IV-III Characterization of miRNA expression profile in different organs

To assess the organ-specificity of these miRNAs, Northern blot analysis were performed to compare the miRNA expression in brain, heart, liver, lung, muscle, kidney, ovary, spleen and thymus, with the expression in MG.

An organ-expression profile was produced for one miRNA for each group of different miRNA profiles : let-7c, miR-26a, miR-24a, miR-30b, miR-16aa and miR-145.

The expression profiles showed that none of the miRNAs detected is expressed only in MG, demonstrating their presence at low to high levels also in some other organs (figure 5): for example miR-26a is more expressed in the muscle, in the lung and in the brain; miR-16a is strongly detected in the lung, spleen and heart, miR-145 is highly expressed in the ovary and in the lung; miR-24a is highly detected in the lung and in the muscle; miR-30b is maximum detected in the lung.

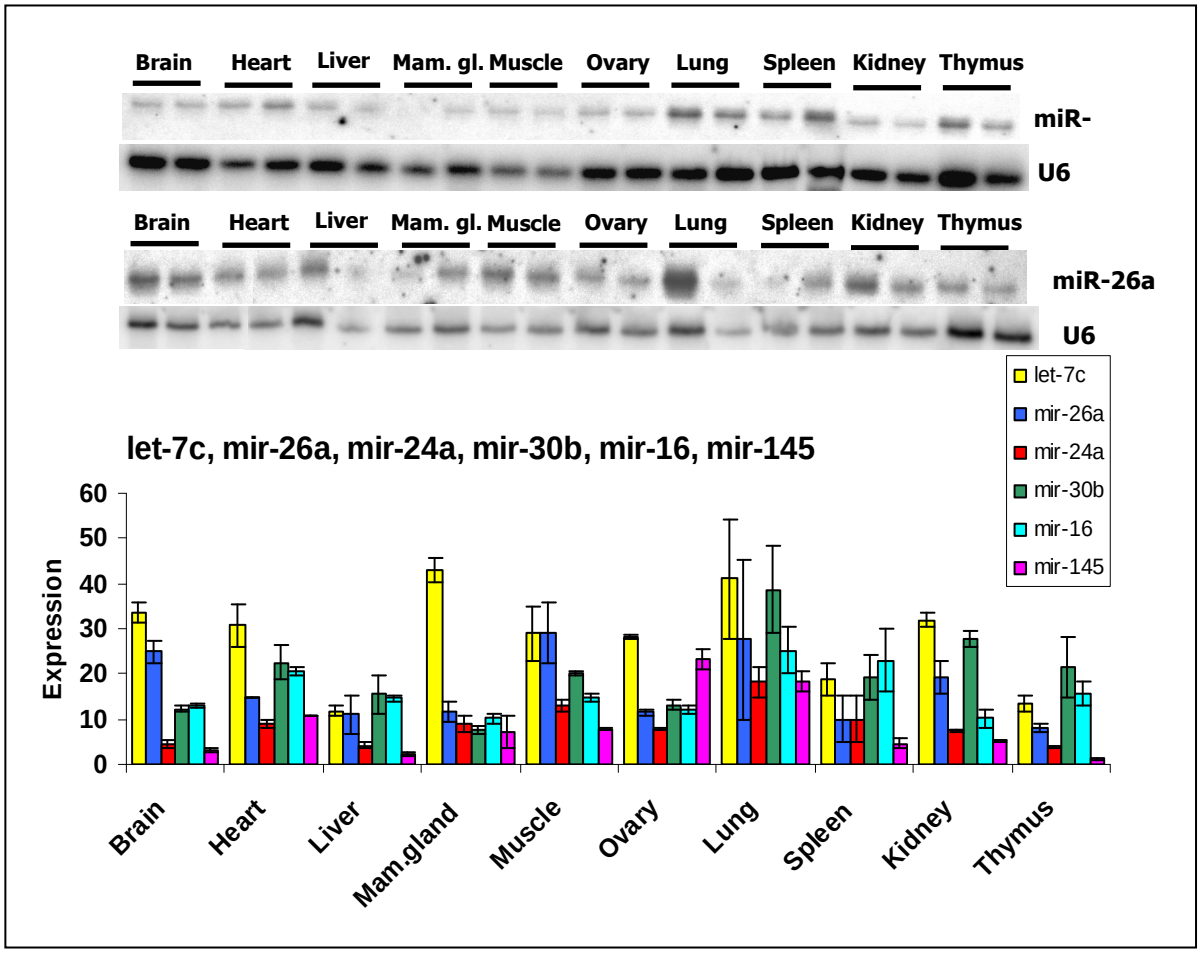
Among the 6 miRNAs considered let-7c shows the highest value of expression in MG comparing to the other organs, while miR-26a and miR-30b are the less expressed in MG. Let-7c is also the more expressed in all the organs considered, while the less represented are miR-24a and miR-145.

The lung could be the organ where all the miRNA tested are expressed at highest levels, followed by the muscle, the heart, the ovary, the kidney, the spleen, the brain, the mammary gland; while the thymus and the liver show lower levels of miRNA expression.

The anatomy and the structure of the lung has some analogies to the mammary gland that could explain the significant expression of the miRNAs detecred in mammary gland in this organ.

Like the mammary gland and kidney the lung is a branched organ in which tissue-specific mechanisms, some of them unique for the function of the organ and some other common to different organs, govern the branching morphogenesis. In the lung the epithelial tissue and its development, like in the mammary gland, has an important role and these observations could suggest a potential cell-type specific role for these miRNAs.

Figure 5. Images of two Northern blot, for miR-16a and miR-26a and expression data of let-7c, miR-26a, miR-24a, miR-30b, miR-16a and miR-145, in ten different mouse organs, after quantifications of Northern blot data. Each column, and the relative error bar, represents the average value (counts per minute) between two individuals.



IV-IV Detecting miRNA cellular origin

The miRNAs expression profiles in the developing MG demonstrate a temporal control of miRNA expression, that could show indirectly a possible miRNA regulatory role on the succeeding stages of MG development.

The MG is composed of two main tissue-compartment : the stroma, or 'fat pad', constituted by different cellular types, such as fibroblast, adipocytes and cells of connective tissue, and the epithelial tissue.

In order to get deeper insight about the expression of the miRNAs in MG it was examined their cellular origin.

The aim of the experiment was to discover if the expression of miRNAs is specific of one of the two main tissue-compartment.

miRNA expression was measured by Northern blot in normal MGs and in MGs where all the epithelial tissue was removed, such as the ‘clear fat pad’ MGs.

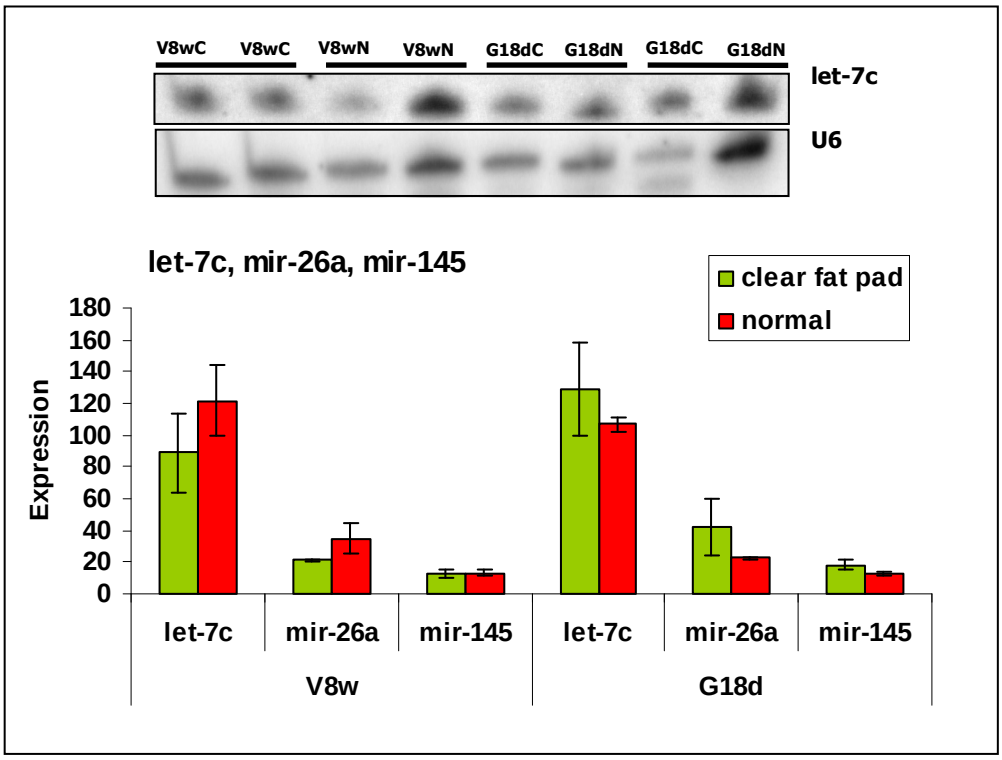
Mice at the stages of virgin were operated at one of the two mammary glands of the fourth couple before the development of the epithelial tissue. The operated mice enter normally in the reproductive cycle and the RNA of their fourth couple of glands, whose one is normal and one is operated, was extracted at the stages of virgin 8 weeks.

A preliminary analysis was effected at the stage of virgin 8 weeks and pregnancy 18 days to test the expression of let-7c, miR-26a and miR-145 (figure 6).

In the stage of virgin the observed difference in expression levels between clear fat pad and normal MGs for let-7c and miR-26a is not significant, while the expression of miR-145 does not show variation between the two MGs. In the gestation the levels of miRNA expression are higher or equal in the clear fat pad MGs comparing to the normal MGs.

