UNIVERSITÀ CATTOLICA DEL SACRO CUORE
MILANO

Dottorato di ricerca in Biotecnologie Molecolari
ciclo XIX
S.S.D. AGR/16

TWO DIFFERENT ASPECTS OF GENOMICS: THE CONSTRUCTION OF A HIGH-DENSITY RADIATION HYBRID MAP AND THE STUDY OF THE INVOLVEMENT OF MIRNAS IN THE MAMMARY GLAND

(DUE DIFFERENTI ASPETTI DELLA GENOMICA: LA COSTRUZIONE DI UNA MAPPA DI IBRIDI DI RADIAZIONE AD ALTÀ DENSITÀ E LO STUDIO DEL COINVOLGIMENTO DEI MIRNAS NELLA GHIANDOLA MAMMARIA)

Tesi di dottorato di : SILVERI LICIA
Matricola : 3280012

Anno Accademico 2005/2006

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…nella speranza che un giorno lei legga e sia
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Sintesi

In questa tesi vengono presentati due studi differenti.
Il primo, svolto sotto la supervisione del professore P. Ajmone-Marsan dell'Università Cattolica del Sacro Cuore di Piacenza, consiste in una ricerca svolta nel quadro di un progetto finanziato dalla Comunità Europea, chiamato 'BovGen', per lo sviluppo di tecnologie utili allo studio del genoma bovino. In particolare il progetto prevedeva lo sviluppo di un *array* contenente 20000 EST (*Expressed Sequence Target*) bovine non ridondanti, di una mappa RH (*Radiation Hybrids*) bovina ad alta risoluzione finalizzata alla costruzione di mappe comparative uomo-bovino, l'assemblaggio dei frammenti bovini genomici già sequenziati in un unico *contig* e il completamento della sequenza dell'intero genoma bovino. Questi strumenti permettono l'identificazione molecolare e lo studio dell'espressione genica di caratteri importanti per il miglioramento genetico dei bovini e una migliore qualità della produzione alimentare.

Nell'ambito di questa tesi inizialmente è stata sviluppata una mappa RH del genoma bovino ad alta densità di marcatori. In particolare, la mappa è stata costruita attraverso la caratterizzazione genica di un pannello 3000-rad di 94 linee cellulari ibride bovino-criceto, precedentemente sviluppato al Roslin Institute di Edimburgo, aggiungendo nuovi marcatori alla mappa RH bovina di prima generazione (William et al., 2002).
Il pannello è stato tipizzato con marcatori EST: dapprima, un set non ridondante di EST è stato selezionato da una libreria di cloni a cDNA derivante da cervello bovino (Herwing et al., dati non pubblicati); tali EST sono state quindi sequenziate e allineate con la sequenza del genoma bovino e, tramite il software *Polyprimers* (http://www.unitus.it/SAG/primers.zip), sono state disegnate coppie di primer in grado di amplificare le EST nel genoma degli ibridi bovino-criceto.
A seguito dello screening tramite PCR del pannello RH 2473, ai 30 cromosomi bovini sono stati assegnati nuovi marcatori che sono stati infine integrati nella mappa precedente: questa conteneva già 1497 marcatori, di cui 262 marcatori AFLP (*Amplified Lenght Polymorphism*) e altri marcatori, tra cui microsatelliti, BAC (*Bacterial Artificial Chromosomes*), *end sequences* e EST già localizzate. La mappa di ogni cromosoma è stata disegnata utilizzando software quali *RH Mapper* (Slonim et al., 1997) e *Carthagene* (Schiex and Gaspin, 1997) ed è disponibile al sito http://www.thearkdb.org (ArkDB public database).
La lunghezza totale della mappa prodotta è 760 Rays e la distanza media tra due marcatori è 19cR.
La mappa di ogni marcatore è stata allineata e confrontata con la mappa RH Illinois-Texas (ILTX) (Everts-van der Wind et al., 2004), precedentemente costruita caratterizzando un pannello RH 5000-rad di 90 linee cellulari ibride (Womack et al., 1997), e con la recente mappa bovina genetica di linkage MARC 2004 ad alta densità di microsatelliti (Ihara et al., 2006). Inoltre, la mappa RH sviluppata durante questo lavoro di ricerca è stata confrontata con la versione più aggiornata della sequenza del genoma bovino (Btau_2.0).

Questa analisi ha evidenziato che l'ordine dei marcatori lungo i cromosomi della mappa RH prodotta è in generale accordo con le prime due mappe, mentre si osservano maggiori inconsistenze tra la mappa prodotta e il recente assemblaggio della sequenza bovina: questo ha permesso di individuarne gli errori e di migliorarne la successiva versione.

Il secondo lavoro di ricerca svolto di questa tesi, svolto in Francia presso l'INRA (Institut National de la Recherche Agronomique) di Jouy-en-Josas sotto la supervisione di F. LeProvost, ha avuto come oggetto il ruolo dei microRNA durante lo sviluppo della ghiandola mammaria.

I microRNA sono una classe di piccole molecole regolatrici, e spesso inibitrici, dell'espressione genica. Dal momento che numerose evidenze sperimentali dimostrano che questi piccoli RNA non codificanti possiedono un ruolo chiave nei processi di proliferazione, differenziazione cellulare e organogenesi (Ambros, 2004; Jason et al., 2006 etc..), è stato ipotizzato un loro coinvolgimento anche nello sviluppo della ghiandola mammaria di topo durante il ciclo riproduttivo.

Tramite la tecnica *Northern blot*, è stata esaminata l'espressione nella ghiandola mammaria di topo di un primo gruppo di 25 microRNA, scelti dalla letteratura tra quelli espressi nella ghiandola mammaria dell'uomo o perchè differenzialmente espressi in tessuti cancerosi della mammella umana.

Fra i microRNA testati, 10 sono risultati espressi anche nella ghiandola mammaria del topo e ne è stata caratterizzata l'espressione durante i diversi stadi dello sviluppo: lo stadio di vergine a 4 e a 8 settimane; durante la gestazione, a 4, a 6, a 9, a 12 e a 18 giorni; durante la lattazione, a 1 e a 3 giorni, e, dopo l'allontanamento dei piccoli, durante lo stadio di involuzione, a 1 , a 3 e a 6 giorni. La quantificazione dell'espressione genica ha dimostrato un andamento variabile nei diversi stadi del ciclo riproduttivo, che denota un controllo dell'espressione genica dei microRNA durante lo sviluppo dell'organo.

Ogni microRNA ha un profilo tipico d'espressione; tuttavia sono state osservate alcune caratteristiche comuni a tutti i piccoli RNA, quali una diminuzione dell'espressione durante la lattazione e un incremento durante l'involuzione. Questo potrebbe suggerire una correlazione con lo sviluppo del tessuto epiteliale, che raggiunge il completo differenziamento durante la lattazione e va in apoptosi allo stadio dell'involuzione, oppure una correlazione con l'andamento di alcuni ormoni importanti nello sviluppo della ghiandola mammaria, quali la prolactina.

L'esame dell'espressione dei microRNA è stato approfondito ricercando l'origine cellulare della loro produzione tramite analisi *Northern blot* di ghiandole mammarie prive di tessuto epiteliale di topi precedentemente operati. Il confronto con ghiandole mammarie normali ha dimostrato che i microRNA analizzati vengono espressi anche in ghiandole mammarie prive di tessuto epiteliale. Inoltre l'espressione genica di questi microRNA è stata ricercata e verificata in 9 differenti organi murini ed è stato riscontrato che essi non sono specifici della ghiandola mammaria.

Una libreria di cloni di microRNA è stata costruita a partire da RNA estratto a differenti stadi dello sviluppo dell'organo (vergine di 8 settimane, gestazione a 2, a6, e a 18 giorni, involuzione a 1 giorno) seguendo il protocollo di Lagos-Quintana et al., 2003.

Sono stati clonati 64 frammenti non-ridondanti della lunghezza tipica di un microRNA (19-25 nucleotidi).

Le sequenze dei frammenti clonati sono state analizzate per identificare la loro eventuale identità o omologia di sequenza con microRNA già depositati nel microRNA Registry (http://www.sanger.ac.uk/Software/Rfam/mirna): la presenza nella libreria di due piccoli RNA noti, *let-7b* e *let-7c*, ha convalidato la tecnica utilizzata in questo lavoro di tesi.

Allo scopo di identificare e convalidare i microRNA presenti nella libreria, le sequenze dei frammenti clonati sono state mappate nel genoma murino (http://www.ensembl.org) e, per una frazione di queste (10 frammenti), è stata predetta, tramite l'uso del programma *Mfold,* (www.bioinfo.rpi.edu/applications/ mfold/old/rna), la struttura secondaria tipica del precursore di un microRNA nella regione genomica di localizzazione.

Successivamente, per 5 potenziali microRNA clonati è stata verificata e caratterizzata l'espressione a diversi stadi dello sviluppo della ghiandola mammaria e in 9 altri organi murini. Questi piccoli RNA hanno dimostrato avere un profilo variabile durante lo sviluppo dell'organo ed essere espressi in tutti i tessuti, anche se prevalentemente nella ghiandola mammaria.

Infine per altri 2 potenziali microRNA è stata indotta e verificata *in vitro* la maturazione a partire dall'espressione del potenziale precursore, dimostrando l'attività dell'enzima che taglia il precursore generando il microRNA maturo, l'enzima Dicer. e confermandoli quali candidati microRNA.

General introduction: The aims of genomics in the 21's century era

Genomics is the scientific study of structure, function and interrelationships of both individual genes and the genome in its entirely.

Recognition of DNA as the hereditary material, determination of its structure, elucidation of the genetic code, development of recombinant DNA technologies and establishment of increasingly automatable methods for DNA sequencing set in the 1990 the stage for Human Genome Project (HGP) and parallely the stage for others genome projects regarding microorganisms, invertebrates, fish and mammals, in particular the mouse, the rat and the farm animals.

Current progress in genetics, comparative genomics, biochemistry and bioinformatics can bring insight into the functioning of organism in health and disease at the cellular and DNA level. The genomics becomes the central and cohesive discipline addressed to biomedical research and the genome sequences, the complex of information that guides biological development and function of organisms, lie at the beginning of any molecular discovery.

The main aim of the genomics after the complete sequencing of some model organism genomes, like, for example, *Caenorhabitis elegans, Drosophila melanogaster, Mus musculus* and, ultimately in 2003, *Homo sapiens*, is to enlarge bases knowledge in order to improve human health and well-being. In particular the genomics needs to extend the knowledge of all the components encoded in the human genome, determine how they function in an integrated manner to perform cellular and organism functions, understand how genome changes and takes on new functional roles.

Actually the human's genome structure is extraordinarily complex and its function poorly understood. Only 1-2% of its bases encode proteins and an equivalent amount of the non-coding genome is under active selection, suggesting an important function in the controlling the expression of 30000 protein-coding genes and myriad other functional elements, like non-coding genes and sequences determinants of chromosome dynamics. Even less is known about the function of half of the genome, that consists of highly repetitive sequences or the remaining non-coding, non-repetitive DNA.

A first objective of genomics is to catalogue, characterize and comprehend the entire set of functional elements encoded in human and other genomes. Comparisons of genome sequences from evolutionary distant species have emerged as a powerful tool for identifying functionally important genomic elements; from the vertebrate genome sequences analyses many previously undiscovered protein-sequencing gene were revealed; mammal-to-mammal sequence comparisons have revealed large numbers of homologies in non-coding regions, defining them in functional terms. Not only the study of genome sequences inter- species is crucial to the functional characterization of the human genome, but also the study of sequence variation intra- species will be important in defining the functional nature of some sequences. As a larger knowledge of genome function is acquired new computational tools for the

prediction of the identity and behaviour of functional elements has emerged. Moreover genomics has to understand the interactions between genes and genes products, the complex networks that give rise to working cells, tissues, organs and organisms.
The finding of the study of simple model organisms, like bacteria and yeast, have been extended to more complex organisms, such as the mouse and the human. Also few well-characterized systems in mammals have been useful to discover biological molecular pathways. A complete understanding of the working cells required information from several levels : it was necessary to simultaneously monitor the expressions of all genes in a cell and to measure in real-time the localization, the modifications and activity of the gene products. For this reason new molecular techniques arose : the microarray, to analyze the transcriptome, the entire set of transcripts of a cell; the in-situ hybridization, to follow the presence of a protein in a tissue in vivo; the bidimensional electrophoresis to study the abundance and the composition of a set of proteins present in a cell or in a tissue, giving birth to the proteomics. Many other techniques that modulate temporally and/or spatially gene expressions *in vitro* or *in vivo*, like gene-knockout methods, knock-down approaches and the recent use of small-molecule inhibitors of specific transcript, developed after the discovery of a new regulatory class of small non-coding RNA and their mechanism of action, generally called the RNA-interference.
The final objectives will be to identify the genes responsible for human phenotypic differences, or traits, and in particular the variations in DNA sequence that are correlated to common diseases and responses to pharmacological agents, even if the expression of a pathology is a condition that has a complex origin, and involves the interplay between multiple genetic factors and non-genetic factors, like environmental influences. For these reasons several projects aimed to identify all the single nucleotides polymorphism (SNP) in the DNA sequence (i.e. single base deletions and insertions) of the human and model organisms genome, have been established along the creation of large-scale genetic association studies.
Moreover it should be considered that the genetic variation responsible of normal and disease state, is also a result of the modifications of the genome subjected to the forces of evolution. Thus, a complete elucidation of genome function requires the parallel understanding of the sequence differences across species, in order to : identify functional elements; provide insight into the distinct anatomical, physiological and developmental features of different organisms; define the genetic basis of speciation; characterize the mutational process, which drives not only long-term evolution, but that is also the cause of inherited genetic disease.
The sequencing of human genome provides an unparalleled opportunity to advance our understanding about the role of genetic factors in human health and disease, and to apply this insight to the prevention, diagnosis and treatment of diabetes, cancer, obesity, heart disease, Alzheimer’s disease, etc. . The actual genomics knowledge and the new molecular tools are able to understand and reclassify all the human illnesses. In fact, the systematic analyses of somatic mutations, epigenetic modifications, genes and proteins expression and protein

modifications should allow the definition of a new molecular taxonomy of illness, that could be the basis for developing better methods for the disease detection and more effective treatments. Such 'sentinel methods' might include analysis of gene expression in circulating leukocytes, proteomics analysis of body fluids, advanced molecular analyses of tissue biopsies. The genetics discoveries will favour also the therapeutic design and the drug development, if we consider that at the present the pharmaceuticals on the market target approximately 500 human products, comparing to the 30000 protein-coding genes present in the human. A particular promising example of the gene-based approach to therapeutics is the application of chemical small molecules that act as positive or negative regulators of individual gene products, pathways or cellular phenotypes, after the screening and the understanding of biological functions of small RNA molecules, like microRNA (Collins et al., 2003).

Genomics now provides more and more powerful tools for unravelling the molecular basis of phenotypic diversity also in domestic animals, but genome research in livestock differs in several respects from that in humans or in experimental organisms, because it is not oriented to the identification of monogenic loci responsible of inherited disease. For decades breeders have altered the genomes of farm animals in search of a desired phenotypic trait and then selecting for it. This genomic work has already facilitated a reduction in genetic disorders in farm animals, as many disease carriers are removed from breeding populations by purifying selection.

Nowadays genomic research in farm animals is oriented to the study of traits of economical interest, like growth, milk production and meat quality, that have a multifactor background and that are controlled by an unknown number of quantitative trait loci (QTL).

Quantitative traits, such as weight and length, show a continuous distribution of phenotype values rather than the discrete values observed for a qualitative trait. They are usually controlled by multiple genes and influenced by environmental factors. A quantitative trait locus is defined as a genomic region that contains one or more genes affecting the same quantitative trait. The number of QTL that controls a given trait is not absolute and, in a statistical model, could be infinite, each genes carrying an infinitesimal effect on the phenotype. The main goal of genome research in livestock is to map and to characterize trait loci controlling various phenotypic traits. This requires powerful genome resources (Andersson, 2001).

Livestock genomics has followed in the footsteps the human genome research, adopting both its successful strategies and technologies. In turn, livestock genomics contributes to inform human genomes and to understand evolutionary history and its underlying mechanisms. Moreover farm animals were shown to be quite valuable resources as models for pathology and physiological studies. For example the reproductive physiology of domestic animals is more similar to humans than that of rodents, because farm animals have longer gestations and

pre-pubertal periods than mice; specific physiological traits, such as the digestive system of the pigs, are similar to those of humans.

In addition agricultural science has a unique responsibility to human health and social stability, that is feeding an expanding world population while minimizing environmental and ecological risks. The identification of DNA variation in livestock genomes that predisposes health and productivity with less reliance on hormones, antibiotics and pesticides, will remain a concern for some time. Ultimately DNA analysis from animal tissue can be used as an inexpensive method for tracking the origin of meat sample, providing the quality assurance for the consumers.

Early attempts to construct whole-genome maps of livestock species were based on the two technologies underlying the first human genome maps : somatic cells genetics and *in situ hybridizations* (Womack and Moll, 1986, Yerle et al., 1995). These early maps defined synteny (genes on the same chromosome but not necessarily linked) and cytogenetic locations of sequences hybridizing specific DNA probes. These finding were extremely important for the first comparative mapping because the markers were genes or gene products highly conserved across mammalian genomes.

Modern genomics in livestock had its formal origins in a series of conferences in the early 1990 in which international teams of animal geneticists launched both formal and informal genome projects for some of the most widely used livestock species. From that moment dense microsatellite maps, large-insert yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC) libraries, radiation hybrid panel (RH) were used for some livestock species, like cattle, pigs, sheep, horses, river buffaloes, goats, rabbit, chicken and some fish like zebrafish, medaka, pufferfish and the sticklebacks in order to localize trait loci. Linkage genetic maps, using microsatellite on the first rough genetic maps, the clonage and the characterization of interesting loci in the BAC and YAC libraries, high-resolution comparative map using the RH strategy, and the first physical maps were developed.

The development of species-specific array and the production of specific transcript profiles started after the development of large collection of sequenced cDNA clones and the corresponding production of the expressed sequence tags (ESTs) for many farm animals. ESTs are small pieces of cDNA sequence (usually 200-500 nt long), which are useful as markers for a desired portion of RNA and DNA that can be used for gene identification and gene localization within a genome. The National Center of Biotechnology Information (NCBI) provides the most comprehensive EST database for many farm animals, while in the Ensembl database (http://www.ensembl.org/) is possible to find a summary of current analyses on coding regions within genomes for selected farm animals. Mapping information are available on the NCBI site http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=? substituting the last '?' sign with the species taxonomic number (i.e. 9031 for the chicken, 9913 for the cow, 9823 for the pig, 7955 for the zebrafish, 9940 for the sheep, etc. ..).

Selection for desirable traits, or conversely, selection against undesirable traits, has been practiced since the domestication of animals begun more than 10000 years ago. There has been a long tradition of collecting and analysing data on phenotypic traits for breeding purpose in farm animals, and the most common strategy for finding trait loci was to use existing pedigree. This approach was easy in farm animals because of the large family size; for example, the artificial insemination in cattle allows to have a 1000 progeny from a single male. The promise of more accurate, efficient and economical selection that will produce offspring with desirable phenotypes, underpins a substantial portion of the founding for livestock genome projects over the past two decades.

The early linkage maps for most livestock species were constructed as tools for mapping traits and for developing molecular markers useful in marker-assisted selection (MAS). However the ultimate goal when mapping trait loci, the ultimate marker for MAS, is the identification of the causative mutations underlying the selected phenotype. Positional candidate cloning is the main strategy for this purpose. High-resolution mapping is necessary to restrict the region of interest that could contain the QTL and the number of potential candidate genes. Information on map location and gene function is then combined to identify more precisely positional candidate genes, which are subsequently evaluated by mutation screening and functional analysis. The difficulty in identifying a QTL could increase if the QTL mutations is situated in regulatory rather than in coding regions and the phenotypic effect is shifty, compared with simple loss-of-function mutations that cause inherited disorders.

Although mapped QTLs in livestock number in the hundreds, very few mutations underlying quantitative trait variation have been identified. The trait loci for which the causative gene and mutation have been identified or for which this is expected in the near future are monogenic traits of economic and biological interest : the coat colour of the pig, in which the dominant white colour is determined by a mutation in the KIT gene, encoding the mast/stem-cell growth factor receptor; the body composition, in particular the relative proportion of muscle to fat tissue, in pigs, cattle and sheep, in which different genes have been proposed as candidate genes provoking particular phenotypes, like the double-muscling phenotypes in cattle or the muscular hypertrophy in sheep; fertility traits are also studied in different species like sheep and pigs; monogenic disorder like the bovine leukocyte adhesion deficiency, caused by missense mutations in *ITGB2*.

Others monogenic disorders have been analysed and the corresponding causative mutations have been catalogued in the 'Online Mendelian Inheritance In Animals (OMIA)' database (http://www.angis.org.au/oma/). In this site is possible to find the list of all the single-locus traits mapped in cattle, pig, sheep, horse, and goat, which counts hundreds of genes, and the relative proportion of genes for which the causative mutations have been identified, approximately one-third of them. Till October 2006 (Womack et al., 2006) there are only two example of the causative mutation underpinning the QTLs, both in dairy cattle, and both controlling the fat composition of milk : the first discovery of quantitative trait nucleotide

(QTN) was found in the DGAT1 locus on chromosome 14 (Grisart et al., 2004), and the second one was found in the *ABCG2* gene on chromosome 6 (Cohen-Zinder et al., 2005).
Ultimately the general disease resistance to pathogens is attracting attention both to improve animal welfare and to reduce losses in production due to disease. Several studies on the relationship between genetic variation and disease resistance have focused on major histocompatibility complex genes. Target diseases are the trypanosomiasis in cattle; the oedema disease in pigs, that is caused by the susceptibility to E*scherichia coli* infections; the Marek's disease (MD) in the chicken, that provoke a lymphoproliferative disease. The identification of QTLs for disease resistance in livestock may be the next frontier for the domestic animal genomics, in order to the understand the host-pathogen interaction and the subsequent improvement of both animal and human health. Linkage disequilibrium mapping will be a very powerful approach for mapping and finding trait loci in domestic animals once dense SNP maps become available and the cost for genotyping is reduced. Current initiative to develop complete BAC contigs of farm animal genomes will produce large-insert contigs covering the region of interest as soon as a trait locus is mapped. Such large-insert contigs can then be used to build a preliminary transcript map of the region by high-resolution comparison with the corresponding region in humans or mice. The completion of the farm animal genome sequencing will provide the researchers with the possibility to analyze the phylogenetic conservation of a causative mutation and its functional role, that will be evaluated later by experimentation. In this way it could be possible to unravel the molecular basis for a variety of phenotypic traits of agricultural, biological and medical significance.

In this thesis two different studies are proposed.
The first part of my work describes a research included in the E.U. funded project 'BovGen', aimed to develop advanced genomic tools useful to study the molecular and genetic control of important traits in cattle. In particular, only an aspect of the project is described : the construction of a high density RH map of bovine genome, which was developed under the initiative and the responsibility of the Institute of Zootechnics of the Faculty of Agriculture of the Catholic University of Piacenza, (Italy), having the professor P. Ajmone Marsan as supervisor.
The second part discusses the involvement of microRNA, an important class of expression regulatory elements in the genome, during the normal development of the mammary gland in a model organism, the mouse. The study of these regulatory elements intends to enlarge bases knowledge about the genetic mechanisms that control the proliferation, differentiation and apoptosis of cells in the tissues composing mammary gland during the reproductive cycle. This work was supported and conducted by the Laboratory of Biochemical Genetic and Cytogenetic (LGBC) at the INRA (Institut National de la Recherche Agronomique) of Jouy-en-Josas (France) under the responsability of F. LeProvost.
The study of some functional elements of the mouse genome, required mouse sequence information available on the Ensembl database, thanks to a previous work of construction of

physical maps and genome sequencing in the mouse, analogous to what has been done for the cattle in the BovGen project. The complete genome sequencing of the bovine was considered an important task in genomic research, a necessary step not only to increase genetic data on this economically important species, but also because of its general utility in the construction of comparative maps and in the identification of new genes or new regulatory conserved elements. Moreover the study of the microRNA function in mammary gland opens the way to the discovery of biological mechanism of cellular proliferation, that could be correlated to the development of breast cancer, but also to the discovery of molecular mechanism that guides epithelial tissue differentiation till the production of milk. In the future it could be possible that the new finding in the mouse could be applied to the bovine, to increase the milk production or to control the timing of lactation.

Recently a new study (Clop et al., 2006) about a QTL controlling meatiness in Texel sheep, demonstrated that the causal mutation in this species is located in the myostatin gene (GDF8) and that a G to A transition in the 3' UTR of the gene creates a target site for two known microRNA, miR-1 and miR-206, which causes translational inhibition of myostatin gene and the muscular hypertrophy, showing how the knowledge of the mechanism of action of microRNA and the use of instruments like genetic map can fuse and focus on particular biological aspects, like the study of economically important QTLs.

First part : A High-density radiation hybrid map construction

I-Introduction

I-I The objectives of livestock genomics

The detection of loci affecting economically important traits represents a major objectives in livestock genomics. It should ultimately lead to more efficient breeding schemes (marker-assisted selection or MAS) and improve the accuracy and intensity of selection programs (Georges and Andersson, 1996; Haley, 1995). In this perspective genetic maps have been constructed in various livestock species, like bovine, sheep and goat, to detect regions containing genes and QTL. The identification of genes and cloning of the corresponding genes may be achieved by standard positional cloning, taking advantage of the existence of large insert libraries and searching for transcribed sequences in these regions.
Cattle are a major economic resource worldwide, therefore there has been considerable interest in the identification of genes that are involved in improved cattle production. Numerous reports have identified genomic regions corresponding to economically important traits in cattle (Georges and Andersson, 1996; Georges, 1999), based on low to medium density genetic linkage maps of the bovine genome.

I-II Genetic maps : brief history

A genetic map shows the relative position and order of markers along the chromosomes of the genome. Genetic mapping is based on the examination of a segregating population, that could be experimental, created for example by cross-breeding experiments, or natural, such as a family, following the principle of inheritance as first described by Mendel in 1865 in his two lows of Genetics, about the segregation of independent genes.
The first genetic maps were constructed in the early decades of the 20th century for organisms such as fruit fly and used simple features inherited on genetic base like markers, even before the discovery that genes are segments of DNA. Genes were looked on as abstract entities responsible for the transmission of heritable characteristics from parents to offspring. To be useful in genetic mapping a heritable characteristic must exist in two alternative forms or phenotypes, each specified by a different allele of the corresponding gene. In the beginning the only genes that could be studied were those specifying phenotypes that were distinguished by visual examinations, like genes for the body color, eye color, wing shape, but soon it was realized that only a limited number of genes has a clear phenotype and in many cases the analyses is complicated because more than one gene affects a single physical feature. It was necessary to find characteristics that were more numerous, more distinctive and less complex than visual ones. The next markers used were biochemical phenotypes, easy to detect in

microbes and humans, like antibiotic resistance or amminoacid request for the bacteria and yeast growth, or the blood groups and immunological proteins such as human leukocyte antigens (the HLA systems) in humans.
Soon it was accepted that a map based entirely on simple phenotypes is not detailed because the genes are widely spaced out in the genome with large gaps between them and moreover only a fraction of the total number of genes exist in allelic forms that can be distinguished conveniently.

I-III Molecular markers

Mapped polymorphisms that are not genes are called DNA or molecular markers. To be useful they must exist in at least two allelic forms.
Many types of molecular markers with different characteristics were developed using different molecular techniques that analyze the variation in the sequence of DNA.
The first ones were the restriction fragment length polymorphisms (RFLP), produced after treating the DNA with a restriction endonuclease. The set of fragments produced can vary if there are single base variations in the DNA sequence of the restriction sites, leading to a length polymorphism of the fragments.
Others molecular markers that are generated from singular base variations of the sequence of DNA were developed later and they can be produced after sequencing of DNA, such as the Single Nucleotide Polymorphism, the SNP markers, or using the PCR (Polymerase Chain Reaction), like the Random Amplification Polymorphic DNA or RAPD markers, or by a combined use of restriction endonuclease and PCR, such as the Amplifyed Fragment Length Polymorphism or AFLP markers.
Another class of molecular markers, widely used in the construction of high-density genetic map, are the Simple Sequence Length Polymorphism or SSLPs markers, that comprise the minisatellites and the microsatellites. The SSLPs are tandemly repeated sequences that show length variation, in the minisatellite the repeats units comprises from tens to a few hundred nucleotides, while in the microsatellite the repeats are shorter, usually di-, tri- or tetranucleotide units. These variations of the number of repeat sequences in the DNA take origin from “errors” during the duplication of DNA during meiosis. It is possible to identify the SSLPs markers by PCR because the sequence flanking them are usually single copy sequence in the genome. Microsatellites are more popular and used compared to the minisatellites, because microsatellites are more conveniently spaced and distributes throughout the genome and because they are shorter and therefore easily to type by PCR.

