1M DTT, 1X first strand buffer, 2 mM dNTPs and $MgCl_2$ for 3 minutes at 50° C and later 150 units of Superscript II reverse transcriptase (Life Technologies) have been added to the final solution of 15 µl. The reaction has been carried on for 30 minutes at 42° C.

The cDNA corresponding to the ligated RNA fragments is then amplified by PCR using the reverse transcription primer and a second 3' primer (table 2) containing the Ban I digestion site.

2 µl of cDNA from the previous step has been used in a final solution of 50 µl containing 5 µl of 10X reaction buffer added with $MgCl_2$ (25mM), 0,2 mM of each dNTPs, 1µM of each primers, and the 2 units of Taq polymerase (Promega).

A PCR of 30 cycles (0: 45 minutes of denaturation at 94° C, 1 minute of annealing at 50° C, 1 minute of extension at 72° C) has been used to amplify the cDNA. The 70 nucleotides in size amplified DNA was tested on 2% agarose gel using 5 µl of the PCR.

Table 2. Name and sequence of the primers used for the reverse transcription and the amplification of the 5'adapter-RNA-3'adapter (bold charachetr : Ban I site of digestion).

Name	Sequence
RT 5' primer	5' GACTAGCTG**GAATTC**AAGGATGCGGTTAAA 3'
RT 3' primer	5' GACTAGCTTGGTGCC**GAATTC**GCGGTAAA 3'

III-III-c Ban I digestion and concatamerization

The PCR fragment has been purified by using Wizard purification kit (Promega) and cloned in pGEM-t plasmid (Promega) or subsequently digested with Ban I enzyme in order to generate fragments able to link all together and to form a concatemer to clone.

The DNA eluted (approximately 30 µl, 500 ng-1 µg of DNA) from the previous step has been used in the digestion, which has been effected at 37° C for 3 hours in a final volume of 200 µl of buffer solution using 200 units of Ban I enzyme (NEB).

The digestion has been tested on 2% agarose gel to compare digested band with undigested PCR product. After the purification of the digested DNA reaction (Promega kit), the concatamerization reaction has been carried on using 80 µl of the purificated digestion reaction mixed with 10 µl of 10X ligase buffer, the two primers (3 µM) and with 1600 units of T4 DNA ligase (NEB), in a final volume of 100 µl. Before the addition of the ligase, the

solution is incubated for 10 minutes at 65° C to denature the short digested fragments and prevent their eventual re-ligation. The solution added with the ligase has been incubated for 5 hours at room temperature and then the concatamerization product has been tested on 2 % low-melt agarose gel.
The DNA of 200-600 base pairs of concatamerization products has been cut from the gel and melted in a 0,3 M NaCl solution at 65° C for 5 minutes and the concatamers purified by the Wizard kit (Promega).
The extremities of the concatamers have been filled incubating for 30 minutes at 72° C in a final solution of 120 µl containing 12 µl of 10X reaction buffer, dNTPs (2 mM each), and Taq polymerase in standard conditions (0,25 µl of Taq at 5U/µl, Promega).

III-III-d Ligation and transformation

1 µl of 10X reaction buffer (Promega) has been added to 1,5 µl of the vector, pGEM-t, (50ng/µl, Promega), with 1 µl of T4 DNA ligase (3U/µl) and 6,5 µl of the DNA fragment to clone in a final volume of 10 µl following the instructions of the kit pGEM-T Vecrtor System I (Promega). The ligation reaction has been incubated at 15° C overnight.
2 µl of the ligation has been used to transform *Escherichia Coli* electrocompetent cells that were previously treated to be transformed by an electric impulse and were stocked at -80° C.
The cells were seeded on dishes containing Luria-Beriani agarose medium (LB, 10 g/l of bacto-tryptone, 10 g/l of NaCl, 5g/l of yeast extract) before seeding them added with IPTG (10 µg/µl), ampicillin (100 µg/µl), X-galactosidase (10 µg/µl). The plates were left in the incubator at 37° C overnight.

III-III-e PCR from colony

To verify that the white colonies contain the fragment and to exclude false positive the white colonies were analyzed by PCR.
The PCR (30 cycles of 94° C for 20 seconds of denaturation, 58° C for 30 seconds of annealing, 72° C for 30 seconds of extension) were effected using two primers (table 3) adjacent to the site of insertion of the fragment, standard conditions for dNTPs and Taq polymerase and the colonies picked up from the plates. The PCR product have been analyzed in 2% agarose gel.
The colonies that showed an amplification of the attended size, approximately 300 base pair, were chosen for a plasmidic extraction.

Table 3. Name and sequence of the two primers used to verify the presence of the inserts in the plasmid.

Name	Sequence
universal	5’ GTTTTCCCAGTCACGAG 3’
reverse	5' CCAGTATCGACAAAGGAC 3'

III-III-f Preparation of recombinant plasmidic DNA and sequencing of the inserts

A bacterial pre-culture have been obtained for each of the colonies of interest seeding the colonies singularly in 2 ml of LB liquid medium auditioned with ampicillin (100 µg/ml). The tubes have been put in agitation overnight at 37° C.
The extraction of plasmidic recombinant DNA is realized following the alkaline lyses protocol (Birnbon et al., 1979), modified by Micard et al. (1985).
The concentration of DNA extraction has been evaluated on 1% agarose gel resulting for the clones in the range of 0,1-0,4 µg/µl.
10 µl of plasmidic DNA of each clones containing both singular fragment or concatamers were sent to be sequenced outside.
The sequences were analysed using the BIOEDIT software (Tom Hall, Departmen of Microbiology, NCSU, USA).

