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DOTTORATO DI RICERCA IN FISIOPATOLOGIA SISTEMICA

XIX ciclo

MICRORNA EXPRESSION PATTERNS IN OSTEOSARCOMAS AND THEIR POTENTIAL ROLE IN TUMOUR PROGRESSION

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“Many people will walk in and out of your life,
but only true friends will leave footprints in your heart”
Eleanor Roosevelt

To my friends who were,
there are,
there will be.

There is an increasing evidence that microRNAs are involved in control of developmental timing, cell proliferation, apoptosis, morphogenesis, fat metabolism. In many studies, performed to investigate the genes and gene products that drive the metastatic process, it has become evident that, in addition to alterations in protein-encoding genes, abnormalities in non-coding genes can also contribute to cancer pathogenesis. Changes in miRNA levels may be related to dysregulated growth in some cancer cells and in this field the differential expression of miRNA may have substantial diagnostic and prognostic value. In the context of microRNAs in tumours, our aim was to evaluate whether pro-metastatic and non-metastatic sarcoma cells, may differ in their microRNA expression, in the effort to identify single pro-metastatic microRNAs in bone and soft-tissue sarcomas. microRNAs were separated from total RNA of MG-63 and 143B osteosarcoma cells with different intravasation behaviour and malignancy degree. Specific libraries were constructed using TopoTA cloning system supported by 5'- and 3' adaptors. Differentially expressed microRNA were identified by sequencing followed by bioinformatic analysis. A number of microRNAs reported in the data base miRNA registry were found to be differential expressed in the two osteosarcoma model cell lines. Of the over 19 microRNAs identified, the majority correspond to "oncomiR", i.e. microRNAs involved in neoplastic transformation and progression. One of the analysed sequences, localized on chromosome 7 of the human genome, showed miRNA configuration and studies are in progress to define its precise characteristics. Two of the differential expressed microRNA, *miR-93* and *miR-210*, for which no functional data were available, were analyzed in different cell lines and tissues and investigated for their potential involvement in motility phenomena by their mis-expression through transduction in to osteosarcoma cells. To identify their molecular targets, different approaches were performed using bioinformatic softwares, through PCR-based strategies and DNA microarray analyses. The expression of another set of four miRNAs (*miR-9*, *miR-183*, *miR-196a*, *miR-484*) differentially expressed, was identified through a global expression analysis, and analyzed in surgical specimens of low- and high-grade osteosarcoma patients. To better define the significance of the expression pattern of these microRNAs, studies on a wider patient cohort are in progress.

INTRODUCTION

1.1 The spreading of neoplastic cells

Tumour cell dissemination and subsequent metastasis formation in adjacent tissues/organs or in anatomical sites distant from the primary lesions are the prevalent cause of death in cancer patients (Kurschat and Mauch, 2000; Liotta and Stetler-Stevenson, 1991; Pepper et al., 2003; Stacker et al., 2002).

The basis for neoplastic cell diffusion is their entrance into haematic and lymphatic vessels, either situated in proximity of the tumour mass or newly generated within it, and subsequent malignant cell transportation trough these streams. Although information has constantly been gathered about the cellular and molecular mechanisms underlying the tropism of various tumour cells for different organs or tissue (bone and lymph nodes being the overwhelmingly most frequent one), we are still rather ignorant about how cancer cells actually decide to stop and extravasate (i.e. egress to the circulation) in pre-selected organ or tissue location.

The importance of many cell surface molecules in the phenomenon of extravasation is still not fully understood (Figura 1.1), neither the relationships among these various components. Selectins (Geng et al., 2004) and their oligosaccharide ligands, various class of integrins and their cell surface-associated molecules (i.e. ICAM1-3 and LFA1-3), integrin-interacting/cooperating surface components (e.g. members of the tetraspan family), various types of surface glycolipids, gangliosides and mucins and metalloproteinase and their inhibitors (Dong et al., 2002) are reported as cell surface elements implicated in tumour transendothelial migration. Several cell adhesion molecules have been considered as both investigative targets for prognosis and diagnosis determination of specific tumour types and exploitable therapeutic targets.