I-IV Genetic linkage maps

A genetic linkage map is based on the principle of genetic linkage, first discovered by Bateson, Saunders and Punnet in 1905, but not fully understood until Thomas Hunt Morgan began his work with fruit flies in 1910-11. This principle sets that chromosome are inherited as intact units and then pair of genes located on the same chromosome are physically linked together and should be inherited together if any crossing-over event recombins homologous portion of two paired chromosomes during the meiosis. The probability that two different genes localized on the same chromosome are inherited together is proportional to the physical vicinity of the two genes considered and inversely correlated to the number of crossing over events that could occur between two genes localized in distant part of a chromosome. The localizations and orders of markers along a chromosome in genetic linkage map reflect a measure of probability. The distance between markers is not physical, but it is measured in centiMorgans (cM), 1 cM corresponding to 1% of frequency recombination between genes. The real distance in base pair, kilobase or megabase between markers and genes is measured only in physical maps, that are not produced using information from breeding experiments or pedigrees, but examining directly the DNA with molecular biology techniques in order to localize markers on different portions of a chromosome.

SNP and microsatellites, due to their high abundance in the genome, are getting more and more importance in linkage genetic maps and identification of QTLs. Microsatellites are excellent genetic markers because of their high polymorphism, different alleles containing different numbers of repeat units, comparing to the SNP, which has only two alleles.

Genetic linkage maps, based primarily on highly polymorphic, anonymous microsatellite markers, have been important in identifying chromosomal regions influencing economically important traits in cattle (Casas et al., 2001; MacNeil and Grosz, 2002; Li et al., 2002).

Cattle genetic linkage maps were constructed in 1997 with 746 markers (Barendse et al., 1997) and 1250 markers (Kappes et al., 1997), the latter one, spanning 2990 cM, was characterized by an average interval of nearly 3.0 cM.

This cattle genetic map was probably sufficient to assign hereditary phenotypes to specific chromosomes, but not to fine-map them. An intensive efforts to develop more markers to narrow the critical region was required. However, the time, labor and cost per marker of isolating DNA markers from a specific chromosomal region was substantially greater than randomly isolating markers.

Thus a random isolation of microsatellite, from microsatellite-enriched libraries (Stone et al., 1995), was chosen to enrich markers across the genome. The microsatellites were genotyped and assigned to chromosomes by multipoint linkage analysis using the CRIMAP software and a new high density bovine genetic map consisting of 3960 markers, including 3802 polymorphic microsatellite and 79 SNPs, with an average marker interval of 1.4 cM, covering 3160 cM for each of the 30 bovine chromosomes, was produced. This map represented a

powerful resource for fine-mapping of QTLs and a genetic backbone for the development of well-annotated gene maps in cattle and other related species.
Recently Ihara et al. (2004) improved this cattle genetic map and developed a microsatellite-based high-density genetic map on the basis of more than 880000 genotypes across the USDA MARC cattle reference families with a potential genetic resolution of 0.8 cM at the 95% confidence level (approximately 800 kb in the bovine genome).

I-V Somatic hybrids and FISH

There are different kind of physical maps, produced with many molecular techniques, that have different degree of resolution in the assignment of genes to chromosomes.
The first crude mapping of genes on chromosomes was obtained in human by Ruddle in 1972 fusing irradiated human cells with rodent cells and observing the generation of mononucleate hybrid cell lines capable of indefinite multiplication that, after the application of selective media, express human biochemical markers in association with the retention of human chromosomes. In the hybrid cells most of the human chromosomes were rapidly and preferentially eliminated and with appropriated stained preparations it was possible to identify the human chromosomes detecting their specific banding patterns (Goss and Harris, 1975).
The correlations between the retention of human biochemical markers in hybrids cells with the retention of identifiable chromosomes permitted to assign 50 human genes to specific chromosomes. The identifications of the position of genes within the chromosome has been achieved in the beginning by exploiting translocations that segregate linked markers (Boone et al., 1972; Gerald et al., 1974), even if this method couldn't be applied to every genes, but only to the genes that are localized into a segment of chromosome large enough to be identified in a translocations.
Recently a bovine/hamster hybrid cell panel consisting of 30 independent hybrids was developed to locate genes (Itoh et al., 2003). The characterization of the panel by typing 279 microsatellites markers revealed the presence of all bovine chromosomes in either entire or fragmented form. The panel was also characterized with EST and 1400 EST were assigned to specific chromosomes, thus making this panel a useful tool to the regional mapping of new genes to cattle chromosomes.

The most direct way to localize a genomic segment on a chromosome is to use locus specific-probes in the *in situ hybridizations*, that is able to visualize the target within a particular banding patterns along chromosomes. The recent development of the in situ hybridization is the fluorescent in situ hybridization, or FISH, able to analyze the position of more than one probe on chromosomes at the same time, by labeling different probes whit different fluorescent molecules and the FIBER-FISH, which gives the possibility to hybridize specific probes directly on a single starnd of DNA attached to a solid support.

However the resulting cytogenetic map has lower degree of resolution compared to other kind of physical map constructed with different techniques, for example analyzing by restriction-based fingerprinting large fragments of DNA, even of megabase, contained in BAC clone library.

I-VI BAC-based physical maps

A BAC (Bacterial artificial chromosome) clone is a bacterial clone that contain one artificial chromosome made fusing casually large fragments of the genome of interest with two arms of the bacterial chromosome, that have to contain the centromer and the telomer, or only the telomer, and which carries a marker of selection on each arms.
The wide use of BAC libraries is due to the clone fidelity, to a low level of cloning artifacts, to the easy of separate the BAC DNA from the host's DNA, to the fact that often individual clones contain complete genes embedded in their genomic environment and then the clones can be used for functional studies in cell lines or transgenic applications.
A bovine artificial chromosome BAC library of 105984 clones was constructed in the vector pBeloBAC11 and organized in 3-dimensional pools in 2001 at the INRA of Jouy-en-Josas (France), (Eggen A. et al., 2001). The average insert size was estimated 120 kb after isolation by field inversion gel electrophoresis (FIGE) of digested fragments of 388 clones. Assuming that the bovine genome contains $3x10^9$ bp the total library corresponded to a four genome coverage. The library was also screened by PCR with 164 microsatellite markers to verify the homogeneous distribution of fragments from all the genome in the clones. FISH was performed for over 50 BAC clones and no one was found chimeric. This bovine BAC library contributed to increase the genome coverage of the cattle of the already existing bovine BAC libraries of 2.7 (Buitkamp et al., 2001), 6 (Cai et al., 1995), 10 (Warren et al., 2000), and 5 (Zhu et al., 1999) genome equivalents, bringing the total coverage of the bovine genome represented in BAC libraries to 28.
An analogous bovine BAC library was constructed and called the 'CHORI 240 cattle BAC library' (http://www.chori.org/bacpac). This library contains approximately 200000 clones and was created by cloning partially digested *Mbo*I genomic DNA isolated from a Hereford bull into the *Bam*HI cloning site of the pTARBAC1.3 vector.

Currently BAC libraries have been extensively used to build numerous chromosome specific or whole genome sequence physical maps by BAC fingerprintings and BAC-end sequencing. Whole genome maps have been constructed for a number of organisms including rat, cow, zebrafish, sorghum, maize and tomato (see www.genome.clemson.edu/fpc and www.bcgsc.edu for links to the corresponding web sites).
A first generation bovine BAC-based physical maps was constructed in 2004 at the INRA of Jouy-en-Josas (Schibler L. et all., 2004). This map was assembled analyzing the totality of the

clones of the bovine BAC library of the INRA and part of the CHORI-240 BAC library (26500 clones) by fluorescent double digestion fingerprinting and sequence tagged site (STS) screening.
DNA preparation was performed using a modified alkaline lyses procedure for each clone. 300-400 ng of BAC DNA was submitted to a double digestion (*Hind*III and *Hae*III), which on average generates about 40 bands of 55 to 750 bp, and simultaneously to a dye labeling. The restriction profiles of the samples was analyzed by capillary electrophoresis using a 1000 automated 96 capillary DNA sequencer. The runs were analyzed with the Genetic Profiler software developed to perform the genotyping analyses on the MEGABACE. The map was constructed starting from an initial stringent build and using an incremental process, which consisted in joining together assembled and ordered part of DNA sequence, contigs, based on end-end comparison. The map was validate and the contigs were anchored using the PCR screening information for a total of 1303 markers (451 microsatellites, 471 genes, 127 EST, 254 BAC ends). The final map, which consisted of 6615 contigs assembled from 100923 clones selected from the two libraries, was considered a valuable tool for genomics research in ruminants, including targeted marker production, positional cloning or targeted sequencing of region of specific interest. This map provided also a good framework to initiate a strategy similar to that of Gregory et al. (Gregory et al., 2002) to establish high-resolution sintenies among ruminant, human and mouse genomes.

I-VII Comparative maps

An important step for efficiently sequencing a new mammalian genome is to have a high-quality, comparatively anchored physical map.
Fujiyama et al. (2002) produced a comparative clone-based map of the human and chimpanzee genomes using paired chimpanzee BAC-end sequences (BESs) aligned by BLAST with the human genome sequences and founding that approximately 98% of chimpanzee BESs has BLAST hits in the human genome that identify putative orthologs. Gregory et al. (2002) produced a detailed comparative physical map of the mouse and human genomes by combining BAC-end sequencing with a whole-genome BAC contig created by BAC fingerprinting, revealing remarkable colinearity of the mouse and human genome.
Larkin et al. (2003) used a large-scale BAC-end sequencing strategy to built the first sequence-based physical and multi-species comparative maps of cattle. They sequenced at both ends a total of 40224 bovine BAC inserts of the CHORI-240 cattle BAC library and generated approximately 60500 high-quality cattle BESs whit an average read length of 515 bp. These BESs comprise more than 14 Mbp of non repetitive cattle DNA, thus providing a resource for anchoring cattle genomic sequences to the human and mouse genomes. The non repetitive cattle BESs were then tested for similarity to human and mouse genome sequence (NCBI Build 30) using BLASTN, revealing 29,4% and 10,1% significant hits, respectively

and howing that random cattle BESs had 3.3-fold higher similarity hits to the human genome than the mouse genome. More than 60% of all cattle BES hits in both the human and mouse genome were shown to be located in within known genes, including coding and non coding regions.

I-VIII Radiation hybrid maps

In order to construct a high-resolution physical map for each specific chromosome, basic tool to assist the final high-quality sequence assembly of the genome, and comparative mapping information from maps of the annotated human and mouse genome can be utilized efficiently. The location of bovine loci that are homologous of human genes may be predicted from the current knowledge about the conservation of synteny between genomes, but comparative mapping can sometimes produce errors, because it is based on the colinearity between two different genomes even if some genomic regions are not colinear, thus the position of a locus has to be actually proven by direct mapping on genome.
Radiation hybrid (RH) mapping has been shown to be a powerful tool to integrate comparative genome data with information from existing genetic and physical maps to generate high-resolution maps (Itoh et al., 2005).
The technology for generating physical maps using irradiation and fusion gene transfer was first developed more than 20 years ago by Goss and Harris (1975). This technology was employed in an isolating mapping experiment of human X chromosome genes ten years later by Williard et al.(1985), but it was not systematically used as a human gene mapping instrument until the work of Cox et al. (1990) of construction of a high-resolution map of the human chromosome 21. This map was constructed using hybrids generated by irradiation fusion gene transfer between a donor somatic cell hybrid containing a single human chromosome and the recipient rodent cell line. Mapping the entire human genome with this approach was impractical because it required a panel of 100-200 hybrids for each chromosome and a screening of over 4000 hybrids to generate a genomic map. For this reason Walter et al. (1994) reverted to the original method of whole genome radiation hybrid (WG-RH) of Goss and Harris, that is the use of diploid cell line like a donor genome at the place of a single chromosome of interest from a somatic cell hybrid, to demonstrate that a panel of hybrids of a diploid human cell line with a rodent recipient line could be used to map any human chromosome. Later Gyapay et al. (1996) and Hudson et al. (1995) demonstrate the emergence of WG-RHs as stand-alone mapping tools publishing two WG-RH maps of the human genome opening the way to the RH maps development.

I-VIII-a Advantages of RH maps

In contrast to linkage maps, which exploit the frequency of natural recombination between markers to calculate distances and orders of markers, RH maps are constructed using the probability of breaks between markers induced by radiation. The retention frequency, that is the measure of the proportion of donor genome retained in hybrids, of two markers is proportional to their vicinity in the genome, and inversely correlated to the number of breaks that could occur between the two markers. The retention pattern of markers for each hybrid is compared to determine linkage and map distances between markers. These distances are measured by centiRay, 1 centiRay (N rad) corresponding to a 1% frequency of breakage between these two markers after exposure to a radiation dose of N rad of X-rays. (McCarthy, 1996).

Radiation hybrids allow a clear determination of a linear order of markers along a chromosome and radiation hybrid mapping has two major advantages over physical mapping and genetic mapping: it has much higher resolution and the markers don't need to be polymorphic to be included in the map. It is an especially powerful tool for comparative gene mapping, since chromosomal order can be established for expressed genes that are usually conserved between species, but often recalcitrant to linkage mapping for lack of allelic variation. Moreover the radiation hybrids maps bridge the gap between genetic and physical maps because they offers the possibility to anchor the large DNA insert of the bacterial artificial chromosome and to identify their orientation.

I-VIII-b Principle of construction of RH panels

To generate RH panels, the donor cell line is irradiated with a lethal dose of X-rays or γ rays, and fused with the recipient cell line, using either Sendai virus or polyethylene glycol (PEG). Non-recombinant donor cells die whitin a week of irradiation. The recipient cell line will contain a selectable marker; the most frequently used are thymidine kinase deficiency (TK-) or hypoxanthine phosphoribosyl transferase deficiency (HGPRT-). Cells containing either of this marker will not grow in media containing HAT (hypoxantine, aminopterin, thymidine). The only post-fusion cells that will grow in HAT medium are recipient cells containing all their complete genome added with casual portion of donor DNA containing both the wild-type TK or HPRT gene. The hybrid colonies are expanded for DNA extraction and 96-well microplates are filled whit the hybrid DNA and the control DNA in order to be screened by PCR for the retention of genetic markers.

I-VIII-c RH panel characteristics and uses

In radiation hybrids the irradiation is utilized both to kill the donor line and to induce chromosomal breaks producing hybrids with the desired fragments size.
Increasing the irradiation dose from 5 to 25 Krads Siden et al. (1992) observed a 5- to 10-fold reduction in the size of the fragments, as well as a dramatic reduction in the retention frequency from 27 to 3%. The optimal radiation doses chosen to construct a panel of radiation hybrids is dependent upon the intended use of the lines. Low dosages results in decreased resolution of a chromosome map, while at very high dosages (greater than 10000 rads) no significant linkage between loci is observed due to extensive fragmentation and loss.
Higher-dosage hybrids which carry small fragments of DNA from a region of biological interest have been used for constructing recombinant DNA libraries and DNA probes (Florian et al., 1991).
It is generally believed that breakage along the chromosome, as well as the rejoining of the broken ends, is a random process (Heddle, 1965). However stabilization of a fragment in the hybrid requires the rejoining of the fragment with elements needed for replication and stable mitotic segregations. The preferential retention of the centromere in radiation hybrids has been observed in a number of radiation hybrids panels (Benham et al., 1989; Goodfellow et al., 1990; Ceccherini et al. 1992; Abel et al., 1993; etc.).
FISH has been used to determine the number and relative size of human fragments carried in hybrids. The number of fragments appeared to be independent of the irradiation dose used to generate the hybrids. FISH was used also as a screening procedure to identify hybrids containing human DNA, which are subsequently used for marker analyses.
The first issue in the design of a radiation hybrid mapping experiment is the number of hybrids required to achieve optimal resolution. This problem has been reviewed by Lunetta and Boehnke (1994). They calculated the resolving power of radiation hybrid panels of varying sizes as a function of retention frequency, assuming that retention frequency is the total number of radiation hybrids retaining a given marker divided by the total number of radiation hybrids tested with the marker. They suggested that a radiation hybrid panels of 90-100 lines is adequate for most mapping experiments.

The protocol for scoring markers on a radiation hybrids panel is a critical step in building the map. Markers scored as present (+) or absent (-) are completely informative; thus, false positives and false negatives bias the map. Ambiguous data can be entered as unknown (?). Testing of the markers is commonly carried out by visual inspections of ethidium bromide-stained PCR products from sequence-tagged site (STS) markers. The problem of scoring many markers across the panel is variation in the relative sensitivity of the marker tested. The problematic markers are those that show abnormally high or low retention frequency and it is normal to avoid them as anchor points in initial radiation hybrid map construction.

The first phase of analyses is a test of each marker against all the other tested markers, or two-point analyses. The two-point analyses can be used to estimate distances between markers, and to identify linkage groups to subject to multipoint analyses, that represent the second phase of the analyses. Multipoint analyses can define the trial orders of markers inside a linkage group and between clusters of markers. Normally this analyses is carried out using as small as possible linkage groups because it is computationally intensive, with N!/2 possible orders to consider for N markers present in each group. It is efficient to subdivide the problem into clusters of markers to be ordered within cluster, then order and orient the ordered clusters (Leach and O'Connell, 1995).

I-VIII-d Software used to construct RH maps

When a marker is tested on the RH panel the pattern of the presence (+) or absence (-) across the panel defines a cytogenetic placement; those markers with the same pattern of + and – are localized in the same cytogenetic 'bin'. Ordering of the bins is carried out either by the ordering of the known cytogenetic breakpoints, or by minimization of the obligate breakpoints under the assumption that the majority of the rearranged chromosomes arise from a single breakage event. These analyses have been carried out in the beginning manually, nowadays analyses packages are available.
One of the software used to produce RH maps for each chromosome is the Microsoft Windows versions of 'Chartagene' (Schiex et al., 2002), available publicly from www.inra.fr/bia/T/CarthaGene'.
The other programs available for building radiation hybrid maps are RH map (Vanderstop et al., 1991), RHMAPPER (Soderlund et al., 1998) and multi-map.
RH, cytogenetic and linkage maps can compared by using Anubis software (www.roslin.ac.uk/cgi-bin/anubis).

I-VIII-e RH bovine panels and maps

Whole genome-radiation hybrid (WGRH) panels have now been used to create medium to high resolution chromosomal maps in several species, including human (Gyapay et al., 1996), mouse (Schmitt et al., 1996; McCarthy et al., 1997), rat (Watanabe et al., 1999), pig (Yerle et al., 2002), horse (Chowdharhary et al., 2002), chicken (Morrison et al., 2004), zebrafish (Geisler et al., 1999), dog (Priat et al., 1998) and cattle (Womack et al., 1997; Rexroad et al., 2000; Williams et al., 2002; Itoh et al., 2005; Band et al., 2001).
Four whole genome radiation hybrid panels available for cattle have been used to construct RH maps: the Womack-5000 rad panel of 90 RH clones (Womack et al., 1997), the Womack-

12000 rad panel of 180 RH clones (Rexroad et al.,1999); the TM112-3000 rad panel of 94 RH clones (William et al., 2002) the SUNbRH 7000 rad panel of 90 RH clones (Itoh et al., 2005).

The first RH bovine panel was developed in 1997 using like a bovine donor cells a normal diploid fibroblast culture established from an Angus bull, JEW38. The cells were irradiated with a cobalt 60 source delivering 185 rad/min for a total dose of 5000 rad. The recipient cell line was the Chinese hamster TK- fibroblast line A23. Six markers were genotyped in all 101 RH lines.

RH panels are generally characterized and anchored to existing genetic maps using microsatellite markers. The Womack-5000 rad panel was screened with six markers spanning each of the linkage maps of bovine chromosome 1, 13 and 19 to create the first whole-genome-RH radiation bovine hybrid map. Later the same RH panel was used to create a cattle-human whole-genome comparative map (Band et al., 2000).

Williams et al. (2002) constructed and characterized a 3000-rad RH panel in order to create an outline bovine RH map. This map was developed testing on the RH panel and incorporating in the map the majority of markers available on published bovine linkage maps.

This RH panel was constructed using like donor cell line a primary bovine fibroblast cell line established from a male Holstein calf by explants culture. Cells were exposed to a 3000 rads of X-rays and fused with the HGPRT-deficient Chinese hamster cell line, Wg3H (Goss and Harris, 1975). 224 cell lines were established and screened with 33 microsatellite markers. A subset of 100 hybrids whit higher average retention frequency was selected and a final panel of 94 hybrids was produced, whose DNA is publicly available for purchase from the Res Gen Invitrogen Corp (cat no. RH10, Huntsville, Ala., USA).

In order to link the 3000-rad RH panel to the genetic (Barendse et al., 1997; Kappes et al., 1997, http://www.marc.usda.gov/genome/genome.html, www.cgd.csiro.au) and physical maps that were published for the cattle till that moment, a total of 1238 markers were typed by PCR on the RH panel (http://www.roslin.ac.uk/radhyb/), of which 1148 are microsatellite loci and 90 are genes or markers within genes. Between them 64 could not be placed, so that 1174 markers were included on the RH-maps of 29 autosomes and the two sex chromosomes.

In most cases the order of markers was consistent between the RH maps, the published linkage maps, the current RH chromosomes maps (chr1: Rexroad et al., 1999; chr 15: Amarante et al., 2000; chr 19: Yang et al., 1998; chr 23: Band et al., 1998) built by using the Womack panel, and the low-density whole genome maps of Band et al. (2000).

Itoh et al. (2005) used the whole genome 7000-rad radiation hybrid (RH) panel, SUNbRH (7000-rad), to build a high-resolution RH map. The Shirakawa-USDA linkage map served as a scaffold to construct a map of 3216 microsatellites on which 2377 ESTs were ordered. The resulting RH map provided essentially complete coverage across the genome, with 1 cR7000 corresponding to 114 kb.

I-VIII-f Integration of bovine RH map data in the construction of comparative maps

RH maps are considered a useful resource for creating comparative maps between bovine and human chromosomes through the alignment of the loci derived from coding sequences (Amaral et al., 2002; Goldammer et al., 2002; Gautier M et al., 2002; Gautier M et al., 2003; Larkin et al., 2003; Everts-van der Wind et al., 2004; Everts-van der Wind et al., 2005).
Larkin DM et al. used the cattle-hamster 5000-rad RH panel of Womack et al.(1997) to confirm *in silico* predictions of cattle chromosome positions of bovine BAC end sequences (BESs). 60547 BESs were previously anchored to the human and mouse genome by BLASTN search, like we have already described, thus the cattle chromosome locations had been predicted for the cattle BESs with significant BLAST hits in the human genome using the COMPASS Perl scripts software (COMPASS III), producing a virtual map of BESs on the cattle chromosomes.
The COMPASS strategy (comparative mapping by annotating and sequence similarity) permits the predictions of chromosome map location based upon sequence similarity of orthologous genes, if comparative map information is available for two species (Band et al., 2000; Rebeiz and Lewin, 2000).
In that case the chromosome location of BESs was predicted using data from the first-generation cattle-human comparative RH map (Band et al., 2000). Furthermore they confirmed *in silico* predictions of cattle chromosome location for a total of 109 BESs having a single high-confidence human hit on HSA11. Oligonucleotides able to discriminate cattle from rodent sequences were designed for these BESs and 89% of them gave distinct PCR product after screening of the RH panel. 84 BESs were mapped on BTA15 or BTA29 after two-point linkage and multipoint map analyses, carried out with RHMAPPER 1.22 (Slonim et al., 1997) software. Thus the high degree of accuracy (approximately 86%) of BLAST-COMPASS approach was demonstrated and a cattle-human comparative map with greater than 1-Mbp resolution was created, 84 BAC ends were added to the existing cattle RH map.
Recently Everts-van der Wind et al. (2005) used the same approach, to construct a high-resolution whole-genome cattle-human comparative map and to add new markers (cattle BESs) to the current high resolution cattle 5000-rad RH map (Band et al., 2000; Everts-van der Wind et al., 2004) collectively known as the Illinois-Texas 5000-rad radiation hybrid panel (IL-TX RH 5000).
They screened by PCR the RH panel of Womach et al. with BES from the CHORI-240 BAC library selected by BLAST for having a single significant match in the human genome, distant one from one other 1 Mbp in the human genome, and having preferentially an orthologous hit in the mouse genome. Approximately 3000 cattle bacterial artificial chromosome end sequences were added to the previous RH map, increasing the number of markers 4 time. The number of comparative points in the human genome was increased 5-fold.

An important advance made possible by mapping cattle BESs is that the RH map could be anchored directly to the whole-genome BAC fingerprinting contig. Comparisons of BES order on the RH maps and whitin the fingerprinting contigs is used to identify inconsistency in the maps and markers or clones that are presumably 'out of place' on the basis of their cattle –human comparative map location. This comparison will ultimately be important in selecting the correct minimum tiling path for the BAC-skim sequencing and correctly assembling the cattle genome sequence. Moreover also the additional mapping information coming from the integration of RH and linkage map would greatly improve the bovine genome sequence assembly (Snelling et al:, 2004; Weikard et al., 2006).

I-VIII-g Integration of bovine RH map data with genetic linkage maps

Linkage maps have been important in identifying chromosomal regions influencing economically important traits in cattle (Casas et al., 2001; MacNeil et al., 2001; Li et al., 2002), but because the lack of recombination between closely linked markers limits resolution, linkage maps are of limited value for ordering closely linked markers and identifying genes underlying quantitative trait loci. The radiation hybrid mapping provides higher resolution for ordering close markers, but high breakage frequency RH data are less reliable than linkage data for ordering widely separated groups of markers (Schiex et al., 2001).
Integrating linkage and RH data into a single map not only will refine marker order to facilitate genomic sequencing, but will also increase the efficiency of identifying genes associated with QTL.
Integration of linkage and RH maps has been reported for a number of species (NIH News Release, http://www.genome.gov/page.cfm?pageID=10506668), like the dog (Breen et al., 2001), the rat (Steen et al., 1999), the feline (Sun et al., 2001) and individual bovine chromosomes (Amarante et al., 2000; Rexroad et al., 1999; Drogemuller et al., 2002). The general approach to integrated mapping has been to score several markers from linkage maps on the RH panel, then align the independent maps via common markers.
While Nadkarni (1998) and White et al. (1999) described procedures to synthesize information from multiple independent analyses into a single merged map, Snelling et al., (2004), differently, used directly data from independent analyses to contribute to the construction of two maps and then merged independent data sets with common markers to built a single integrated map.
Agarwala et al. (2000) developed procedures for integrating RH maps, where markers common to independent RH panels contributed to the solution of a comprehensive RH map, while Schiex et al. (2001) developed and released CarthaGene software (CarthaGene home page, http://www.inra.fr/bia/T/CarthaGene) to merge and solve integrated maps representing multiple linkage and RH data sets.

The bovine chromosome (BTA) 15 was considered interesting and was chosen from Snelling WM et al. (2004) to study the integration of linkage and RH data and to compare the bovine and human genome because a QTL for meat tenderness was reported to be present on this chromosome (Keele et al., 1999; Rexroad et al., 2001) and because comparative mapping indicated that alternative segments of human chromosome (HAS) 11 are conserved on BTA 15 and 29 (Amarante et al., 2000; Rexroad et al., 2001; Gautier et al., 2002). They used the second-generation linkage map of bovine genome (Kapper et al., 1997), and the radiation hybrid data for 109 markers from the ComRad project radiation hybrid panel (94 cells lines, Williams et al., 2002; Gautier et al., 2002) to construct an integrated BTA15 map representing 145 markers, whose 42 shared by both data sets, 36 unique to the linkage data and 67 unique to RH data.

Another study that aimed to the construction of a high-resolution map of a specific chromosome was carried out from Weikard et al. (2006) on the bovine chromosome 6 (BTA 6) because a number of different QTL for various phenotypic traits, including milk production, functional, and conformation traits in dairy cattle as well as growth and body composition traits in meat cattle, have been mapped consistently in the middle region of this chromosome (Bovine QTL Viewer at texas A&M University 2005, http://bovineqtl.tamu.edu/ ; Reprogen QTL Mapof Dairy Cattle Traits 2005 http://www.vetsci.usyd.edu.au/reprogen/QTL_Map/).