III-III-g Analyses of the cloned fragments

The 19-25 nucleotides cloned fragments have been multi-aligned by the Clustalw software (http://www.ebi.ac.uk/clustalw/) to exclude the redundant sequences and to observe the partial sequence homology between them.
The non redundant cloned fragments have been analyzed by nucleotide-nucleotide BLAST (http://www.ncbi.nlm.nih.gov/blast) in the EST-mouse database, in the miRNA registry (http://www.sanger.ac.uk/Software/Rfam/miRna/) and they have been mapped in the mouse genome (http://www.ensembl.org/Mus_musculus/index.html).
A genomics sequence of 120 nucleotides containing the cloned fragments was analyzed using the mfold software (http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi, 1995-2006, Michael Zuker, Rensselaer Polytechnic Institute) to predict possible secondary structure of miRNA precursors in this genomic region.

III-IV-Validation of the potential miRNA

III-IV-a Construction of the precursor sequences

The precursor sequences were reconstructed using two different methods: for the longest sequence (in this case G18n27 and LSII39) two long oligonucleotides of approximately 80 nucleotides, overlapping one on the other and pairing to the two extremities of the potential precursor were mixed in a filling reaction followed by a PCR with the same couple of primers ; for the shortest sequences (FLP31 and LSII15) two primers of 40 nucleotides were used to amplify the mouse genome (table 4).

The primers bring at the external extremities a site of digestion, (Bam HI in this case), of the enzyme used to clone the precursor in the plasmid. The sequences of the precursors were analyzed to verify the absence of any site of digestion of this enzyme before beginning the protocol.

In the filling reaction 4 µl of the 80-nt size primers (100 pmol/µl) were mixed to 24 µl of distilled water and placed first at 95° C for 5 minutes to avoid complementary hybridization of primers. After their cooling in ice 2 µl of dNTPs (10mM), 4 µl of 10X Klenow Buffer and 2 µl of Klenow enzyme (5U/µl) (Biolabs) were added to obtain a final reaction of 40 µl which went on for 30 minutes at 37° C and for 15 minutes at 65° C.

The PCR was performed in a finale volume of 50 µl using, in one case, 2 µl of the paired 80-nt primers, and in the other case 1 µl of mouse DNA (100 ng/µl), with 0,5 µl of each primers (30 pmol/µl), in a PCR mix containing 2 mM dNTPs and 2,5 mM $MgCl_2$ and 0,5 µl of Taq Polymerase (5U/µl). The PCR ran for 40 cycles : 94° C for 10 seconds of denaturation, 60° C for 20 seconds of annealing, 72° C for 20 seconds of extension.

5 µl of the PCR were tested on 1% agarose gel with a DNA size marker to verify the correct length of the precursors.

Table 4. Names and sequences of primers used for the construction of G18n27 precursor, LSII39 precursor, FLP31 precursor, LSII15 precursor (bold characters: site of Bam HI digestion)

Name	Sequence
preG18275'	**CGGGATCCCG**TCCAGAACACGGACACCGCAGGGTGAGGTAGTAGGTTGTGTGGTTTCAGGG
preG18273'	**CGGGATCCCG**TCCCTGGGCGCCTCAGGGAAGGCAGTAGGTTGTATAGTTATCTTCGGTGG
preLSII395'	**CGGGATCCCG**GGCAGTCTCAGAAATCCAAACTTTTCAGTTTCCTGGATTTTTCTCTCTATTAA
preLSII393'	**CGGGATCCCG**GGCAGGCGAGAATTCTACCACTGAACCACCAATACTACCTTTATTTTATCTTAA
preFLP315'	**CGGGATCCCG**GTTTCAAAGTTTTGATAGGTTCTACGCATG
preFLP313'	**CGGGATCCCG**GCTTCAGCTTTGACTTTCAGAGCACTGGG
preLSII155'	**CGGGATCCCG**AGCGCCGAATCCCCGCCGCGCGTCGCGGCGTG
preLSII153'	**CGGGATCCCG**GGTCTTCCGTACGCCACATTTCCCACGCCGCGACGCGCGGCGG

The remaining 45 µl of the PCR were mixed to 2,5 volumes of Ethanol 100%/Acetate of Na (2,5 mM) and let at -20° C overnight to precipitate. The day later the DNA was centrifuged for 15 min at 15000 rpm at 4° C and washed one time with ethanol 70%. The pellet was dried at the room temperature and resuspended in 10 µl of distilled water leaving the DNA for 5 minutes at 65° C.
The precursor sequence was ligated in the pGEM-t vector and the correct sequence was verified like described in the paragraphs III-IV to III-VII.

III-IV-b Construction of the expression vector

For the in vitro expression studies the precursors were cloned in the Bam HI digestion site of the 5021 base pair pUHG 17-1 plasmid which contains an ubiquitous promoter, CMV. rtTA and B-globin sequences are adjacent to the site of insertion. The precursor sequence and the vector were prepared independently before the ligation.