These results demonstrate that miRNA are expressed in the stroma of the mammary gland, without excluding their expression in the epithelial tissue. There are evidences (results not published) of miRNA expression *in vitro* in mouse mammary epithelial cell lines (HC11). Moreover the expression profile of all the miRNAs analyzed show that there could be an association between the inhibition and activation of miRNA expression, at the lactation and involution, respectively, and the simultaneous proliferation /differentiation and apoptosis of epithelial cells in the corresponding stages.

Figure 6. Image of the Northern blot for let-7c and expression data of let-7c, miR-26a and miR-145, represented like column after normalization of Northern blot data, in clear fat pad, C, MGs, and in normal, N, MGs at the stage of virgin 8 weeks and gestation 18 days. Each column, and the relative error bar, represents the average value (counts per minute) between two individuals.



IV-V Cloning new miRNA in mammary gland

The mammary gland is an evolutionary recent organ that accomplish the lactation, a unique function that characterizes mammals.

Recent evidences about organ- and tissue-specific miRNAs (Lagos-Quintana et al., 2002; Liu et al., 2004; Sempere et al., 2004; Pay et al., 2004; Frederikde et al., 2006; Ryan et al., 2006; Chen et al., 2006, Ramkisson et al., 2006; Coutinho et al., 2006; Xu et al., 2006; Gu et al., 2006) and primate-specific miRNAs (Devor, 2006) suggested the existence of miRNAs specific of the MG, other than the conserved miRNAs already noted in the microRNA registry and/or discovered in other species.

For this reason libraries of miRNAs extracted from mouse mammary gland at some stages of its development have been built. These libraries were screened in search for inserts corresponding to reliable miRNAs; ultimately different approaches were used to validate these 'candidate miRNAs', describing their expression in mammary gland and their maturation from the potential precursor *in vitro.*

The cloning protocol was performed on RNA samples of mammary glands of mouse at the stage of virgin 8 weeks, gestation 2-, 6- and 18-days and involution 1-day, in order to cover as much as possible the cycle of MG development.
Between the colonies potentially transformed, 340 of them (13,6%) resulted positive to a PCR test for the insertion in the vector of an insert of the attended size (70 nucleotides).
The inserts of these 340 colonies were sequenced.
The nucleotide sequences were analyzed and, after a preliminary alignment (CLUSTAL W, attached 1), which was performed to identify the redundant sequences, 88 clones (26% of the total sequenced), whose 24 were redundant, showed an insertion fragment of the characteristic length of miRNAs (19 to 25-27 nucleotides) (table 6).
In the multiple alignment some fragments have very similar sequence (90% of identity) between them, but all the inserts having at least 1 nucleotide different were considered like independent fragments. Many fragments share a significant part (25-90% : 5-18 nucleotides on an average sequence of 22) of the sequence, positioned in the beginning, in the center or in the end of the sequence.
In the microRNA registry miRNAs with identical mature sequence or with sequences different for one or two nucleotides have the same name, even if they are cloned from different species or if they originate from separate genomic loci. These miRNAs are grouped in the same family, thus it could be possible to consider only a small number of the inserts cloned belonging to the same family.
The inserts with more different sequences, but which share an homology of 8-18 nucleotides could have sequence similarity in the hairpin portion of the primary transcript and could originate from the same hairpin precursor, for example from the two arms of it, like it is reported in literature (Lau et al., 2001; Lagos-Quintana et al., 2002).
A miRNA common target in animals could be present for all the miRNAs which have a consecutive string of 7-8 nucleotides, the ‘seed’ or ‘nucleus’ string, at the 5’end of their sequence, which is responsible of the pairing to the mRNA target.
The sequences aligned often share a ‘seed sequence’ and it could be suggested a similar role for some of them.

The 340 cloned fragments were divided in : 55 clones whose insert was extracted from the total RNA at the stage of virgin at 8 weeks, 93 from the gestation at 2 days, 54 from the stage of gestation at 6 days and 91 at 18 days, 47 from the involution at the first day (table 5).
The half of the 64 clones selected were extracted from the stage of gestation at 18 days, while the other half is divided in equal fractions in clones from the stages of virgin 8 weeks, gestation 2 days, involution 1 day, and only a minority (2 clones) come from the stage of gestation 6 days.

It is evident from these data that not all the stages contributed at the same level to the production of a cDNA library of potential miRNAs. In particular, in relations to the number of clones sequenced, a significant fraction of clones from the stages of gestation at 18 days and the involution at 1 day contains potential miRNAs, the stages of virgin and gestation at 2 days contributed at inferior level, while the stage of gestation at 6 days does not contribute substantially to the construction of miRNA libraries.
It is not known why most part (25 and 33%, respectively) of the 64 clones derived from the stages of gestation 18 days and involution 1 day, and only in modest part from the stages of virgin and gestation 2 days. It is possible to suppose that miRNAs are more abundant in late gestating and involuting mouse mammary glands, but it is also important to note that the cloning protocol was not applied at the same time for all the samples, increasing an already high number of variables present in this long and difficult technique.

Table 5. Distribution of the number of sequenced clones, including the redundant and the clones bringing the insertion fragment of the correct length, and the relative percentage of this category in each of the stage of extraction.

Stage	N°clones sequenced	N° clones inserts of 19-27 nt	%clones relative to 64
Virgin 8w	55	8	11%
Gest. 2d	93	12	19%
Gest. 6d	54	2	3%
Gest. 18d	91	30	48%
Invol.1d	47	12	19%
Total	340	64	100%

The remaining sequenced clones (252) are : 180 clones (53% of the total sequenced) that contain only primers used for the cloning; 65 clones (19.1% of the total sequenced) that contain short inserts of 12-18 nucleotides, that could be RNA fragments coming from degradation of RNA transcripts or ribosomal, like it was verified for some of them blasting their sequence in the mouse genome database; 2 clones that have a longer (35-40 nt) insertion fragments (0.6% of the total sequenced) and 5 clones (1.5% of the total sequenced) that contain a fragment not sequencable.

IV-VI Validating potential miRNAs

Different approaches have been described in literature to confirm a miRNA, based on the definition of its most important features. The following characteristics need to be demonstrated : the miRNAs should have a precursor of 60-80 nucleotides, with a typical imperfect stem-loop structure in which the miRNAs is located in the stem part of one of the two arms (1); a miRNAs should be expressed like a transcript of 19-25 nucleotides

(2); the miRNA should be matured from the stage of miRNA precursor (pre-miR) by the activity of Dicer enzyme in the cytoplasm (3).
It was planned a combinatorial strategy, in part using bioinformatics tools and in part experimental, to confirm some of the fragments cloned like candidate miRNAs.

IV-VI-a Evaluating the precursor secondary structure

A necessary requirement to confirm a short RNA sequence as a miRNA is the detection of the typical pre-miR having a particular length and secondary structure.
An *in silico* analysis was performed for each of the 64 cloned inserts to localize them in the mouse genome and to find the presence of a miRNA precursor in this identified genomic region.
First the sequences of these cloned fragments were blasted in the murine EST database, to search if they are part of studied genes. Secondly the sequences were searched in the miRNA registry, to know if they are already annotated and discover eventual similarities or identities (table 6).
In the EST-mouse database the 64 fragments matched, with higher or lower similarity, inside cDNA clones of different kind of mouse cDNA library.
A second analysis in the miRNA registry was effectuated to verify that the cloned fragments were not miRNA already known and annotated and to search eventually similarity with others miRNAs.
Blasting the short sequence of each insertion fragment the software provided a list of the most similar miRNA present in the registry. In the table is reported for each cloned inserts the most similar match found, and the sequence homology to the corresponding miRNA.
For 24 fragments (37% of the total) the software did not find any match or found some miRNA that were weakly similar to the fragment of interest (sequence homology < 50%), that were not inserted in the table.
For 40 fragments (63% of the total) it is reported a match with a miRNA, even if the degree of sequence homology is highly variable, ranging from the 50% to the 86%, with a 100% of identity for G18n273 and V11 (from the stage of gestation 18 days and virgin 8 weeks), respectively to the sequence of let-7b and let-7c, two ubiquitous miRNAs belonging to the same family, finding which constituted a positive control of the cloning protocol.
Amongst the 40 more interesting fragments 14 (33%) match with mouse miRNAs, 16 (41%) with humans miRNAs, the remaining 10 (26%) match with miRNAs of different species, such as *Drosofila* or *Caenorabditis elegans* and others.
Some fragments with similar sequence match with the same miRNA.

The cloned fragments were mapped in the mouse genome using the public mouse genome map available on the www.ensembl.org site : for each potential miRNAs were annotated all the genomic localization corresponding to the highest percentage of probability (table 6).
It was considered the chromosome of localization, the genomic sequence in which they are embedded, the position relative to the gene surrounding and/or the exact position inside a gene, in exons or introns.

Table 6. Names, sequences, stage of origin, length in nucleotides, match with known miRNAs and corresponding percentage of sequence homology, chromosome localizations and mapped positions inside genes of the 64 non-redundant inserts cloned.