The objective of the study was to construct a high-resolution 'gene rich' RH map for the target chromosomal region of BTA6 containing candidate genes underlying the QTL for milk production traits (Cohen-Zinder et al., 2005; Olsen et al., 2005; Schnabel et al., 2005; Weikerd et al., 2005) in order to dissect the different QTL at the gene-based level.

A total number of 237 loci including 115 genes and expressed sequence tags (ESTs) and markers from the recently published bovine genetic map (Ihara et al., 2004) were typed on the cattle-hamster 12000-rad WG-RH panel (Rexroad et al., 2000) and the new RH map, with a total of 234 loci, displayed a substantial increase in loci density compared to existing physical BTA6 maps. The average retention frequency of the markers was 15.2% and the average inter-loci interval on the targeted BTA6 region covered on the RH map was 17.8 cR12000, corresponding to approximately 300 kb. The order of loci determined in the new map for the targeted BTA 6 region was generally consistent with that reported on previous published RH (Itoh et al., 2005, Everts-van der Wind et al., 2004) and linkage map (Ihara et al., 2004; Snelling et al., 2005).

High-resolution RH maps integrate anonymous markers, ESTs, and genes from currently available bovine linkage and RH maps as well as high number of comparative anchor loci derived from the orthologous human chromosomes. Although a number of links to the currently existing genetic, cytogenetic, and RH maps are possible, a multitude of contigs and scaffolds of the available bovine genome sequences resources still have to be anchored and/or oriented on the chromosomes. Connecting animal phenotypes associated with the QTL anchored on genomic level with putative underlying genes would accelerate the identification

of sequence polymorphisms and gene variants and the development of SNP markers for validation of association substantially.

I-IX International bovine projects

I-IX-a International physical map and Bovine sequencing projects

An international bovine physical map project (www.bcgsc.bc.ca/projects/bovine_mapping, http://www.livestockgenomics.csiro.au/cattle.shtml) was proposed to analyze single digest fingerprintings obtained from 280000 BAC clones to identify new fusions between contigs from the two BAC-based physical maps derived from the BAC library of the INRA (Schibler et al., 2004) and the CHORI-240 BAC library. Additional mapping information can facilitate the ordering of fingerprinting contigs for the construction of physical BAC maps covering whole chromosomes and ultimately provides a valuable starting point for whole genome sequencing projects, like it happened for the human (Cao et al., 1999), for the mouse (Gregory et al., 2002) and in Drosophila (Hoskins et al., 2000).
The ultimate map for a species is the correctly assembled genome sequence.
The U.S. National Institute of Health (NIH) has given high priority to the complete genome sequencing of two Cetartiodactyl species, *Bos taurus* (cattle) and *Sus scrofa domestica* (pig; http//www.genome.gov/page.cfm?pageID=10002154) to make progress the mammalian comparative genomics because the mammalian order Cetartioactyla comprises a philogenetically distant clade of eutherian mammals relative to primates, having diverged from a common ancestor approximately 85 million years ago (Kumar and Hedges, 1998), and, on the basis of a limited amount of sequence information for orthologous regions in a number of mammals (Thomas et al., 2002), it is clear that a Cetartiodactyl genome will play an essential role in informing the human genome for conserved non coding structural and regulatory elements, for properly annotating exon/intron boundaries, and for the identification of novel genes.
The bovine genome sequencing project started in 2003 and used a combination of whole genome shotgun sequencing (WGS) and sample sequencing of a minimum tiling path of BAC clones spanning the genome. An international Bovine Genome Sequencing Consortium was established.
In october 2004 the initial draft of the bovine genome sequence using was released (NCBI Bos taurus Genome Resources 2005-http://www.ncbi.nlm.nih.gov/genome/guide/cow/; Human Genome Sequencing Center at Baylor College of Medecine_Bovine Genome project 2005-http://www.hgsc.bcm.tmc.edu/projects/bovine).
Preliminary assemblies of the current bovine genome sequence update representing a 6x coverage were established and announced in October 2005 (Pre! Ensembl (Btau 2.0) in NCBI Bos taurus genome mapview (build 2.1), (Pre! Ensembl Bos taurus Genome Assembly Site 2005, http://www.ensembl.org/Bos_taurus/index.html; NCBI Bos taurus Map Viewer Site

2005, http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9913), while recently a new assemblies (build 3.1) is available (www.livestockgenomics.csiro.au/perl/gbrowse.cgi/bova3/).

I- IX-b The BovGen project

The BovGen project started the first January 2003 and involved the work of European and extra-european scientific groups belonging to different institutes (Rosline Institute-UK, University of Alberta-Canada, INRA-France, Catholic University of Piacenza-Italy, Tuscia University of Viterbo-Italy, Max Planck Institute for Molecular Biology of Berlin, Germany).
The project had the objectives to develop advanced genomic tools to provide the necessary infrastructure for researchers to study the molecular and genetic control of important traits in cattle. Information on these traits could then be applied to the selection of cattle that are best suited to producing healthier food products of the desired quality in appropriate production systems.
As the project progressed the international project to sequence the bovine genome made very rapid progress and an addition priority objective was included in the BovGen project: to work closely with the international Bovine Genome Sequencing Consortium to aid the assembly of a high quality bovine sequence.

In details the intended molecular tools to improve or create were:
1) the best characterised bovine expression array available with around 20,000 unique expressed sequences (ESTs) to give the possibility to examine gene expression profiles in target cells under various physiological conditions such as, fed or starved, healthy and diseased as an important route to gene discovery and understanding gene function.
It was planned that the expression arrays should contain a non-redundant , or “unigene” set of 20000 unique ESTs identified in cDNA clones from a bovine brain cDNA library. The non-redundant set of ESTs was created by the Max Planck Institute starting from the brain as it was supposed that this organ expresses the greatest diversity of genes in the body and the 20000 ESTs were estimated, from the human sequence, to represent about 30-40% of all genes in the genome.
2) a high resolution RH bovine map which let the construction of cattle-human comparative map that could contain not only more than 300 and 400 links between bovine and human genomes, like the actual RH comparative maps has, but at least more than 3000 links, as the actual mouse-human comparative map, in order to place cattle genomics information on a par with the mouse-human comparative information.
3) the construction of long genome spanning BAC contigs. In this project the INRA bovine BAC library, with 105000 clones, including 20000 clones from the CHORI 240 BAC library, was available and was characterised with the ESTs sequenced to increase the immediate

utility of the BAC library and provide access points to BAC clones for local sequencing objectives.

4) the ultimate bovine genome sequencing. An international consortium competing with the international Bovine Genome Sequencing Consortium was established to sequence the bovine genome. The corner stone to the sequencing work was a whole genome BAC contig. The characterisation of the BAC library in this project was an important input to the assembly of the genome wide BAC contig. An additional contribution of the Bovgen Project was the ordering of Sequence scaffolds on chromosomes, which was achieved using markers identified within the sequences to align them with the chromosomal maps.

Almost the totality of objectives of the project were achieved and 30 publications and numerous international conference presentations were produced from this work, that made significant contribution to the international bovine sequencing project.

II-Objective

Several approaches can be used to determine the order of loci on chromosomes and hence develop maps of the genome. However, all mapping approaches are prone to errors either arising from technical deficiencies or lack of statistical support to distinguish between alternative orders of loci. Errors in maps can greatly affect the ability to map and isolate genes for complex and Mendelian traits (Risch and Giuffra 1992; Feakes et al., 1999; Goring and Terwilliger 2000), for the identification of QTL.

Inaccuracies in genetic maps can result from genotyping errors, as well as from the use of a limited number of informative meiosis to generate maps. A higher confidence in genetic-map order can be obtained by creating maps using a likelihood-ratio criterion of >= 3, as opposed to using a minimum-recombination map (Morton 1955).

Errors in the order of markers on physical maps can be due to problems with assembly or to incorrect identification of marker positions. Even when the order of markers is known to be without error, accurate estimates of recombination fractions will play an important role in linkage and associations studies (Clerget-Darpoux et al., 1986; Risch and Giuffra, 1992; Goddard et al., 2000; Collins et al., 2001; Reich et al., 2001).

The accuracy of the genome maps could in principle be improved if information from different maps (genetic, comparative with other species, RH submitted to different radiation intensity, physical, sequence assembly) was combined to produce integrated maps.

The publicly available bovine genomic sequence assembly is a draft that contains errors. Correcting the sequence assembly requires extensive additional mapping information to improved reliability of ordering of sequence scaffolds on chromosomes.

RH panels represent a powerful tool to construct high-resolution maps.

RH panels are generally characterised using microsatellite markers; however the number of these markers is often insufficient to join all the linkage groups and assemble complete maps, particularly for high-resolution panels. The development of additional anonymous markers can be a time-consuming task, and generally other types of markers, particularly ESTs, are used to saturate RH maps. These ESTs also serve to link the RH map with maps in other species (Schlapfer et al., 2002; Weikard et al., 2002).

The objective of the work described is the construction of a bovine high-density RH map, one of the main aim of the BovGen project, which could be used for the construction of an integrated map and could contribute to the International Sequencing Project to aid the final assembly of the bovine genome sequence.

It is discussed the presence of possible errors in the RH map comparing with other recently published RH and genetic maps (the Illinois-Texas (ILTX) RH map and the MARC 2004 linkage map) aligning the sequence of the corresponding mapped markers. All the bovine maps were aligned with the 6x bovine assembly (Btau_2.0 sequence) to identify its potential inconsistencies.

III-Material and Methods

III-I Sequencing of ESTs

A non-redundant “unigene” set of ESTs was selected by oligo-nucleotide fingerprinting and clustering of cDNAs from a brain library (Herwig *et al.*, manuscript in preparation). This non-redundant cDNA clone set contains 23040 bovine clones grouped by sequence assembly of ESTs into 14989 unique cDNA clusters and singletons. The cDNA clones of the “unigene” set were amplified in a 384-well microplate format by PCR consisting of an initial denaturing for 2 min at 95°C, denaturing for 45 sec at 94°C, annealing and elongation for 4 min at 65°C in 30 cycles. PCR primers were complementary to the insert-flanking vector sequences. The PCR mix contained 5 pmol of forward and reverse primers (table 1), 0,1 mM dNTP’s, 1,5 M Betain, 1x PCR buffer, 0,1 mM Cresol Red and 1 U per reaction Taq DNA polymerase. PCR buffer consisted of 0,5 M KCl, 1% Tween20, 15 mM $MgCl_2$, 350 mM TrisBase, 150 mM Tris/HCl pH 8,3. PCR fragments were subjected to sequence analyses using BigDye-terminator chemistry (Applied Biosystems) and a 3700 DNA sequencer (Applied Biosystems). Average sequence read length was 750 bp. The individual EST sequence data were submitted to GenBank and are publicly available under accession numbers CO871676-CO897060.

Table1. Sequence of primers used to amplify the cDNA inserts

forward primer	**GGATCTATCAACAGGAGTCCAAGCTCAGCT**
reverse primer	**TCACCATCACGGATCCTATTTAGGTGACAC**

III-II Primer design

Maximum sequence information for annotation was achieved by aligning the ESTs data with available public cattle transcript sequences contained in the TIGR bovine gene index. TIGR clusters and corresponding ESTs cattle sequences produced were aligned and the resulting 14989 cluster sequences (consensus) were used for the subsequent construction of primers. Cluster sequences were aligned with bovine genomic sequences and only those showing clear splicing were used to define the precise exon-intron boundaries for the final primer selection. The primer design was carried out using dedicated software now in the public domain (Polyprimers, http://www.unitus.it/SAG/primers.zip). The software uses the nearest-neighbour method (SantaLucia et al., 1996) to predict the complementarity of primers and secondary structures (dimers, hairpin etc.) and is able to process large number of sequences in batches, picking primers in designated regions. To minimize the amplification of hamster DNA contained within the RH panel cell lines, primer pairs were designed with one primer within exon, the other within the adjacent intron or non coding sequence. The primer design was standardized to achieve a maximum of uniformity in their amplification conditions.

Primer details are available to the public in the ArkDB database (ArkDB Public database browser, http://www.thearkdb.org).

III-III Screening of the Roslin RH panel

2473 marker loci were successfully typed on the 94 cell lines of a 3000-rad bovine/hamster RH panel as described by Williams *et al.* (Williams et al., 2002). Vectors of 262 AFLP markers (Gorni C et al., 2004) were added to the dataset.

III-IV RH data analyses

RH vectors were assigned to chromosomes by analysing 2-pt linkage with mapped loci (Gorni et al., 2004) using RH mapper (Slonim et al., 1997). Multipoint maps were constructed using the default algorithm of the Carthagene software (Schiex and Gaspin, 1997). The initial multi-point map was improved by an iterative process of inspection of marker loci and removal and alternative addition of badly linked or disrupting loci. This process resulted in the removal of 122 loci that could not be reliably fitted into the chromosome maps with highest probability. The best maps generated by this process were compared to the ComRad RH-map (Gorni et al., 2004) and the MARC 2004 linkage map (Ihara et al., 2004) and regions showing discrepancies were examined in detail to identify the presence of problem markers. Marker positions on the maps of each chromosomes are available from the ArkDB database at http://www.thearkdb.org.

III-V Mapping of marker associated sequences against the bovine sequence assembly

ESTs sequences used to design the primers for mapped loci were aligned with the assembled 6x bovine sequence assembly (Btau_2.0) using BLAST (Altschul et al.,1990) and Spidey (Wheelan et al., 2001). To filter out incorrect alignments the BLAST e-value was set to a maximum of 1e-20 and minimum percent identity to 90%. In addition, the relative length of the BLAST hit (i.e. coverage, or length of the hit divided by the length of the query sequence) had to be at least 80%. Where ambiguous alignments were observed higher stringency filters were applied (sequence similarity higher than 97.5% and coverage higher than 90%).

III-VI Diagrammatic representation of chromosomal maps

Visual representation of map alignments for figures 2-5 was achieved using cMap (GMOD Generic Software Components for Model Organism Database, http://www.gmod.org/cmap/). For figure 1, a custom ruby script was used in combination with the bioruby toolkit (BioRuby http://www.bioruby.org).

IV-Results

IV-I Radiation hybrid map

A total of 2735 markers were added to those, 1231 markers, on the first-generation whole-genome RH maps (Williams et al., 2002), of which 2473 are newly mapped loci and 262 are previously reported AFLP markers (Gorni et al., 2004), giving a total of 3966 markers, of which 1999 are within genes, 1072 are microsatellite loci, 262 are AFLP markers, 376 are BAC end sequences and 257 are from ESTs sequences that do not show convincing similarity to the annotated bovine sequence (table 1). The RH maps for the 30 bovine chromosomes constructed from this data can be viewed and information can be downloaded from the ArkDB database (http:// www.thearkdb.org).

The total length of the RH map, including all bovine autosomes and the X chromosome is 760 Rays (R). The map of BTA 28 is the shortest one, 1141 cR, and the longest one is that of BTA7, 4408 cR. The average marker interval over the whole genome is 19 cR ranging between 12 cR (BTA29) to 29 cR (BTA20). Distance comparisons between common markers on the RH map, MARC linkage map and the bovine sequence suggests, on average, that 1 cR on the BovGen RH map is equivalent to 0,04 cM and 23 Kbp respectively, although this varies considerably across the genome.

Table1. Statistics of the RH maps by chromosome.
[a] BAC end sequences; [b]ESTs which could not be assigned to an annotated sequence; [c]average over whole genome

BTA	marker numbers		marker types					map length			marker density	
	no of markers	new mapped	AFLP [26]	BES[a]	genes	micro-satellites	unknown ESTs[b]	cR	cM	Mbp	cR/ marker	Mbp/ marker
1	158	83	8	2	69	70	9	3695.80	154.67	102.83	23.39	0.65
2	169	112	11	3	98	46	11	3487.70	128.88	86.54	20.64	0.51
3	206	160	10	3	134	35	24	4405.40	128.90	85.36	21.39	0.41
4	113	56	10		64	36	3	2605.20	119.93	69.56	23.05	0.62
5	275	220	9	94	105	40	27	4210.30	135.60	76.43	15.31	0.28
6	59	28	6		33	20		1699.40	134.42	69.62	28.80	1.18
7	217	153	13		141	45	18	4408.80	135.56	69.14	20.32	0.32
8	93	51	7	10	35	38	3	2458.60	128.62	62.12	26.44	0.67
9	133	62	8		58	64	3	3004.10	116.17	64.65	22.59	0.49
10	162	108	17	2	91	40	12	2760.50	118.83	70.00	17.04	0.43
11	178	123	14	3	102	48	11	3658.90	130.97	87.17	20.56	0.49
12	89	37	16	1	34	36	2	1553.30	109.95	48.61	17.45	0.55
13	128	76	14		64	36	14	2275.40	105.38	62.72	17.78	0.49
14	223	189	1	169	16	33	4	2667.10	103.95	50.71	11.96	0.23
15	147	43	12	2	77	38	18	2434.10	109.75	53.82	16.56	0.37
16	120	79	9	1	72	33	5	2957.20	94.46	56.99	24.64	0.47
17	115	65	15		68	27	5	2654.10	95.86	45.92	23.08	0.40
18	200	155	9	5	141	25	20	3182.10	84.38	56.51	15.91	0.28
19	156	104	7	53	58	35	3	2136.70	109.61	56.39	13.70	0.36
20	58	26	9		26	23		1712.20	82.94	42.95	29.52	0.74
21	62	18	9		18	33	2	1449.00	83.79	49.72	23.37	0.80
22	111	74	5	5	69	31	1	2031.90	88.10	48.33	18.31	0.44
23	130	78	8	1	80	20	21	2345.70	80.05	41.60	18.04	0.32
24	68	15	6	5	19	35	3	1588.70	78.13	45.25	23.36	0.67
25	133	93	6	1	89	31	6	2256.20	68.42	41.41	16.96	0.31
26	75	34	6		36	30	3	1715.10	79.39	35.72	22.87	0.48
27	65	31	3	11	18	29	4	1191.20	67.37	31.57	18.33	0.49
28	65	31	5	4	25	22	9	1141.30	61.66	34.89	17.56	0.54
29	150	110	4		103	34	9	1895.70	69.73	45.82	12.64	0.31
X	108	59	5	1	56	39	7	2390.70	146.50	47.90	22.14	0.44
Total	3966	2473	262	376	1999	1072	257	75972.4	3151.97	1740.25	19.16[c]	0.44[c]

IV-II Comparison with the ILTX RH map

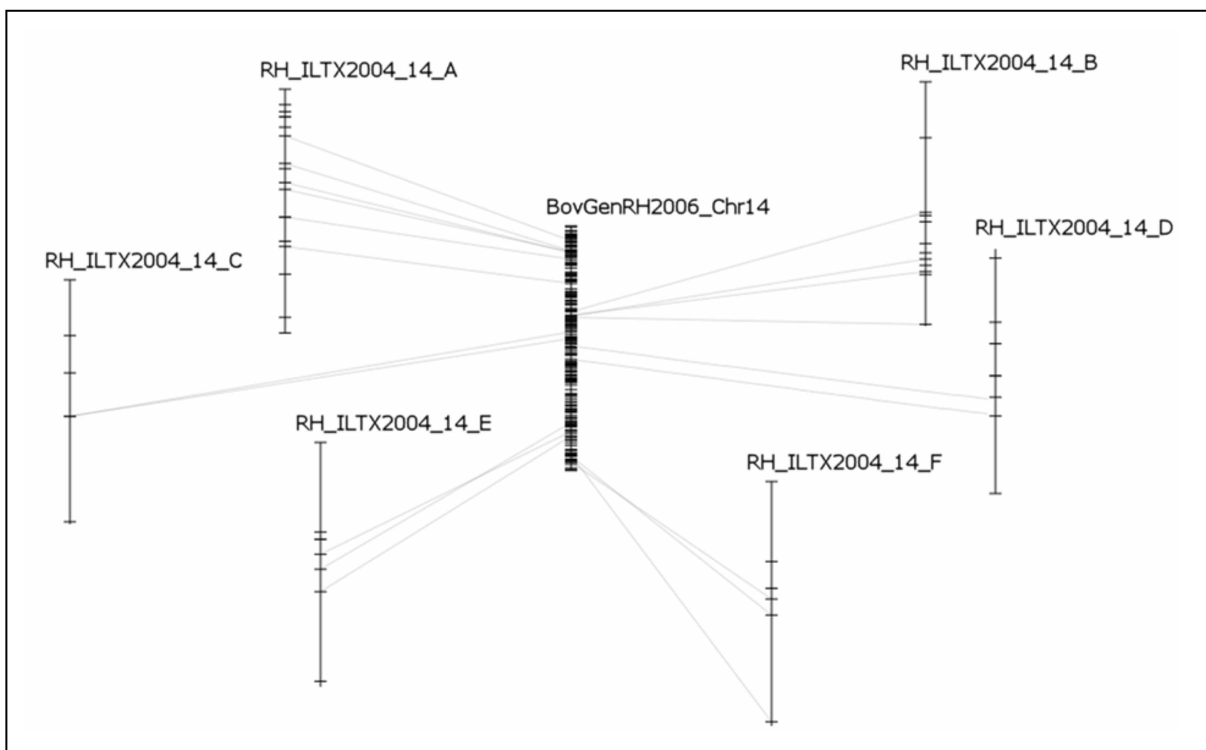
There are 241 marker loci in common between the BovGen RH map described here and the Illinois-Texas (ILTX) RH map, comprising 71 linkage groups (Everts-van der Wind et al., 2004). All of these common loci were assigned to the same chromosomes on both maps.

Correspondences in 32 linkage groups cannot be assessed for consistency of their order because the groups contain only one or two markers common between these maps. For the remaining 39 linkage groups 21 are in perfect agreement with the BovGen RH map and 14 have only one inconsistently positioned marker.

For example, the BovGen RH map of chromosome 14 has 20 markers in common with the ILTX RH map. These are divided into six linkage groups (14_A to 14_F), which are located consecutively along the chromosome. The groups contain 2 to 6 markers which are in common and the order generally agrees between both maps (figure 1). In four linkage groups (5_A, 7_A, 27_B and X_C) discrepancies between the maps are observed with more than one displaced marker. One of those, 5_A is relatively consistent despite four discrepancies in

order, as it contains 26 correspondences and covers a complete chromosome, and the discrepancies are minor. In contrast 7_A, 27_B, 30_C contain fewer correspondences (6 each) but all have several inconsistencies. Each of the three groups cover approximately half a chromosome and differ from the BovGen RH map in their marker order at 4, 4 and 5 correspondences, respectively.

Figure 1. BovGen RH map of the chromosome 14 compared with the corresponding six linkage groups of the ILTX RH map. Lines between maps connect markers common in both maps. Marker names were omitted to improve perceptibility.



IV-III Comparison with MARC 2004 linkage map

There are 885 marker loci in common between the BovGen RH and the MARC 2004 linkage maps (Ihara et al., 2004) which allows a detailed comparison of map orders and chromosome assignment.

Inconsistencies in chromosomal assignment are found for 5 of these 885 loci. In all these cases only individual markers are involved. The marker order on 13 chromosomes (BTA 4, 10, 11, 13, 14, 16, 18, 21, 23, 24, 25, 27 and 28) is in very close agreement between the BovGen RH maps and MARC 2004 maps. For example the order of the 27 markers on chromosome 4 which are in common shows only minor inversions of two pairs of linked loci (*BMS1840* and *MAF70* and also *BMS2571* which appear on the different sides of the co-mapping markers *BMS779* and *BMS3002*) (figure 2). Despite of the similarity in both cases the marker order as suggested by the MARC map is inconsistent with the multipoint map BovGen RH data, as the MARC order gives a much lower p-value.

On a further 13 chromosomes minor discrepancies between these maps are observed. On BTA 3, 5, 8, 9, 12, 17, 19, 22 and X the order of markers is essentially the same, but with a number of individual markers at different positions. For BTA 1, 2, 6 and 26 differences are observed involving the orientation of linkage groups, but with the order of markers within the linkage group is conserved. For example on BTA 26 the marker order is in general consistent between the BovGen RH and the MARC 2004 linkage map, however two small linkage groups 26_A (*BMS882*, *TGLA429*, *BMS2567* and *BM6041*) and 26_B (*MAF36*, *ILSTS091*, *MAF92* and *BM804*) have the same marker order in both maps, but are inverted with only one marker (*BM7237*) at divergent position (figure 3).
On four chromosomes major inconsistencies are observed, where complete linkage groups map to different chromosomal positions (BTA 7, 29) or where the order of markers differs within several linkage groups (e.g. BTA 7, 15 and 20). On BTA 7 for example, the position of two linkage groups 7_A (limited by the markers *CSKB071* and *TGLA303*) and 7_B (limited by the markers *BM6105* and *BM2607*) is exchanged. In addition 7_A is in a different orientation in both maps, while the marker order in 7_B is inconsistent (figure 4). Nevertheless, these discrepancies only involve about a quarter of the chromosome, and 12 out of the 38 common markers. The map positions of the other 26 markers are in close agreement between the two maps.

Figure 2. BovGen RH maps of the chromosome 4 compared to the MARC 2004 linkage map. The number of markers in each map is indicated in brackets. Lines between the maps connect markers common in both maps. Only marker names common in both maps are displayed.

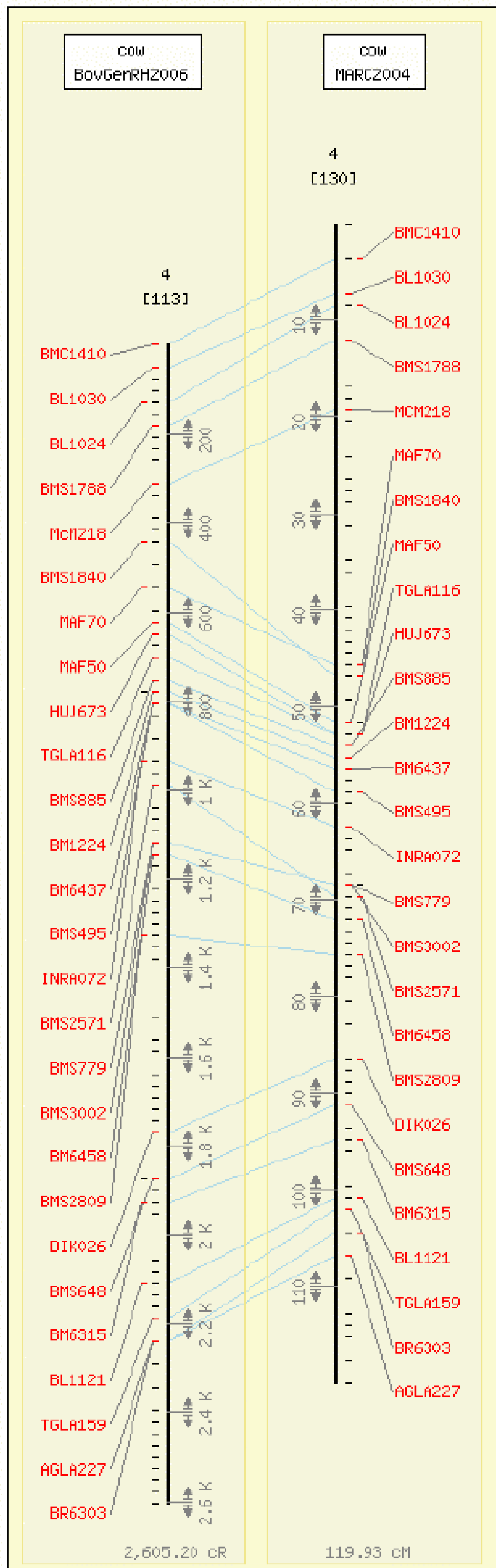


Figure 3. BovGen RH map of chromosome 26 compared with the corresponding MARC 2004 linkage map. The number of markers in each map is indicated in brackets. Lines between the maps connect markers common in both maps. Only markers names common in both maps are displayed. Locations of discussed linkage groups and their orientation are indicated by arrows.