The precursor sequence was recovered digesting the pGEM-t plasmid of the sequenced colonies containing the correct precursor sequence. In a final volume of 120 µl 20 µl of the plasmidic DNA (1 µg/µl) were digested using 10 µl of Bam HI enzyme (20u/µl, NEB)and 12 µl of 10X reaction buffer (NEB). All the digestion reactions were charged on a 2% low-melt agarose gel and the precursor fragments (from 110 to 200 nucleotides, depending on the precursor) were recovered from the gel with a scalpel. The DNA was eluted from the gel like described before and purified by the aid of Wizard kit (Promega). 40 µl of purified DNA were recovered and concentrated under vacuum (speed-vac) to a final volume of 17 µl, whose 4 µl were tested on a 2% agarose gel (10 ng/µl).

2 µl of pUHG plasmid (1,5 µg/µl) were digested in a final volume reaction of 200 µl with 20 µl of Bam HI enzyme (20U/µl) and 20 µl of 10X reaction buffer (NEB). 5 µl of the digestion were tested on a 1% agarose gel together with 0,2 µl of non digested plasmid to verify the different run of the two plasmid.
To confirm the plasmid linearization 15 µl of the digested plasmid were subjected to a second digestion with Hind III in a finale volume reaction of 100 µl with 5 µl of enzyme (20U/µl, NEB). 6 µl of this second reaction were tested on a 1% agarose gel along with a marker size and the two fragment of digestion of 940 and 4081 base pair were observed at the correct position.
The remaining 180 µl of the Bam HI digestion were then purified by the Wizard kit (Promega) and eluted in 140 µl of distilled water.
The digested and purified plasmid was then subjected to a dephosphorilation in a finale volume reaction of 200 µl with 9 U of alkaline phosphatase(Roche) and 20 µl of 10X reaction

buffer (Roche) at 50° C for 40 minutes. The reaction was purified and eluted in a volume of 33 µl of distilled water. 3 µl were tested on a 1% agarose gel with a size marker and 0,3 µl of non digested pUHG and the concentration of the digested dephosphorilated plasmid was estimated to be approximately 150 ng/µl.

III-IV-c Ligation, transformation and sequencing

6 µl of each purified insert (the precursor) were mixed to 2 µl of the dephosporilated and linearized pUHG plasmid in a finale volume reaction of 10 µl adding 1 µl of 10X reaction buffer and 1 µl of ligase (3U/µl). The reaction went on for 10-15 hours at 37° C. A negative control was provided from a tube in which the fragment of insertion was not added.
Escherichia Coli cells were transformed with the engineered plasmid and cultivated on plates of LB agarose medium added with ampicillin (100 µg/ml) like it is described in the paragraphs III-III-d.
A PCR test (30 cycles : 94° C for 30 seconds of denaturation, 58° C for 30 seconds of annealing and 74° C for 40 seconds of extension) was effected like described in the paragraph III-III-e on a series of colonies to select the transformed ones using primers pairing on the rtTA and Bglobin sequences.
The plasmidic DNA was prepared like described in the paragraph III-III-f.
The plasmidic DNA were tested by PCR to determine the orientation of each precursor in the site of insertion of the plasmid.
1 µl of each DNA plasmidic preparation was subjected to a couple of PCR with primers (table 5) pairing on the precursor and, respectively, one primers pairing on rtTA or on B-globin sequences. The PCR of 30 cycles (in the same condition as for testing the positive colonies, but with an extension temperature of 72° C) was effected in a finale volume of 25 µl using 0,5 µl of each primers (30 pmol/µl) and standard buffer and Taq polymerase conditions.
The PCR was tested on 2% agarose gel and the different size of PCR product indicated the orientation of the precursor in the plasmid.
10 µl of recombinant plasmidic DNA from the positive colonies for both orientations of each precursor were sent to sequencing using both the rtTA and the B-globin primers to sequence the insertion fragments in both directions. The sequences were analysed by BIOEDIT.

Table 5. Primers used to test the orientations of the inserts

rtTA/1	**GATGCCCTTGGAATTGACGAG**
B-glob/2	**TATAACATGAATTTTTCAATAGCG**

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III-IV-d DNA plasmidic preparation

After the analyses of the sequences 1 positive colony for each precursor in both orientation was chosen for a first pre-bacterial culture and a subsequent bacterial culture in 100 ml of LB liquid medium added with ampicillin (100 µg/ml), which was put at 37° C overnight seeding inside the 2 ml of pre-culture.
Large amount of plasmidic DNA was extracted using the alkaline lyses method following the protocol of the Nucleobond PC100 plasmid DNA purification kit (Marcherey Nagel). The DNA was tested on a 1% agarose gel with a circular gamma range of plasmidic DNA and was estimated to be in the range of 0,5-1 µg/µl.