Insert name	Sequence	Stage	N°nt	miRNA registry answer	Homology with miR	Chromosome localizat.	Belonging to known genes	Intragenic position
V817	TATGGAGACAGATGGCAGG	Virg. 8w	19	mmu-miR-422b	63%	/	/	/
V119V8s	CCTAGCTCTCTGTCGGGGTGTCG	Virg. 8w	23	/		/	/	/
V11	TGAGGTAGTAGGTTGTATGGTT	Virg. 8w	22	mmu-let-7c	100%	chr. 15, 16	mmu-let-7c-1, 2	exon
V819	AAAGGGTGTGGGTCAGGTTAAAA	Virg. 8w	23	/		/	/	/
FLP_40s	GCGCCCAAAGGTTCCCTCAGAAC	Virg. 8w	24	mmu-miR-351	58%	/	/	/
FLP_46s	TGGTGCTCTTGACTGAGTGTCTCGG	Virg. 8w	25	/		/	/	/
FLP-51	AGGGTTCGTGTCCCTTCGTGGT	Virg. 8w	22	/		/	/	/
LSI-3s	AACGGGCACCCTCACTAAA	Virg. 8w	19	hsa-miR-659	68%	/	/	/
G217	CATTATTAGCTTTTGGTACGCG	Gest. 2d	22	mmu-miR-126-5p	86%	/	/	/
G23nuo	CCGTGACGGGTCGGGTGGGT	Gest. 2d	20	/		/	/	/
G2n22	GGCGGACGGCGGGAGAGGG	Gest. 2d	19	/		/	/	/
Gd43p5	TCACATCGCGTCAACACCCGCC	Gest. 2d	22	/		chr. X, 2, 3, 18	Not known, gene Camk1d; not known,	no exon
Gd43p4	GCACCACCACCCACGGAATC	Gest. 2d	20	/		/	/	/
Gd43p2	TTCCACTCGGCCACCTCGTC	Gest. 2d	20	/		/	/	/
Gd43p1	CCCCCGGCCCCGCCCGCGCG	Gest. 2d	20	mmu-miR-671	70%	/	/	
Gd43p3	GGCCCCCACCCCCACGCCCGCC	Gest. 2d	22	hsa-miR-638	63%	/	/	/
G28	CCCGGGGCCGCAAGTGCGTTCG	Gest. 2d	22	/		/	/	/
Gd19	ACATGAAGTGCGAATGCTGAC	Gest. 2d	21	ebv-miR-BART2		/	/	
G1	GCCCCCGGCCGTCCCTCTT	Gest. 2d	19	mmu-miR-714	74%	/	/	/
Gd8p	TTCGGGCCCCGCGGGACACTC	Gest. 2d	21	hsa-miR-663	62%	chr.6	not known	no exon
Gd6	CGGGGAGCCCGGCGTGTGCCGGC	Gest. 6d	23	hsa-miR-638	74%	/	/	/
LSIG636	CGTCGGGGGTGATCCGCTCTGA	Gest. 6d	22	mmu-miR-712*	55%	/	/	/
G18n273	TGAGGTAGTAGGTTGTGTGGTT	Gest. 18d	22	mmu-let-7b	100%	chr. 15	ncRNA predicted	exon
G18n13	GAAAATCCGGGGGAGAGGGT	Gest. 18d	20	mghv-miR-M1-2	55%	/	/	/
B30G18s	AATGTAGGTAAGGGAAGTCGGC	Gest. 18d	22	/		/	/	/
B40G18s	AAGAGGGACGGCCGGGGGC	Gest. 18d	19	mmu-miR-714	74%	/	/	/
LSIG1818u	TCCGAAGGGACGGGCGATGGC	Gest. 18d	21	/		/	/	/
G18242	TCGGGGGGGCCGGCGGCGGCGGC	Gest. 18d	23	hsa-miR-638	57%	/	/	/
G1871	GGGGTCGGCACGCGGCAGCGG	Gest. 18d	21	osa-miR-531	67%	/	/	/

G1828	GGGGGAGGGAGGC GGAGGG	Gest. 18d	19	/		/	/	/
G1814p 1	ACGACGGGGCCCCG CGGGG	Gest. 18d	19	hsa-miR-663	74%	/	/	/
G1812d	GGGCCCCGCGGCGA CACTCAGCT	Gest. 18d	23	hsa-miR-664	61%	/	/	/
G1814p 2	CGCTTCGGGCCCCG CGGGA	Gest. 18d	19	hsa-miR-665	63%	/	/	/
G18n27 2	TAAACGGGTGGGGT CCGCGCA	Gest. 18d	21	/		/	/	/
A47G18 s	AGAGCTGGAGGTGT CCCGGTGT-	Gest. 18d	22	ame-miR-317	68%	/	/	/
G18	AAGCCTACAGCACC CGGTA	Gest. 18d	19	/		/	/	/
G1821d	CATCGCGTCAACAC CCGCC	Gest. 18d	19	/		chr. X, 2, 3, 18	not known; chr. 2: gene camk1d	intron
G18302	AGTCTGGTGCCAGC AGCCGC	Gest. 18d	20	/		chr. 6, chr. 17	not known	exon
G18212	CTCGGGCCGATCGC ACGCC	Gest. 18d	19	mmu-miR-714	63%	/	/	/
G18n30	AAACGGCGCCCATC TC-CGCCAT	Gest. 18d	22	mmu-miR-674*	59%	/	/	/
LSIG182 u	GCTCGCCGAATCCC GGGGCCGAGG	Gest. 18d	24	rlcv-miR-rL1-1	50%	chr. 17	gene: mediator of DNA damage checkpoint	intron
G1836	TTTTGCCGACTTCCC TTACCTACATT	Gest. 18d	26	/		chr.17	gene Mdc1	no exon
G1810	GTCTTGGGAAACGG GGTGC	Gest. 18d	19	kshv-miR-K12-1	58%	not known, chr 9, chr 13, chr 9	not known, chr 9: gene Itga9; chr.9: gene RIKEN	intron
G1812	AGAGGTCTTGGGGC CTGAAAC	Gest. 18d	21	hsa-miR-635	62%	/	/	/
G1831	CTGGGTGTTGACTG CGATGTG	Gest.18 d	21	/		/	/	/
G1833	GGGTGCGAGAGGTC CCCGGGTTC	Gest. 18d	23	mmu-miR-712*	61%	/	/	/
LSIG1u S	AGTGGTGGTGGCGC GCGGG	Gest. 18d	19	cel-miR-251	74%	/	/	/
FLP_20s	TCCCGGGGAGCCCG GCGGG	Gest. 18d	19	hsa-miR-596	79%	/	/	/
FLP_26s	CGGGGGGGCCGGC GGCGGCG	Gest. 18d	20	hsa-miR-638	65%	/	/	/
FLP_33s	GTCCCGCGGGGCCC GAAGCGTT	Gest. 18d	22	hsa-miR-663	59%	chr. 6, chr. 17	not known	intron
FLP_22	AAACGGGTGGGGTC CGCGC	Gest. 18d	19	/		/	/	/
FLP_31	GGCGGGTGTTGACG CGATGTGA	Invol.1d	22	/		chr. X, 2, 3, 18	not known, gene Camk1d, not known,	intron
LSII15	CGCGGCGTGGGAAA TGTGGCGT	Invol.1d	22	osa-miR-808	50%	not known	not known	intron
LSII39u	TGGGTGGTTCAGTG GTAGAATTCTCGC	Invol.1d	27	mmu-miR-183	52%	chr.:1,6,13,2,3,7,3,, 8,10,17	often not known gene; chr. 3: Trim2; chr.7: Nalp4a; chr.8: Mrc I; chr. 10: Kit 1; chr.8: Vac 14;	intron
I210I1s	ATAATTTGTGGTAGT GGGGGAC	Invol.1d	22	/		chr.12, 2, 13, 3, 10, 18, 1, 19, 3, 4	chr.13: gene Cdyl; chr. 3: Cyp7b1; chr.4: U1 spliceosomal RNA; chr.18: gene U1, Fcgr3a; chr.3,19,6,4: gene U1;	all in exons
LSI-8s	CGTGGGGGTGGGGG CCGTCAACT	Invol.1d	23	hsa-miR-638	57%	/	/	/
I228I1s	AGTCAGCGGAGGAA AAGAAACTAAA	Invol.1d	25	hsa-miR-511	60%	not known	not known	intron
LSII26us	AAGGGAACGGGCTT GGCGGAAT	Invol.1d	22	/		/	/	/
I116I1s	AAGAGGGCGTGAAA CCGTTAAGAGGTA	Invol.1d	27	hsa-miR-519b	52%	chr.5, 1, 3, 2, 16	gene Cdk8,not known, not known, Abi1, not known	intron
LSII7u	AGCTGCGCTGCTCC TGGTAACTGC	Invol.1d	24	dme-miR-275	63%	chr. 4	gene: novel kelch domain containing protein	intron

LSI-11s	TCGGTCGCGTTACC GCACTGGACGCCTC	Invol.1d	28	/		chr. 11	gene: XM_894976.1	exon
LSII11u	AAGGAGCCTAACGC GTGCGCGAGTC	Invol.1d	25	dre-miR-125c	48%	chr.17, chr.16	not known	intron, not exon
LSII45u	TGAGTGTCCCGCGG GGCCCGAA	Invol.1d	22	hsa-miR-663	59%	/	/	/
I120I1s	GCCGGCGGGAGTCC CGGGGAGA	Invol.1d	22	mmu-miR-711	55%	/	/	/

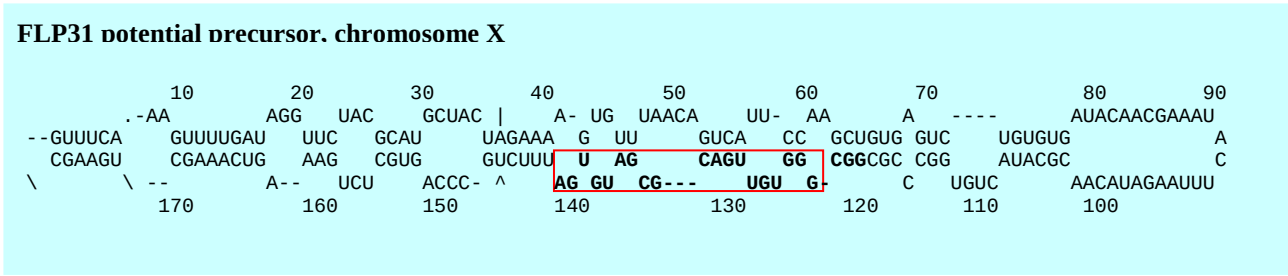
18 of the 64 cloned inserts (28.6%) were mapped and were found dispersed in all the chromosomes of the mouse genome except the chromosome 14 and the chromosome Y.
The 18 cloned insert which have high homology to specific genomic region show sometimes more than one genome localizations. Clones with highly similar sequence mapped in the same positions.
Half of the mapped clones are localized in known gene, while the other half are in genomic regions not studied.
It could be intriguing to analyze the meaning of the genetic localization of the inserts: the genes where the inserts mapped are sometimes correlated to the regulation of the cell cycle, to transmembrane protein, to nuclear receptor, and others…One of the inserts results part of U1 gene, that codes for a spliceosomal RNA (I210I1s) and only G18n273, actually let-7b, results to be located in a genomic fragment known for the presence of non-coding small RNA, V11, let-7c, was correctly mapped on this miRNA gene.
The 18 mapped insert are divided in : 2 insert deriving from the stage of gestation 2 days, 7 from gestation 18 days, 9 from the involution at the first day, no one from the stage of virgin.