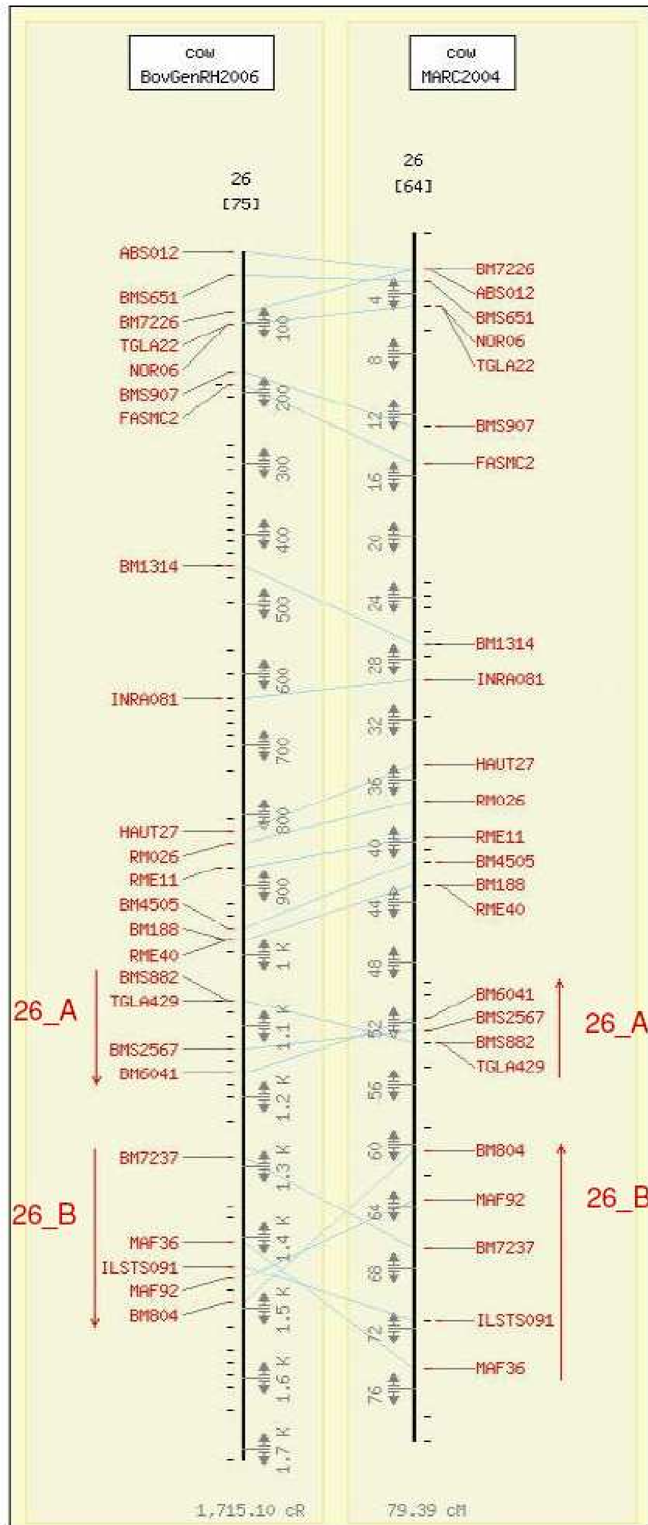
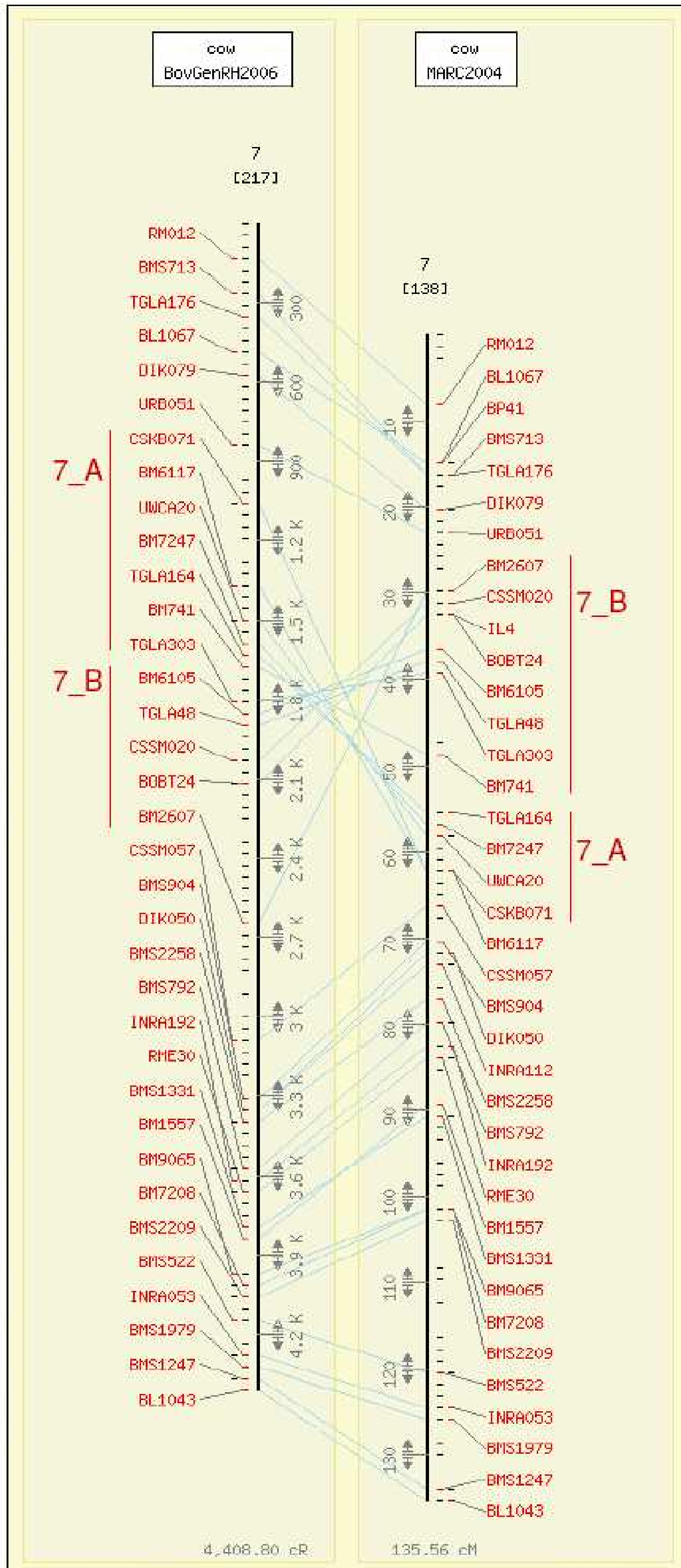


Figure 4. BovGen RH map of chromosome 7 compared with the corresponding MARC 2004 linkage map. The number of markers in each map is indicated in brackets. Lines between the maps connect markers common in both maps. Only markers names common in both maps are displayed. Locations of discussed linkage groups and their orientation are indicated by arrows.



IV-IV Comparison with the 6x bovine assembly

Of the 3966 markers successfully included in the RH map, 2898 could be unequivocally assigned to a position in Btau_2.0 bovine sequence, 2767 were assigned to the same chromosome, but 131 mapped on different chromosomes between the BovGen RH map and the sequence. On seven chromosomes inconsistent assignments involving groups with three or more markers were observed (table 2).

On most chromosomes there were many differences between the map order and the sequence: only on two chromosomes, BTA 9 and 14, the discrepancies were minor, involving a few individual markers in a different order. On most chromosomes large discrepancies involving complete linkage groups and/or large numbers of individual loci were seen particularly on chromosomes 5, 7, 16, 22, 25 and 29. On chromosome 16, six linkage groups are located at different position on the sequence compared with the BovGen RH maps (figure 5).

When markers that were at inconsistent positions between the BovGen RH and either the ILTX or MARC linkage maps were removed, 217 common markers with the ILTX RH map and 771 common markers with the MARC2004 linkage map remained where the available mapping data were in agreement. The mapping order of these markers was then compared with the order in the bovine sequence. Using only the markers that are consistent between the BovGen and other RH or linkage maps, the comparison with the Btau_2.0 sequence reveals considerable discrepancies across the whole genome. On chromosome 5 six markers which could be assigned to positions in the sequence assembly appeared with inconsistent positions (*BP1*, *AGLA293*, *ILSTS022*, *CSSM022*, *ILSTS066*). The remaining markers are in close agreement between the three maps and reveal significant inconsistencies with the sequence assembly (figure 6).

Table 2. Inconsistent chromosome assignments between the BovGen RH map and Btau_2.0 sequence. Only the seven most significant cases are listed, involving at least three linked markers. HSA4 is a homologue to BTA6, MM15 and HSA8 to BTA8, HSA14 to BTA21 and HSA17 to BTA19. Most 8 likely assignments are indicated by bold font.

Case	Markers involved	Assignment BovGen RH BTA	Assignment Btau_2.0 BTA	Other assignments species, chromosome [reference]
1	*BMS4030* *BOVGEN_158* *BOVGEN_91*	**1**	4	**BTA1** [40]
2	*MAF23* *BZ855103* *BZ864360*	**5**	4	**BTA5** [41]
3	*ADH7* *ADH1A* *ADN1C*	**6**	21	**HSA4**[a] [42, 43, 44]
4	*PTK2B* *BZ948637* *B4GALT1*	**8**	5	**BTA8** [1], **MM15**[a] [29], **HSA8**[a] [30]
5	*KIAA0284* *Q9Y4F5* *KNS2* *BTBD6*	11	**21**	**HSA14**[a] [31]
6	*BZ850749* *CC517527* *CC471629*	14	25	-
7	*ACLY* *KLHL11* *SC65* *JUP* *E0362G17*	**19**	23	**HSA17**[a] [45, 46]

Figure 5. BovGen RH map of the chromosome 16 compared with the 6x bovine assembly and the MARC 2004 map. The corresponding sequence similarity hits are connected by lines. The number of markers in each map is indicated in brackets. Only marker names common in both maps are displayed. Locations of discussed linkage groups are indicated.

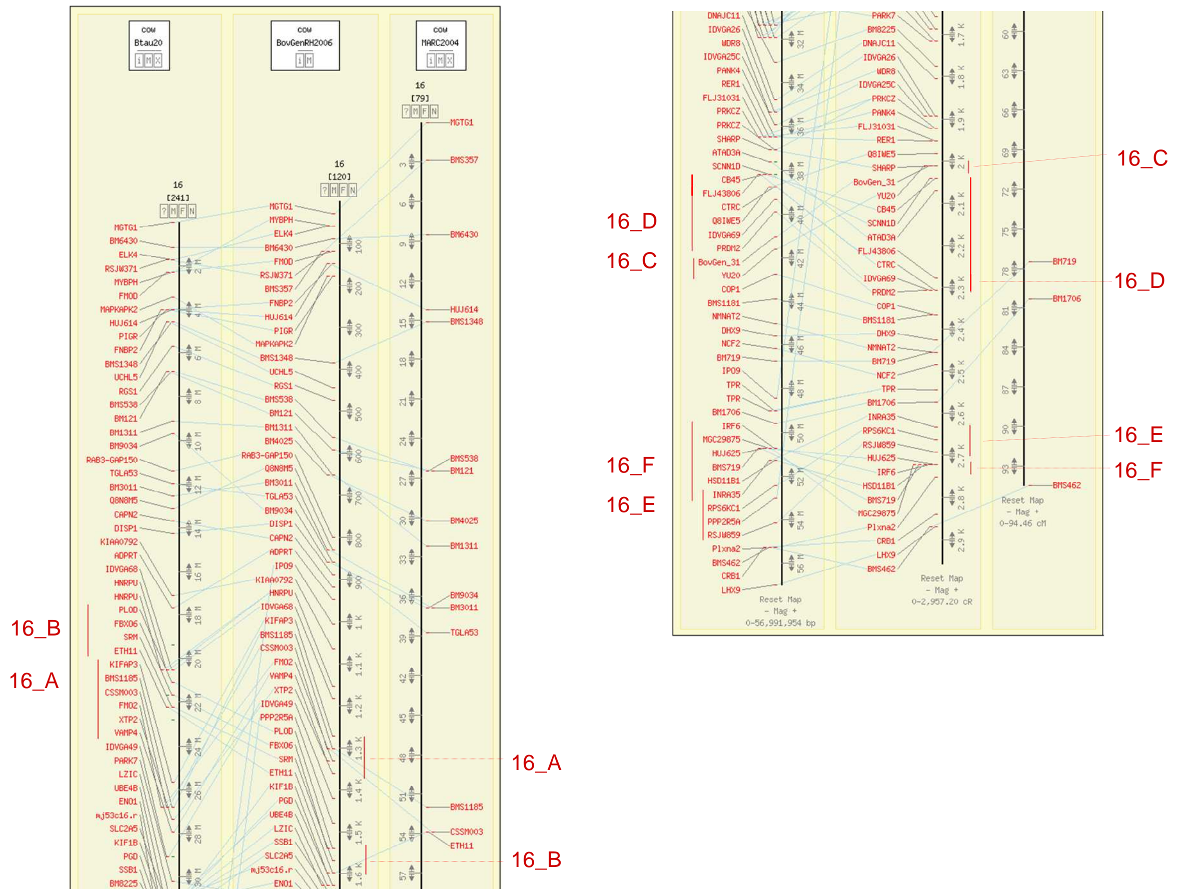
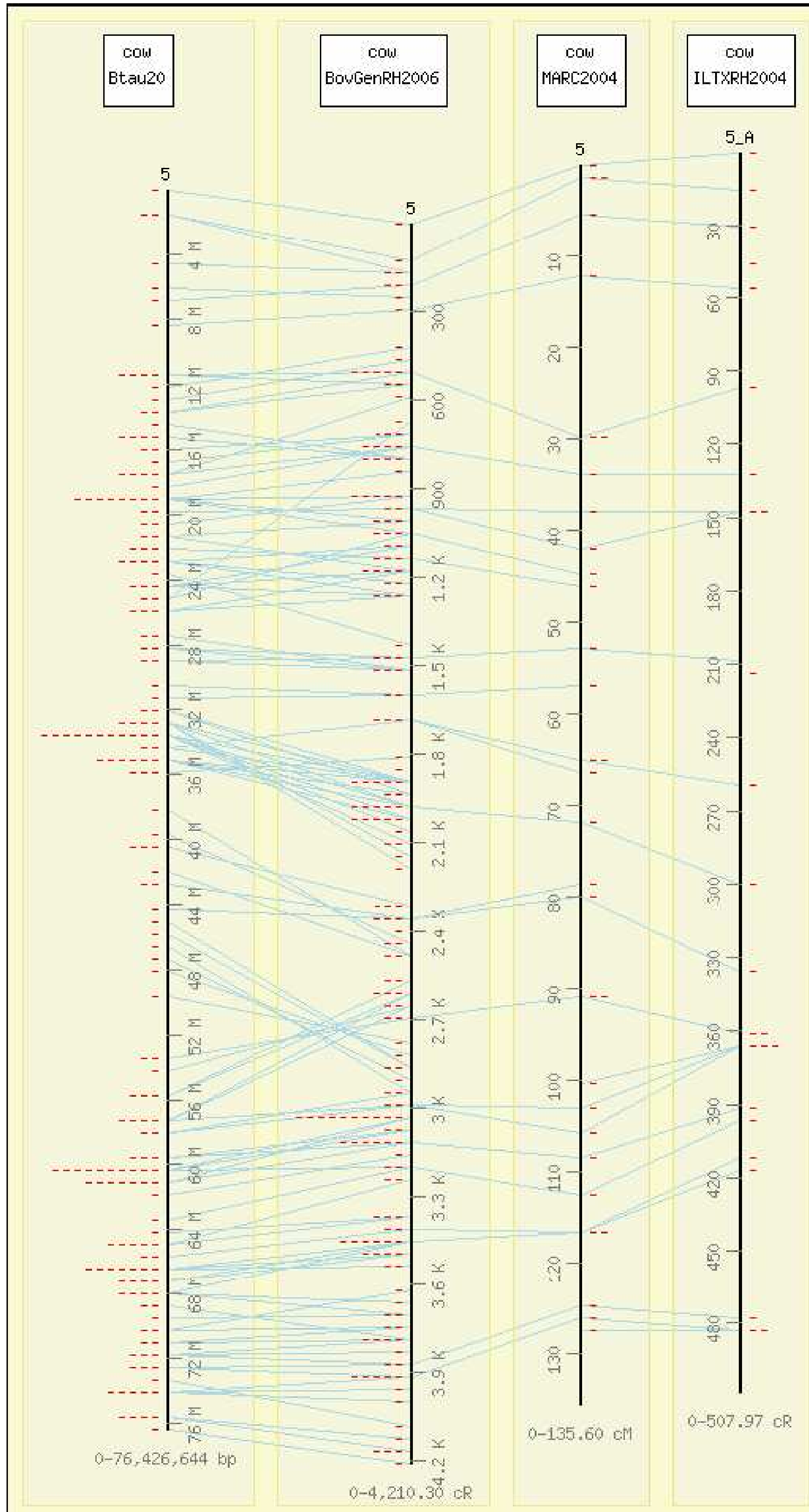


Figure 6. BovGen RH map of chromosome 5 compared with the 6x bovine assembly and with the MARC 2004 and the ILTX RH map. Markers which were inconsistently mapped between the two RH and the MARC linkage mp and also assigned to a position of the sequence assembly were removed. Lines between the maps connect common markers.



V-Discussion

The resolution of genome maps differs between approaches, and all approaches, including the assembly of a whole genome sequence, are prone to errors: in some cases insufficient information is available to assign the correct order or positioning of loci, while data errors can introduce distortions in the maps. The ultimate genome map of a species is the correctly ordered DNA sequence. Achieving the correct sequence assembly uses several levels of information. Sequence information from other species, including the human genome could be used as a template, but should be treated with extreme caution as local species specific variations are known (Ranz et al., 2001).

Direct sequence information is used for local assembly of shot-gun sequence reads into contigs, and these contigs are then assembled into scaffolds using additional information, such as overlapping clones, and sequences from paired clone ends. The ordering of these scaffolds on chromosomes and assembly of the final sequence relies on additional mapping information, including BAC fingerprint contig maps, linkage maps and RH maps.

In this work it was described a RH map with approximately 4000 mapped loci which will contribute to the assembly of the bovine genome sequence.

V-I Comparison with other linkage and RH maps

The reliability of different maps can be assessed by examining consistency in alignment of common loci, however it is important that the information used when assembling the maps is independent, as circular arguments can give a false measure of agreement. In contrast to the approach of Itoh et al. (2005) it was not used a linkage map as template for the construction of the RH maps presented here because the aim was to assemble the most likely maps using only the RH information. This independent data can then be used to assess potential errors across different maps. It was carried out an alignment of the BovGen RH maps with the other available maps of the bovine genome and with the Btau_2.0 sequence assembly, but only after the maps were constructed. This approach could result in maps that are less consistent with other published information, but it is important to realise that is the only way to contribute new information. This independent mapping information can be used to develop a combined map which carries a measure of map confidence, based on similarity and differences between maps using independent data.

The BovGen and ILTX RH maps (Band et al., 2000; Everts-van der Wind et al., 2004; Everts-van der Wind et al., 2005) appear to be more consistent with each other than with the MARC 2004 linkage map. Some inconsistencies between linkage and RH maps may be due to the different mapping approaches, however; the observation of the apparent higher consistency between the RH maps must be treated with care. The BovGen RH map has fewer loci in common with the ILTX map than with the MARC 2004 linkage map and so fewer

discrepancies could be detected. Moreover, the ILTX map consists of 71 unordered linkage groups which are a major source of the inconsistencies.

V-II Comparison with the sequence assembly

Sequence similarity search algorithms used to align maps with Btau_2.0 have a considerable risk of errors as the algorithms might also detect gene duplications or similar motifs in different genes. To minimize this problem it was used very stringent parameters for minimum homology and maximized the required length of overlap between sequences. In addition sequence matches were assessed manually before accepting hits as correct. Thus the loci aligned between the different maps and the sequence carry a very high probability of correctly assigned homology. Differences in the position of individual markers in different maps could be simple technical variations explained by using different parameters and algorithms to construct the multipoint maps. Inconsistencies in the chromosomal assignment of individual markers may also have simple explanations, such as poor primer design resulting in amplification of related loci, and not the target locus. Of greater importance for the interpretation of the map information are inconsistencies affecting whole linkage groups. To minimise the propagation of errors in individual maps we eliminated markers that were inconsistently mapped from further analyses against the sequence assembly.
While the BovGen RH map is in general agreement with the ILTX map and the MARC 2004 map, chromosomal regions of high agreement with the Btau_2.0 sequence are quite rare. Many differences in the marker order between the Btau_2.0 sequence and the BovGen RH map cannot be detected when comparing the two RH and the MARC linkage map. Therefore, after eliminating regions and markers that were inconsistent between these maps, we found that there was poor overall consistency between the RH and linkage maps with the Btau_2.0 bovine sequence assembly. For example on chromosome 4 the marker order on the BovGen RH map is in agreement with the MARC 2004 and ILTX map, but is inconsistent with the sequence assembly. The extent of the inconsistencies detected with the sequence assembly reveals the need for improvement by inclusion of further combined mapping information (figure 6).
If we consider regions where there are inconsistencies between the different mapping methods, e.g. on chromosomes 7, 25 and 29, the assembled sequence is most consistent with the linkage map. Recalculating the maps for these three chromosomes using only markers that can be located in the bovine sequence gives a map length for chromosomes 7, 25 and 29 of 3780,7 cR, 1788,5 cR and 1683,1 cR respectively, when the markers are ordered according to the original BovGen RH maps. If the common markers are forced into the order they appear in the sequence assembly: the map lengths increases to 567,.6 cR for chromosome 7, 2680,5 cR for chromosome 25 and 2683,3 cR for chromosome 29, and the $\log_{10}$ likelihood decreases from -1306,58 to -1615,01 (BTA 7), from -763,13 to –982,82 (BTA 25) and from -741,18 to -976,64 (BTA 29). The marker order suggested by the bovine assembly and the MARC

linkage map is therefore incompatible with the data underlying the BovGen RH maps for these chromosomes.

V-III Assignment of markers to different chromosomes

The most significant problem in the genome assembly is that of erroneous chromosome assignments. By comparing assignments among different RH and linkage maps and also using comparative human or mouse information, it seems likely that the assignment in the bovine assembly is most often at fault (table 2). For example the markers *PTK2B*, *BZ948637* and *B4GALT1* (table 2, case 4) are closely linked on the BovGen RH map of BTA 8 and the linkage map of Barendse et al. (1997) which also locates the genes on BTA 8. This is also consistent with data from Fiedorek and Kay (1995) who mapped *PYK2B* (alias *PTK2B* or *Fadk*) on murine chromosome 15 and Inazawa et al. (1996) who mapped the gene on human chromosome 8 at positions which share conservation of synteny with BTA 8 (Everts-van der Wind et al., 2005).

In contrast these marker loci are placed on chromosome 5 in the Btau_2.0 sequence assembly. All three markers are located on a single sequence scaffold (chr 5.80), suggesting that the chromosomal assignment of this scaffold is wrong.

The linkage group formed by the markers *KIAA0284*, *Q9Y4F5*, *KNS2* and *BTBD6* was assigned to chromosome 11 on the BovGen RH maps; however the assignment is not consistent with other mapping data (table 2, case 5). The human homologues of these loci map to human chromosome 14 (Goedert et al., 1996) suggests that this group is correctly assigned in the Btau_2.0 sequence to chromosome 21 and that the BovGen RH assignment is incorrect. Nevertheless the linkage of this group to other markers on BTA 11 is convincing with LOD linkage values up to 13,8 between the extreme marker *KIAA0284* and the neighbouring markers on the BovGen RH map. If this linkage group is tested with markers located on BTA 21 using the BovGen RH datasets it shows no linkage. In the Btau_2.0 assembly this linkage group is at an extreme telomeric position and suggests that the statistical support for this assignment is weak and may have been made on the expected position derived from the supposed conservation of synteny between human and cattle chromosomes.

The markers *BZ850749*, *CC517527* and *CC471629* are assigned to chromosome 14 on the BovGen RH map and to chromosome 25 in the Btau_2.0 sequence assembly (table 2, case 6). These markers are derived from BAC end sequences of clones from the CHORI-240 library and are not present on other maps which could be used for comparison. All these markers are assigned to the scaffold Chr25.84 and are in a chromosomal region of the assembly with a low density of corresponding markers. In contrast on the BovGen RH map, the markers in the same region are at a higher density. This suggests that these markers are more tightly linked on the BovGen RH map and correctly positioned. No further information is available to resolve this inconsistency.

VI-Conclusions

There is striking consistency between the RH maps presented here, the MARC linkage map and the ILTX RH map. Using this data it is possible to identify possible errors in the assembly of the current bovine genome sequence and hence aid the improvement of the next sequence build. The inconsistencies between the BovGen RH, the Illinois-Texas RH and the MARC linkage maps fall into three categories, markers that are assigned to different chromosomes, which are few, minor rearrangements, which account for the majority of discrepancies, and major rearrangements of marker, or linkage group order, which also are few. When the major discrepancies between these maps are removed a large number of inconsistencies still remain with the bovine sequence assembly. Using the combined mapping information available from the high-resolution RH maps presented here together with the additional map data available from publicly available RH and linkage maps should allow the next assemble of the bovine genome sequence to be improved considerably.

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Second Part: The microRNA in the mammary gland

I-Introduction

I-I The miRNA

I-I-a RNA silencing and miRNA

From the discovery of the structure of DNA (1953, Watson and Crick) to our days, big steps have been done in discovering the biological mechanisms with which DNA can carry the genetic information, can transmit it from one cell to one other and transfer it in a molecule of RNA and later in the structure of a protein, the final functional actor of the biology of a cell.
Even if the recent completion of the human, mouse and other eukaryotic genomes were important scientific milestones towards the understanding of eukaryotic biology, it's not easy to assess which regions of DNA have simply structural functions, which are really transcribed and code from a protein, how many genes are presents in a genome. In the human the last reported genome annotation has identified only 20000-25000 protein-coding genes (International Human Genome Sequencing Consortium, 2004), in contrast with previous higher estimate (Fields et al., 1994) and this raise some questions about the real definition of an eukaryotic gene. A possible answer could be found in the presence of alternative-splicing and of many non-coding RNA gene that do not have any clear "Open Reading Frame"(ORF) and are very difficult to predict from genomic sequences (Costa, 2005).
For many years RNA was considered to be just accessory molecules involved in mediating transcription and translation. RNA molecules are very versatile and their chemical properties allow them to form complex tertiary structures capable of performing several roles that were thought to be under the exclusive domain of proteins (Szymanski et al., 2003). They can interact with different proteins forming ribocomplexes, they can associate with specific DNA and/or other RNA sequences, controlling several aspects of gene regulation and different molecular connections in cells that are only partially discovered (Mattick, 2004).
In 1969, Britten and Davidson for the first time proposed that RNAs can solve the problem of eukaryotic gene regulation determine which genes are turned off and on by base-pairing against DNA (Britten and Davidson, 1969). The idea was abandoned with the discovery of a large class of protein transcription factors.
It was only in the 1990 that two different groups discovered for the first time the mechanism of RNA silencing like an internal mechanism of defense in petunia observing a 'cosuppression' of a transgenic and an endogenous gene after the introduction of the first one in the plant (Napoli et al, 1990; van der Krol et al., 1990). In some years this phenomenon was discovered in a broad spectrum of eukaryotes, from fungi to flies (Zamore and Haley, 2005) and it was shown to be involved in a plethora of mechanism like the regulation of

transcription, of chromatin structure, of genome integrity and, most commonly, of mRNA stability.

More precisely double stranded RNA-mediated gene silencing is a general term that refer to several pathways by which double stranded RNA can orchestrate epigenetic changes, repress translation, and direct mRNA degradation in a sequence-specific manner. These diverse effects of non-coding RNA on gene expression have been termed RNA interference (RNAi) (Rao and Sockanathan, 2005). It is carried out by tree different class of small non-coding RNA: small interfering RNAs (siRNAs), repeated-associated small interfering RNAs (rasiRNAs) and microRNAs (miRNAs) that are distinguished by their origin, but that share a common set of proteins in the mechanism of production and action.

In the main lines RNA interference is triggered by double strand RNA (dsRNA) precursor that vary in length and origin and that is processed in the cytosol by a specific ribonuclease called Dicer into a short RNA duplex of 21 to 28 nucleotides in length which determines in a sequence-specific way which mRNA should be degradated. This short double stranded RNA guides a protein complex to the recognized mRNA target that is silenced by cleavage or translational repression.

In nature double stranded RNA can be produced by RNA polymerization starting from a viral RNA, for example, or by hybridization of overlapping transcripts, for example from repetitive sequence such as transgene arrays or transposons. Such dsRNA give rise to siRNAs or rasiRNAs which generally guide mRNA degradation or chromatine modification.