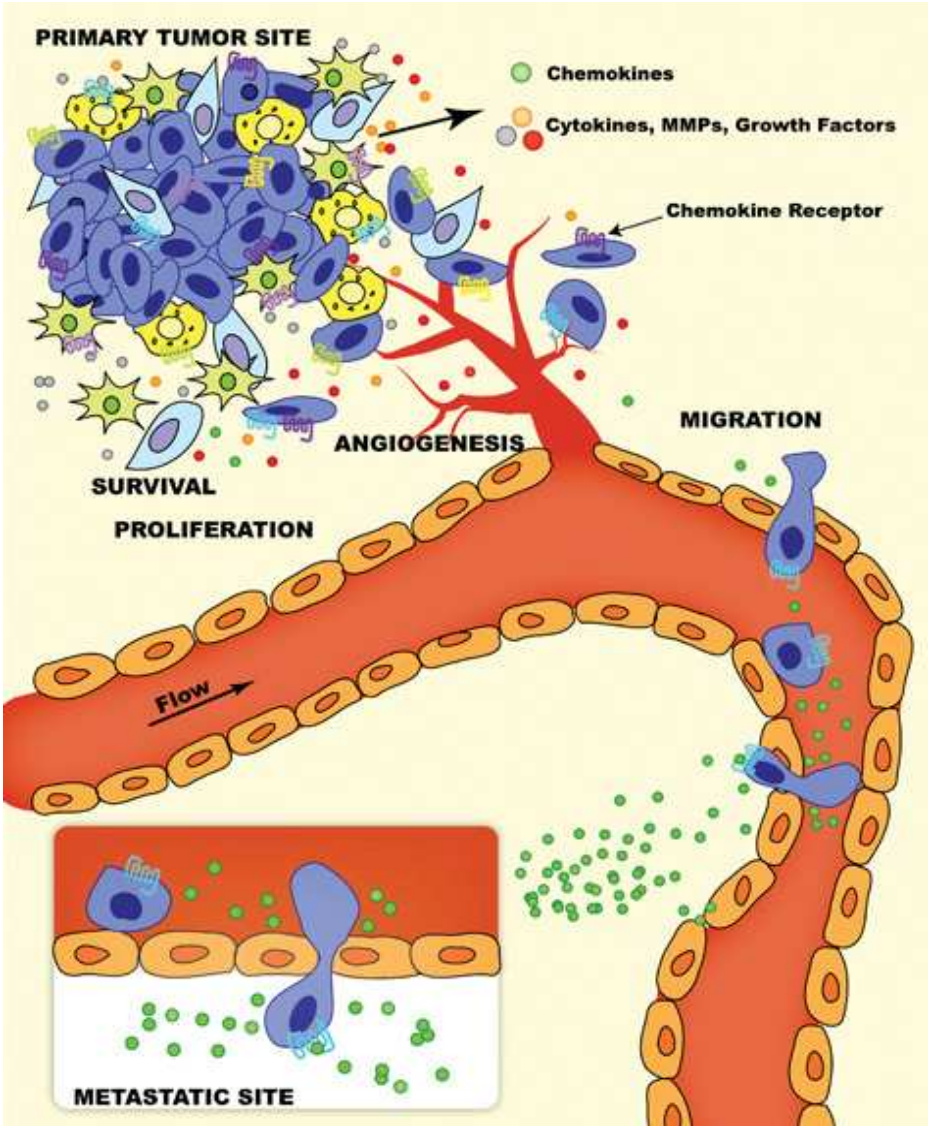

Figure 1.2 *Metastatic spreading from primary tumour site*

This figure illustrates many aspects of cancer progression where chemokines/receptors may play a role: growth of the primary tumour, angiogenesis (cell migration of endothelial precursors), metastasis, and survival/growth of the metastasized cells, probably the least efficient step in the metastatic process. In the primary lesion, tumour cells (dark blue) are supported by a network of cells in the microenviroment including fibroblasts (light blue), Dendritic Cells (green) and Tumour Associated Macrophages (yellow). Chemokines, produced by the tumour cells, serve to recruit endothelial cells, thereby promoting angiogenesis. They also recruit leucocytes, which produce other cytokines, growth factors and metalloproteinasis that enhance growth, proliferation and angiogenesis. Fibroblasts also produce angiogenic and survival/growth-promoting chemokines. Tumour metastasis is facilitated by the up-regulation of particular chemokine receptors (such as CXCR4) on the tumour cells, which enables them to migrate to secondary tissues where the ligands are expressed. Similar to the primary site, paracrine and autocrine chemokine/cytokine signalling among cells within the microenvironment may be especially important for survival and growth of the metastasized cells (O'Hayre et al., 2008).

1.2 Osteosarcomas

Sarcomas, whose origin is believed to be associated with both hard and soft connective tissue, account for almost 5% of the total malignancies. The predominant class is represented by soft-tissue sarcomas (Registry, 2007; Ries, 1999). Comparison of the incidence rate shows that osseous neoplasms occur at a rate approximately one fifth of that of the closely related group of soft-tissue sarcomas (Dorfman and Czerniak, 1995; Higginson et al., 1992; Mack, 1995). Osteosarcoma is the most common primary malignant tumour of bone, accounting for approximately 35% of these tumours and occurs predominantly in patients younger than twenty years of age (Ries, 1999). This sarcoma type appears in different anatomical sites, mostly in the extremity, and the characteristic metastasis areas are lungs and other skeleton parts.

The genetic alterations playing an important role in triggering the osteosarcoma neoplasm seem to be mutations of tumour suppressor genes. For example, the deletion of retinoblastoma gene (RB) on chromosome 13q14 has been found in 60% of osteosarcoma patients. However, this tumour is not linked to a specific alteration since a lot of karyotypes show different chromosomal amplification (Man et al., 2004). Till today, only the histotype analysis is used to identify the various subtypes and establish the tumour stage.

Low grade osteosarcomas- In almost 80% of the cases, they are located in the long bones with peculiarity for the distal femur and proximal tibia (Kurt et al., 1990). They are generally classified in different sub-types the most frequent of which are afterwards briefly described.

Low grade central osteosarcoma (Figure 1.2A) is composed of a hypo- to moderately fibroblastic cellular stroma with variable amounts of osteoid production (Franceschina et al., 1997);

Parosteal osteosarcoma (Figure 1.2B.) is the most common type of osteosarcoma of bone surface (Okada et al., 1994) and has the tendency to wrap around the involved bone (Bertoni et al., 1985);

Periosteal osteosarcoma (Figure 1.2C) that arises on the surface of a bone, displays non-homogeneous, calcified spiculation that are disposed perpendicularly to the cortex and give an overall sunburst appearance. It has a conspicuous fusiform appearance when it involves the entire circumference of the shaft of a bone (Unni et al., 1976). Histologically, periosteal osteosarcoma has the appearance of a moderately differentiated chondroblastic osteosarcoma (Unni et al., 1976).

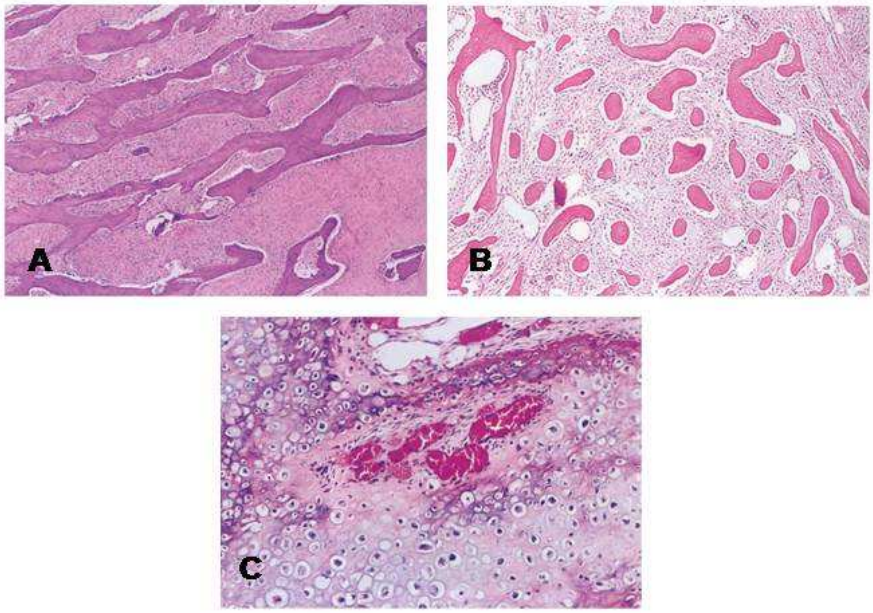

Figure 1.1 *Low grade osteoarcoma*

***A. Low grade central osteosarcoma**. At low magnification, long, parallel seams of bone surrounded by hypocellular spindle cell stroma are seen (Inwards and Unni, 1995). **B. Parosteal osteosarcoma**. Well-formed bone trabeculae in a hypocellular spindle cell stroma. (Unni, 1996). **C. Periosteal osteosarcoma**. Typical appearance of chondroblastic grade 3 sarcoma. There are lobules of malignant –appearing cartilage with bone formation in the centre of the lobules (Unni et al., 1976).*

High grade osteosarcomas – The term “high” is mainly referred to the grade of malignancy. The most recurrent types are then in brief described.