After having mapped the cloned inserts, it was searched, for all the most probable genomic positions of the potential miRNAs, the presence of a miRNA precursor in the genomic region of 120 nucleotides flanking the cloned fragments.
The 'mfold' software was used (www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi). 'mfold' software is able to predict the secondary structure of a given RNA sequence showing all the most probable conformations and their thermodynamic stability, giving the values of free energy of each conformation.
10 of the 18 potential miRNAs mapped (table 7), are located in a genomic region that is able to fold in a structure that has the features of a miRNA precursor : an hairpin structure of at least 60-70 nucleotides constituted by two strands pairing imperfectly, flanked by a large loop (longer than 5-6 nucleotides), which contains the potential miRNA in the linear part of one of the two strands (figure 7). For the cloned insert that mapped in more than one position in the genome sometimes more than one typical miRNA precursor was observed, each coming from a different chromosome.

Table 7. Data coming from the mfold analysis of the genomic region where each cloned insert mapped. Name of the inserts, and stage of origin, for which it was found a miRNA precursor in the genomic region in which they are embedded. Number of precursor found for each insert and their chromosome localization.

Name	Stage	Number of precursor	Chromosome localization
LSII15	Involution 1d	1	chr.17
LSII39	Involution 1d	7	1, 6, 13, 7, 3, 8, 10
Gd8p	Gestation 2d	1	chr. 6
FLP33	Gestation 18d	2	chr.6, 17
FLP31	Gestation 18d	1	chr. X
I116I1	Involution 1d	3	chr.3, 2, 16
Gd43p5	Gestation 2d	2	chr. X, chr. 18
LSII11u	Involution 1d	1	chr. 16
G1836	Gestation 18d	1	chr.17
G1821	Gestation 18 d	1	chr. X

Figure 7. Example of one secondary structure observed for FLP31on the chromosome X with the
typical conformations of a miRNA precursor. The position of the potential miRNA (FLP31) is
indicated by the red rectangle.



IV-VI-b Searching for miRNAs expression

After having identified *in silico* a potential miRNAs precursor for a group of 10 cloned fragments, their expression in mouse mammary gland was searched experimentally to validate them like it is proposed in literature reference.

The expression of 5 potential miRNAs was evaluated by Northern blot analysis in RNA samples of mammary gland at the same stages from which they were cloned : virgin 8 weeks, gestation 18 days, involution 1 day, using like probes the complementary sequences of the corresponding 5 potential miRNAs.

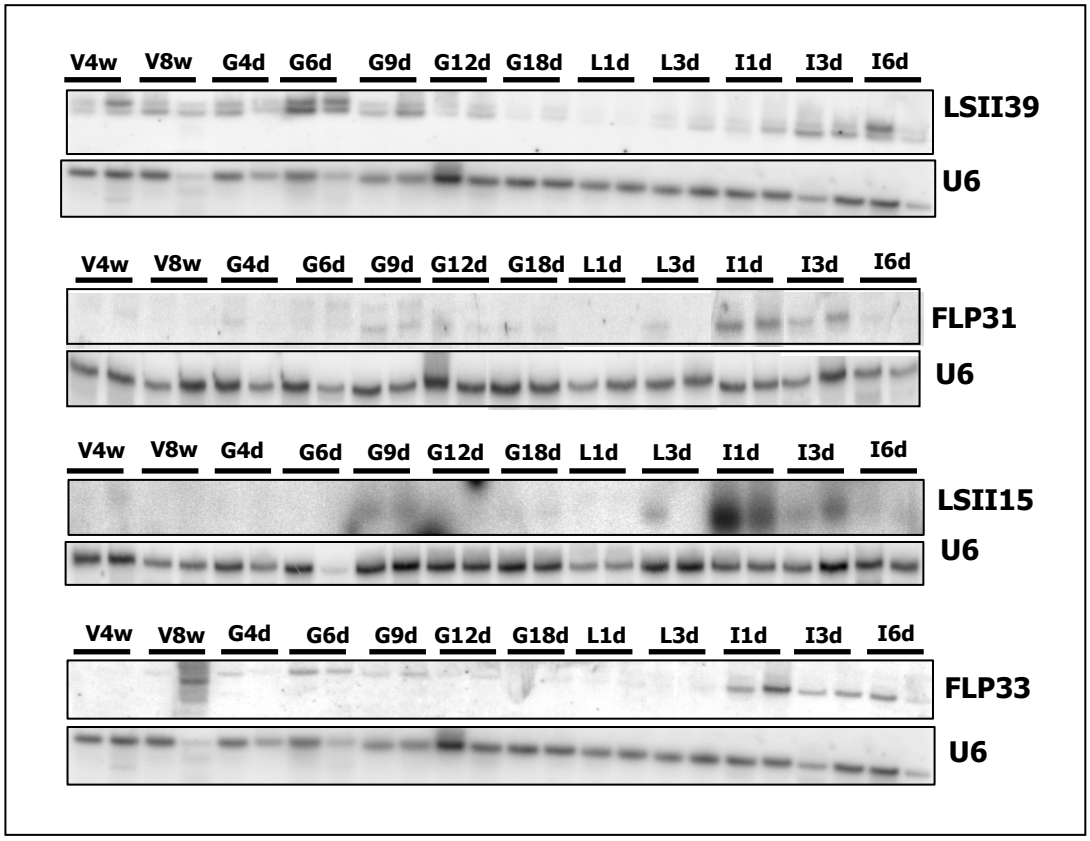
All the cloned fragment tested, LSII39, FLP31, LSII15, FLP33, I116 (table 8), showed the detection of a band of the size attended that was more evident in the stages of involution and gestation.

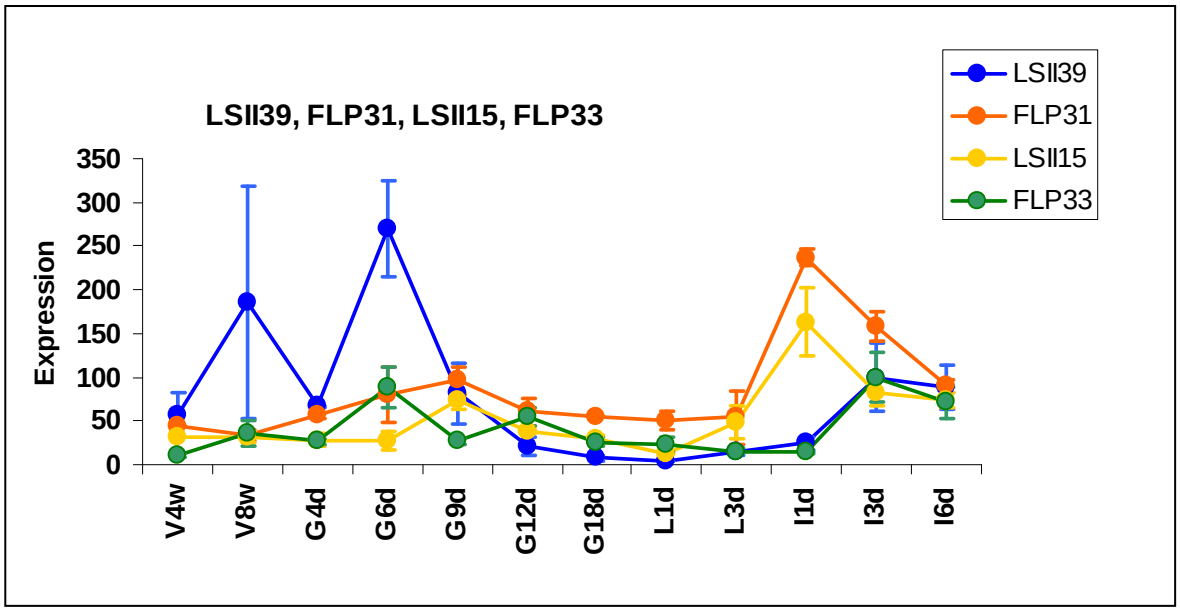
Table 8. Name, stage, sequence and complementary sequence used like probes of the 5 cloned fragments whose expression was tested by Northern blot.