IV-IV-e Transfection test

For the *in vitro* studies COS-7 cells (cellular line derived from 'afric green' monkey kidney transformed by the SV40, this line express the T antigen, it was established from CV-1 monkey cells and has a fibroblasts morphology) have been used.
The cells have been grown in 10 cm of diameter dishes (Orange Scientific) in Dulbecco's Modified Eagle's medium (DMEM) with the addition of 10% of SVF, 2 mM glutamine, penicillin 10U/µl and streptomycine 100U/µl incubating them in a thermostat at 37° C (5% of CO_2) until a confluence of 40-60% was observed at the optical microscope after 24/48 hours.
Transfection tests have been made using the jetPEI^{TM} cationic reagent (PolyPlus transfection) and following the instructions relative to the reagent.
The cells have been seeded in 6 multi-wells plates and when a 40-60% confluence is noticed each wells has been transfected with plasmidic recombinant DNA carrying a different precursor in one orientation. The negative control was provided by a wells of cells non transfected. Both DNA and the jetPEI^{TM} were diluted in two independent 100 µl 150 mM NaCl sterile solution. 6 µl of jetPEI^{TM} were necessary to transfect 3 µg of plasmidic DNA. The jetPEI^{TM} solution was added to the DNA solution and the mixed solution was left 15-30 minutes at room temperature before being added to 2 ml of the medium of each well. The cells are left in the incubator at 37° C and the transfection went on for 2 days.
0.8 ml of RNA NOW reagent were added to the medium containing cells of each wells and the total RNA was extracted, tested on 1% agarose gel (1-2 µg/µl) for its quantity and quality and used to analyze by Northern blot, like it is described in the paragraph III-II, the *in vitro* expression of the miRNAs corresponding to the transfected precursors.

IV-Results and discussion

The involvement of miRNAs in the mouse mammary gland was analyzed in two different ways: i) it was examined the expression of a set of known miRNAs during the stages of development of this organ, ii) in order to identify organ-specific miRNA, libraries of microRNA, extracted from different stages of the developing mammary gland, were constructed and some new 'candidate miRNAs' were characterized.

IV-I Detecting miRNAs in mouse mammary gland

In order to defined the role of miRNA during the development of mammary gland (MG), first the expression of some miRNAs already described in literature and present in the microRNA registry was analyzed.
A group of 25 miRNAs was selected : between them 12 are human miRNAs (Lagos-Quintana et al., 2001) 100% identical to part of sequences of cDNA derived from the mammary gland in the EST mouse bank; 9 are highly expressed in human breast tissue (Liu et al., 2004), the remaining show an involvement in tumor breast tissue (Iorio et al., 2005; Lee et al., 2005; Cimmino et al., 2005). For the mammary human miRNAs the homologous murine miRNAs were searched and the complementary sequences were ordered and used like probes for the hybridizations (table 1).

Table 1. Mouse miRNA tested, their bibliography origin, their corresponding probes.

Name miRNA	Sequence	Origin	Name probe	Sequenze
mmu-miR7.1	TGGAAGACTAGTGATTTTGTT	Lagos-Quintana et al, 2001	miR7.1as	AACAAAATCACTAGTCTTCCA
mmu-miR92.1	TATTGCACTTGTCCCGGCCTGT	"	miR92.1as	ACAGGCCGGGACAAGTGCAATA
mmu-miR130a	CAGTGCAATGTTAAAAG	"	miR130aas	CTTTTAACATTGCACTG
mmu-miR140	GTGGTTTTACCCTATGGTAG	"	miR140as	CTACCATAGGGTAAAACCAC
mmu-miR142	CATAAAGTAGAAAGCACTAC	"	miR142as	GTAGTGCTTTCTACTTTATG
mmu-miR145	GTCCAGTTTTCCCAGGAATCCC	"	miR145as	GGGATTCCTGGGAAAACTGGAC
mmu-miR203	TGAAATGTTTAGGACCAC	"	miR203as	TGGTCCTAAACATTTCA
mmu-miR212	TAACAGTCTCCAGTCAC	"	miR212as	GTGACTGGAGACTGTTA
mmu-miR216	CTCAGCTGGCAACTGTG	"	miR216as	CACAGTTGCCAGCTGAG
mmu-miR217	CTGCATCAGGAACTGATTGG	"	miR217as	CCAATCAGTTCCTGATGCAG
mmu-let7b	TGAGGTAGTAGGTTGTGTGGTT	"	let7bas	AACCACACAACCTACTACCTCA
mmu-let7c	TGAGGTAGTAGGTTGTATGGTT	"	let7cas	AACCATACAACCTACTACCTCA
mmu-let7a	TGAGGTAGTAGGTTGTATAGTT	Liu et al., 2004	let7aas	AACTATACAACCTACTACCTCA
mmu-miR23b	ATCACATTGCCAGGGATTACCAC	"	miR23bas	GTGGTAATCCCTGGCAATGTGAT
mmu-miR24-2	TGGCTCAGTTCAGCAGGAACAG	"	miR24-2as	CTGTTCCTGCTGAACTGAGCCA
mmu-miR26a-1	TTCAAGTAATTCAGGATAGGCT	"	miR26a-1as	AGCCTATCCTGGATTACTTGAA
mmu-miR26b	TTCAAGTAATTCAGGATAGGTT	"	miR26bas	AACCTATCCTGAATTACTTGA