Classic osteosarcoma (Figure 1.3A) shows a profound propensity for involvement of the long bones of the appendicular skeleton; in particular, the distal femur, proximal tibia and proximal humerus. It is often a large, metaphyseally centered, fleshy or hard tumour which may contain cartilage and it frequently transgresses the cortex; it is associated with a soft tissue mass. It tends to be a highly anaplastic, pleomorphic tumour in which the tumour cells may be epithelioid, plasmacytoid, fusiform, ovoid, small round cells, clear cells, mono- or multinucleated giant cells, or spindle cells. Most cases are complex mixtures of two or more of these cell types (Campanacci, 1990).

High grade surface osteosarcoma (Figure 1.3B) comprises less than one percent of all osteosarcomas. The tumour radiographically presents as a surface, partially mineralised, mass extending into the soft tissue; the histopathology shows the same spectrum of features seen in classic osteosarcoma (Wold et al., 1990).

Telangiectatic osteosarcoma (Figure 1.3C) occurs in most cases in the metaphyseal region of long tubular bones. The tumour contains blood-filled or empty

spaces separated by thin septa simulating aneurismal bone cyst. The tumour cells are hyperchromatic and pleomorphic with high mitotic activity including atypical mitoses. This tumour tends to make osteoid matrix when it metastasized (Matsuno et al., 1976).

Small cell osteosarcoma (Figure 1.3D) is composed of small cells associated with osteoid production; the matrix shows flocculent dense material in close apposition to tumour cell membrane with subplasmalemmal densities in the adjacent cells.

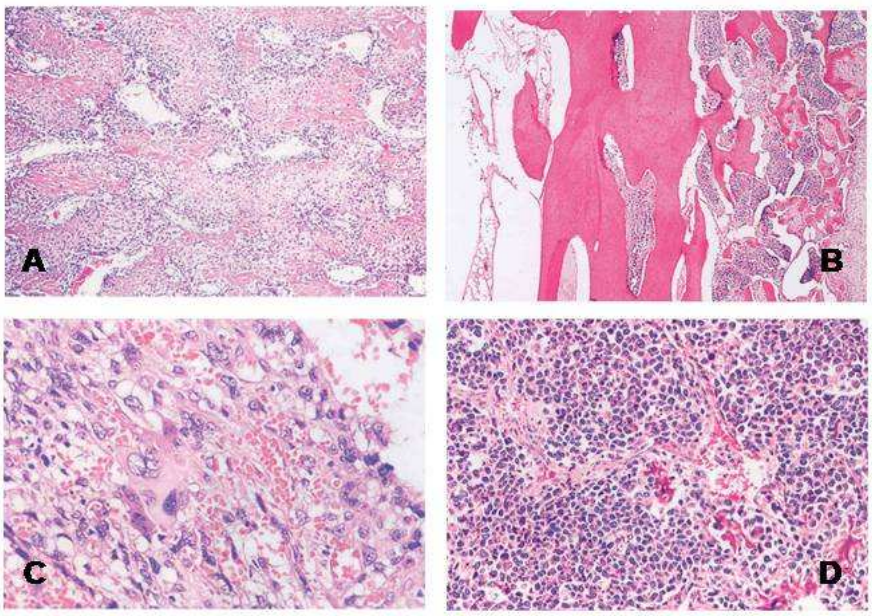

Figure 1.1 *High grade Osteosarcoma*

***A. Classic osteosarcoma**. Frequently occurring angiocentric pattern of growth may impart a basket-weave appearance while combined with abundant osteoid production (Raymond et al., 1987). **B. High grade surface osteoarcoma**. The tumour produces large amounts of bone (right). The cortex (middle) and the medullary cavity (left) are uninvolved. (Wold et al., 1990). **C. Telangiectatic osteosarcoma**. Highly malignant tumour cells produce minimal amounts of fine, lace-like osteoid. (Matsuno et al., 1976). **D. Small cell osteosarcoma**. Osteoid production at lower right (Fletcher et al., 2002).*

1.3 Biomarkers in human cancer

A tumour marker can be detected in the tumour lesion, in tumour cells in circulating peripheral blood, in metastasis formation or in the body fluids. Markers may also be used to detect the presence of occult metastatic disease, to monitor response to treatment or to detect recurrent disease (Lindblom and Liljegren, 2000).

Oncofetal antigens, which are normally expressed in cells during embryological development, can be considered in several cases as tumour markers when abnormally expressed in neoplasms and result very useful to control the state of patient health. In particular, carciembryonic antigen, the most commonly oncofetal antigen, is expressed

in all gastrointestinal tumours as well as in many other tumours (Hunerbein, 1998). The α fetoprotein, mainly used in the diagnosis of hepatocellular cancer, is also expressed in testicular and ovarian cancer (Lamerz, 1997).

The best-characterized markers are described for the most frequent cancer types such as breast, colorectal carcinoma and lung for which genetic alterations and serum analyses are routinely carried out. In sarcomas, important markers are those associated with chromosomal translocation and fusion proteins. The identification of several specific fusion products in very infrequent sarcoma types has allowed for a diagnostic improvement. In fact clinical pathological diagnosis is often difficult, while the demonstration of a specific fusion protein is diagnostic for these rare sarcomas (Lindblom and Liljegren, 2000).

However, when the application of some clinical markers is limited by their apparent lack of specificity or sensitivity, additional biomarkers are required to implement a tailored individualized therapy. Many studies are currently conducted with this perspective and in the last years the increasing importance of small non-coding RNAs in gene regulatory networks has address many researches to unravel their roles in cellular processes and to understand their potential as new and additional markers in tumours of different kind.