Name	Stage	Sequence	Sequence of probes
LSII39	Involl. 1d	TGGGTGGTTCAGTGGTAGAATTCTCGC	TGGGTGGTTCAGTGGTAGAATTCTCGG
FLP31	Gestat 18d	GGCGGGTGTTGACGCGATGTGA	TCACATCGCGTCAACACCCGCC
FLP33	Gestat 18d	GTCCCGCGGGGCCCGAAGCGTT	AACGCTTCGGGCCCCGCGGGAC
I116I1	Involl. 1d	AAGAGGGCGTGAAACCGTTAAGAGGTA	TACCTCTTAACGGTTTCACGCCCTCTT
LSII15	Involl. 1d	CGCGGCGTGGGAAATGTGGCGT	CGCGGCGTGGGAAATGTGGCGT

Later the expression of the 5 potential miRNA was characterized by Northern blot analysis in total RNA samples during the development of mouse MG (figure 8).
The expression profile was produced only for four potential miRNA : LSII39, FLP31, FLP33 and LSII15. For I116 many bands were observed and the quantification of the expression was not possible.
The expression profile obtained for these potential miRNAs was not similar to the previous ones of the first 10 known miRNAs analyzed and even if there are common characteristics each potential miRNA has its typical profile that shows different levels of expression at different stages, denoting a characteristic regulation of expression and eventually a specific role.
The level of expression is not high during the stages of virgin, in the early-mid gestation the expression increases, for LSII39 and FLP33 the pick of intensity is at the day 6, for FLP31 and LSII15 at the day 9; in the late gestation and during the lactation the expression decreases, like it happens for the first miRNA studied, reaching a minimum value at the first day of lactation for LSII39 and LSII15, while for FLP33 and FLP31 the levels of expression does not change between the first and third day. In the involution the levels of expression are higher, even if for FLP31 and LSII15 the values reach the maximum pick of the profile at the first day of involution and later progressively decrease, while for LSII39 and FLP33 at the day 1 of involution the expression is still low and in the last two stages increases, without reaching the levels of FLP31 and LSII15.

Figure 8. Images of Northern blot and expression profiles during the development of mouse mammary gland of 4 potential miRNA cloned, after normalization and quantifications of the Northern blot data. Each point, and the relative error bar, represents the average miRNA expression between two individuals.





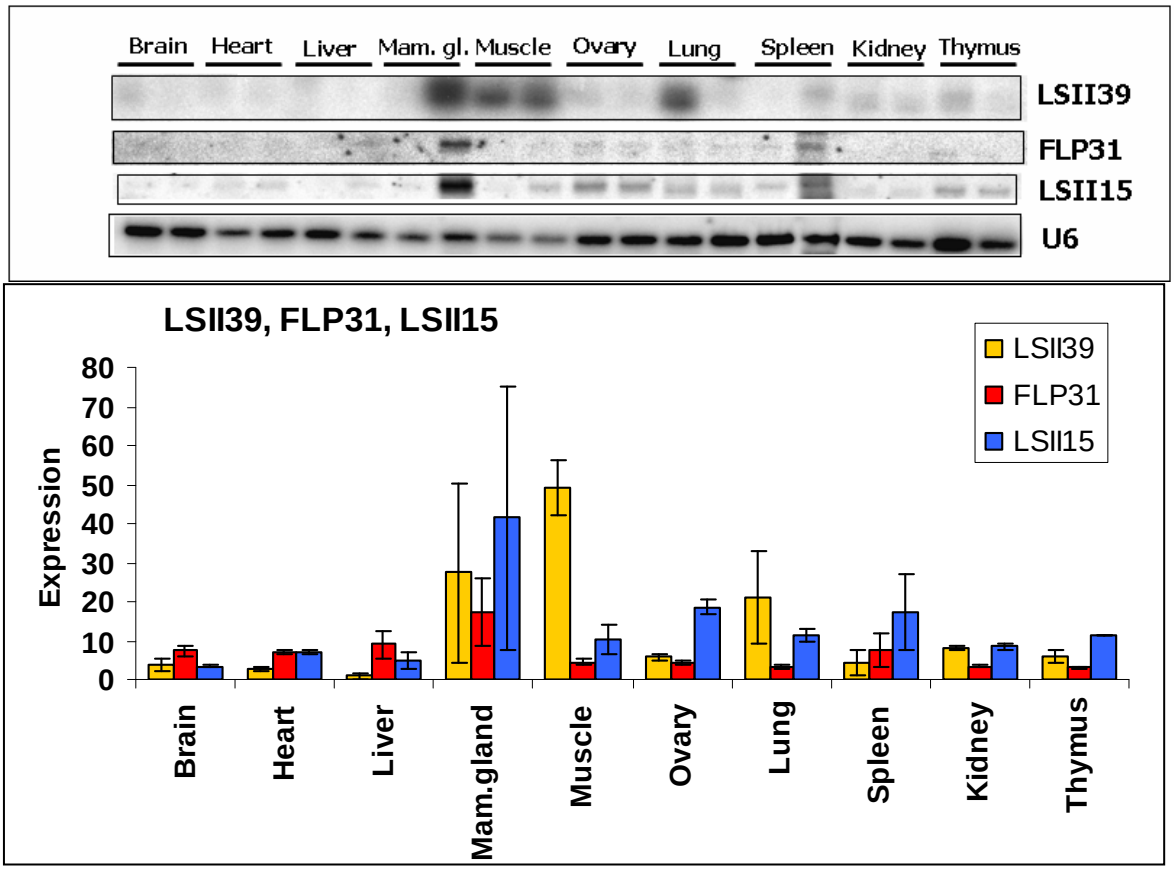
The aim of the experiment was to discover miRNAs specific of the mammary gland.

The mammary gland specificity was examined analyzing the expression of the potential miRNAs in nine mouse organs, other than in mammary gland : total RNA samples were extracted and analyze from brain, heart, liver, lung, muscle, kidney, ovaries, spleen and thyme, like it was performed for the expression characterization of the 10 known miRNAs studied. The resulted bands were quantified and the corresponding histograms were produced for LSII39, FLP31 and LSII15 (figure 9).

The expression profiles showed that LSII39, FLP31 and LSII15 are highly present in the mammary gland comparing to the other organs, even if LSII39 is strongly expressed also in the muscle.

The profile of LSII39 shows a big difference in expression between the mammary gland, muscle and lung, and the other organs. FLP31 is most expressed in mammary gland, while the level of expression in brain, heart, liver and spleen is half-reduced and is some more reduced in the others organs. LSII15 is most expressed in mammary gland and its level is significantly higher comparing to ovary and spleen, where it is half-reduced, and to the others organs, where the levels is even lower. These expression profiles are different from those of the known miRNA studied and show a modest degree of expression specificity for the mammary gland.

Figure 9. Images of Northern blot and expression profiles of LSII39, FLP31 and LSII15 in nine mouse organs and in MG, after normalization and quantifications of the Northern blot data. Each point, and the relative error bar, represents the average expression between two individuals.



IV-VI-c Testing miRNA maturation

A validation of the potential miRNA precursor was performed *in vitro.* COS cells were transfected with a plasmid containing a strong promoter (CMV) and the miRNA precursor sequence in order to measure the potential miRNA expression .

For each miRNA analyzed, the potential precursor have been cloned in the 'sense' direction, and in the 'antisense' direction,

The total RNA extracted from : cells transfected with a plasmid containing the 'sense' precursor sequence, cells transfected with a plasmid containing the 'antisense' precursor sequence and from cells non transfected was analyzed by Northern blot with probes complementary in sequence to the miRNA cloned and to the the miRNA*, to detect an over-expression of one of these molecules comparing to the non transfected cells.

The expression of 3 potential miRNA have been analyzed in transfected cells : LSII39, FLP31 and LSII15 and let-7b was examined as positive control of the technique (figure 10).

The expression of the corresponding miRNA and miRNA* have been visualized and measured in cells transfected with the plasmid containing the sense and antisense precursor sequence after normalization with the probe U6.

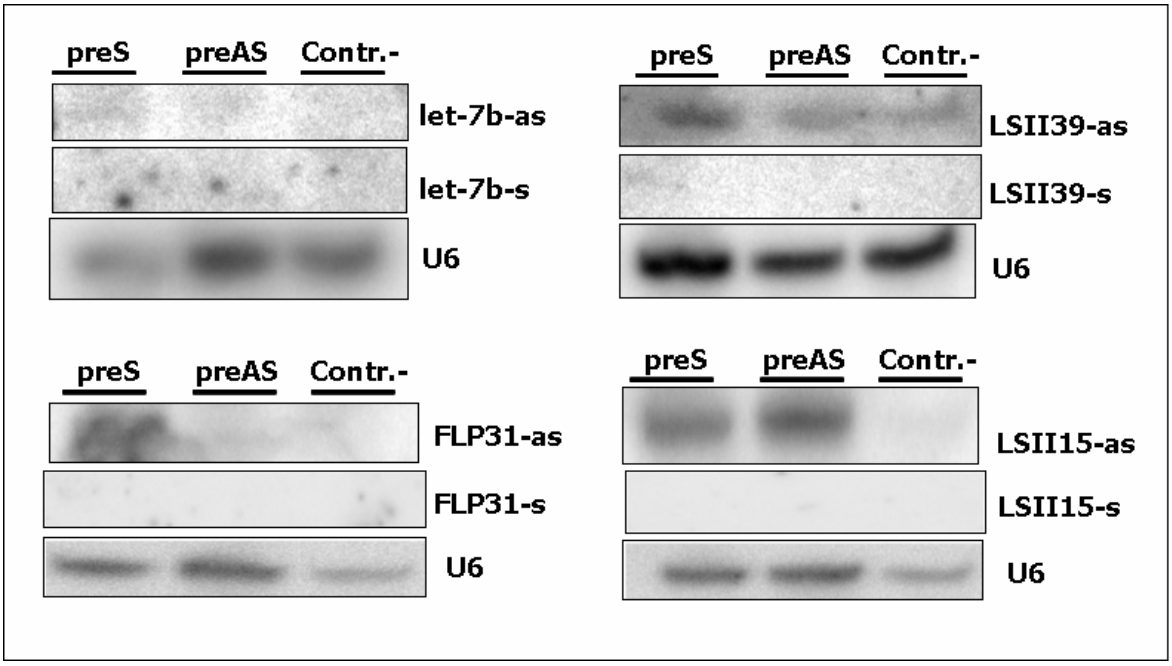
For let-7b the levels of expression of the miRNA is augmented in cells transfected with the 'sense precursor' comparing to the cells transfected with the 'antisense precursor' and to the non transfected cells, considered the negative control. Let-7b* is not expressed at a sufficient level both in cells transfected and non transfected to quantify its expression and to produce a graph of comparison.