mmu-miR30b	TGTAAACATCCTACACTCAGC	"	miR30bas	GCTGAGTGTAGGATGTTTAC
mmu-miR30c-1	TGTAAACATCCTACACTCTCAGC	"	miR30c-1as	GCTGAGAGTGTAGGATGTTTACA
mmu-miR30d	TGTAAACATCCCCGACTGGAAG	"	miR30das	CTTCCAGTCGGGGATGTTTACA
mmu-miR100	AACCCGTAGATCCGAACTTGTG	"	miR100as	CACAAGTTCGGATCTACGGGTT
mmu-miR15a	TAGCAGCACATAATGGTTTGTG	Iorio et al, Lee et al, Cimmino et al, 2005	miR15aas	CACAAACCATTATGTGCTGCT
mmu-miR16a-1	TAGCAGCACGTAAATATTGGCG	"	miR16as	CGCCAATATTTACGTGCTGCTA
mmu-miR125a	TCCCTGAGACCCTTTAACCTGTG	"	miR125aas	CACAGGTTAAAGGGTCTCAGGGA
mmu-miR205	☒☞☞☒☒☞☛☒☒☞☞☛☞☞✌✌☛✌☒☞☒✌	"	miR205as	CAGACTCCGGTGGAATGAAGGA

The expression of these 25 miRNAs was analyzed by Northern blot in several stages of mouse mammary gland before and after the fecundation.

The expression of 10 miRNAs was detected : let-7a, let-7b, let-7c, miR-26a, miR-26b, miR-16a, miR-24-2, miR-145, miR-30b, miR-30d.

For 6 miRNas, 3 belonging to the let-7 family, 2 to the miR-26 family and for miR-16aa, the expression was strong, for the remaining of them was weak (table 2).

Apart let-7b, let-7c, miR-145, chosen after selection in the EST murine database, and miR-16a, analysed as presented in breast tumour tissue, the others miRNAs detected in mouse are homologous to human miRNAs highly expressed in the mammary gland (Liu et al., 2004).

It is not known either if the undetected miRNAs are not expressed in the mouse mammary gland or if the sensibility of the technique is not sufficient to reveal the less expressed miRNAs. For Liu et al. (2004) the Northern blot analysis has been used to confirm microarray data and they have shown that there is a high concordance between the results of the two techniques, then it was not surprising to detect let-7a, miR-26a, miR-26b, miR-24-2, miR-30b, miR-30d, but also the other human homolog miRNAs were attended to be detectable in mouse mammary gland. The lack of expression of miR-23b, miR-30c-1 and miR-100 could be explained by the difference, in the two studies, of the species in exam (human versus mouse) and/or by differences in the samples analyzed, like the stage of development of the mammary gland (virgin or lactating or involuting..) or the tissue analyzed (epithelial cells or connective tissue or both), conditions that are not described in the work of Liu et al.. ; as well the fact that miR-15a, miR-125a, miR-205a, differentially expressed in the neoplastic human breast tissues comparing to normal breast tissues (Iorio et al., 2005; Lee et al., 2005; Cimmino et al., 2005), and the remaining human homolog miRNAs, miR-7.1, miR-92.1, miR-130, miR-140, miR-142, miR-203, miR-205, miR-216 and miR-217, were non detected, could be due to the same reasons.

Table 2. miRNA tested and their level of expression.

miRNAs analyzed	detection level
let-7a, let-7b, let-7c, miR-16aa, miR-26a, miR-26b,	+++
miR-24-2, miR-30b, miR-30d, miR-145	+
miR-7.1, miR-15, miR-23b, miR-30c1, miR-92.1, miR-100, miR-125b, miR-130, miR-140, miR-142, miR-203, miR-205, miR-212, miR-216, miR-217	-

IV-II Characterization of miRNA expression profile in MG

An extensive experiment was set to obtain a complete and accurate expression profile of the miRNA in different stages of the development of MG.

Total RNA was extracted from mouse MG at the stages of 4 and 8 weeks of virgin; 4-, 6-, 12-, 18-days of gestation; 1-, 3-days of lactation; and 1-, 3-, 6-days of involution. The levels of miRNA expression, analyzed by Northern blot, were measured in each stage, after normalization of the quantity of RNA. A reliable expression profile during the development of MG was obtained for each miRNA tested.

The expression of all the 10 miRNAs examined is detected at all stages studied and is variable along the MG development. The profiles are different, but a strong decrease of expression during the lactation and a relevant increase during involution is observed for all of them.

let-7a, let-7b, let-7c and miR-26a and miR-26b are strongly detected (figure 1 and 2). let-7b and let-7c have similar profiles, which show common features whit the miR-26a profile, while let- 7a is more similar to miR-26b.

At the stages of virgin, 4 and 8 weeks, the expression of let-7a and miR-26b is some more higher than in the gestation. During this stage, at 4, 6, 9, 12 and 18 days, their expression is stable. At lactation the expression of let-7a and miR-26b decreases, reaching the minimum of the profile at the first and third day, respectively. During involution the levels of let-7a and miR-26b progressively increase touching the maximum of the profile at the sixth day.

The expression of let-7b, let-7c and miR-26a at the stages of virgin is comparable to or higher than the expression of let-7a and miR-26b, respectively. In the early gestation, 4 and 6 days, the levels of let-7b, let-7c and miR-26a remain high, while these levels progressively decrease during the stages of mid and late gestation till reaching the levels

of expression of let-7a and miR-26b at 12 and 18 days of gestation. During the lactation (day 1 and day 3) the expression of let-7b, let-7c and miR-26a is low and comparable to the expression of let-7a and miR-26b. Also in the stages of involution the profiles of the 5 miRNAs are comparable, undergoing a progressive increase.