In the contrary endogenous transcripts that contain complementary or near-complementary 20-to 50-base-pair inverted repeats fold back on themselves to form dsRNA hairpins. These are processed in miRNA, that in the most part of cases repress translation, but that can also guide the degradation of mRNA (see review: Meister and Tuschl, 2004). This class is predicted to regulate alone one third of all human genes.

I-I-b The discovery of miRNAs

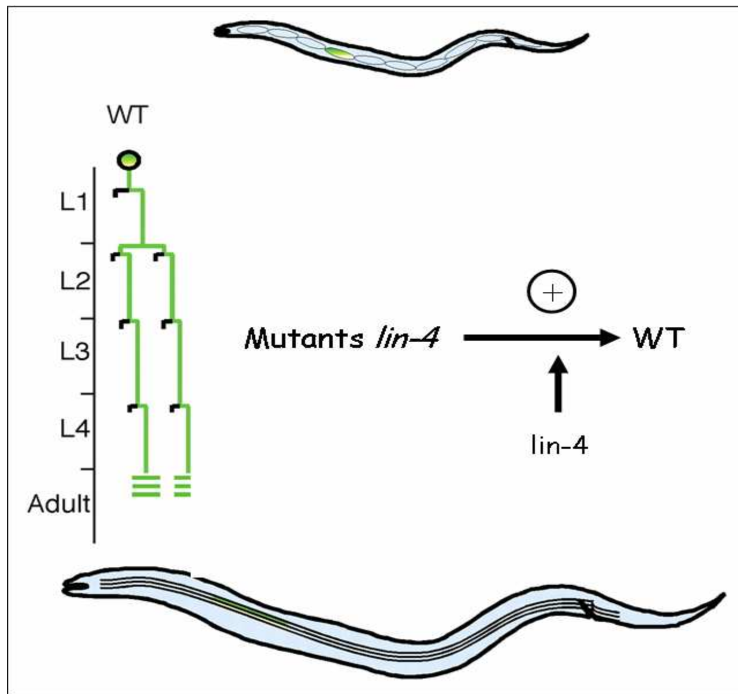
MiRNA are a class of evolutionary conserved, small (19-25 nt) non-coding RNAs that negatively regulate gene expression at the post-transcriptional level.

The finding member of miRNA family*, lin-4*, was identified in *C.elegans* through a genetic screen of mutants for defects in the temporal control of post-embryonic development (Chalfie et al., 1981; Ambros, 1989).

In *C.elegans* the post-embryonic development pass through four different larval stages (L1-L4) in which cell lineages have distinct characteristics. *Lin-4* encodes a 22-nt non coding RNA that is partially complementary to a short (7 nt) conserved site in the 3'-untranslated region (3'UTR) of the *lin-14* gene, its target (Lee et al., 1993; Wightman et al., 1991). *Lin-14* encodes a nuclear protein that is normally downregulated at the end of the first larval stage to

allow the developmental progression into the second larval stage (Ruvuk and Giusto, 1989) (figure 1).

Figure 1. The stages of development of *C.elegans* and the mechanism of action of lin-4.



Mutants for *lin-4* do not progress in the second larval stage showing reiteration of specific cell-division pattern of first larval stage even late in the development. Opposite phenotypes were observed in mutants deficient for *lin-14* and even before the molecular identification of *lin-4* and *lin-14* these genes were placed in the same regulatory pathway on the basis of opposite phenotypes and antagonistic genetic interaction. After a series of molecular and biochemical studies was demonstrated that the direct and imprecise binding of *lin-4* to the 3'UTR of *lin-14* was able to reduce the amount of the LIN-14 protein without changing in the level of *lin-14* mRNA (Olsen and Ambros, 1999). These evidences supported a model in which the *lin-4* RNA pair to the 3'UTR of *lin-14* to specify translational repression of it as part of the regulatory pathway that control the timing of development in the worm. Also another target of *lin-4* was discovered, *lin-28*, a cold-shock-domain protein that initiates the developmental transition between L2 and L3 stages (Moss et al., 1997).
For seven years any others miRNA was identified in nematodes and there was no evidence of any similar non coding RNAs beyond nematodes.
In 2000, the second miRNA was discovered, *let-7*, also using forward genetics in *C.Elegans*. *let-7* encodes a temporally regulated 21- nt RNA that binds to the 3' UTR of *lin-41* and *lin-57*, inhibiting their translation (Lin et al., 2003; Abrahante et al., 2003; Slack et al., 2000; Vella et al., 2004). *let-7* controls the transition from L4 stage to the adult stage (Reinhart et al., 2000). The identification of *let-7* not only suggested the existence of a new class of molecular regulator of the timing of developmental transition, but also it opened the way to the

discovering of many *let-7* homologs in other species. Pasquinelli et al. (2000) found, first through BLASTN searches, the existence of homologs of *let-7*, later experimentally by Northern blot, their expression in all stages of development of *D.Melanogaster* and in all tissues of human. They went on to find homologs in all vertebrates studied and in the same time siRNA were discovered and it was shown that components of the siRNA apparatus processing RNA are also involved in *lin-4* and *let-7* expression. This suggested that these small RNA could be more common than just *lin-4* and *let-7*. In less than one year, thanks to the work of three different labs, approximately one hundred of miRNA were cloned from flies (20 in *Drosophila*), worms (60) and human cells (30) (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). This first group of miRNA identified showed the same length, the same way of production from an endogenous precursor, and they were generally evolutionary conserved, some quite broadly, others only in more closely related species such as *C.elegans* and *C.briggsae*. Even in this first group some showed a tissue or cellular-specific expression, differently from *lin-4* and *let-7*, whose expression is temporally-regulated. Intensified cloning efforts have revealed numerous additional miRNA genes in plants, mammals, fish, worms, flies and even virus (Lagos-Quintana et al., 2002, 2003; Mourelatos et al., 2002; Ambros et al., 2003; Aravin et al., 2003; Dostie et al., 2003; Houbaviy et al., 2003; Kim et al., 2003; Lim et al., 2003) giving origin to the first microRNA registry, a public list to catalog miRNA and to facilitate the naming of newly identified genes (Griffiths-Jones, 2004). More than 330 miRNA have been cloned in humans (Griffiths-Jones et al., 2006; Hsu et al., 2006) and bioinformatic tools predict that they could be 1.000 and can represent up to the 3-5% of all the genes in the human genome.
Their high number, their spatiotemporal, tissue- and cell-type expression and the extensive conservation strongly indicated an important role in development, like it was supported later (Bartel 2004; He and Hannon, 2004).

I-I-c Biogenesis and mechanism of action

MiRNAs genes are widespread in the genome and their genomic localization and organization vary together with their mode of transcription (Bartel, 2004).
Most mammalian miRNA genes come from regions of the genome quite distant from previously annotated genes and are considered as independent transcription units with specific promoter core elements and polyadenylation signals (Pasquinelli, 2002; Cullen, 2004; Kim and Nam, 2006).
The remaining are located part in long non coding RNA transcript or, in the majority of cases, in introns of protein encoding genes. These are not transcribed from their own promoter, but they are transcribed together with their host genes, for example processed from introns by alternative splicing (Aravin et al., 2003; Lagos-Quintana et al., 2003; Lau et al., 2001; Lee and Ambros, 2001).

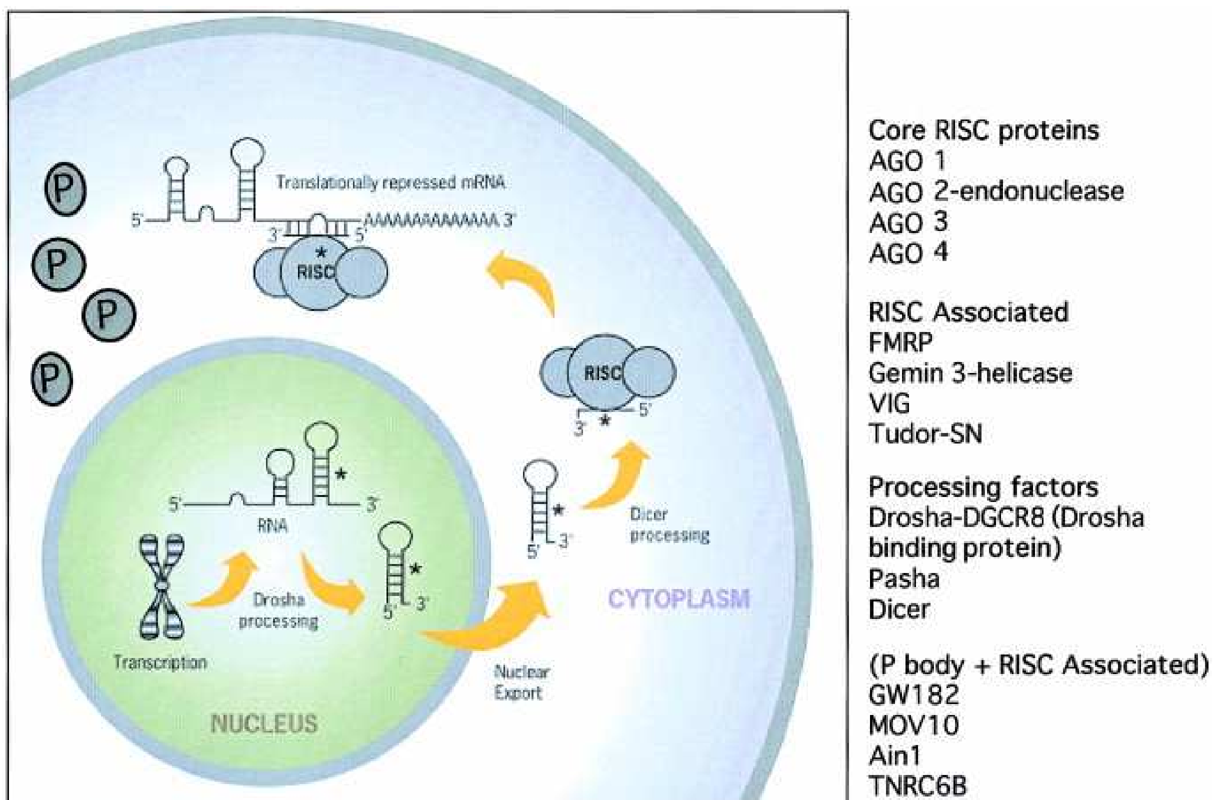
They could be present singularly or in cluster. The most part of human and *Drosophila* miRNA are clustered. These clusters are single transcription units and produce poly-cistronic transcripts. Often the miRNAs within the same genomic cluster are related to each other, like it happens, for example, for the orthologs of *C.Elegans lin-4* and *let-7*, that are coexpressed from the same cluster in fly and human genomes (Aravin et al., 2003; Bashirullah et al., 2003; Sempere et al., 2003). This suggests that in the same group miRNAs with no apparent sequence homology could have functional relationship.

Not all the molecular steps that elucidate the biogenesis of a miRNA starting from its transcription to its maturation are well established and the general model often refers to the biogenesis of the first miRNA identified, *lin-4* (Conrad et al., 2006).

The generation of miRNAs is a complex multistep process that occurs in two separate cellular compartement, the nucleus and the cytoplasm, and during which miRNA pass four different stages and structure: primary miRNA, precursor miRNA, duplexed miRNA and active miRNA (figure 2).

A primary miRNA (pri-miRNA) of 100 to more then 1000 nucleotides in length, is transcribed from the genome by RNA polymerase II in the nucleus (Song et Tuan, 2006).

Figure 2. Biosynthesis and mechanism of actions of miRNAs and the main molecular components involved



Initially RNA polymerase III was the candidate for the transcription of miRNAs, like it happens for some of the shorter noncoding RNAs, including tRNAs, 5S ribosomal RNA and

the U6 snRNA, but numerous evidences supported the activity of the RNA polymerase II (see review: Di Leva et al., 2006). In the beginning several long transcripts comprising miRNAs were identified in expressed sequence tags and their complex expression control was typical of those transcribed by RNA polymerase II, later the association with this enzyme was demonstrated clearly. Until now only a few different pri-miRNA have been isolated and characterized, three from human, one from *C.Elegans*, one from plants and they all are capped, polyadenylated and apparently noncoding (Cullen, 2004).

In the nucleus the pri-miRNA is converted to precursor miRNA or pre-miRNA , a 60-70 nucleotides stem loop intermediate, through the cleavage activity of the Drosha enzyme, a nuclear Ribonuclease III endonuclease which cleaves the flank regions of pri-miRNA (Lee et al., 2002, 2003; Zeng and Cullen, 2003).

Drosha can cut only pri-miRNAs that have a large terminal loop (>10 nt) in the hairpin and a stem region one turn bigger than the precursor, 5' and 3' single-stranded RNA extensions at the base of the future miRNAs (Filipowicz et al., 2005, Tomari and Zamore 2005). The hypothesis is that Drosha recognizes the primary precursor through the stem-loop structure and then cleaves the stem at a fixed distance from the loop, liberating the pre-miRNA and determining one end of the mature miRNA. It's not clear in which way Drosha recognizes the pri-miRNA stem-loop from stem-loop of other RNAs. The pre-miRNA presents a 5' phosphate and 3'hydroxy termini and two or three nucleotides with single-stranded overhanging ends, classic characteristics of Ribonuclease III cleavage of dsRNAs (Di Leva et al., 2006). It is 60-70 nucleotides long with an imperfect stem-loop structure. In the stem part of one of the two arm is present the sequence of the mature miRNA and in the other arm the near complementary miRNA* that will be later eliminated.

The pre-miRNA is actively transported from the nucleus to the cytoplasm. Pre-miRNA interacts with the export receptor Exportin-5 (Exp5) (Kim, 2004) and RanGTP forming a nuclear heterotrimer that promote the stabilization of pre-miRNA and is exported to the cytoplasm. Once the heterotrimer reach the cytoplasm through the nuclear pore, the RanGTP is hydrolyzed to RanGDP and the pre-miRNA is released (Di Leva et al., 2006).

In the cytoplasm the pre-miRNA is processed into 18-22 nucleotide imperfect double strand RNA duplex (miRNA: miRNA*) by the cytoplasmic Ribonuclease III, Dicer, that acts, in humans, with the trans activator RNA (tar)-binding protein , TRBP (Chendrimada et al. 2005). Dicer contains a putative helicase domain, a DUF283 domain, a PAZ (Piwi-Argonaute-Zwille) domain, two tandem RNase-III domains and a dsRNA-binding domain 8 (dsRBD). The PAZ domain is responsible of the interaction of Dicer with the 2-nucleotide 3' overhangs of dsRNA such as the pre-miRNA. The efficient Dicer cleavage also requires the presence of the overhangs and a minimal stem length. The model assumes that the PAZ domain of Dicer recognizes the end of the pre-miRNA and can position the site of the second cleavage on the stem of the precursor. The variable size of the product, from 18 to 22 nt, results from the

presence of bulges and mismatches on the pre-miRNA. Efficient cleavage requires dimerized RNase III domains because the functional catalytic site resides in the interface of the dimmer (see review: He and Hannon, 2004).
Like Dicer also Drosha has two tandem RNase-II domains. The exact biochemical mechanism that guides the cleavage has not been elucidate, but it's probable that it shares closely related mechanism for processing miRNA.
In plant no Drosha homologues have been found and it suggests that the maturation of miRNA from long primary transcript should occur differently comparing to the animal model. However there are four Dicer homologues in *Arabidopsis thaliana*, DCL1, DCL2, DCL3, DCL4, two of which contain nuclear localization signals. It seems possible that in plant the Drosha function is carried out by one or more specialized Dicer. In plants deficient for DCL1 not only the production of some miRNA is reduced, but also is not detected the accumulation of the corresponding pre-miRNA. A model in which Dicer specialised enzymes catalyse both Drosha and Dicer cleavage for the maturation of miRNAs inside the nucleus has been built. The functional specificity of different Dicer enzymes in organisms with multiple Dicer homologues has recently been indicated also in *Drosophila* and the function of Dicer seems not simply restricted to the cleavage, but also correlated to the initiation of RNA-silencing in the effector complex (see review: He and Hannon, 2004).

Only one strand of the dsRNA contains the miRNA that preferentially enters the RNA-induced silencing complex (RISC), the effector protein complex in which the miRNA pairs the mRNA target and produces its degradation or the inhibition of its translation into a protein (see review: He and Hannon, 2004).
This effector complex shares so much core components with that of siRNA that it's generally called RISC for siRNA and miRNA even if in humans it's called miRNP, miRNA ribonucleoprotein, after the identification of the proteins that constitute it (Hutvagner and Zamore, 2002; Mourelatos et al., 2002). Several proteins have been purified and identified as essential components of RISC, but only a few have been functionally characterized (see review: Di Leva et all., 2006). RISC has been purified in many organisms and it always contains a member of the Argonaute protein family, which is thought to be a core component. Many Argonaute proteins were already identified in worms, fungi and plants and shown to be implicated in the mechanism of RNA silencing. These evolutionary conserved proteins of approximately 100 kDa are called also PPD proteins because they all share the PAZ and PIWI domains. The first one domain has the function to bind weakly to
single-stranded RNA and also to double stranded RNA; this suggest that this protein can have the ability to bind miRNA before and after its association with the mRNA target. Structural and biochemical studies have proved that the Argonaute proteins are the target-cleaving endonuclease of RISC, and that the complex is coordinated also by other proteins whose functions are not really understood, like the RNA-binding protein VIG, the Fragile-X related protein in *Drosophila*, the exonuclease Tudor-SN, and many other putative helicases (Nelson

et al., 2003). In humans miRNP is constituted by the Argonaute protein called eIF2C2 (Martinez et al., 2002), and other two helicases, Gemin3 and Gemin4.

When the miRNA strand of the miRNA: miRNA* duplex is loaded into the RISC the miRNA* is unwind and rapidly degraded. The target specificity and probably also the functional efficiency of a miRNA requires that the mature miRNA strand from the duplex be selectively incorporated into the RISC for target recognition (see review: Bartel, 2004). What is the mechanism for choosing which of the two strand enters the RISC? Some evidences show that the strand that enter is nearly always the one whose 5' end is less tightly paired (Khvorova et al., 2003; Schwarz et al., 2003). After the cleavage of Dicer the stability of the 5' ends of the two strand of the duplex is usually different. It seems that helicases present in the RISC take with the same frequencies both the end of the two strand before beginning to unwind the duplex and that finally the relative ease of unwind the less stable facilitate its preferential incorporation into the RISC, determining the asymmetrical assembly of the complex.

The precise mechanism that underlies post-transcriptional repression by miRNAs still remain unknown. We know that two processes exist for miRNA-mediated gene regulation: degradation of the target mRNA and translational repression, depending largely on the degree of the complementarity between the miRNA and the target. The miRNA will specify cleavage if the mRNA has sufficient complementarity to the miRNA or it will repress productive translation if the mRNA is only partially complementary, but has a sufficient number of miRNA complementary sites (Hutvagner and Zamore, 2002; Zeng et al., 2002, 2003; Doench et al., 2003). This model is supported from many evidences, but it can not be considered a general rule because there was at least one case of a plant miRNA, miR-172 in *A.thaliana*, that regulates APETALA2 via translational repression despite the near-perfect complementarity between the miRNA and the target (Aukerman and Sakai, 2003; Chen, 2003).
When miRNA guides the cleavage the cut happens in a precise position, between the nucleotide pairing to residues 10 and 11 of the miRNA, like it happens for siRNA (Kasschau et al., 2003; Hutvagner and Zamore, 2002; Llave et al., 2002) and it appears to be determined relative to miRNA residues, not to miRNA: target base pairs. After cleavage of the mRNA the miRNA remains intact and can guide the recognition and destruction of additional mRNA (Tang et al., 2003).
The mechanism of translational inhibition was first observed and studied looking at the RNA-silencing of *lin-4* over *lin-14* in *C.Elegans*. It was observed that in the animal kingdom miRNA typically mediate translational repression rather than mRNA cleavage, that is more common in plants, even if recently one miRNA in *Drosophila*, miR-196, was found to direct mRNA cleavage of its target, Hoxb8 (Yekta et al., 2004). It seems that in animal generally the miRNAs have a lower degree of complementary to mRNA targets comparing to the nearly

perfect base pairing of plant miRNA to the corresponding target, that in plants generally guides to its destruction (Hake, 2003). It was observed that the cooperative action of multiple RISC provide the most efficient translational inhibition (Doench et al., 2003). This correlates with the presence of multiple miRNA complementary sites in most genetically and computationally identified targets of metazoan miRNA. It has been proposed that different miRNA can regulate the same target and that exists a combinatorial control (Reinhart et al., 2000; Abrahante et al., 2003; Lin et al., 2003).
We know that the complementary sites for the known metazoan targets reside in the 3' UTR of mRNA, in contrast with the target complementary sites in plants, that are located throughout the transcribed regions of the target gene (see review: Bartel, 2004). It was demonstrated that in metazoan the most important site of complementarity to the target on the miRNA sequence is a short portion at the 5' end of seven nucleotides, from residues 2 to 8. Actually this short sequence is the most conserved among homologous metazoan miRNA (Lewis et al., 2003; Lim et al., 2003), it was observed to be perfectly complementarity to multiple 3' UTR sites involved in post-transcriptional repression also in invertebrate (Lai, 2000), moreover this heptamer seems to be the most useful to productive prediction of target mRNA and is the most important complementary site also in plant miRNA. We don't know why the complementarity to the 5'end is so universally important, but understanding the mechanism of pairing of miRNA to the mRNA in the RISC will also help to reveal the process of translational repression.

I-I-d Approaches to microRNA discovery (see review : Berezikov et al., 2006)

The first step to discover and understand the biology of miRNA is to isolate and identify the miRNAs expressed in the cells and organisms of interest. Since the discovery of the first miRNA, *lin-4*, in *C. Elegans*, many miRNAs have been identified or predicted in a wide array of organism. In 2003, the rapid growth of the number of miRNA genes led to the creation of a comprehensive an searchable database of published miRNA sequences via a web interface : miRNA Registry (http://microrna.sanger.ac.uk/sequences) (Griffiths-Jones, 2004). This was created with two objectives: to avoid to assign unique names to distinct miRNAs and to provide a database for all miRNAs sequences, including the stem-loop sequences, the genomic location, homologous sequences and possible target predictions. The miRNAs are annotated with numerical identifiers based on sequence similarity. For homolog miRNAs in different organisms, itm is usual to assign the same nameon the similarity of the 22-nt mature sequence. Identical mature forms are assigned the same name and, if identical miRNAs are produced from different genomic loci they are differentiated by suffixes, such as "miR-16-1" and "miR-16-2"; if there are differences in one or two bases they are denominated with a final different letter such as "miR-181a" and "miR-181b". If two miRNA derive from the two arms of the same precursor it is added to the miRNA name the suffix 5p and 3p to identify the

two different arms, until the data will confirm which form is predominantly expressed, while the species less expressed is normally denoted by an asterix (Di Leva et al., 2006).
In October 2006 the miRNA Registry contained 4.361 entries from 40 organism including viruses and mammals, counting 332 human miRNA (Croce, 2006).

It was necessary a uniform definition of miRNA to annotate new "candidate miRNA" like true miRNA to prevent misclassification of other types of small non coding RNA like miRNAs (see review: Berezikov et al., 2006).
MiRNAs were defined as non coding RNAs that fulfill the following combination of expression and biogenesis criteria:

1) mature miRNA should be expressed as a distinct transcript of approximately 22 nt that is detectable experimentally (by Northern blot analyses, cloning, real-time quantitative PCR. in situ hybridisation, primer extension..);
2) mature miRNA should originate from a precursor with a characteristic secondary structure, such as hairpin or fold-back, without any large internal loops or bulges, and miRNAs should occupy the stem part of the hairpin;
3) mature miRNA should be processed by Dicer (Ambros et al., 2003).

The definition implies that miRNA should have a demonstrated function, however biological function has been elucidated only for a few miRNA and the criteria established for miRNA classification (Ambros et al., 2003) do not include the requirement of a biological role. Instead, an optional but commonly used criterion is the phylogenetic conservation of the mature miRNA, an indirect indication of a possible function. Strictly speaking the term "candidate miRNA" should be used as long as the function of miRNA is unknown, but practically evidences of expression of a 22-nt transcript and of the presence of an hairpin precursor are sufficient to classify a sequence as a miRNA.
All approaches to discovering miRNAs are based on these definition and can be split in two groups:

1) experiment-driven methods, in which the expression of small miRNA is first experimentally established and structural requirements for the precursor are searched later by bioinformatic tools;
2) computation-driven approaches, in which candidate miRNA are first predicted in genome sequence using structural features and expression is demonstrated later experimentally (Berezikov et al., 2006).

In the beginning forward genetics methods were able to identify the first miRNAs genes, *lin-4* and *let-7*, but since then only four additional miRNAs, *bantam*, *miR-14* and *miR-278* in *Drosophila melanogaster* (Brennecke et al., 2003; Xu et al., 2003; Teleman and Cohen, 2006) and *lys-6* in *C.elegans* (Johnston and Hobert, 2003), have been discovered by forward genetics approaches. The inefficiency of this methods can be explained by the difficulties in targeting by spontaneous or induced mutagenesis the miRNA sequence and specially the "seed sequence" of 7 nucleotide determinant for their functionality, considering their

redundancy (Abbott et al., 2005), which often tolerate mutations that does affect the 'seed sequence' and does not result in strong variation in the phenotype of the mutants.

The preferred approach to the identification of miRNAs is to sequence size-fractionated cDNA libraries. An initial protocol useful for cloning small interfering RNA (Elbashir et al., 2001) was shown to be adapt also for identifying many miRNA (Lagos-Quintana, 2001). Later little variations of it were developed independently (Lau et al., 2001; Lee and Ambros, 2001) but all these successful protocols follow the same principles (Aravin and Tuschl, 2005). In the main lines an RNA sample is separated in a denaturing polyacrylamide gel and the size fraction corresponding to the miRNA is recovered. Then 5' and 3' adapters were attached to the RNAs and a RT-PCR is carried out. The fragments are cloned into vectors to create a cDNA library. The clones are sequenced and analyzed to search the genomic origin of the small RNAs. Bioinformatic tools are required to check if the hairpin precursor is encoded in the genomic regions where the small RNAs have been localized and if this precursor is conserved in other species. This analyses is complicated because hairpin structure are common in eukaryotes and are not a unique features of miRNAs (Lin et al., 2006; Shen et al., 1995) and moreover miRNAs should be distinguished from others types of endogenous small RNAs (Aravin and Tuschl, 2005; Kim and Nam, 2006).

The limit of cloning protocol is the difficulty to discover miRNAs that are expressed at a low level, in specific stages or specific cell types. Moreover some miRNAs could be hard to clone due to physical properties correlated to sequence composition or post-transcriptional modifications, such as editing or methylation (Luciano et al., 2004; Yang et al., 2006).

Surveying genomic sequences to predict miRNAs became popular after initial cloning efforts generated sufficient information about miRNA properties to recognize a set of distinctive miRNA features (Berezikov and Plasterk, 2005; Bentwich, 2005). On the basis of the particular features of miRNAs different approaches have been developed to predict miRNA, but all of them use secondary structure information, many rely on phylogenetic conservation of both miRNA sequence and structure, other methods asses the thermodynamic stability of hairpins and refers to sequence and structure similarity of known miRNAs, or search miRNAs on the genome near known miRNAs already localized.

Many software, like MiRScan, snarloop, miRSeeker were developed to search miRNA on the basis of conservation criteria referring to hairpins structure. The genome of *C. Elegans* (Lim et al., 2003; Grad et al., 2003), of *D. melanogaster* (Lai et al., 2003) and the human (Lim et al., 2003) were analysed and the number of predicted miRNA strongly extended on the basis of haipin sequence similarity to experimentally confirmed miRNAs. The potential target sequences of miRNAs have been analyzed in the 3'UTR of genes in search of complementary sequence to 'seed sequences' of known miRNAs. Using conserved motifs that did not match to any known miRNAs it was possible to predict other miRNAs candidates in human (Xie et al., 2005). Similar approaches have been recently applied to the prediction of miRNAs in *A.Thaliana* (Adai et al., 2005), flies and worms (Chan et al., 2005).