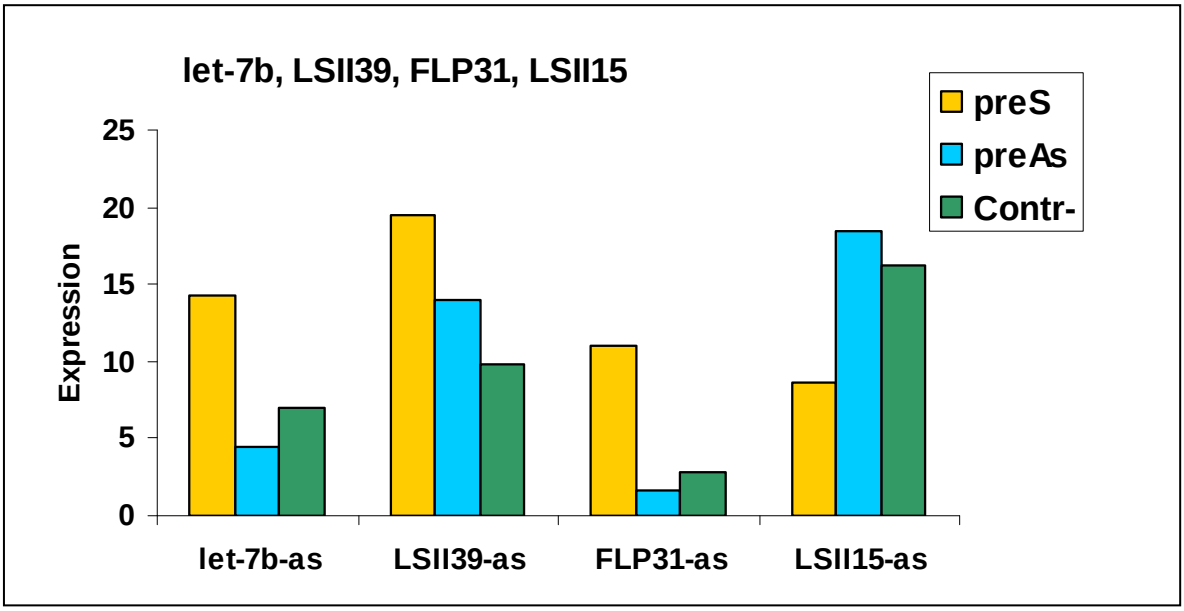
LSII39 and FLP31 are over-expressed in cells transfected with the 'sense precursor' comparing to the cells transfected with the 'antisense precursor ' and to the non transfected ones. No expression of LSII39* and FLP31* was detected in any kind of cells.

LSII15 was not found over-expressed in cells transfected with the 'sense precursor' comparing to the non transfected ones, but it was found expressed at comparable values in cells transfected with the 'antisense precursor' and in non transfected cells. The presence of LSII15* was not detected.

In conclusion between the 3 potential miRNAs tested it was found for 2 of them a band of the attended size and their expression was higher in cells transfected with the potential precursor sequence. The positive control, let-7b, validated the technique. It was thus shown that the this 2 candidate miRNAs are matured from the precursor transcript, like it is attended in the last stage of the biosynthesis of miRNAs.

Figure 10. Images of Northern blot and expression comparisons of 3 potential miRNA, LSII39, FLP31, LSII15 and of let-7b of the corresponding miRNA* in cells transfected with the 'sense precursor', (preS), the 'antisense precursor', (preAS), and in non transfected cells, after normalization with the probe U6.





V-Conclusions

V-I State of art about miRNA involvement in mammary gland

The study of the tissue-specific miRNA expression signatures began few years ago in mouse and humans (Lee and Ambros, 2001; Lagos-Quintana et al., 2002; 2003; Lim et al., 2003; Krichevsky et al., 2003; Liu et al., 2004; Nelson et al., 2004; Thomson et al., 2004; Sun et al., 2004; Miska et al., 2004; Sempere et al., 2004; Babak et al., 2004; SmiRnova et al., 2005; Monticelli et al., 2005) with the development of techniques and tools like tissue-specific cloning, microchip containing oligonucleotides corresponding to known miRNAs, and using Northern Blot analysis and real-time RT-PCR.

It was found that in some tissues or in some organs, or in a precise physiological or pathological state of a tissue, one or a group of miRNAs is exclusively expressed or differentially over-/under-expressed comparing to the expression levels of all the others studied miRNAs analysed : for example the miR-1 family in heart, the miR-122 family in liver, miR-124 variants in brain, etc...

In humans the tissues of the eye (Ryan et al., 2006), the brain, the lung, the liver, the skeletal muscle (Sempere et al., 2004), the haematopoietic cell (Ramkissoon et al., 2006), the pancreatic endocrine cells (Poy et al., 2004) etc.. has recently been analysed, while with the development of recent genome sequence assembly of other mammalian species of economical interest, like for example chicken and bovine, new tissue-specific miRNAs have been discovered (Xu et al., 2006; Coutinho et al., 2006).

The research of tumour-specific miRNAs has attracted more attention and many works dealing about differential miRNA expression in normal to cancerous tissue or cell lines have been published (Calin et al., 2002; Michael et al., 2003; Takamizawa et al., 2004; He et al., 2005 ; O'Donnell et al. 2005; Iorio et al., 2005; Lu et al., 2005; Ciafre et al., 2005; Chan et al., 2005; Calin et al., 2005) most of all focusing on humans.

Among the tumours analysed the breast cancer was investigated as well. It was proved that the miRNA were present in genomic regions involved in human breast cancers (Calin et al., 2004), that some miRNAs showed differential expression in human breast cancer cell lines (Jiang et al., 2005; Scott et al., 2006; Hossain et al., 2006) and in human cancer breast tissues (Iorio et al., 2005; Zhang et al., 2006; Mattie et al., 2006; Volinia et al., 2006; tsuchiya et al., 2006).

Till now the researches of miRNA expression in normal breast tissue are few : the breast was analysed together with other human tissues using a miRNA-specific microchip (Liu et al., 2004) and a set of differentially over-expressed miRNA was identified, while in the mouse only one work compares the miRNA expression in mammary gland tissue with the miRNA expression of other mouse and human tissues (Gu et al., 2006).

V-II miRNA expression in mammary gland

In this thesis the attention was focused on the miRNAs expressed in the normal mammary gland and, for the first time, their regulation in the different stages of its development.
The work was devoted to the study of a possible miRNA involvement in the regulation of the development of this organ. The mammary gland of mouse was chosen like model to dissect its developmental cycle in several stages and investigate miRNA activity during the progression of this cycle. The mouse mammary gland consists of two main tissues, the stroma and the epithelial tissue. The epithelial compartment occupying, from the stage of gestation till the involution, an important physical portion of this organ and developing into a secretor tissue whose differentiation reflects the functional state of the organ. Considering the strong association of miRNA expression to general mechanism such as cell differentiation and organogenesis (Song and Tuan, 2006) it was suspected to discover different expressed miRNA at different physiological and functional states of this organ.
In total the 40% of the miRNAs tested, 10 over the 25, showed a detectable expression by Northern blot analyses.
Moreover the most part of miRNAs found to be highly expressed in human breast by microarray analysis (Liu et al., 2004) are also detectable in mouse breast, demonstrating a similar miRNA activation in the two species. The efficacy of the technique used is validated from the fact that the microarray data of Liu et al. were confirmed by Northern blot and this technique is normally used to verify the singular miRNA expression and the quantification of expression after a first screening of differential activity of a large group of miRNA (Ryan et al., 2006; Frederikse et al., 2006; Sempere et al., 2004; Xu et al., 2006; ec..). In this case a first experimental selection of expressed miRNA from a wide group of miRNA was avoided and the literature data were used to chose a little group of 25 miRNAs and to proceed directly towards a singular analysis of the expression levels in mammary gland.

Some miRNAs were detected at high levels (let-7a, let-7b, let-7c, miR-26a, miR-26b, miR-16a), other at weak levels (miR-24-2, miR-30b, miR-30d and miR-145). Let-7a, b, and c were highly detected, this was not surprising because the miRNA of the let-7 family are ubiquitously expressed at high level in many tissues (Lagos-Quintana et al., 2003). MiR-16a was found down-regulated in patients with B cell chronic lymphocyte leukaemia (Calin et al., 2002, Cimmino et al., 2005) and in tumour breast cell lines (Jiang et al., 2005), while in the normal mammary gland its expression results easily detectable. A deeper comparison of quantification data of miR-16aa expression in tumoral and healty mammary gland could reveal a cell-differentiation state specific mechanism of expression . Surprisingly miR-125b was not detected in mammary gland, in disagreement with recent evidences about its high expression in human differentiated cells and tissue (Lee et al., 2005; Iorio et al., 2005), including the mammary gland.

These results were compared to those (Jiang et al., 2005) of the quantification by real-time RT-PCR of pre-miRNA in 32 commonly used human cell lines, including 5 breast cancer ones. Some miRNA detected in the normal mammary gland were weakly expressed in the tumour breast cell lines, whereas others not detected in the normal mammary gland were strongly expressed in the tumour cells. This may suggests the existence of miRNAs which could be used like molecular markers of the healthy or cancerous state of the breast tissue, like it was proposed for some miRNAs in other tissues (Iorio et al., 2005). However the detection techniques used and the detected RNA (pre- versus miRNA) are different, making the evaluation of the significance of this observation difficult.

It was supposed for the 15 miRNAs not detected in the mammary gland that they could be not expressed or, that they could be expressed at not sufficient level to be detected.