Figure 1. Image of the Northern blot for let-7c and expression profiles of let-7a, b, c during the development of mouse mammary gland, after normalization and quantifications of the Northern blot data. Each point, and the relative error bar, represents the average miRNA expression (counts per minute) between two individuals.

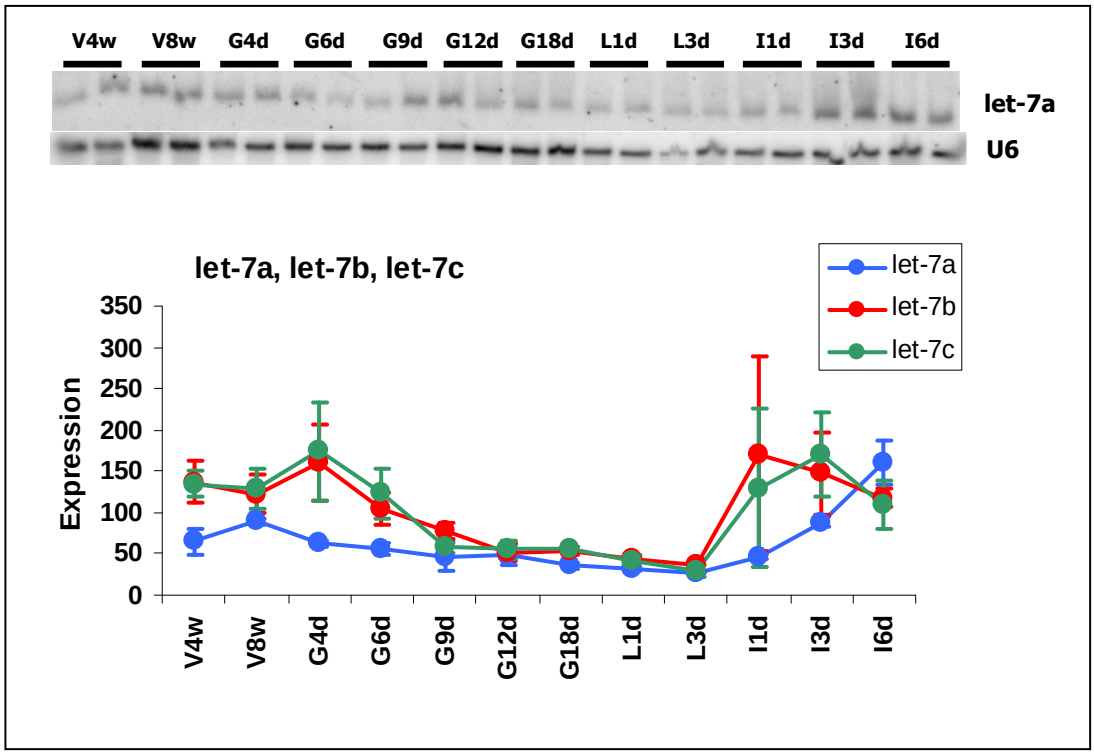
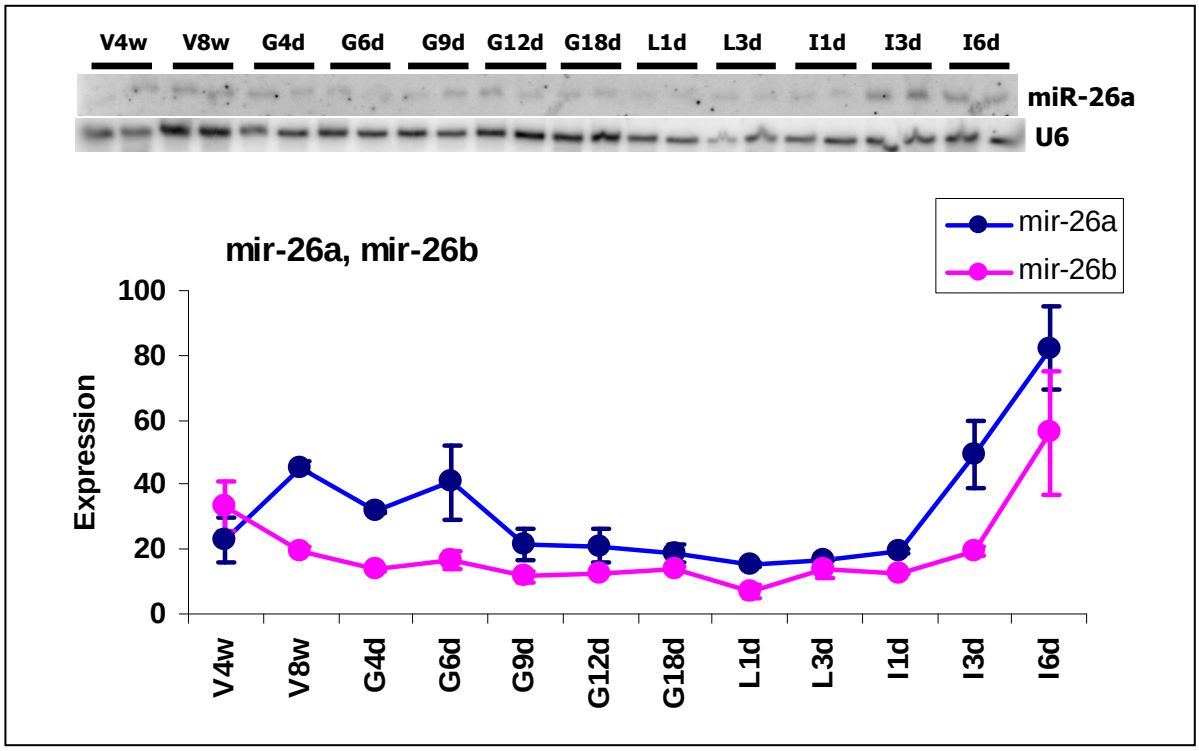