1.4 The small non-coding RNA world

Four types of gene silencing-related small RNA have been found in animals over the past few years: small interfering RNAs (siRNAs), microRNAs (miRNAs), and, more recently, repeated associated small Interfering RNAs (rasiRNA) and Piwi-interacting RNAs (piRNAs) (Kloosterman and Plasterk, 2006).

siRNAs, also found in plants and yeast, are usually derived from double-stranded RNA which originates from virus. Generally, siRNAs trigger mRNA degradation by binding their targets with perfect complementarity (Meister and Tuschl, 2004).

Genomic repeats and retrotrasposons give rise to **rasiRNAs**. They seem to function through a distinct small RNA pathway involving Piwi proteins in *Drosophila* (Saito et al., 2006; Vagin et al., 2006).

A germline-specific class of vertebrate small RNAs is formed by **piRNA**, 29-30nt-long RNAs that act by binding to Piwi proteins. piRNAs are not associated with repeats, even if the encoding genes are localized in clusters in the genome (Girard et al., 2006).

miRNAs are a well established class of ~22 nt endogenous, noncoding small RNAs that influence mRNA stability and translation. Although the first miRNA, *lin-4*, was found in 1993 (Lee et al., 1993; Wightman et al., 1993), the miRNA field did not take off until the discovery of the highly conserved *let-7* small RNA (Reinhart et al., 2000).

Since then, several research groups have applied small-RNA-cloning strategies to identify new small RNAs in vertebrates and invertebrates (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). More recently, hundreds of miRNAs have been found in many animal species by in silico analysis, experimental approaches, or combined strategies (Berezikov et al., 2006).

1.4.1 Origin and maturation of microRNA

miRNA genes are transcribed by RNA polymerase II as capped and polyadenylated primary miRNA transcripts (pri-miRNA) (Cai et al., 2004; Lee et al., 2004). The RNAse III enzyme Drosha initiates the nuclear processing of the pri-miRNA into an ~70nt precursor miRNA, pre-miRNA (Lee et al., 2003). The double-stranded RNA binding protein DGCR/Pasha interacts with Drosha to form the microprocessor complex (Kim, 2005). Pre-miRNAs are exported from the nucleus by binding to the nucleocytoplasmic transport factor Exportin 5 (Kim, 2004). Maturation of the pre-miRNA into an imperfect RNA duplex, with 2nt 3' overhangs, is mediated by the cytoplasmic enzyme Dicer (Hammond, 2005). The strand of the duplex with the weakest base pairing at its 5' terminus is preferably loaded into the RNA-induced silencing complex (RISC) (Hutvagner, 2005). The miRNA guides the RISC complex to the 3'UTR of target mRNAs (Figure 1.3). Animal miRNAs usually pair with imperfect complementarity to their target. The seed region (nucleotide 2-8) of miRNAs guide strand is the most important for target recognition and silencing (Doench and Sharp, 2004; Lewis et al., 2005). However, other studies report that miRNA targeting is also influenced by additional factors such as the presence of miRNA-recognition elements (MREs) and their cooperation (Doench et al., 2003), the spacing among MREs (Saetrom et al., 2007), the proximity to the stop codon, the position within the 3'UTR, the AU composition (Farh et al., 2005) and the secondary structure of mRNA target (Long et al., 2007). The association of miRNAs with their mRNA targets inhibits translation. Both the initiation and elongation steps of translation are hypothesized to be affected even if the exact mechanism of the process remains unclear (Petersen et al., 2006; Pillai, 2005; Valencia-Sanchez et al., 2006). Moreover, repressed mRNAs

are present in cytoplasmic foci called P-bodies known as sites for mRNA destabilization (Bruno and Wilkinson, 2006). Recent data suggest that, indeed, miRNA-mediated repression might alter the levels of target mRNA, mainly via mRNA de-adenylation (Bagga et al., 2005; Giraldez et al., 2006; Lim et al., 2005; Valencia-Sanchez et al., 2006).

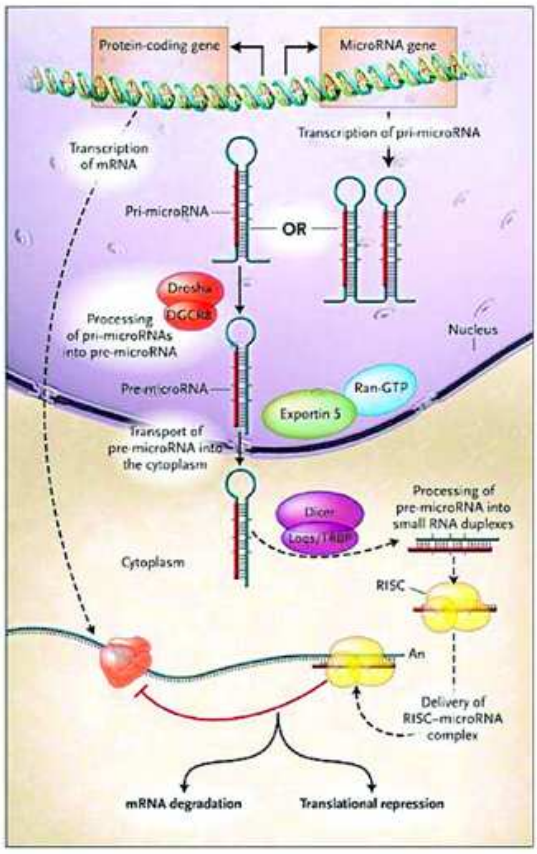

Figure 1.3 ***Biogenesis of microRNA and microRNA-mediated gene regulation in animal cells***

MicroRNAs are produced from full-length RNA polymerase II transcripts (pri-miRNA) after cleavage by two RNAse III enzymes (Drosha and Dicer) in association with accessory proteins (RISC). The actions of miRNAs are mediated by the RISC that is believed to block mRNA translation, reduce mRNA stability or induce mRNA cleavage, after imperfect binding to MREs within the 3' and 5' untraslated region (UTR) of target mRNA genes (Waldman and Terzic, 2008).

Recent advances in analyzing the spatial expression of miRNAs have shown that they are expressed in a very tissue-specific manner during the development (Aboobaker et al., 2005; Ason et al., 2006; Kloosterman and Plasterk, 2006; Wienholds et al., 2005). In particular *miR-1* is expressed in heart, *miR-124* in the central nervous system and *miR-10* in anterior-posterior axis. This suggests microRNAs could be involved in the molecular watch controlling the organism development (Figure 1.2).