All the 10 miRNAs detected were present at each developmental stage studied. This could be interpreted in different ways : these miRNAs could have no direct implication on the development and /or differentiation of the mammary gland, or the overall miRNA expression patterns observed might hide cell-type specific miRNA regulation, considering the different cell-type of the tissues composing the mammary gland.

V-III Characterization of miRNA expression profile

A potential variation of miRNA expression during different stages of development of mammary gland, according to the hypothesis of miRNA spatial / temporal control has been studied for 10 miRNAs. The profiles obtained demonstrate that the presence of each miRNA is variable along the development of the organ and it

suggests the existence of a precise control of miRNA expression. This indirectly could suggest a role for these miRNAs in regulating a set of target mRNAs with specific functions in the development of mammary gland.

The miRNAs expression profiles produced are different, someone showing stronger level variations in virgin comparing to gestation stages, but they all share a common characteristic : in the lactation the miRNA expression is always low and during involution is high.

The miRNAs analyzed belonging to the same family, like let-7a, let-7b, let-7c and miR-26a, miR-26b have highly similar profiles and very similar sequences between them, thus it is probable that they act regulating the same targets.

Among the 10 miRNAs analyzed the profiles more variable comparing to those of the let-7 and miR-26 family are those ones of miR-145 and of miR-30b and miR-30d. The different profiles of miR-30b and miR-30d, even if belonging to the same family and sharing the 80% of the sequence, is intriguing and suggests a potential different target of action.

The identification of the miRNA targets is a difficult task. The *in silico* prediction of the possible targets is not sufficient to indicate a potential candidate gene, but it can be useful only to identify a large group (till 200 genes) of candidates. It is now (Yoon and De Micheli, 2006) accepted that each miRNAs could have a large spectrum of mRNA targets and, vice versa, the activity of an mRNA could be negatively repressed by a set of different miRNAs. The non-univocal mechanism of action and the redundancy of miRNAs could explain the similarity of the expression profiles and it could indicate a common target for all the miRNAs analysed, or at least a common task to inhibit a set of mRNA during the involution and to release this inhibition during the lactation. In the mammary gland the endocrine control of some physiological states of the development it is well known. The profile of secretion of the prolactin, strictly correlated to the proliferation and differentiation of epithelial cells, seems to be inversely associated to the miRNA expression profile, thus suggesting a potential target gene of miRNAs. The activity of estrogen and of progesterone has some common features, at least in the first part of the developmental cycle till the lactaion, with the miRNA expression profile and this could suggest a miRNA synergistic mechanism during the development of mammary gland.

It could be intriguing to identify one or a set of mRNAs which show an expression profile inversed compared to that one of the miRNAs, during the mammary gland development, even if in the animals the miRNAs show more often a mechanism of inhibition of translation of mRNAs more than a degradation of the target mRNAs (Bartel, 2004), making more difficult the observations of a decrease of mRNA levels and more probable a decrease in the corresponding protein levels. In the mammary gland the genes for the caseins are highly

expressed during the lactation, as well some inhibitors of the prolactin activity, such as Stat5, are inhibited in the lactation, thus suggesting a cellular necessity to control important mechanisms of gene expression, like the inactivation of Stat5, in critical stages of the mammary gland development. The individuation of the miRNA targets is addressed to genotype-phenotype association studies in transgenic animals. Up to now it is not possible to obtain a knock-out animal for a specific miRNA, but it is possible to block the production of a set of miRNAs, blocking the activity of Dicer enzyme (Bernstein et al., 2003; Murchison et al., 2005), and over- or under-express a miRNA using, respectively, adapted construction with strong promoters or RNA silencing specific constructions (Krutzfeldt, 2006).

V-IV Analysis of organ- or tissue- miRNA specificity

It was also investigated the potential organ- and tissue-specificity of miRNA expression. Their presence in other 9 mouse organs, and the tissue-compartment of their production in the mammary gland was analyzed.

The miRNA levels of expression in brain, heart, liver, lung, muscle, kidney, ovary, spleen and thymus showed that almost all of them are not differentially over-expressed in mammary gland, with the exception of let-7c, which shows the highest expression in mammary gland.

The tissue-specificity was investigated in order to have deeper insight about miRNA expression that could help in the interpretation of their profile.

The comparison of let-7c, miR-26a and miR-145 expression in normal and ‘clear fat pad’, such as devoid of epithelial tissue, mammary glands reveals that the miRNA expression is not specific of the epithelial tissue.

The mammary gland accomplish the lactation with a milk-secretor epithelial tissue that characterizes this organ and develops during the mammary gland cycle passing through stages of cell proliferation, differentiation, apoptosis. It was supposed a miRNA expression in this tissue, and eventually a epithelial-specific expression, supported from recent findings about miRNA expression in mouse epithelial mammary cell lines (HC11, results not published) and from all the evidencespresent in the literature of cell-differentiation and tissue-specific miRNA expression. This hypothesis was not confirmed. However it is known that miRNAs are responsible of the regulation of fundamental mechanism like cell-proliferation and differentiation also acting far from the molecules that promote directly these phenomenon. Moreover the epithelial tissue and the stroma are two tissue-compartments that interact between each others and there are many evidences of molecular signals passing between them (Parmar and Cunha, 2004). It is not excluded a miRNA expression in both of the two tissues.

V-V Construction of miRNA libraries

Lactation is a late emerging function during evolution. miRNA implication in the control of such a physiological process could involve the regulation by evolutionary conserved miRNA of new genes or alternatively by cell- and stage-specific miRNA (Wienholds and Plasterk, 2005) not evolutionary conserved. The existence of new miRNA specie-specific, such as primate, was supported by recent evidences (Bentwich et al., 2005; Devor, 2006). cDNA cloning permits to identify this class of miRNAs specific of an organ or a tissue of interest in the more evoluted species, like mammals.
It was constructed the first cDNA library of mammary gland potential miRNAs following a cloning protocol (Lagos-Quintana et al., 2003)
64 non-redundant cloned fragments in the libraries have a sequence length of 19-25 nt, typical of miRNAs.
This strategy to isolate miRNAs was validated comparing the 64 potential miRNAs with the annotated miRNAs present in the microRNA registry and finding, among them, let-7b and let-7c, already detected by Northern blot in the mammary gland. All the remaining cloned fragments did not identify any other known miRNA, even if the a significant portion showed a sequence similarity of the 50 to the 86% to known miRNAs, most of them belonging to the human and mouse genome. The sequence similarity of most of them to known miRNAs was encouraging. Some of them with similar sequence matched to the same known miRNA and it confirmed the abundance of a fragment or a group of similar fragments in the cells, underlying their importance. The miRNAs which did not match to any known miRNAs were not excluded from the library of potential miRNAs because they could be not-evolutionary conserved miRNAs typical of the mammary gland.

V-VI Validation of potential miRNA

Following the examples present in the literature (Lagos-Quintana et al., 2003; Yi et al., 2005; Xu et al., 2006; Guy et al., 2006; Cummins et al., 2006) the potential miRNAs cloned were submitted to an *in silico* and to a preliminary experimental analysis in order to identify which ones between them fulfill the criteria (Wienholds and Plasterk, 2005) to be classified as newly identified miRNAs.

Their characterization proceeds mapping the potential miRNAs in the mouse genome. Only 28% of fragments (18) of the library were mapped in the mouse genome, the others were excluded because their sequence did not correspond to any known genomic sequence.

For the fractions of mapped cloned fragments it was found their chromosome localizations, the genes where they are hosted and the intragenic localization, in intron or exons. In the 80% of cases they are localized in introns and in some few cases in exons. This is in agreement with the literature data (Bartel, 2004), that show that in mammals, and in particular in humans, at least the 50% of miRNAs are localized in introns. In literature several models have been described (Hornstein and Shomron, 2006) based to experimental evidences to interpret the miRNAs mechanism of expression in association to the expression of the regulated genes : miRNAs could be coexpressed with the target gene or the target gene could be under-expressed in the tissue where the miRNA is specifically activated. MiRNAs are sometimes coexpressed with the gene to which they belong from the activity of the same promoter, but this is not an evidence that their target is the gene in which they are hosted, so that it is difficult to give a precise meaning to their genetic localizations. Often miRNAs in mammals are localized in the genome in the same cluster and these miRNAs are coexpressed (Lagos-Quintana et al., 2001; 2002; Lau et al., 2001; Lee et al., 2002) and show similar activity and target, but in this case any cloned fragment was found in cluster in the mouse genome.

Among the 18 potential miRNAs mapped in the mouse genome 11 showed *in silico* the presence of at least one characteristic miRNA precursor stem-loop secondary structure in the genomic flanking sequences. For some potential miRNAs localized in more than one positions in the mouse genome more than one typical miRNAs precursor was identified. The set of precursors obtained from the same potential miRNAs is variable in sequence, at contrary in the works of Xu et al. (2006) it is discussed the presence of 3 copies for a miRNA gene (*miR-757*) in the chicken genome, 2 of which have the same precursor sequence and structure.

The phylogenetic conservation of the identified precursor sequences was not searched in other species, while in other studies (Xu et al., 2006; Cumminis et al., 2006; Guy et al., 2006) homologous sequences have been found in human, chimpanzee, cow, mouse, rat, dog, chicken, pufferfish and zebrafish genome. In the work of Guy et al. (2006) it is proposed the existence of a rodent specific cloned miRNA on the basis of the lack of its sequence conservations in other species, supporting the idea that by cloning size selected RNAs it is possible to isolate new specie-specific miRNAs.