Figure 2. Image of Northern blot for miR-26a and expression profiles of miR-26a and miR-26b during the development of mouse mammary gland, after normalization and quantifications of the Northern blot data. Each point, and the relative error bar, represents the average miRNA expression (count per minute) between two individuals.



miR-24, miR-30b and miR-30d are weakly detected.

The expression profile of miR-24 (figure 3) is similar to the profiles described before : the expression at the stages of virgin is higher comparing to the early and mid gestation, at 6 and 9 days, like for let-7a and miR-26b. At the day 12 of gestation is observed a small increase in expression, while during the late gestation and the lactation the level decrease and reaches the minimum values of the profile. During the involution the expression progressively increases as observed for the first 5 miRNAs.

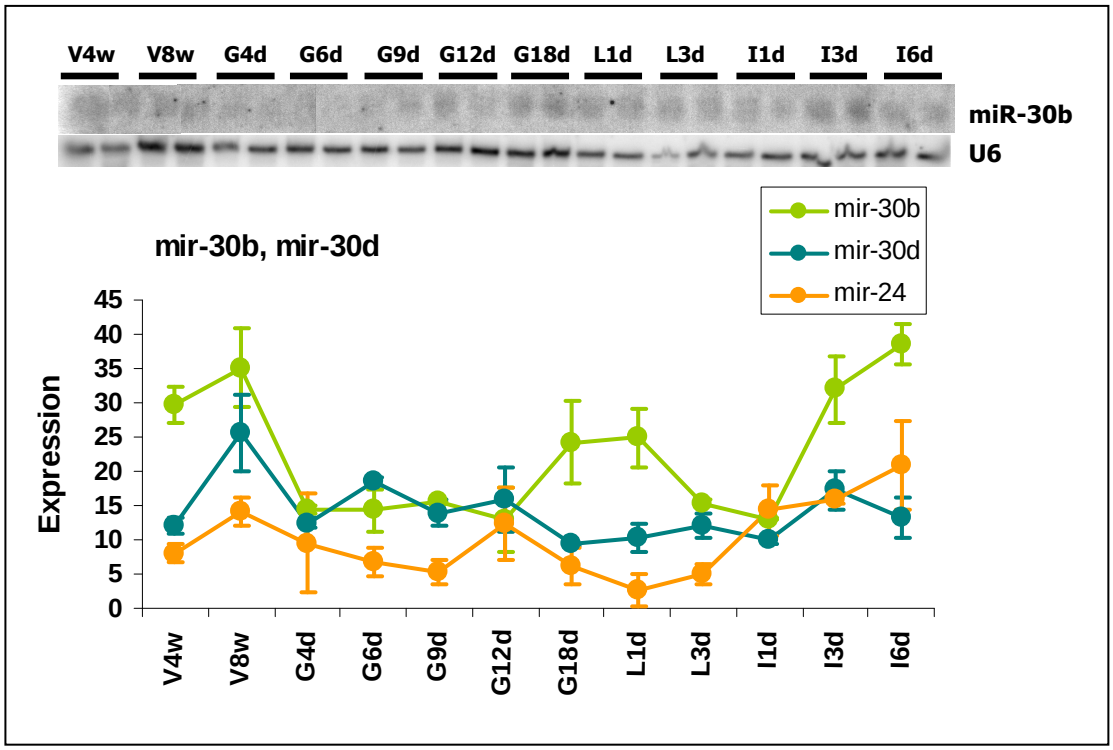
miR-30b and miR-30d show different expression profiles between them : comparing to miR-30b, the activity of miR-30d is more similar to that one of the first miRNAs described.

miR-30d shows higher expression at the stages of the virgin comparing to the gestation. The minimum of the profile is observed at the end of this stage, at 18 days, and during the lactation, when the levels of expression are low and constant. The expression remains low also in the beginning of involution (day 1) and increases some more at 3 and 6 day of involution.

miR-30b is highly expressed at 4 and 8 weeks of the virgin, while during the gestation its expression strongly decreases, remaining constant till the 12 day of gestation. Interestingly the miR-30b expression increases at the day 18 of gestation and it is high at the first day of lactation. The miR-30b expression level significantly decreases at the

day 3 of lactation and at the beginning of involution, even if it remains comparable to the levels of early and mid gestation. At the third and sixth day of involution miR-30b is more expressed, at the same levels measured at the stages of virgin.