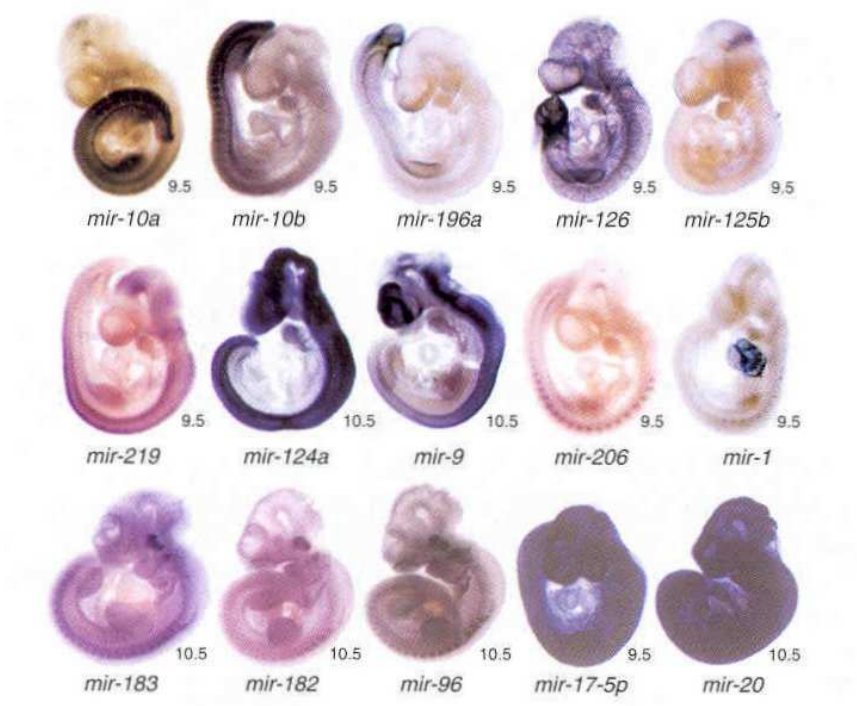

Figure 1.4 ***Expression of different microRNA***

Expression of fifteen miRNAs in 9.5 and 10.5 d.p.c. mouse embryos: miR-10a and miR-10b, posterior trunk; miR-196a, tailbud; miR-126, blood vessels; miR-125b, midbrain-hindbrain boundary; miR-219, midbrain, hindbrain and spinal cord; miR-206, somites; miR-1, heart and somites; miR-183, miR-182 and miR-96, cranial and dorsal root ganglia; miR-17-5p and miR-20 are ubiquitously expressed (Kloosterman et al., 2006).

1.4.2 miRNA gene clusters

A prominent characteristic of animal microRNA is that their genes are often organized in tandem and are closely clustered on the genome (Houbaviy et al., 2003; Lau et al., 2001), but they are processed into multiple individual mature miRNA (Stefani and Slack, 2008). In many cases, such clustered miRNAs seem to be processed from the same polycistronic precursor transcript (a single mRNA molecule produced from the transcription of several sequential genes). The genomic organization of these miRNA clusters is often highly conserved, suggesting an important role for coordinated regulation and function of a set of mRNAs targets (Stefani and Slack, 2008). For example, a mammalian miRNA gene-cluster of particular interest encodes the six closely related genes *miR-290-* to *miR-295* in mouse. These are expressed specifically in embryonic stem (ES) cells. Although it is not yet clear whether the human genome contains an orthologous cluster of ES-cell-enriched miRNAs, these findings suggest that translational repression of gene expression by miRNAs contributes to the maintenance of stem-cell potency (Houbaviy et al., 2003). Clusters can also contain miRNAs of distinct sequences and, in this case, each distinct miRNA type may in a

coordinate manner deploy towards its target. For example, in mouse, *miR-1* localizes in a genomic cluster with *miR-133* but these two miRNAs differ in their seed sequence, and have distinct functions in contrast to *miR-1*, which promotes myogenesis, *miR-133* inhibits muscle differentiation and induces proliferation by repressing serum response factor (Chen et al., 2006).

The *miR-17-92* cluster is a prototypical example of polycistronic miRNA gene. In the human genome, the *miR-17-92* cluster encodes six miRNAs (*miR-17, miR-18a, miR-19a, miR-20a, miR-19-b-1, and miR-92-1*) which are tightly grouped within an 800 base-pair region of human chromosome 13 (Figure 1.5A). Both the sequences (Figure 1.5B) of these mature miRNAs and their organization are highly conserved in all vertebrates. The human *miR-17-92* cluster is located in the third intron of a ~7 kb primary transcript known as C13orf25 (Ota et al., 2004).

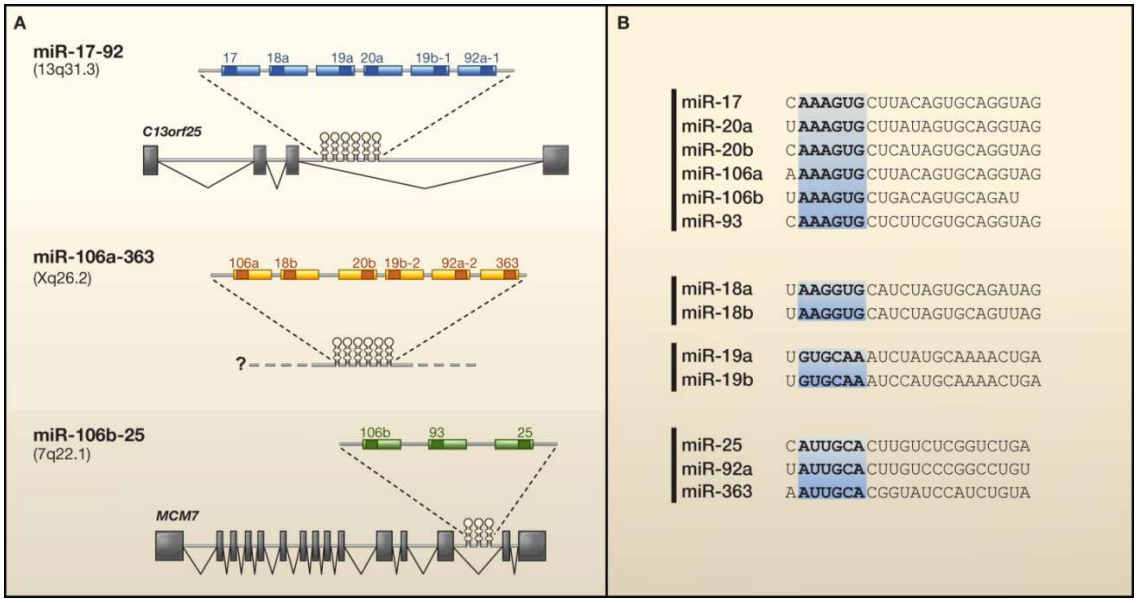

Figure 1.5 ***Organization of the miR-17-92 Cluster and its paralogs (Mendell, 2008)***