On the basis of the lower free energies of folding of miRNAs comparing to tRNAs and rRNAs (Bonnet et al., 2004) was set a new software that combines thermodynamic stability criteria with conservation criteria, Rnas (Washietl et al., 2005a, 2005b), that was successful to predict additional miRNAs in various organisms (Hsu et al., 2006; Missal et al., 2006).
Recently other alignment-type methods have been develop to identify homologs of known miRNAs "aligning" potential miRNA with known one at both sequence and structural level (Legendre et al., 2005; Nam et al., 2005; Wang et al., 2005).
All methods that rely on phylogenetic conservation can not predict non conserved miRNAs. For this reason some new *ab initio* approaches were developed that use only intrinsic structural features of miRNAs and not external informations (Bentwich et al., 2005; Sewer et al., 2005; Xue et al., 2005; Pfeffer et al., 2005). Each of these methods builds classifiers that can measure how a candidate miRNA is similar to known miRNAs on the basis of several features, such as free energy of folding, length of the perfect longest stem, average size of symmetrical loops, proportion of different nucleotides in the stem, etc..(Sewer et al., 2005), to which a model assigns different weights and an overall score result is measured for each candidate miRNA.
All these computationally predicted miRNAs need to be validate experimentally. Validation approaches can be split in two categories: those that demonstrate only miRNAs expression, like Northern blot analyses (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001) and *in situ* hybridization (Wienholds et al., 2005; Kloosterman et al., 2006; Nelson et al., 2006), and others determine the exact sequence of the mature miRNA in the precursor sequence, like cloning strategy and primer extension assay (Seitz et al., 2004) or RNA-primed array-based Klenow extension (RAKE) (Nelson et al., 2004).

I-I-e Strategy to determine biological functions (see review : Krutzfeldt et al., 2006)

The elucidation of the general mechanism of miRNA function in the regulation of gene expression suggests a gene regulatory model in which nuclearly encoded genetic information is not only transcribed and translated into proteins, but at the same time regulates these processes through non coding miRNA. This paradigm adds a new level of regulation and fine control of gene expression that is likely to be important for the maintenance of many, if not all, cellular functions.
In spite of our ability to identify miRNA and elucidate their biogenesis and the basic mechanism of actions, very little is known regarding miRNA function. With the near completion of the miRNA inventory the focus is shifting to elucidation of their biological role. For this purpose other scientific aspects have been studied, and the corresponding technology have been developed, including the analyses of the possible miRNA target by bioinformatics prediction algorithms, preliminary inspections of the localization and the effects of their

expression by *in vitro* or *in vivo* expression studies, by reporter assays, *in situ* hybridizations, over expression and silencing technologies.
Different complementary strategies can be useful to begin to study a miRNA of interest: it is possible to examine the profile of its expression in different cellular contexts, and indirectly make a first hypothesis on its possible role, predict or identify its molecular target, strictly connected to its function, before over expressing or silencing a miRNA *in vivo* and *in vitro*, that is a task that requires a defined model and precise technical competences, even if the technology is now available.

From the first study in *C. Elegans* and in *D. Melanogaster* was evident that miRNA has not only-spatio-temporal, but also tissue and cell-type specific expression. For this reason many commercially miRNA microarrays including the content found in Sanger miRBase 7.0 (http://microorna.sanger.ac.uk) were developed making it possible to monitor tissue-specific miRNA expression and regulatory changes in developmental, physiological and disease states. First results using oligonucleotide microarray confirmed the existence of several tissue-specific miRNAs (BaskerVille and Bartel, 2005; Barad et al., 2004; Nelson et al., 2004; Thomson et al., 2004) that may suggest that some of them have an organ- or cell type-specific functions. Microarrays have also been used to study miRNA expression profile during differentiation of cells, such as myoblasts (Chen et al., 2006) and preadipocytes (Esau et al., 2004) or in disease state, most notably human cancer, such as in B cell chronic lymphocytic leukemia (Calin et al., 2002), in colon carcinomas (Michael et al., 2003), in small lung carcinomas (Johnson et al., 2005; Takamizawa et al., 2004). It was shown for few specific miRNA that their level of expression can decrease or increase typically in a particular differentiation cellular stage or neoplastic tissue raising the idea that miRNA profiling could contribute to more precise tumor classifications and predict therapeutic outcomes in the future. Several techniques have been developed to visualize miRNA expression *in vivo*. In *C. elegans,* it was possible to study the activity of the miRNA promoter of miRNAs using reporter cassette that contains the miRNA promoter regions fused with the sequence of the green fluorescence protein (GFP) or β-galactosidase, the reporter gene (Johnson et al., 2003). A method to detect the presence of a specific miRNA in tissues uses the "sensor" transgene, which constitutively express a reporter gene that contains sequences complementary to the miRNA of interest in its 3' UTR region (Mansfield et al., 2004). In this case in the tissues or cells in which the miRNA is expressed the activity of the reporter gene will be blocked. This method has potentially excellent spatial temporal resolution, but it is not known if it can be used for the detection of low expressed miRNAs. The most frequently applied method to visualize miRNA expression to date is *in situ* hybridization and in particular a variation of it that uses special probes Locked Nucleic Acid- (LNA) modified probes able to detect short sequences like miRNAs. This technique has already been applied successfully to identify miRNA expression in mouse embryos (Kloostermam et al., 2006).

Even if several independent groups have established computational algorithms designed to predict target genes of miRNA sequences (John et al., 2004; Kiriakidou et al., 2004; Krek et al., 2005; Rajewsky, 2006; Lewis et al., 2003) there is a big lack of experimental evidence that validate this sequences like the corresponding target of miRNAs.

Moreover, also due to the functional mechanism of miRNA, that do not require necessary a strong degree of sequence complementarity to the target and that do not exclude binding of multiple miRNA to the same mRNA, the computational prediction of a target is difficult and on average 200 genes have been predicted to be regulated by a single miRNA (Krek et al., 2005; Lewis et al., 2003).

To date several methods have been established to show experimentally the miRNA regulation of a putative target. One of the most used is the luciferase reporter construct, containing the target 3'UTR with the putative binding site downstream of the reporter coding region. These constructs are used to transfect cells expressing the relevant miRNA, along with vectors carrying mutant versions of binding sites. Evidence for miRNA activity can be demonstrated if wild-type reporters have less activity than their respective mutants. One other approach uses antisense 2'-O-methyl modified oligoribonucleotides to inhibit miRNA expression and provokes some loss-of-function effect (Chen et al., 2006; Poy et al., 2004; Schratt et al., 2006). Another approach tries to determine miRNA target increasing the intracellular concentration of a miRNA by transfection of homologous synthetic short interfering RNAs or recombinant adenoviral infection and measuring differential gene expression by microarray (Krutzfeldt et al., 2005; Lim et al., 2005).

Induced expression of miRNAs was the initial step that identified miRNA function in many model organisms or mammalian cell systems.

Transient overexpression in cell-based assays is easily achieved by transfection of a double-stranded RNA similar to Dicer cleavage product, but long-term overexpression in cultured cells or mouse models depends on the transfection of a plasmidic vector that carry a specific construct for the expression of a miRNA. This construct is relatively simple and is the same used for protein-coding mRNA; introducing the sequence of the precursor together with a strong constitutive promoter is sufficient to overexpress a miRNA (He et al., 2005; Hayashita et al., 2005). It is possible to introduce these vectors into adenovirus or retrovirus (Chen et al., 2004) system and then transfect cultured cells or inject them in mouse tissues *in vivo*. Tissue-specific overexpression of a miRNA *in vivo* can be obtained also generating transgenic mice, even if the technology requires time and higher competences.

Studies that are based only on overexpression must be taken with caution because misexpression of miRNA could target genes otherwise not affected in physiological context and the results should be confirmed by loss-of-function experiments.

The technologies to silence miRNA and generate loss-of-function mutants can be divided in genetic and nongenetic approaches.

The first class developed with the parallel development of DNA recombinant technology and the recent generation of mutant mice has been invaluable in the elucidation of miRNA gene function.

In particular three class of experiment can be design to disrupt miRNA-mediated gene regulation and infer their possible role.

1) The generation of mice with mutated alleles of Dicer1 leads to the deficiency of all mature miRNAs (Kanellopoulou et al., 2005; Harfe et al., 2005; Harris et al., 2006); the phenotypes analysed show the important collective functional role of miRNAs in many developing tissues, but can not provide information on the exact role of individual miRNA. For this reason the injection of a singular miRNA in Dicer-null mutant can restore the expression of a specific miRNA and discover the contribution of individual miRNA. This approach has been successful in zebrafish (Giraldez et al., 2005).
2) The generation of a knockout mutant for a miRNA that exist in cluster can interfere with the

proper folding and processing of the polycistronic transcript, affecting the expression of neighboring miRNAs and provoking a visible changing in the phenotype (Ying et al., 2005). To date there is no evidence for any miRNA knockout in any animal models.

3) The generation of mutants with mutating binding sites in the 3'UTR of the target gene, but also this approach is not actuated till now.

Nongenetic approaches to silence miRNA function use the transfection or injection of synthetic oligonucleotides that act like chemical inhibitors of miRNAs.

1) 2'-O-methyl-modified oligoribonucleotides complementary to the miRNA act irreversibly like stoichiometric inhibitors and have been used in cell lines (Meister et al., 2004), *C. elegans* (Hutvagner et al., 2004) and *D. melanogaster* (Leaman et al., 2005).

2) 'antagomiRs', cholesterol-conjugated single-stranded RNAs complementary to miRNAs have been injected in mouse tissues *in vivo* (Krutzfeldt et al., 2005). This silencing is dose-dependent, can be observed within 24 hours and last 3 weeks. Even if it is not clear till now in which way the cells take up antagomiRs this approach can allow the study of individual miRNA. This technology was successful in the mouse for the functional study of miR-122.

4) Recently new antisense oligonucleotides, (ASOs) (Esau et al., 2006), unconjugated single-stranded RNAs that carry complete phosphorothioate backbones and 2'-O-methoxyethyl modifications, have been developed to target miRNAs *in vivo* and for the moment they have been used only in liver tissues, confirming the results about miR-122 obtained with the 'antagomiRs'.

I-I-f MiRNAs and Cell differentiation in mammalian development (see review : Song and Tuan, 2006)

Animal development requires the establishment of a highly regulated spatiotemporal gene expression network in order to convert the totipotent zygote into an animal containing various specialized tissues functioning in a concerted manner (Lee et al., 2006). One important feature of this regulatory scheme is the specific expression of factors that are required for each developmental window. However, it is also crucial to inhibit the expressions of genes that are not required for particular developmental stages or which may promote alternative differentiation pathways (O'Rourke et al., 2006).
At a cellular level the tissue development is produced by cell differentiation. The ability of a precursor cell to differentiate into different cell types actually depends on upregulation of factors required for one lineage combined with the downregulation of others specific for a different fate.
It has long been thought that this process is regulated primarily at the level of transcription, but it is also possible that posttranscriptional mechanism are required to drive cell commitment. miRNAs may serve as a switch to determine the developmental program of precursor cells; alternatively they may function to maintain the identity of differentiated cell types (O'Rourke et al., 2006).
Here I will summarize the recent data on miRNA involved in cellular differentiation during mammalian development.

Embryonic stem (ES) cells are totipotent cells that resides in the inner cell mass of the blastocysts that have the capacity to generate precursor cells of endoderm, ectoderm and mesoderm. In vitro, ES murine and human cells lines can be induced to generate germ and somatic cells of both the three layers, reproducing part of the in vivo embryonic development. ES cells present great potential in clinical and biological applications, even if the molecular mechanism governing their differentiation is poorly understood. A possible role for miRNA came from the identification of ES cell-specific miRNAs in mouse screening of two miRNA libraries from undifferentiated and differentiated mouse ES cells (Houbaviy et al., 2003). 6 miRNAs (miR-290, miR-291-as, miR-292-as, miR-293, miR-294, miR-295) were found expressed in pluripotent cells and silenced or downregulated after differentiation. Later miRNA libraries from human ES cells were analysed and 17 novel miRNA were identified (Suh et al., 2004). 12 of them were found localized in two genomic cluster that are transcribed in two polycistronic primary transcript whose level decreases when ES cells develop into embryonic bodies, suggesting the specificity of these miRNAs for undifferentiated cells.
miRNAs play a key role also in maintaining the differentiation state of pluripotent ES cells as demonstrated by the loss of stem cells and early lethality of Dicer-null mice embryos

(Murchison et al., 2005). In vitro Dicer-null ES cells did not differentiate indicating that lack of Dicer and endogenous miRNAs compromise the differentiation potential of ES cells.

The first indication of the involvement of miRNAs in limb development in mammals came from an *in situ* hybridization study on mouse embryos (Schulman et al., 2005). An ortholog of the worm *lin-41* was detected and localized in the embryo in the posterior region of the limb bud, while in embryo at the same stage *let-7*, the corresponding negative regulator, is expressed in the anterior region of the limbs. Their reciprocal expression patterns implied that they play a role in limb development.
Using microarray analyses Hornstein et al. (2005) identified a miRNA, miR-196, which is preferentially expressed in the hindlimb. It was shown that miR-196 can direct the cleavage of a known transcription factor that mediate anteroposterior polarity in fore- and hindlimb buds, Hoxb8, only in the hindlimb, but not in the forelimb, regulating the limb development.

Adipocyte differentiation can be reproduced *in vitro* using primary subcutaneous preadypocytes
that exposed to hormonal stimuli develop in mature adipocytes. Esau et al. (2004) use this systems to assess the effects of several miRNAs on adypocyte differentiation transfecting preadipocytes with 2'-O-methoxy-ethyl phosphorothioate-modified antisense RNA oligonucleotides targeting specific miRNAs. They found that miR-143 is involved in the maturation of preadipocytes, like it was confirmed by its upregulation in differentiated adipocytes. ERK5 is probable the gene target and its protein level is elevated in cells with decreased miR-143 expression. This was the first time that antisense oligonucleotides were used successfully to determine the function of miRNAs.

Cardiomyocyte differentiation and cardiogenesis require sequential activation and repression of transcriptional factors such as serum response factor (SFR), MyoD, Mef2. Zhao et al. (2005) identified, by a combination of *in silico* and experimental approaches, miR-1-1 and miR-1-2, whose expression was specific of in the heart and skeletal muscle of adult mice. Overexpression of miR-1 demonstrated is essential role in cardiogenesis and its molecular target, Hand2. It was also analysed the regulation of its expression and it was found that potential binding sites exist in the enhancer region of miR-1 for the transcription factors Mef2, SFR and MyoD. A model was proposed in which miR-1 functions in the SFR-myocardin-dependent pathways in cardiac progenitor cells and is responsive to MyoD/Mef2 in skeletal muscle precursor. Recently another miRNA, miR-181, was discovered to be upregulated during terminal differentiation of myoblasts (Naguibneva et al., 2006). Loss-of-function assays *in vitro* using antisense oligonucleotide against miR-181 completely abolished the myoblast differentiation of cells. Since miR-181 expression was not detected in resting muscle cells *in vivo* it is probable that it plays a role only in the establishment of the differentiated phenotype, but not in its maintenance.

Regulation of hematopoietic differentiation is a complex process that involves the commitment, proliferation, apoptosis and maturation of hematopoietic stem/progenitor cells and a variety of regulatory molecules including miRNA. Several miRNA were detected preferentially in specific hematopoietic cell lineages: miR-181 in differentiated B lymphocytes, miR-142s in B-lymphoid and myeloid, miR-223 in myeloid (Chen et al., 2004). In particular miR-181 seems to be a positive regulator for B-cell differentiation, as its ectopic expression led to a doubling of cells in the B-lymphoid lineage without changing of T-lymphoid lineage both in vitro and in vivo.

Mice with defective Dicer function show abnormal epithelium morphogenesis, both in the skin and in the lung. Yi et al. (2006) saw that mice with defective Dicer activity in their skin progenitor cells exhibited abnormal epidermis and hair follicles. In a other study Harris et al. (2006) observed that Dicer inactivation led to dramatic branching effects in the lung. An increased and prolonged cell death was observed in both skin and lung epithelia in the mutant mice, but it is not known how this
contributes to the abnormal morphogenesis and which are the miRNA responsible.

Several miRNAs were found exclusively (miR-9, miR-142a, miR-124b, miR-135, miR-153, miR-183, miR-219) or highly expressed (miR-9*, miR-125a, miR-125b, miR-128, miR-132, miR-137, miR-139) in mouse and human brain tissues and some are also upregulated in embryonal carcinoma cells (Sempere et al., 2004). Overexpression and inactivation of three of them, miR-124a, miR-9, miR-125b, in neuronal progenitor cells dramatically change the relative fraction of astroglial-like cells and neuronal cells, confirming their critical role in neuronal differentiation.
To explain their mechanism of function it was proposed that they promote neuronal differentiation by suppressing the expression of non-neuronal transcripts. Experiment of overexpression and inactivation of the same miRNAs led to trace a model in which miR-125a and miR-125b are responsible of neuronal differentiation targeting the 3’UTR of lin-28 and altering both its translational efficiency and its mRNA levels. It was also demonstrated (Krichevsky et al., 2006) that the phosphorylation state of STAT3, a transcription factor that when phosphorylated inhibits neuronal terminal differentiation and promotes glial-like cells differentiation, is controlled by miR-9. To balance the formation of neuronal and glial cells in mammalian brain, expression of miRNA is tightly controlled. It was shown that the RE1 silencing transcription factor, REST, can switch the differentiation lineages cells between neuronal and glial cells and that it is correlated to the expression of the miRNA studied. Moreover miR-134 was found to be a negative regulator of dendritic spine development in hippocampal neurons and the protein kinase Limk1 was proposed as its target (Schratt et al., 2006).

I-I-g MiRNAs and cancer (see review : Esquela-Kerscher and Slack, 2006)

Cancer is caused by uncontrolled proliferation and inappropriate survival of damaged cells, which results in tumour formation. Many regulatory factors switch on or off genes that direct cellular proliferation and differentiation. Damage to these genes, which are referred to as tumor-suppressor genes and oncogenes, is elected for in cancer. Recent evidence indicates that miRNAs might also function as tumor suppressor and oncogenes. They have been shown to control cell growth, differentiation and apoptosis, consequently impaired miRNA expression has been implicated in tumorigenesis (Esquela-Kerscher and Slack, 2006).

Components of the miRNA-machinery have been found involved in tumorigenesis. For example, expression of Dicer has been shown to be downregulated in lung cancer (Karube et al., 2005), suggesting a potential indirect role of Dicer in tumor formation that result from the collective reduction of miRNAs. The Argonaute proteins have also been associated with various cancer. Three
human Argonaute genes are frequently deleted in Wilms tumors of the kidney and have been also associated with neuroectodermal tumors. In particular it is supposed that Argonaute 1 (AGO1) is involved in developing lung, kidney and renal tumors. An additional human argonaute gene, HIWI, maps to a genomic region associated with testicular germ-cell cancer and might normally control the proliferation and maintenance of germ cells.

The biological role of only a small fraction of identified miRNAs have been elucidated to date. These miRNAs regulate cancer-related processes such as cell-growth and tissue differentiation and therefore might themselves function as oncogenes.
Interestingly the mammalian homologues of *lin-4* and *let-7* have been shown to control cell proliferation in human cell lines (Lee et al., 2005; Takamizawa et al., 2004) and are also associated with various cancer (Johnson et al., 2005; Calin et al., 2004; Iorio et al., 2005; Sonoki et al., 2005). In *D. Melanogaster, bantam* induces tissue growth by both stimulating cell proliferation and inhibiting apoptosis (Brennecke et al., 2003; Hipfner et al., 2002), *miR-14* suppress strongly apoptosis (Xu et al., 2003), and these are features of oncogenes. Other characterized miRNAs have essential functions during development and differentiation of cells into various tissues.

A recent study showed that about 50% of annotated human miRNAs are located in areas of the genome, known as fragile sites, that are associated with cancer. For example miR-125b-1 is located in a region that is deleted in a subset of patients with breast, lung, ovarian and cervical cancers (Calin et al., 2004) and recently it has also been associated with leukaemia.
The first indication that miRNAs could function as tumor suppressors came from a report that showed that patients diagnosed with the B-cell chronic lymphocytic leukaemia, (CLL), often

have deletions or downregulation of two clustered miRNA genes, miR-15a and miR-16-1 (Calin et al., 2002). Deletions within this locus occur also in 50% of mantle cell lymphomas cases, 16-40% of multiple myelomas and 60% of prostate cancers cases. It was predicted that a tumor-suppressor gene reside in this region. Later Cimmino et al. (2005) showed that miR-15 and miR-16-1 negatively regulate BCL2, an anti-apoptotic gene that is often overexpressed in many types of human cancers, including leukaemias and lymphomas, supporting a tumor-suppressor role for these two miRNAs.

Additional studies have shown a strong correlation between abrogated expression of miRNAs and oncogenesis. For example, miR-143 and miR-145 are significantly reduced in colorectal tumours (Michael et al., 2003).

The let-7 miRNAs family were the first group of miRNAs shown to regulate the expression of an oncogene, the Ras gene. Ras protein are membrane associated GTPase signalling proteins that regulate cellular growth and differentiation. About 15-30% of human tumors possess mutations in Ras genes. The 12 human homologous miRNAs of let-7 family map to fragile sites associated with

lung, breast, urothelial and cervical cancers (Calin et al., 2004). In particular the transcripts of certain let-7 were downregulated in human lung cancer (Takamizawa et al., 2004). Later studies in *C. Elegans* found that the 3'UTR of Ras genes contains multiple complementary sites for the let-7 family and that let-7 and Ras expression is inversely correlated in tumours (Grosshans et al., 2005; Johnson et al., 2005).

The MYC oncogene, which encodes a basic helix-loop-helix transcription factor, is often mutated or amplified in human cancers and has been shown to function as an important regulator of cell growth owing to its ability to induce both cell proliferation and apoptosis (Pelengaris et al., 2002). It seems that there is a correlation between miRNAs and the increased expression of MYC in the development of B-cell malignancies. MiR-142 and miR-155 are associated to MYC overexpression in the development of B-cell cancers, in Burkitt and Hodgkin lymphoma. MiR-155 is also involved in breast carcinomas, indicating other roles for this miRNA outside of the hematopoietic system. Recently He et al. (2005) and O'Donnell et al. (2005) describe a more direct relationship between miRNAs, MYC and cancer identifying a transcript that was preferentially upregulated in cancers and that encode the miR-17-92 clusters. By overexpression experiment it was shown that miRNAs within the miR-17-19b-1 cluster function cooperatively as oncogenes, possibly by targeting apoptotic factors activated in response to MYC overexpression and thus indirectly provoking uncontrollable cell proliferation. Surprisingly two miRNA gene in this cluster were shown to block indirectly the cell proliferation acting on the transcription factor E2F1. The double nature of the miR-17-92 cluster, the tumor-suppressing and the oncogenic one, emphasizes the complexity of cancer progression as well as the intricacies of miRNA-mediated gene regulation. These results might also reflect the fact that a single miRNA can control many unrelated gene targets, resulting in the control of opposing activities such as cellular proliferation and differentiation. A recent report (Felli et al., 2005) describes the ability of

miR-221 and miR-222 to downregulate the KIT oncogene and future studies will reveal that miRNA function as key regulators of many cancer-related genes like BCL2, Ras, E2F1, MYC and KIT. Therefore miRNAs might be powerful drug target that could be used in a broad range of cancer therapies.

As Northern blot and microarrays analyses have already been used to determine tissue specific 'signatures' of miRNA genes in humans (Pasquinelli et al., 2000; Lagos-Quintana et al., 2003; Lim et al., 2003; Liu ret al., 2004; Nelson et al., 2004; Thomson et al., 2004; Krichevsky et al., 2003; Miska et al., 2004; Sempere et al., 2004; SmiRnova et al., 2005; Sun et al., 2004; Monticelli et al., 2005; Babak et al., 2004), researchers are now using miRNA-expression signatures to classify cancers and to define miRNA markers that might predict favourable prognosis (Takamizawa et al., 2004; Iorio et al., 2005; Calin et al., 2002; Lu et al., 2005; Ciafre et al., 2005; Chan et al., 2005; He et al., 2005; O'Donnel et al., 2005; Calin et al., 2005).
A recent report from Lu et al. (2005) found that the expression profile of a relatively few miRNAs (200) can be sufficient to accurately classify human cancers.
Following comparison of the expression level of miRNAs in normal and tumorous tissues it was shown that in general miRNA are downregulated in tumorous tissues, supporting a model in which miRNAs drive cells in more differentiated state and can be marker of the degree of cell differentiation. These studies define miRNA more like oncomiRs and imply that abnormalities in miRNA expression might directly result in de-differentiation of cells, allowing tumour formation (Esquela-Kerscher and Slack, 2006).

The emergence of miRNAs as important cancer-prevention genes is likely to have a large effect on gene therapies designed to block tumour progression. Large-scale expression screen to compare miRNA levels in tumours versus normal tissues will be useful in identifying novel miRNAs involved in cancer. In the future the administration of synthetic anti-sense oligonucleotides that encodes sequences complementary to oncogenic miRNAs, the anti-miRNA-oligonucleotides (AMOs), could inactivate miRNAs in tumours or slow their growth. The antagomiRs, that are AMOs conjugated with cholesterol, have already used to inhibit miRNA activity in various organs after injection into mice (Krutzfeldt et al., 2005), and might be a promising therapeutic agents. At contrary, techniques to overexpressed tumor-suppressor could be used to treat specific tumours. More development of these methods is needed before miRNAs treatment can move from the laboratory bench to the bedside. Even if we do not know if miRNAs will become a 'magic bullet' in the future, research in this area will undoubtedly provide insight into the underlying mechanism of oncogenesis.

I-II The mammary gland

I-II-a The mammary gland: structure and cellular composition

Mammalian evolution has been accompanied by the formation of a unique organ: the mammary gland. In fact, on a phylogenetic scale, this organ is a recent acquisition: it appeared 200 million years ago with the appearance of mammals to provide nourishment to the newborn in the form of milk (Hennighausen and Robinson, 1998). Unlike other branched organs, the most part of its development takes place post-natal rather than in embryonic life to accomplish the unique capacity of producing and secreting milk during the lactation (Sternlicht et al., 2006).

The number and location of mammary glands vary strongly between different species, but the structure and cellular composition is very similar. This organ is constituted from two tissue compartments: the epithelial one, that will give origin to ducts and to milk-producing alveolar cells, and the connective one (stroma or mammary fat pad) composed of adipocytes, fibroblast, cells of the haematopoietic systems, blood vessels and also neurons (see review: Hennighausen and Robinson, 2005) (figure 3).

Figure 3. Carmine-stained whole mounts of a section of mammary gland : in violet the epithelial tissue and its ducts, in white the stroma.

In general the epithelial tissue, at the parturition, is differentiated in cells constituting ducts, elongated canals transporting milk, and luminal secretory and myoepithelial cells that together constitute the central lumen and the outer layer of the alveoli, the functional secretory structural unit of mammary gland. Many grouped alveoli constitute a lobule and many

lobules are grouped in many bigger lobuloalveolar units. This branched structure is similar to the lung structure.
Each alveoli has a spherical structure inside which a monolayer of epithelial cells secrete milk in the central lumen. The milk is transported into the ducts by the contractile actions of myoepithelial cells, and is delivered to the body surface through the nipple. The extensive system of ducts and alveoli is embedded in the stroma, that supports the epithelial tissue and provides nourishment to epithelial cells (see review: Hennighausen and Robinson, 2005).

In the mouse there are five pairs of mammary gland located just below the skin, which extend from the thoracic (three pairs) to the inguinal (two pairs) regions of the animal along what is termed the milk or mammary line (Richert et al., 2000). Apart a nipple and a ductal-alveolar system, each gland has a limph node that is often used as a landmark when examining histological sections or whole mounts (Russo IH and Russo J, 1996). There is a gradient of differentiation among the glands, with the first thoracic gland being the least differentiated and the fifth inguinal gland the most (Bolander, 1990).

I-II-c The development of mammary gland

The mammary gland is a dynamic organ the structure of which changes throughout the female reproductive cycle. The development of the gland occurs in distinct stages, defined fundamentally by hormones, that are connected to the sexual development and reproduction: embryonic, prepubertal and pubertal stages, pregnancy, lactation and involution (see review: Hennighausen and Robinson, 1998).
In the main lines at birth the anlage consists of a few rudimentary ducts in the vicinity of nipple, pronounced ductal outgrowth and branching commences at puberty, in pregnancy and expanded lobulo-alveolar compartment develops. Functional differentiation of the secretory epithelium coincides with parturition and large amounts of milk are produced and secreting during lactation.
After weaning of the young the entire alveolar epithelium compartment is remodelled to resemble a virgin-like state. With each pregnancy a new round of lobulo-alveolar development occurs.