The ‘biogenesis’ and ‘expression’ criteria recently put forward by Ambros et al. (2003) for miRNA annotation were fulfilled for some of the 11 potential miRNA by examining their expression in mammary gland. 5 potential miRNA analyzed by Northern blot were expressed and visualized like short transcripts, for 4 of them the expression patterns

during different stages of development of mammary gland was characterized. The quantification of the level of expression demonstrate a typical profile for each potential miRNAs and variable presence in different stages, thus underlying a mechanism of expression regulation and a potential role in the developing mammary gland, like it was verified for the known miRNAs. For 3 potential miRNA the expression is high during the involution, most of all at the first day, and all of them have the lowest expression in the lactation, thus showing also a similar 'behaviour' comparing to the known miRNA found expressed in the mammary gland and confirming their reliability like new miRNAs.

The organ-specificity was verified examining the expression pattern of 3 potential miRNAs in 10 different mouse organs, included the mammary gland : even if it is not possible to define them mammary-gland specific, they are over-expressed in mammary gland comparing to the other organs, with the exception of a high expression in the muscle for one of them.

To further validate these potential miRNA an *in vitro* experiment to over-express the miRNAs, by transfecting its precursor associated with a strong promoter, was performed for 3 of the potential miRNAs. Preliminary results confirmed the augmentation of the expression in transfected cells for 2 of them, thus implicating the activation of part of the miRNA biosynthetic mechanism, in particular the activity of the Dicer enzyme, able to cut the precursor after its transcription from the plasmid, and to produce the mature form of the cloned miRNA.

V-VII Perspectives

In this work it was verified the expression of some miRNAs in normal mouse mammary gland and their temporal regulated expression could suggest a role in the development of this organ. Their expression was described also in other organs.

A cDNA library of mammary gland potential miRNA was build and four cloned potential miRNA were validated by identifying the precursor hairpin-like structure and by verifying experimentally their expression in mammary gland. Their expression profile was also characterized at different stage of the mammary gland cycle.

The temporal and spatial characterization of the expression pattern is the first step towards the understanding of the role of these molecules. The next step towards a better understanding of the known and new miRNAs function in mammary gland could be the *in situ* localization of their activity in the different cell-type of the mammary gland tissues, like it was recently done in other studies (Guy et al., 2006) with the development of new probes, locked nucleic acid (LNA) probes, for the detection of small size RNA, such as the miRNA.

An eventual miRNA cell-type specific expression could help in the selection of a set of candidate target genes both in their *in silico* predictions and in experimental studies.

The analysis of the transcriptome and of the proteome of the cells in which the miRNAs are expressed during the developing mammary gland could identify the less present transcript or protein, without the necessity of the time-consuming production of transgenic animals and/or Dicer conditional mutant animals.

The validation of other potential miRNAs present in the cDNA library and their expression characterization could add important information to go deeper in the understanding of an eventual common mechanism of actions which all the miRNAs analyzed seems to share during some stages of the development of mammary gland. The characterization of other potential miRNA is in course.

In this work we described the identification of four candidate genes starting from a miRNA library, thus depicting the mammary gland like a miRNA enriched organ, while before the attention was focused more on other organs, and only recently (Gu et al., 2006) the tissue of mouse mammary gland, together with the brain and the mouse eye, appears to be enriched of expressed miRNAs like the brain, the lung, and the human eye. However the breast is an interesting subject of study in relation to the appearance of the cancerous pathologies, being the breast tumor one of the most frequent cancer in the woman and one of the most studied. Further understanding of the synergistic interaction of the miRNAs with other regulatory pathways of gene expression in this organ will lead to more in-depth knowledge on the regulation of cellular function and differentiation, as well it can facilitate the development of clinical applications and of drugs based on the mechanism of RNA interference.

Currently the study of miRNAs in mouse normal mammary gland are proceeding towards a better understanding of their biological functions.

In vitro experiments with epithelial mammary cell lines derived from rabbit are aimed to examine miRNA expression in un-differentiated cells versus differentiated cells, after the induction of this stage by hormonal stimulation.

The microinjection technology applied to mice give the possibility to obtain transgenic animals expressing or over-expressing a gene of interest. Currently a lineage of transgenic mouse over-expressing in the mammary gland some interesting miRNA genes, such as the gene for miR-145 or for let-7c, under the action of a strong mammary-gland specific promoter, is in preparation. The phenotype, the transcriptome and the proteome of these transgenic mice will be evaluated in search of a-typical characteristics, differential expressed molecules and different set of protein comparing to normal mice. The same studies will be performed on mice bringing a mutated allele for the Dicer gene, mutation that should interfere with the maturation of a large set of miRNAs, producing a global disruption in the miRNA mechanism of biosynthesis and relevant changes at morphological and physiological level. The study of th transcriptome will also profit of the miRNAs specific microarray now available on the market.

Attached 1. Multiple sequence alignment (CLUSTAL W) of the 64 cloned inserts selected on the bases of their nucleotide length.

```
FLP_40s          -----GGCGCCCAAAGGTTCCCTCAGAAC-------- 24
LSI-3s           -----------AACGGGCACCCTCACTAAA------- 19
Gd43p1           -------CCCCCGGCCCCGCCCGCGCG---------- 20
Gd43p3           -----GGCCCCCACCCCCACGCCCGCC---------- 22
G1               ------GCCCCCGGCCGTCCCTCTT------------ 19
G18212           --------CTCGGGCCGATCGCACGCC---------- 19
G18n30           ------AAACGGCGCCCATCTC-CGCCAT-------- 22
G1836            -TTTTGCCGACTTCCCTTACCTACATT---------- 26
Gd43p5           -----TCACATCGCGTCAACACCCGCC---------- 22
G1821d           --------CATCGCGTCAACACCCGCC---------- 19
Gd43p4           ------------GCACCACCACCCACGGAATC----- 20
G18302           ------AGTCTGGTGCCAGCAGCCGC----------- 20
G18              ---------AAGCCTACAGCACCCGGTA--------- 19
LSII7u           ---------AGCTGCGCTGCTCCTGGTAACTGC---- 24
Gd43p2           -------------TTCCACTC-GGCCACCTCGTC-- 20
LSI-11s          ----TCGGTCGCGTTACCGCACTGGACGCCTC----- 28
B40G18s          ---------AAGAGGGACGGCCGGGGGC--------- 19
LSIG1818u        -------TCCGAAGGGACGGGCGATGGC--------- 21
V817             ---------TATGGAGACAGATGGCAGG--------- 19
G1810            ------GTCTTGGGAAACGGGGTGC------------ 19
LSII15           ---CGCGGCGTGGGAAATGTGGCGT------------ 22
B30G18s          ------AATGTAGGTAAGGGAAGTCGGC--------- 22
I228I1s          --AGTCAGCGGAGGAAAAGAAACTAAA---------- 25
G217             ----CATTATTAGCTTTTGGTACGCG----------- 22
V11              --TGAGGTAGTAGGTTGTATGGTT--------------22
G18n273          --TGAGGTAGTAGGTTGTGTGGTT------------- 22
LSII39u          --TGGGTGGTTCAGTGGTAGAATTCTCGC-------- 27
LSIG1uS          ---------AGTGGTGGTGGCGCGCGGG--------- 19
LSI-8s           ---------CGTGGGGGTGGGGGCCGTCAACT----- 23
G1812            ---------AGAGGTCTTGGGGCCTGAAAC------- 21
I116I1s          --------AAGAGGGCGTGAAACCGTTAAGAGGTA-- 27
G18n272          TAAACGGGTGGGGTCCGCGCA---------------- 21
FLP_22           -AAACGGGTGGGGTCCGCGC----------------- 19
V819             --AAAGGGTGTGGGTCAGGTTAAAA------------ 23
FLP-51           ----AGGGTTCGTGTCCCTTCGTGGT----------- 22
G23nuo           ----CCGTGACGGGTCGGGTGGGT------------ 20
LSII26us         ---AAGGGAACGGGCTTGGCGGAAT------------ 22
G2n22            ----------GGCGGACGGCGGGAGAGGG-------- 19
G1828            ----------GGGGGAGGGAGGCGGAGGG-------- 19
G18242           ------TCGGGGGGGCCGGCGGCGGCGGC-------- 23
FLP_26s          -------CGGGGGGGCCGGCGGCGGCG---------- 20
G1871            -------GGGGTCGGCACGCGGCAGCGG--------- 21
Gd6              -------CGGGGAGCCCGGCGTGTGCCGGC------- 23
FLP_20s          ----TCCCGGGGAGCCCGGCGGG-------------- 19
Gd8p             --------TTCGGGCCCCGCGG-GACACTC------- 21
G1812d           -----------GGGCCCCGCGGCGACACTCAGCT--- 23
G1814p2          -----CGCTTCGGGCCCCGCGG-GA------------ 19
G1814p1          ----ACGACGGG-GCCCCGCGGGG------------ 19
I120I1s          ---GCCGGCGGGAGTCCCGGGGAGA------------ 22
FLP_33s          -------------GTCCCGCGGGGCCCGAAGCGTT-- 22
LSII45u          --------TGAGTGTCCCGCGGGGCCCGAA------- 22
A47G18s          ---AGAGCTGGAGGTGTCCCGGTGT------------ 22
G1833            --GGGTGCGAGAGGTCCCCGGGTTC------------ 23
G18n13           ---------GAAAATCCGGGGGAGAGGGT-------- 20
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```
I210I1s                  ----------ATAATTTGTGGTAGTGGGGGAC----- 22
LSIG182u                 ------------GCTCGCCGAATCCCGGGGCCGAGG 24
LSII11u                  --------AAGGAGCCTAACGCGTGCGCGAGTC---- 25
V119V8s                  -------CCTAGCTCTCT-GTCGGGGTGTCG------ 23
FLP_46s                  ---------TGGTGCTCTTGACTGAGTGTCTCGG--- 25
G1831                    -------CTGGGTGTTGACTGCGATGTG--------- 21
FLP_31                   ------GGCGGGTGTTGAC-GCGATGTGA-------- 22
LSIG636                  -----CGTCGGGGGTGATCCGCTCTGA---------- 22
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