A. *The genomic organization and primary transcript structures of the human miR-17-92, miR-106a-363, and miR-106b-25 clusters. The miR-106a-363 primary transcript has not been characterized.* ***B.*** *Based on their seed sequences- which are the regions considered most important for target selection (nucleotides 2-7; shown in blue)- the miRNAs of these clusters can be grouped into four families: the miR-17 family (miR-17, miR-20a/b, miR-10a/b, and miR-93); the miR-18 family (miR-18a/b); the miR-19 family (miR-19a/b); and the miR-25 family (miR-25, miR-92a and miR-363). MicroRNAs of the miR-17-92 cluster and its paralogs have been implicated in normal development of the heart , lungs, and immune system (Koralov et al., 2008; Ventura et al., 2008; Xiao et al., 2008) as well as in tumorigenesis.*

Ancient gene duplications have given rise to two *miR-17-92* cluster paralogs in mammals: the *miR-106b-25* cluster (located on human chromosome 7) and *miR-106a-363* cluster (located on the chromosome X) (Figure 1.5A). The *miR-106b-25* cluster is located within the 13th intron of the protein-coding gene MCM7. Unlike the *miR-17-92* and *miR-106b-25* clusters, which are both abundantly expressed across many tissue and cell types, *miR-106a-363* cluster was dectable only in same pathological tissues such as T-cell leukemia (Landais et al., 2007). Perhaps this polycistronic miRNA provides a very specialized function in cell type yet to be studied.

1.4.3 Functions of animal microRNAs

Different studies have been taken to elucidate miRNA function and the potential phenotypes induced by miRNA perturbations have placed these molecules at the centre of critical cellular and developmental pathways. The important role of miRNAs in different physiological process in both cells and organisms is supported by their conservative sequences and tissue-related expression. Although miRNAs have only been studied intensively in the last years, important function for miRNAs in animal development and potentially, human disease, have already emerged (Alvarez-Garcia and Miska, 2005; Xiao et al., 2008). Indeed, animals without miRNAs cannot live or reproduce (Bernstein et al., 2003; Ketting et al., 2001; Wienholds et al., 2003).

Some miRNAs are regulators of developmental timing controlling proper transition among *Caenorhabditis elegans* larval stages (Wightman et al., 1993) or, as *let-7*, regulate different transcription factors during the larval-to-adult transition acting as a master switch that control temporal patterning (Abrahante et al., 2003; Grosshans et al., 2005; Lin et al., 2003).

Although not directly related to signalling cascade, miRNAs often are in feedback loops to control their own expression. For example, granulocytic differentiation is enhanced by *miR-223* expression that is controlled by a feedback mechanism (Fazi et al., 2005): before the differentiation process, the transcription factor NFI-A allows weak expression of *miR-223*; upon stimulation with retinoic acid, NFI-A is replaced by the transcription factor C/EBPα. That induces high expression of *miR-223*, which, in a reciprocal negative-feedback loop, post-transcriptionally represses the expression of NFI-A.

Many miRNAs show a cellular or region specific expression, in particular in the brain, *miR-134* is found to localize in synaptic sites of rat hippocampal neurons (Schratt et al., 2006), *miR-132* is expressed in cortical neurons (Vo et al., 2005), *miR-124a* is

involved in the differentiation of neuronal progenitors into mature neurons by degradation of non-neural transcripts (Conaco et al., 2006). The importance of microRNA in the functioning brain (Wienholds et al., 2005) is suggested by some miRNAs that are implicated in specific asymmetric gene expression in chemosensory neurons (Chang et al., 2004; Johnston and Hobert, 2003). For example, *lys-6* miRNA is expressed in left hemisphere neurons, and *miR-273* is expressed in right hemisphere neurons.

Many microRNAs are conserved from worms to mammals. One of the best characterized is *miR-1*, a microRNA involved in formation and maintenance of muscles. It is highly expressed in the muscles of larva and *Drosophila* flies (Aboobaker et al., 2005; Sokol and Ambros, 2005) and in the muscles and heart of mice (Zhao et al., 2005). Although *miR-1* genetic knockout is not available, over-expression of *miR-1* results in developmental arrest, thin-walled ventricles, and heart failure due to myocyte premature differentiation and proliferation defects.

Other miRNAs appear to be involved in energy homeostasis, in particular *Drosophila* flies with mutated *miR-14* display peculiar phenotype: they are obese and have elevated levels of triacylglycerol. Thus, *miR-14* seems to play a role in fat metabolism. In a similar gain-of-function screening for genes affecting tissue growth, the *miR-278* locus was identified (Teleman et al., 2006): *miR-278* mutants are lean and have elevated insulin production.

1.4.4 miRNAs and cancer

The important role of various miRNAs in the determination of various physiological patterns can suggest that deregulations of this control system can lead to different diseases and tumour formation and progression (Table 1.1). Specific changes in miRNA expression were found in tumour tissues with respect to normal tissues (Liu et al., 2004; Lu et al., 2005; Volinia et al., 2006). The hypothesis that miRNA loss leads to a de-differentiation process and less-differentiated tumours was supported by evidence that miRNA induction is closely related to normal cellular differentiation (Lu et al., 2005). Since the prognostic power of miRNA profiles is astonishing, their importance in developing and maintaining cellular fate is easily drawn. Specific roles of miRNAs in certain cancer are established (Table 1.1) (Schickel et al., 2008) and many miRNA targets are emerging, explaining and predicting some activities of miRNAs in cancer. The microRNA involved in cancer progression are called oncomiRs (Figure 1.6) (Esquela-Kerscher and Slack, 2006). Oncogenic miRNAs include *miR-155* and

miR-17-5p. The miRNAs with tumour-suppressing activity include *miR-15*, *miR-16* and *let-7* (Table 1.1) (Schickel et al., 2008).