The epithelium and the surrounding stroma are derived from ectoderm and mesoderm, respectively (Parmar and Cunha, 2004).
In mice the mammary gland first appears embryonically as an epithelium bud that penetrates the underlying mesenchyme. The first morphological signs of mammary rudiments are lens-like placodes that form around embryonic day 11 and protrude slightly from the body wall (Robinson, 2004). This rudiment becomes bulb-shaped, they elongates and invades the

mesenchym to form a simple ductal tree with several branching ducts. This first phase of development is indipendent of hormonal signals (Richert et al., 2000).

In mice at birth the mammary gland consists of the epithelial cords and the stroma. While the first one is rudimentary (Topper and Freeman, 1980; Russo IH and Russo J, 1996) the stroma is thick and dense around epithelial structures and consists of eosinophilic fibrous connective tissue and fibroblast and in the early stage of development is filled with large adipocytes. Also present are lymphatics and blood vessels, the last will increase in number during pregnancy and lactation (Matsumoto et al., 1992).

The period of most rapid growth occurs during puberty from approximately 3-6 weeks of age in the mouse. The ducts lengthen and branch to form secondary and tertiary ducts that ultimately extend to fill the mammary fat pad by approximately 3 months of age. The terminal end buds (TEBs) appear at 3 weeks at the tips of growing ducts and are the sites of highest epithelial proliferation in the gland (Richert et al., 2000). From this bulbous structure cells are capable to migrate, to proliferate and differentiate in luminal and ductal epithelial cells (Daniel and Silberstein, 1987). This migration and proliferation result both in elongation of ducts and invasion of the fat pad; the differentiation in the TEBs is also responsible of branching (Gordon and Bernfield, 1980; Silberstein and Daniel, 1982) and formation of lateral and alveolar buds, that eventually subdivide to form rudimentary alveolar structure in the post-pubertal glands, after 10-12 weeks of age, in response to cyclic secretion of ovarian hormones at each estrous cycle (Andres and Strange, 1999).

The peak of mammary differentiation occurs during the 19-21 days of pregnancy and culminates with formation of alveoli and a fully lactating gland at parturition (Nandi, 1958).

In the beginning of pregnancy, a massive proliferation of ductal branches and the formation of alveolar buds, like in the postpubertal stage, could be observed. The epithelial to adipocyte ratio increase.

During the second half of pregnancy the alveolar buds progressively cleave and differentiate into individual alveoli that in the late pregnancy fill the majority of the fat pad. By the day 18 of pregnancy the alveolar epithelial cells are producing milk proteins and lipid, in preparation for lactation. The amount of stroma is greatly decreased, allowing more contact of the epithelium with adipocytes (Neville et al., 1998; Elias et al., 1973).

As lactation begins the milk in the lumen of alveoli is forced into the ducts (Asch HL and Asch BB, 1985; Richardson, 1949; Dulbecco et al., 1986), the fat in the adipocytes is metabolized and the alveoli expand to completely fill the gland (Neville, 1999). In normal condition the process of lactation continues for approximately 3 weeks, until the pups are weaned. At this moment the gland goes through a process of death and remodelling, the involution. This process is initiated by milk stasis once milk removal has ceased (Quarrie et al., 1996).

Forced weaning is often chosen as a model for involution because it is more controlled than natural weaning and allows for more precise timing of structural changes.

In the first day of involution big morphological changes is not observed, except for the flattening of the epithelium due to engorgement of the alveoli with milk. After 2 days the gland begins the irreversible sequence of cell death and remodelling: the secretory epithelial cells of the alveoli go in apoptosis and can be cleared by neighbouring epithelial cells or invading macrophages (Burwen and Pitelka, 1980; Richards and Benson, 1971; Fadok, 1999). At the day 4 the alveoli collapse into clusters of epithelial cells, while the adipocytes appear to be refilling. The epithelium progressively disorganize and decrease while adipocytes and stroma increase (Richert et al., 2000). At the day 6 of involution all the alveoli have collapsed and both epithelium and stroma are rearranged (Strange et al., 1992) as the majority of cell death has already occurred; the involution of alveoli continue till the day 21 of involution, when the gland resemble the prepregnant mature gland.
With each pregnancy a new round of lobulo-alveolar development occurs, together with the cycle of proliferation-secretion and involution of the epithelial tissue.

I-II-d Endocrine control on mammary development

While in embryo the initial stages of mammary development are independent of systemic endocrine signals and rather depends on reciprocal signalling between the epithelium and the mesenchym, the most part of development, that occurs after birth during pregnancy, is under control of steroid and peptide hormones.
Both the role of systemic hormones and the influence of the stroma on mammary epithelium have been recognized for some time (Mackie et al., 1987), actually the study of endocrine control of mammopoiesis and lactogenesis began more than 100 years ago.
The first demonstration that ovarian steroids and pituitary hormones can determine breast development and lactation came from an experiment of ovariectomy and transplantation of ovaries in mouse in 1900 (Halban and Knauer, 1900). The responsible bioactive compounds extracted were the progesterone and estrogen (Allen, 1924).
Later it became clear that other factors than ovarian hormones were required for mammopoiesis and in 1928, Stricker and Grueter induced milk secretion artificially in castrated virgin rabbits by injection of pituitary extract (Stricker and Grueter, 1928). Five years later Riddle and collegues purified the prolactin from this extract (Riddle et al., 1933).
From the 1906 it was known that also the placenta can secrete mammotrophic substances (Lane-Claypton and Starling, 1906), like placental lactogens, estrogens, progesterone and gonadotrophins. It was shown for the first time in 1980, by the introduction of *in vitro* mammary organ cultures, that it is a synergy of insulin, hydrocortisone and prolactin that controls the differentiation of secretory mammary epithelium (Topper and Freeman, 1980). In

the same year steroid and peptide hormone receptors were cloned and in 1990 downstream signalling components were identified, providing a basis for the understanding of signal transduction pathways.

Ductal elongation in the first days after birth originates from a few small TEBs and is probably the result of residual effects of maternal and fetal hormones (Hennighausen and Robinson, 1998). The acceleration of ductal growth during puberty and the strong lobulo-alveolar proliferation during pregnancy are controlled mainly by ovarian steroid hormones (Daniel and Silberstein, 1987), respectively by the oestrogen and progesterone, that act regulating cell proliferation and cellular turnover.

Progesterone is secreted in the beginning of pregnancy from the yellow body and its level is low in the beginning, increases during this phase and decreases brutally near the parturition, when the placenta and the yellow body involute (Martinez and Houdebine, 1994, chap.1).

The level of estradiol is high during puberty, in pregnancy the concentrations of estrogens secreted from the placenta is lower, but sufficient to cooperate with the progesterone in inducing the growth of lobulo-alveolar systems till the parturition, when estrogen level decreases rapidly (Martinez and Houdebine, 1994, chap.1).

Both estrogen and progesterone have pleiotropic actions in the uterus, ovaries and the hypothalamic-pituitary axis in regulation of sexual development. Since the need for a functioning mammary gland is dependent on a successful pregnancy, the evolutionary process use the same set of hormones for both developmental process (Hennighausen and Robinson, 2001).

The primary mechanism of steroid hormone action is through their specific nuclear receptors, which function as transcription factors when bound to their ligands (Hennighausen and Robinson, 2005).In post-natal mammary tissue not only most epithelial cell express receptors for estrogen (ER) and progesterone (PR), but also cells of stroma.

Both ER and PR have two isoform, ERα and β, PR-A and -B, that have different functions during the development of mammary gland.

Studies from knockout mice for ERα demonstrated that both stromal and epithelial ERα are required for normal ductal elongation and outgrowth during puberty (Bocchinfuso et al., 2000), even if ERα is not necessary for pregnancy alveolar expansion (Mueller et al., 2002).

Recombinant tissue experiment showed that estradiol elicits epithelial mitogenesis indirectly through ER stromal cells (Cunha et al., 1997).

Knockout mice showed that is the PR-B form responsible of proliferative effects on mammary epithelium, in particular to expansion of the alveolar compartment, and only in minor part to ductal elongation and branching (Mulac-Jericevic et al., 2003).

In early pregnancy PR cells are found in closely proximity to proliferating cells, suggesting a paracrine effects for progesterone. Progesterone seems to induce the production of a signal that guides the proliferation of neighbouring cells. One possible candidate is the receptor

activator of nuclear factor kB (NF-kB)-ligand or RANK-L (Mulac-Jericevic et al., 2003), belonging to the tumor necrosis factor (TNF) family.
It is now clear that estrogen induce the receptor for progesterone in epithelial cells, increasing the sensibility of cells to this hormone.

Prolactin (PRL) signalling is essential for the proliferation and functional differentiation of lobulo-alveolar structures during pregnancy (Topper and Freeman, 1980).
PRL is produced mainly by the lactotrophs in the anterior pituitary gland, even if also local production of PRL by mammary epithelium has been reported (Vonderhaar, 1999).
Its level is relatively low during the most part of the pregnancy, but in the last part increases and reaches high level at the parturition (Martinez and Houdebine, 1994, chap.1).
It has two roles in reproduction : the maintenance of corpus luteum, through which the secretion of estrogen and progesterone is ensured, and the induction of mammary development. After birth PRL is essential for maintaining lactation.
By the use of knockout mice the four independent components of prolactin pathway have been identified : the ligand itself (Horseman et al., 1997), the receptor (PRLR) (Ormandy et al., 1997), a transmembrane protein of the class I cytokine receptor family, the transcription factors Stat5a (Liu et al., 1997) and Stat5b (Udy et al., 1997).
Binding of PRL to its receptor leads to receptor dimerization and the activation of the Janus kinase 2 (JAK2), Fyn, a specific tyrosine kinase associated to the PRLR. JAK2 phosphorylates the two Stat5 isoforms that dimerize and migrate in the nucleus to induce transcription of target genes, such as genes for the caseines and genes containing γ-interferon activation sites (GAS). As well as Stat5, PRLR can signal through the mitogen-activated protein kinase (MAPK) pathways and others that are dependent of JAK2 (Hennighausen and Robinson, 2005).
Current evidence indicates that PRL present a generic signal that activates transcriptional programmes that are shared between several cytokine receptors, and even if these pathways have some cell-specific components they mediate general responses like proliferation and cell survival. Moreover not only PRL activate STAT5, leading to a developmental program that ends with the production of milk-secreting cells, but also other placental lactogens and members of the EGF family, whose effecte is mediated by EGF receptor such as ERBB1 and ERBB4, both necessary for mammalian development during pregnancy. In particular ERBB4 was shown to have a more prominent role in the functional luminal cell during lactation than PRL has (Long et al., 2003).
The signalling pathway activated by hormones is quite understood, but the mechanism by which it is negatively modulated is not well known. Recently evidences suggest that member of the SOCS family are involved in the inhibition of PRL signalling (Linderman et al., 2001; tonko-Geymayer et al., 2002).

Mammary development is not only controlled by systemics hormones, like estrogen, progesterone and PRL, but also by peptide that are produced either in the stromal or epithelial compartment, such as the osteoclast differentiation factor RANKL (Fata et al., 2000), inhibinβB (Robinson et al., 1997) and member of the TGFβ family (Nguyen and Pollard et al., 2000).
Several evidence from knockout mice suggest that RANKL, compared to PRL, induces identical or related developmental programs during pregnancy (Humphreys et al., 1999; Fata et al., 2000).
The growth factors, like transforming growth factor α and β, TGFα and TGFβ, mammary derived growth factor 1, MDGF1, and epidermal growth factor, EGF, are present in the mammary epithelium, secreted by the epithelial cells. The MDGF1, TGFα and the TGFβ are autocrine and mitogenic factors secreted by epithelial cells in order to stimulate the production of the collagen IV, an essential component of the basal membrane, where epithelial cells lie and proliferate in a polarized way during the alveolar development (Martinez and Houdebine, 1994, chap.1). Estrogen control indirectly the synthesis of collagen IV and the activity of the growth factors throughout the degradation of the basal membrane which supports epithelial cells.

At the moment of parturition strong changing in the concentrations of hormones occurs :
The progesterone, that negatively controls the PRL secretion and the local synthesis of caseins and other milk components, disappears, while PRL reaches high concentration; the level of estrogen increase progressively and stimulate the secretion of PRL; glucocorticoids are produced to amplify the PRL action; other hormones not specific of the lactation are involved, like the growth factor, (GH), and thyroid hormones (Martinez and Houdebine, 1994, chap.1).

I-II-e Role of extracellular matrix on mammary development

The multihormonal control on the mammary epithelium development and on the secretion of milk proteins was observed and studied relatively early due to the fact that the glands could be analysed easily in vivo (Dembinski and Shiu, 1987; Houdebine et al., 1985; Neville and Neifert, 1983; Topper and Freeman, 1980). However it must be recognized that a substantial proportion of epithelial hormonal responses reflects the modulation imparted by a complex extracellular compartment, that can exert its influence on mammary epithelium through several mechanisms : the mediation of hormonal signals via stromal hormone receptors; the local elaboration of soluble agonist/antagonist factors; the provision of a supporting vascular network; the contribution to a bed of basement membrane proteins on which epithelial cells are positioned (Russell and Vonderhaar, 2002). Moreover the importance of cell to cell interactions and cell to extracellular compartment interactions is gaining importance since functional cell cultures in vitro were developed (Martinez and Houdebine, 1994, chap.4).

At every stage of mammary development the duct or the alveoli lie on a basal membrane. It is possible that the interactions stroma-epithelium in vivo are mediated through the structure and composition of this extracellular matrix (MEC), that is the surface and the region of contact between the two tissues. The study of the biochemical composition and the structure of the MEC (Hassell et al., 1985; Kleinman et al., 1986; Miller and Gay, 1987) show that this basal membrane is not a passive layer, but in the contrary is an active membrane that receive structural and functional message to direct the behaviour of stromal and epithelial cells (Bissel and Aggeler, 1981; Bissel and Hall, 1987; Bissel aet al., 1982; Hay, 1981; IngBer and Jamieson, 1985; Wicha, 1984) .

This basement membrane underlying epithelial cells in vivo consists of 3 separate layers (Sakakura, 1991) : in contact with epithelium there is the lamina lucida, a thin space under which is located the lamina densa. Together they constitute the basal lamina. Adjacent to the basal lamina is the stroma-associated layer of variable thickness, the reticula lamina.

Based on in vitro study and immunolocalization experiments it was long assumed that components of the basal lamina, such as laminin, heparin sulphate proteoglycans and type IV collagen, were all derived from epithelial cells and that components of reticula lamina, such as collagen types I and III, fibronectin and tenascin, were derived from the stroma (Russell and Vonderhaar, 2002). Recent studies have assessed that the stroma is the primary source of extracellular matrix proteins and that also collagen I, IV and laminin derived from stroma (Keely et al., 1995). These finding define even the time of production of these macromolecule: collagen I is expressed in early puberty and early pregnancy, collagen IV during pregnancy and laminin during lactation. Moreover the expression of fibronectin from the stroma seems to be regulated by ovarian steroid hormones in association with epithelial-stromal interactions (Woodward et al., 2001). It is not clear if this dynamic construction of basal membrane during the mammary gland development is the result or the cause of the epithelial morphogenesis.

It is clear that various components of this basal membrane regulates the formation and function of epithelial cells and their response to external signals, such as ovarian steroid hormones or growth factors (Woodward et al., 2000). Even if we do not know the exact contribution of these extracellular proteins at a cellular level a general model establishes that the extracellular matrix exert its influence interacting with transmembrane proteins, able to communicate with the cytoscheleter and the nucleus of epithelial cells (Martinez and Houdebine, 1994, chap.4).

I-II-f The miRNAs in the mammary gland

An implication of miRNA in mammary gland biology is suggested from the data of some few recent reports, most of them focusing more on pathological situations, such as the appearance of breast cancer, than on the normal mammary development.

Liu et al. (2004) analyzed the gene expression profile of 18 adult and 2 fetal normal human tissues using a microchip containing the oligonucleotides for 248 miRNA (161 derived from human, 84 from mouse, 3 from Arabidopsis). They showed that each tissue has a specific pattern of miRNome expression (defined like the totality of miRNA present in a cell) that can be quantified. The mammary gland was one of the tissue analyzed and it was revealed that its specific signatures is characterized by the expression profile of only 23 miRNAs, the lowest number of miRNA detected in any tissue.

Other indirect evidences of miRNA involvement in the biology of mammary gland come from studies about breast tumors.

It was analyzed the genomic localization of 186 human miRNA (Calin et al., 2004), 52,5 % of them are present in cancer-associated genomic regions or in fragile sites and between them 15 miRNA are located in regions involved in human breast cancers. It was quantified (Jiang et al., 2005) by real-time PCR the expression of 222 pre-miRNA in 32 human cancerous cell lines, 5 derived from breast cancer, and it was observed that let-7f-1 expression was 7-fold higher in epithelial-derived breast, lung and colorectal cancer cells comparing to the mean of the remaining cell lines. Moreover another study used microarray technology to measure the differential expression of miRNA in normal and neoplastic human breast tissue and 29 miRNAs were found to be differentially regulated, 15 of which could be used with 100% accuracy to predict the tumor (Iorio et al., 2005). In particular miR-125b have a decreased expression level in samples derived from breast cancer primary tumors comparing to normal breast tissue (Lee et al., 2005).

To date any reports deals about the expression of miRNAs in normal mammary gland during the stages of its development.

II-Objective

To establish the genetic and functional network of a more comprehensive developmental model of the mammary gland the genomics approaches should identify new putative control genes and gene manipulation, in combination with tissue transplants, should evaluate their physiological role. It should be important to evaluate also the time windows during which a particular gene product is needed.

Taking in mind that many genetics pathways that control the development of mammary tissue are used in organ systems that appeared earlier in evolution and considering the big evolutionary conservation of miRNAs throughout every kingdoms and their involvement in various mechanism of organogenesis, it was chosen to address the attention to miRNAs, in order to discover putative regulatory molecule of the mammary gland development.

The study of miRNA in the mammary gland began analysing the expression of a first group of conserved miRNAs, during different stages of mammary development in mouse; then the expression profile during all the gland development has been studied in search for their potential regulatory role in determining the passage from one phase to one other. Later it was examined the cellular origin of their production.

The second objective of this work was the identification of mammary gland specific miRNA, the idea was supported from the finding of organ- and tissue- specific miRNA (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 also from the recent discovery of new specific primate miRNAs (Devor, 2006). After having constructed a cDNA library of small RNA extracted at different stages of mouse mammary gland, the expression of 'candidate miRNAs' was characterized and a composite analyses, in part using bioinformatics and experimentally tools, has been realized in order to validate them like miRNAs.

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).

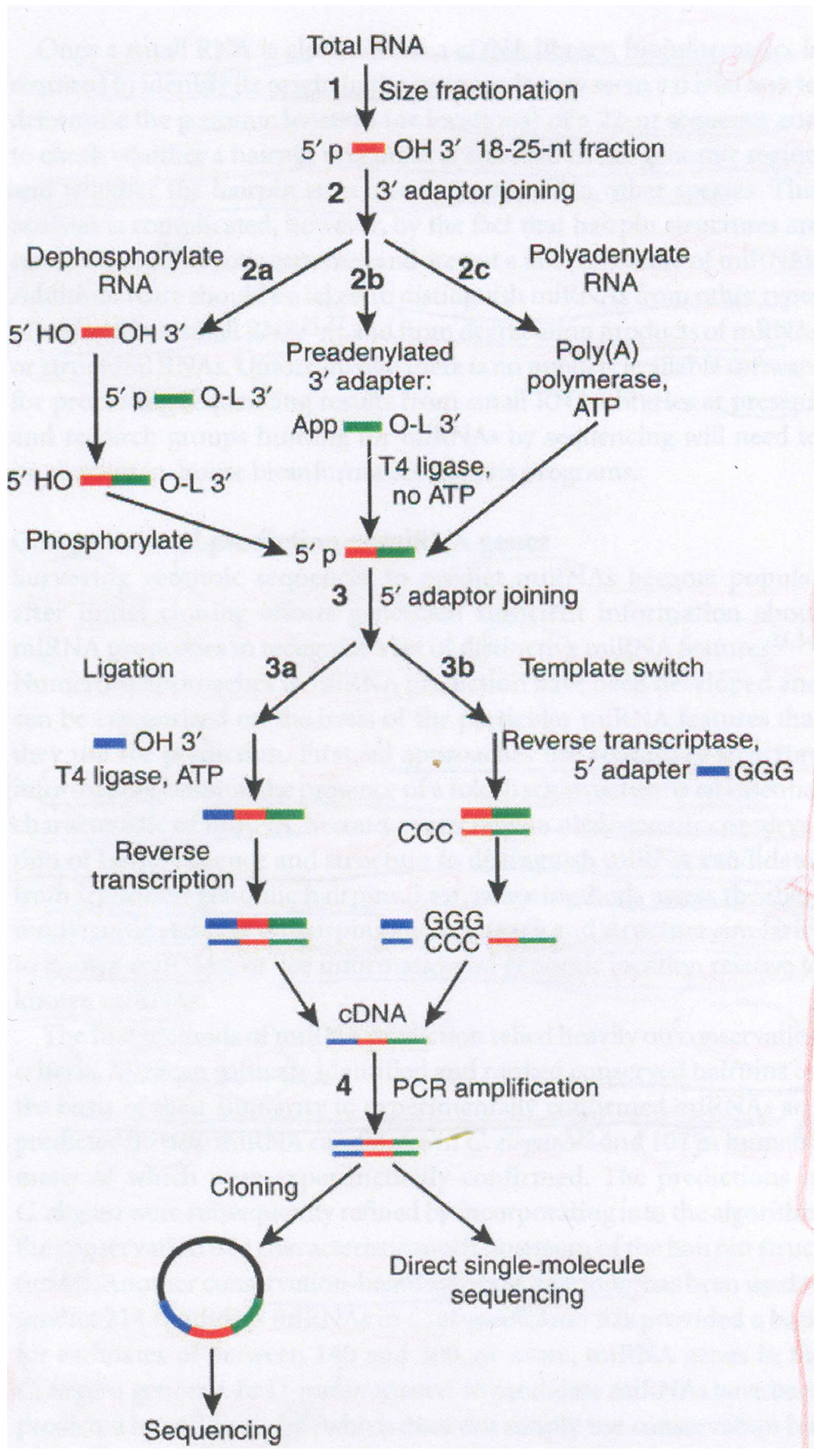
The reagent includes a cocktail of chaotropic agents, such as guanidinium salt derivative compound, which works synergistically to effectively alter the secondary and tertiary structures of proteins and polysaccharides and permits the extraction of RNA from other organic components.

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′
5′ HO O-L 3′
T4 ligase, no ATP
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™ 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™ were diluted in two independent 100 µl 150 mM NaCl sterile solution. 6 µl of jetPEI™ were necessary to transfect 3 µg of plasmidic DNA. The jetPEI™ 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	[illegible]	"	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.

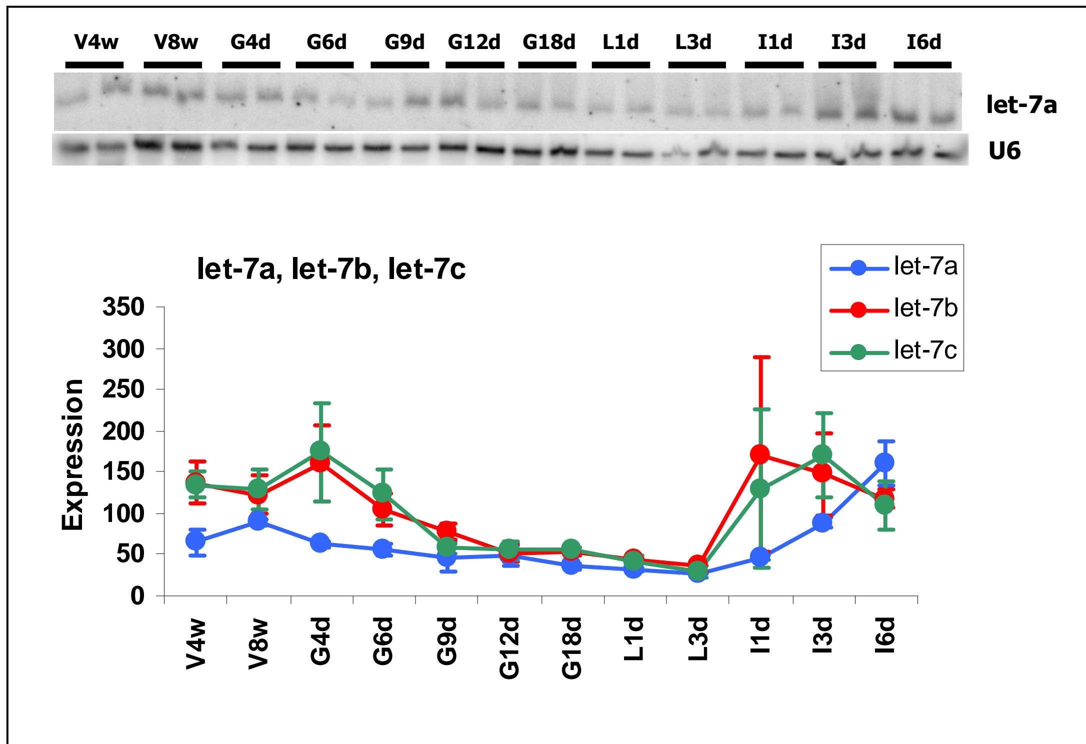
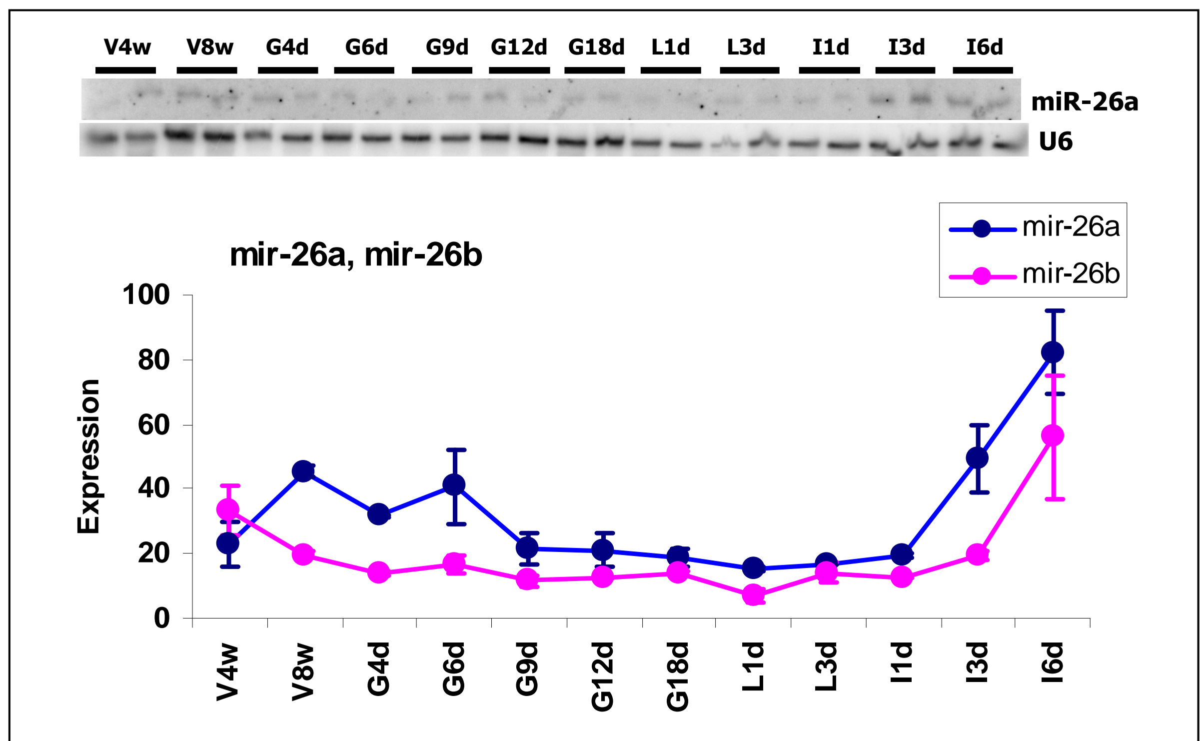


Figure 2. Image of Northern blot for miR-26a and expression profiles of miR-26a and miR-26b during the development of mouse mammary gland, after normalization and quantifications of the Northern blot data. Each point, and the relative error bar, represents the average miRNA expression (count per minute)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

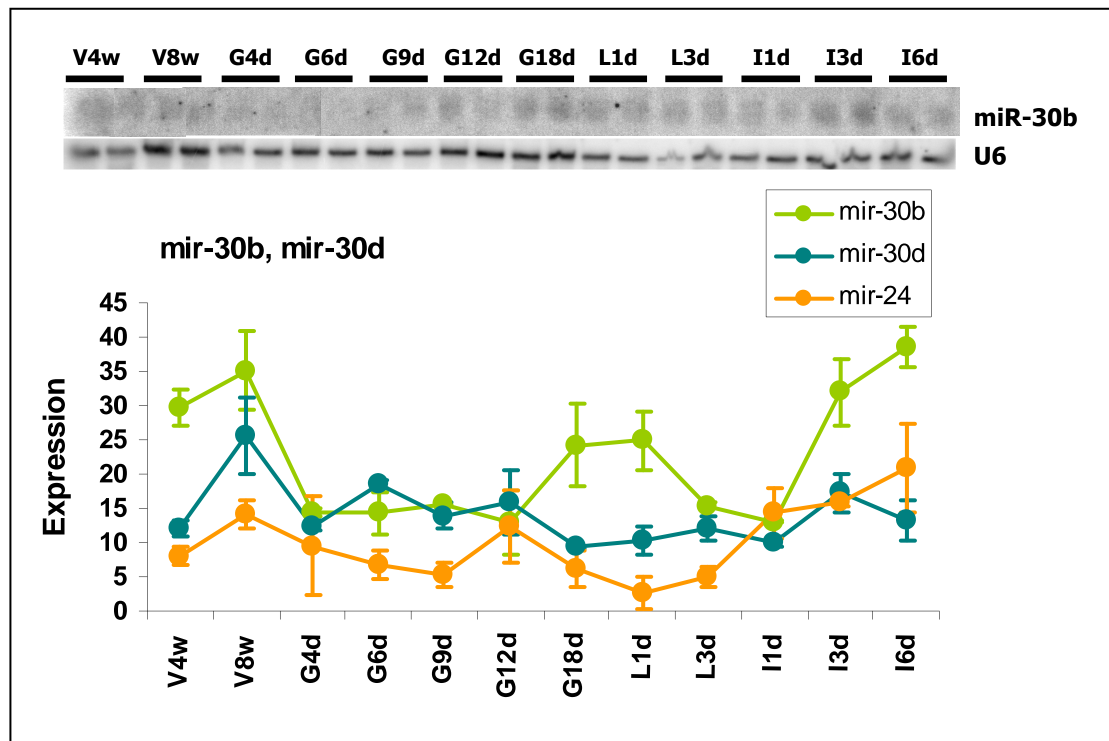
miR-30b and miR-30d show different expression profiles between them : comparing to miR-30b, the activity of miR-30d is more similar to that one of the first miRNAs described.