Figure 3. Image of the Northern blot for miR-30b and expression profiles of miR-30b, miR-30d and miR-24 during the development of mouse mammary gland, after normalization and quantifications of the Northern blot data. Each point, and the relative error bar, represents the average miRNA expression (counts per minute) between two individuals.



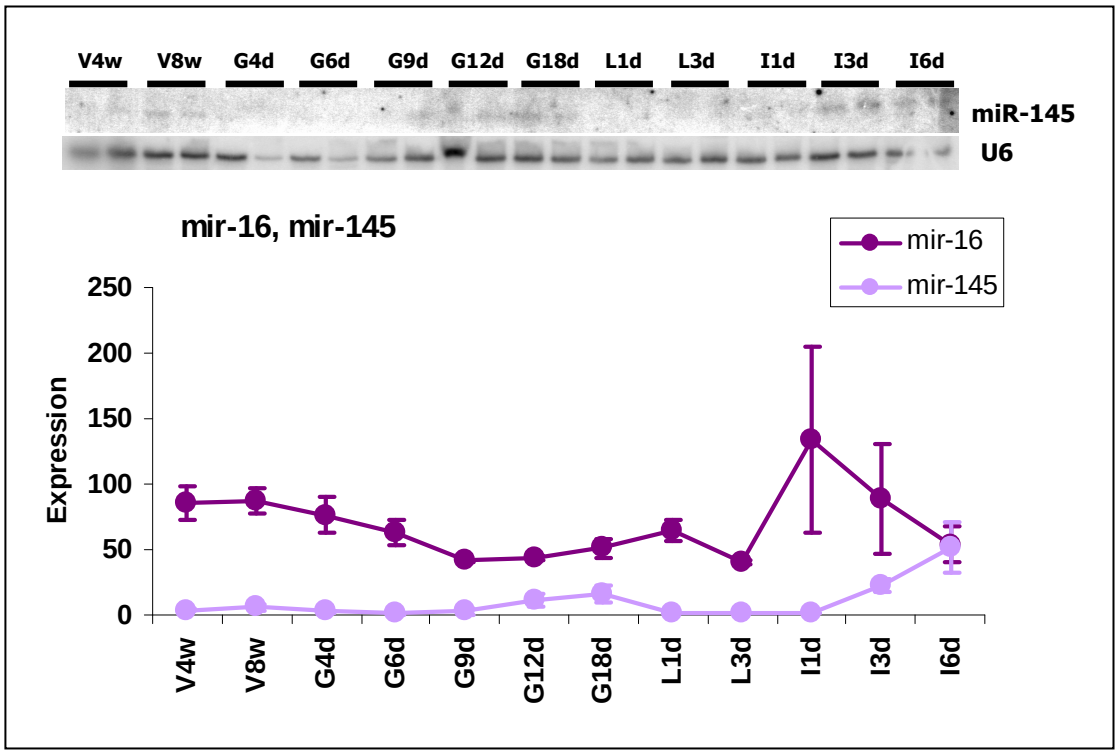
miR-145 is weakly detected and it shows an expression profile different from the other miRNAs analyzed. Also miR-16aa, detected at higher values, as a singular profile comparing to the first ones (figure 4).

The miR-145 expression is low during the first stages of virgin and it decreases some more during the early and mid gestation, till the day 9. At the day 12 and 18 of gestation miR-145 is more expressed, at levels higher than in the virgin. During lactation and in the beginning of involution its expression decreases significantly till reaching the minimum value of the profile. At the third and sixth day of involution its level of expression strongly increases reaching the maximum level at the day 6.

miR-16a is highly expressed at the stages of the virgin. These levels progressively diminish during the early and mid gestation (4, 6, 9 days) reaching a first minimum of the profile at the day 9 of gestation. Interestingly during the mid and late gestation (12 and 18 days) till the beginning of lactation (1 day) its expression progressively increases, while at the third day of lactation a significant decrease in expression is

observed. As for the most part of miRNAs analyzed, miR-16aa expressions increases during the stages of the involution.

Figure 4. Image of the Northern blot for miR-145 and expression profiles of miR-16 and miR-145 during the development of mouse mammary gland, after normalization and quantifications of the Northern blot data. Each point, and the relative error bar, represents the average expression (counts per minute) between two individuals.



The miRNA profiles could be correlated with the hormonal control of some physiological phases of mammary gland development or whit the differentiation of the epithelial tissue during the developmental cycle of the organ : for example the progesterone is secreted in the beginning of pregnancy and its level increases during this phase till the parturition, when the secretion decreases brutally; the concentrations of estrogens are high during puberty and low during pregnancy, while the levels rapidly decrease at parturition. The miRNAs profile could reflect a combination of more than one regulatory pathways of these hormones. Moreover the concentration of prolactin is low during the most part of the pregnancy, increases in the last part of this stage, reaching higher levels during the lactation, while it decreases in the involution. This profile shows to be inversed correlated to that one of different miRNAs analysed (for example let-7a, let-7b, let-7c, miR-26a, miR-26b). The secretion of prolactin promotes the proliferation and differentiation of the epithelial tissue, and the growing number of cells and differentiated structures for the milk secretion during the gestation, at parturition and during the lactation, is also inversely correlated to the profile of some miRNAs.