The first evidence for a direct link between miRNAs and human cancer was provided by the observations that two microRNA genes, *miR-15* and *miR-16* located in a 30Kb region on chromosome 13, were found deleted or their expression reduced in chronic lymphocytic leukemia (CLL) cases (Calin et al., 2002). Another study found that *miR-143* and *miR-145* expression levels were reduced with the more advanced stages of colorectal neoplasia (Michael et al., 2003). Both studies were focused on a small number of miRNA and based on miRNA cloning and northern blotting approaches. The subsequent development of miRNA microarray technologies increased the number of miRNA expression studies in human cancer. Crucially, q-PCR for mature miRNA also became available for the analysis of small tissue samples and microarray miRNA validation (Lu et al., 2005). A current map of all miRNA loci involved in at least two different human cancers through their expression profiling lists 56 loci (Figure 1.7) (Blenkiron and Miska, 2007).

In contrast to *miR-15/miR-16* expression, whose expression is often reduced in cancers, the *miR-17-92* cluster (Figure 1.4) is over-expressed in many cancer types (Hayashita et al., 2005; He et al., 2005b; Ota et al., 2004). Recently it was found that the inhibition of *miR-17-5p* and *miR-20a*, two miRNAs of this cluster, address lung cancer cells towards apoptosis (Matsubara et al., 2007). *miR-20a* negatively regulates E2F1 (O'Donnell et al., 2005) and E2F3 (Sylvestre et al., 2007), whereas all the three E2Fs regulate the expression of *miR-17-92* cluster binding its promoter, in an auto regulatory feedback loop. The E2F transcriptional networks appear to link cell cycle progression to apoptosis. *miR-20a* over-expression protects cancer cell lines from apoptosis, suggesting that oncogenesis promotion by over-expression of *miR 17-92* cluster could be derived by perturbing the E2F network in a way that induces proliferation and inhibits apoptosis (Sylvestre et al., 2007). Therefore, perturbation of expression levels can induce either proliferation or apoptosis depending on the cellular context.

Table 1.1 *miRNAs with relevance in cancer and cell death (Schickel et al., 2008)*

miRNA	*Normal function*	*Cancer relevance*	*Cell death relevance*	*Targets*	*References*
Let-7/miR-98	Upregulated late during *Caenorhabditis elegans* development; increased expression in differentiated cells; may act to maintain differentiated states	Downregulated in lung colon and ovarian cancer; expression inversely correlates to patient survival in ovarian cancer, NSCLC and adenocarcinoma; possible growth suppressor in colon carcinoma (let-7a-1)	Contributes to IL-6-directed survival signaling and reduces chemotherapy-mediated cell death in human malignant cholangiocytes (let-7a)	HMGA2, RAS, IGF2BP1, c-Myc, NF2 and LIN28B[a]	Akao *et al.* (2006); Boyerinas *et al.* (2008); Gramantieri *et al.* (2007); Guo *et al.* (2006); Hebert *et al.* (2007); Iorio *et al.* (2005); Johnson *et al.* (2005); Lee *et al.* (2006a); Lee and Dutta (2007); Lu *et al.* (2007a); Mayr *et al.* (2007); Meng *et al.* (2007b); Park *et al.* (2007); Pasquinelli *et al.* (2000); Reinhart *et al.* (2000); Sampson *et al.* (2007); Shell *et al.* (2007); Yanaihara *et al.* (2006)
miR-1	Involved in cardiogenic and myogenic differentiation; involved in maintaining proper cardiac depolarization		Pro-apoptotic effect in response to oxidative stress	HSP60, HSP70, KCNJ2 and GJA1	Chen *et al.* (2006); Xu *et al.* (2007); Yang *et al.* (2007)
miR-9	Upregulated in primary brain tissue upon introduction of reactive oxygen species production	Hypermethylated in breast cancer		Granuphilin (Slp4)	Bredenkamp *et al.* (2007); Lehmann *et al.* (2008); Lukiw and Pogue (2007); Plaisance *et al.* (2006)
miR-10a	Possible inhibitor of megakaryocytic differentiation			HOXA1	Garzon *et al.* (2006)
miR-10b		Inversely correlates with prognosis and initiates invasion and metastasis in breast cancer cell lines		HOXD10	Ma *et al.* (2007)
miR-15a	Regulation of pancreatic regeneration	Frequently deleted or downregulated in CLL patients	Pro-apoptotic effects mediated through targeting BCL2	BCL2 and NGN3[a]	Calin *et al.* (2005); Cimmino *et al.* (2005); Joglekar *et al.* (2007)
miR-16	Involved in the regulation of pancreatic regeneration; negatively regulates cell cycle progression and cell growth	Frequently deleted or downregulated in CLL patients	Pro-apoptotic effects mediated through BCL2	BCL2 and NGN3[a]	Calin *et al.* (2005); Cimmino *et al.* (2005); Joglekar *et al.* (2007); Linsley *et al.* (2007)