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.



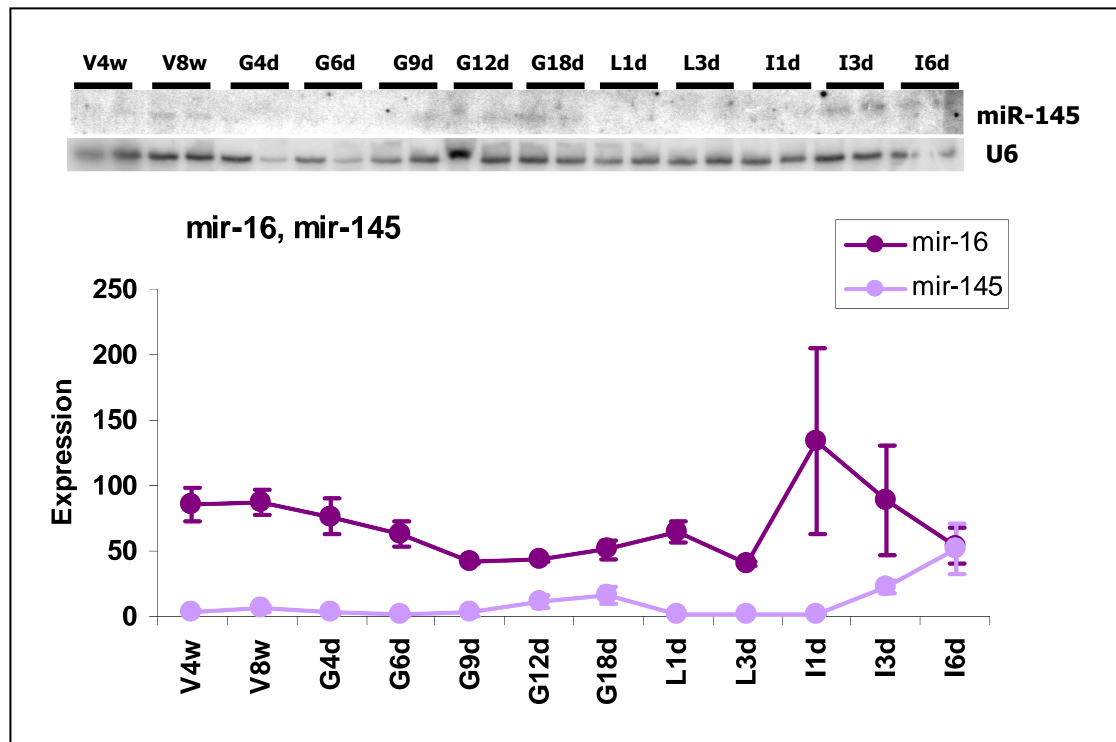
miR-145 is weakly detected and it shows an expression profile different from the other miRNAs analyzed. Also miR-16aa, detected at higher values, as a singular profile comparing to the first ones (figure 4).

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.

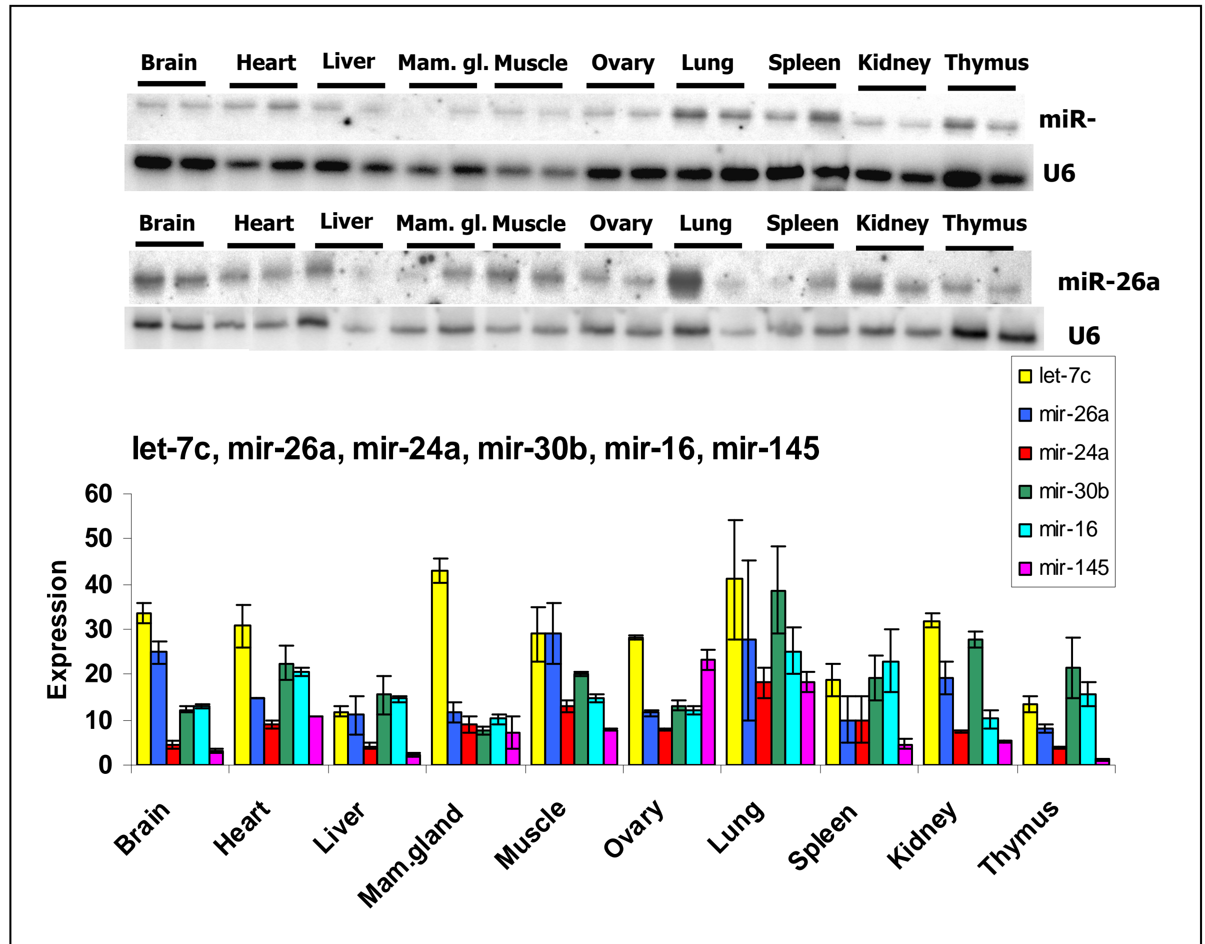


The miRNA profiles could be correlated with the hormonal control of some physiological phases of mammary gland development or whit the differentiation of the epithelial tissue during the developmental cycle of the organ : for example the progesterone is secreted in the beginning of pregnancy and its 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.

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.

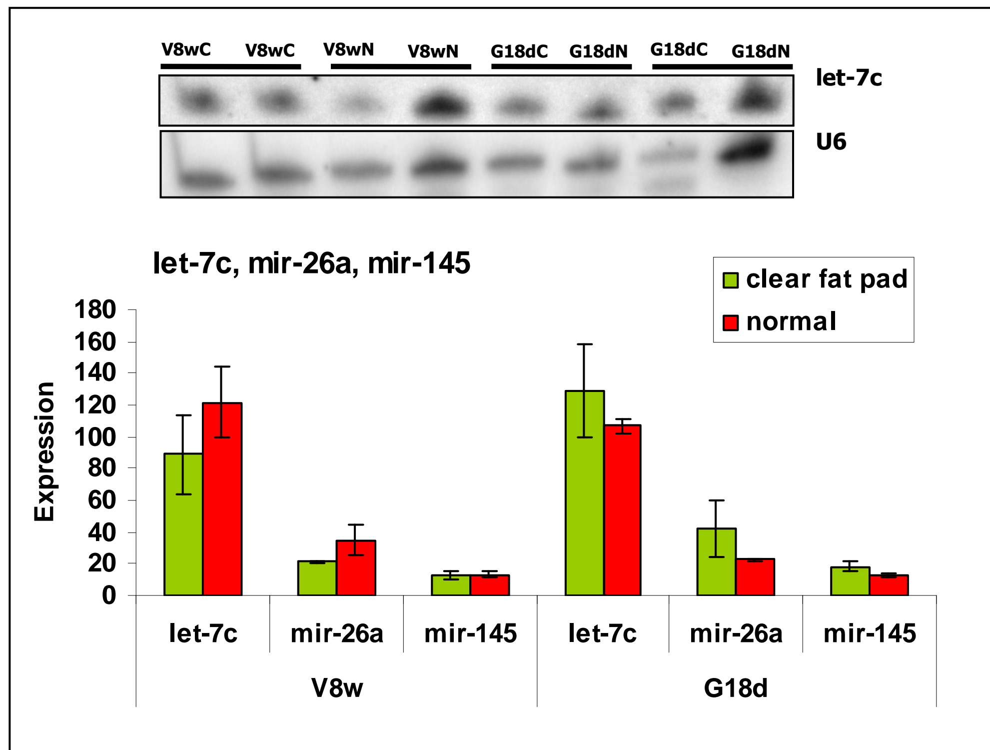
The MG is composed of two main tissue-compartment : the stroma, or 'fat pad', constituted by different cellular types, such as fibroblast, adipocytes and cells of connective tissue, and the epithelial tissue.

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%	/	/	/

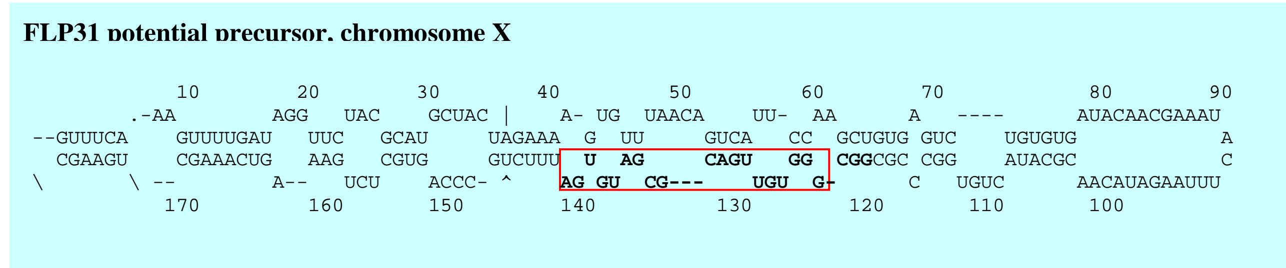
18 of the 64 cloned inserts (28.6%) were mapped and were found dispersed in all the chromosomes of the mouse genome except the chromosome 14 and the chromosome Y.
The 18 cloned insert which have high homology to specific genomic region show sometimes more than one genome localizations. Clones with highly 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	Invol. 1d	1	chr.17
LSII39	Invol. 1d	7	1, 6, 13, 7, 3, 8, 10
Gd8p	Gestat 2d	1	chr. 6
FLP33	Gestat 18d	2	chr.6, 17
FLP31	Gestat 18d	1	chr. X
I116I1	Invol. 1d	3	chr.3, 2, 16
Gd43p5	Gestat 2d	2	chr. X, chr. 18
LSII11u	Invol. 1d	1	chr. 16
G1836	Gestat 18d	1	chr.17
G1821	Gestat. 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	Invol. 1d	TGGGTGGTTCAGTGGTAGAATTCTCGC	TGGGTGGTTCAGTGGTAGAATTCTCGG
FLP31	Gestat 18d	GGCGGGTGTTGACGCGATGTGA	TCACATCGCGTCAACACCCGCC
FLP33	Gestat 18d	GTCCCGCGGGGCCCGAAGCGTT	AACGCTTCGGGCCCCGCGGGAC
I116I1	Invol. 1d	AAGAGGGCGTGAAACCGTTAAGAGGTA	TACCTCTTAACGGTTTCACGCCCTCTT
LSII15	Invol. 1d	CGCGGCGTGGGAAATGTGGCGT	CGCGGCGTGGGAAATGTGGCGT

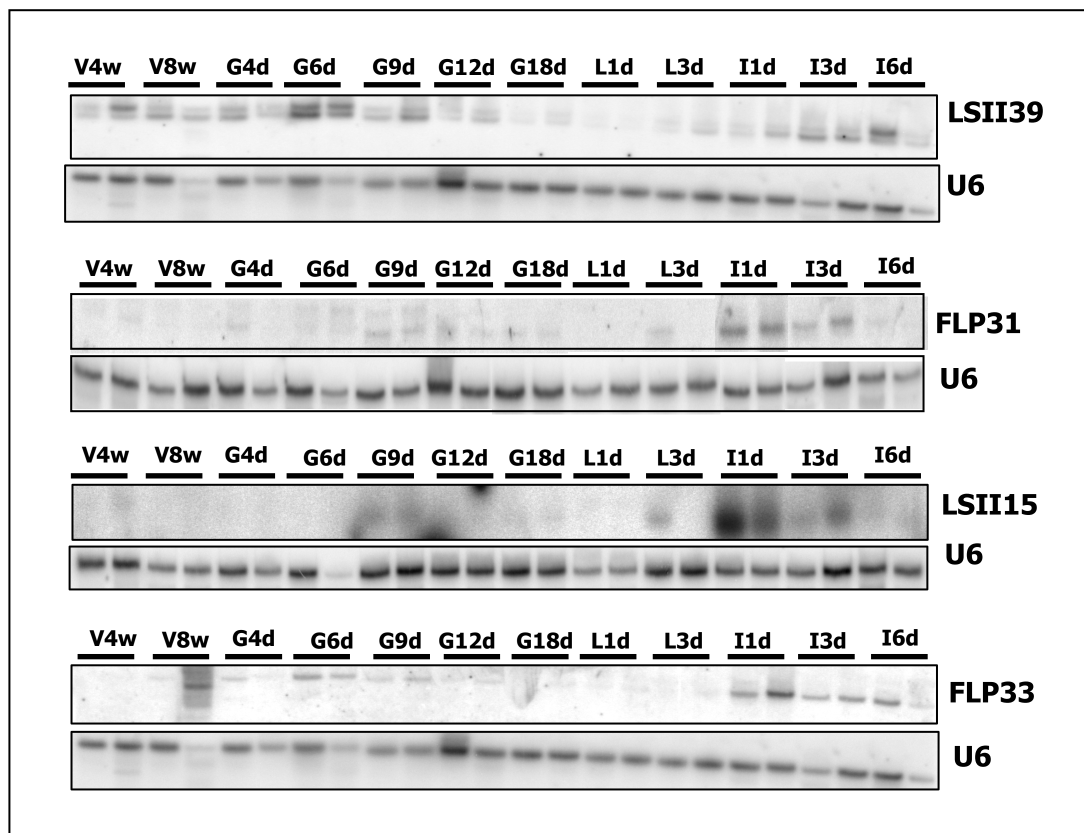
Later the expression of the 5 potential miRNA was characterized by Northern blot analysis in total RNA samples during the development of mouse MG (figure 8).

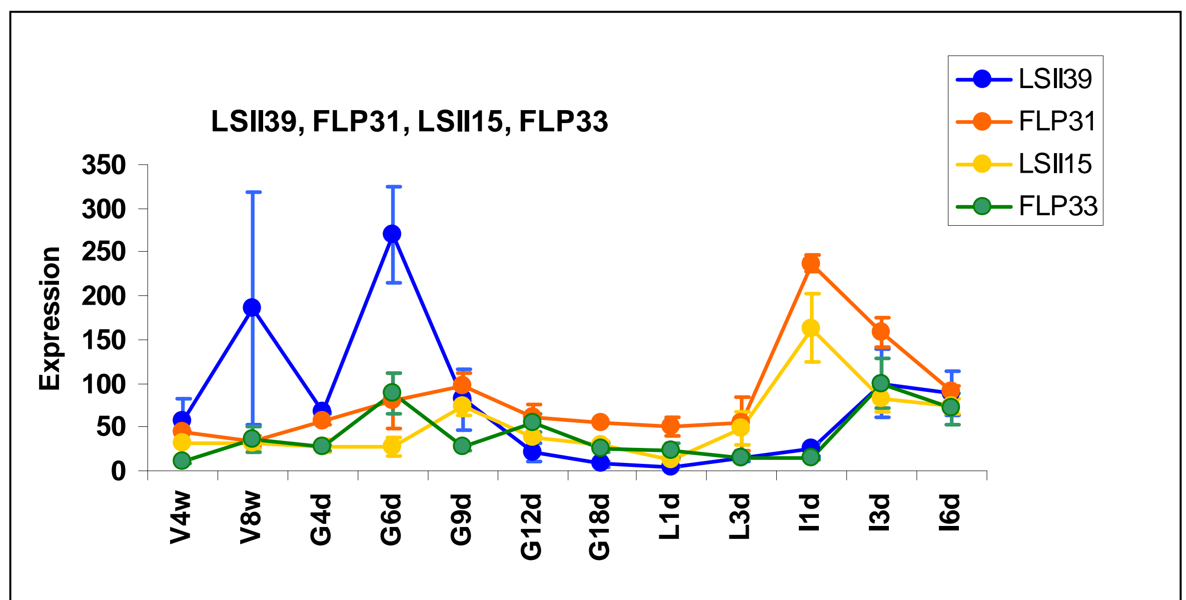
The expression profile was produced only for four potential miRNA : LSII39, FLP31, FLP33 and LSII15. For I116 many bands were observed and the quantification of the expression was not possible.

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.





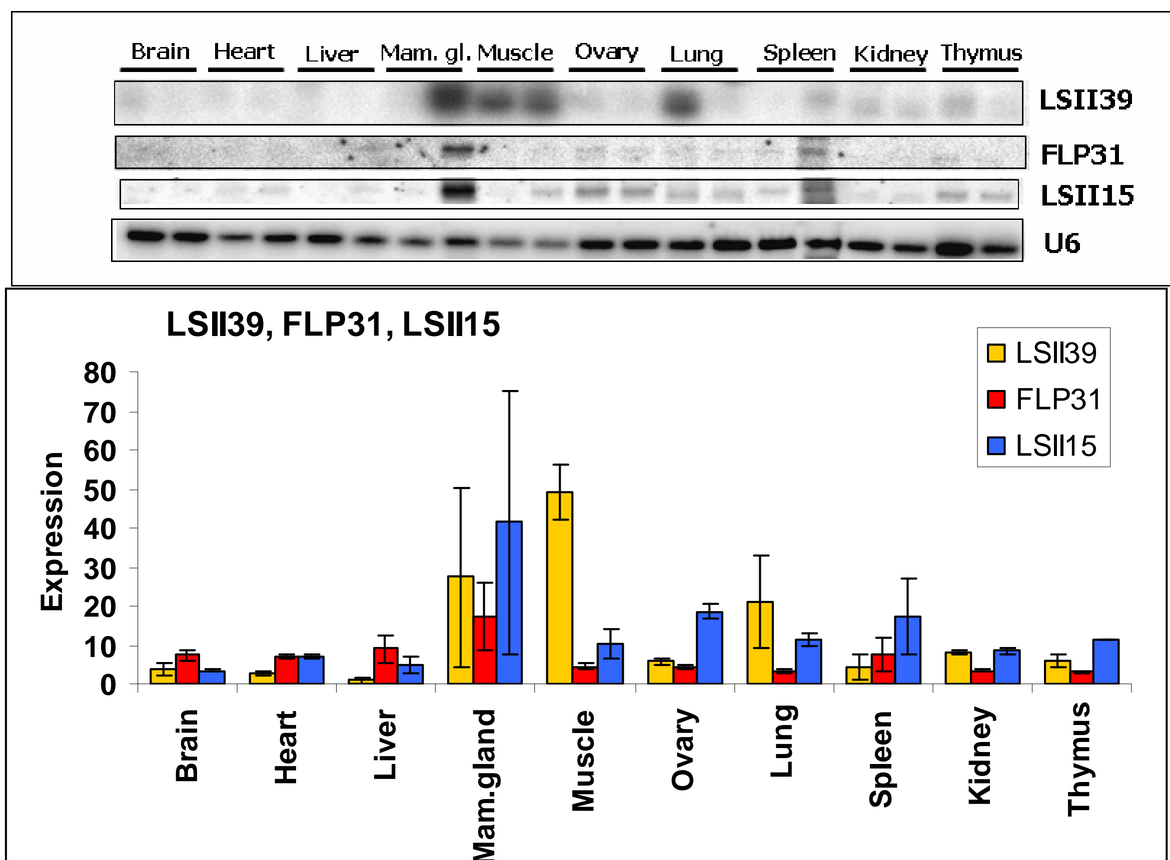
The aim of the experiment was to discover miRNAs specific of the mammary gland. The mammary gland specificity was examined analyzing the expression of the potential miRNAs in nine mouse organs, other than in mammary gland : total RNA samples were extracted and analyze from brain, heart, liver, lung, 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.

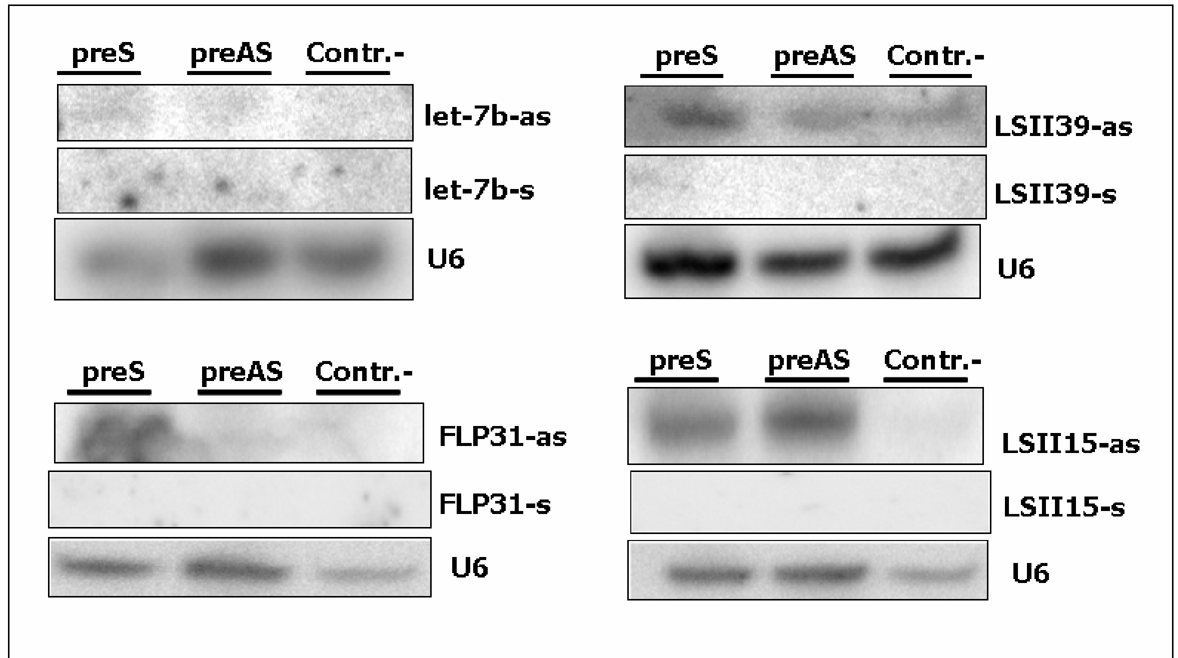
For let-7b the levels of expression of the miRNA is augmented in cells transfected with the 'sense precursor' comparing to the cells transfected with the 'antisense precursor' and to the non transfected cells, considered the negative control. Let-7b* is not expressed at a sufficient level both in cells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

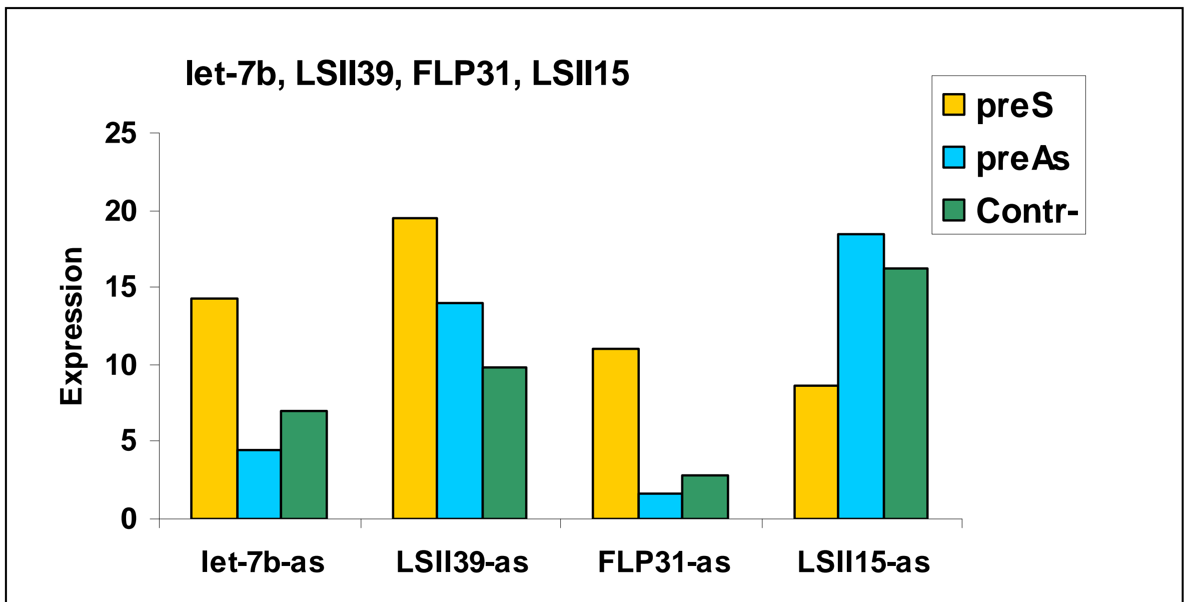
LSII39 and FLP31 are over-expressed in cells transfected with the 'sense precursor' comparing to the cells transfected with the 'antisense precursor ' and to the non transfected ones. No expression of LSII39* and FLP31* was detected in any kind of cells.

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 the 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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