Table 1.1 *Continued (Schickel et al., 2008)*

miRNA	Normal function	Cancer relevance	Cell death relevance	Targets	References
miR-21		Widely overexpressed in cancer; promotes growth; shown to induce a metastatic phenotype	Shown to inhibit apoptosis	PTEN, TPM1 and PDCD4	Asangani *et al.* (2008); Chan *et al.* (2005); Frankel *et al.* (2007); Loffler *et al.* (2007); Meng *et al.* (2007a); Roldo *et al.* (2006); Si *et al.* (2007); Zhu *et al.* (2007)
miR-29	Suggested to participate in maintaining proper DNA methylation	Reduced levels in B-CLL and NSCLC, enforced expression restores normal methylation in NSCLC and reduces tumorigenicity *in vitro* and *in vivo*	Increases apoptosis sensitivity to TRAIL	TCL1 and Mcl-1	Fabbri *et al.* (2007); Mott *et al.* (2007); Pekarsky *et al.* (2006)
miR-34a	p53 inducible; translationally regulates cell cycle, anti-apoptotic and check point genes; shown to induce growth arrest	Decreased expression in lung and colon cancer, and neuroblastomas; shown to be highly expressed in CLL but not in ALL; gene locus is subject to heterozygous deletions in some tumors	Increases p53-mediated apoptotic sensitivity	CDK4, E2F3 and BCL2[a]	He *et al.* (2007); Raver-Shapira *et al.* (2007); Tazawa *et al.* (2007); Versteeg *et al.* (1995); Welch *et al.* (2007); Zanette *et al.* (2007)
miR-34b/c	p53 inducible; translational regulation of proliferation and adhesion-independent growth	Decreased/loss of expression in p53 null tumors and described to inhibit neoplastic growth; often deleted in NSCLC	Increases apoptotic sensitivity possibly through translational or indirect repression of anti-apoptotic factors	CDK6, c-Met[a] and c-Myc[a]	Bommer *et al.* (2007); Corney *et al.* (2007); Raver-Shapira *et al.* (2007)
miR-122	Hepatocellular-specific miRNA highly expressed in the normal liver and involved in fatty acid and cholesterol metabolism	Commonly downregulated in HCC; loss suggested to facilitate genomic instability due to the loss of translational repression of CCNG1, a p53 pathway antagonist		CCNG1 and CAT-1b[a]	Chang *et al.* (2004); Esau *et al.* (2006); Gramantieri *et al.* (2007); Kutay *et al.* (2006)
miR-126	Expressed in endothelial cells	Downregulated in metastasizing breast cancer		VCAM-1	Harris *et al.* (2008); Tavazoie *et al.* (2008)
miR-130a	Involved in megakaryocytic differentiation; suggested to inhibit differentiation by targeting transcription factors that mediate megakaryocyte differentiation in an ERK-dependent manner	Inhibits pro-angiogenic factors, suppressing angiogenesis in vascular endothelial cells		GAX, HOXA5 and MAFB	Chen and Gorski (2008); Garzon *et al.* (2006); Sevinsky *et al.* (2004)

Table 1.1 *Continued (Schickel et al., 2008)*

miRNA	Normal function	Cancer relevance	Cell death relevance	Targets	References
miR-133	Promotes myoblast proliferation and inhibits cardiac hypertrophy		Inhibits intrinsically activated apoptosis mediated through caspase-9	SRF and caspase-9	Care *et al.* (2007); Chen *et al.* (2006); Xu *et al.* (2007)
miR-142	Highly expressed in hematopoietic tissues and fetal liver	Locus is a common translocation site in aggressive B-cell leukemia generating a miR-142-myc fusion transcript, producing high levels of pre-miR-142			Chen *et al.* (2004); Gauwerky *et al.* (1989)
miR-143	Required for adipocyte differentiation	Downregulated in B-cell leukemias and colorectal neoplasia; reduced expression led to an increase in miR-21 expression; strongly downregulated in multiple tumor types			Akao *et al.* (2007); Esau *et al.* (2004); Lui *et al.* (2007); Michael *et al.* (2003); Yanaihara *et al.* (2006)
miR-146	NF-κB inducible, attenuates the TLR4 signaling pathway	Upregulated in papillary thyroid carcinoma		IRAK1 and TRAF6	He *et al.* (2005a); Taganov *et al.* (2006)
miR-150	Controls B-cell differentiation *in vivo*; highly expressed in mature B and T cells			c-Myb	Xiao *et al.* (2007); Zhou *et al.* (2007)
miR-155	Regulates cytokine expression, optimizing germinal center T-cell response; JNK-induced expression during the macrophage response and in activated B cells; involved in maintaining erythroblasts	Overexpressed in multiple tumor types, particularly in leukemia; suspected oncogenic effect due to translational repression of a known tumor suppressor	Inhibits TP53INP1-mediated apoptosis	AGTR1 and TP53INP1	Eis *et al.* (2005); Georgantas *et al.* (2007); Gironella *et al.* (2007); Lawrie *et al.* (2007); Masaki *et al.* (2007); Metzler *et al.* (2004); O'Connell *et al.* (2007); Ramkissoon *et al.* (2006); Sethupathy *et al.* (2007); Thai *et al.* (2007)
miR-181a	B-cell linage; increases T-cell sensitivity to antigens and exhibits increased expression in mature T cells	Induces polyclonal premalignant expansion; loss of expression noted in transition to aggressive B-cell chronic lymphoma		TCL1, HOXA11, SHP-2, DUSP5, DUSP6 and PTPN-22	Chen *et al.* (2004); Ciafre *et al.* (2005); Costinean *et al.* (2006); Li *et al.* (2007); Marton *et al.* (2008); Naguibneva *et al.* (2006); Pekarsky *et al.* (2006)
miR-195	Stress-responsive miRNA involved in cardiac tissue growth and hypertrophy; mediates pancreatic postoperative regeneration	Highly expressed in CLL		NGN3[a]	Joglekar *et al.* (2007); van Rooij *et al.* (2006); Zanette *et al.* (2007)

Table 1.1 *Continued (Schickel et al., 2008)*

miRNA	Normal function	Cancer relevance	Cell death relevance	Targets	References
miR-196	Involved in developmental patterning and myeloid differentiation			HOXB8	Hornstein *et al.* (2005); Kawasaki and Taira (2004); Yekta *et al.* (2004)
miR-221/222	Maintains progenitor cells in erythropoiesis; suggested to promote proliferation	Inhibits cell growth in erythroleukemic cell lines; upregulated in glioblastoma, papillary thyroid carcinoma, prostate and pancreatic tumors		c-Kit and p27(Kip1)	Ciafre *et al.* (2005); Felli *et al.* (2005); Galardi *et al.* (2007); Gillies and Lorimer (2007); He *et al.* (2005a); le Sage *et al.* (2007); Lee *et al.* (2007)
miR-223	Regulator of myelopoiesis; suggested involvement in chorioamniotic membrane development	Epigenetically silenced by AML1/ETO, an oncogenic protein; increased expression in bladder cancer			Fukao *et al.* (2007); Garzon *et al.* (2006); Gottardo *et al.* (2007); Ramkissoon *et al.* (2006)
miR-335		Downregulated in metastasizing breast cancer; inhibits metastatic cell invasion	Pro-apoptotic factor antagonizing miR-21	SOX4 and Tenascin C Jagged-1[a]	Sathyan *et al.* (2007); Tavazoie *et al.* (2008)
miR-372/373		Inhibits p53 directed CDK signaling, promoting tumorigenesis in testicular germ cells		LATS2	Voorhoeve *et al.* (2007)
miR-106-363 cluster					
106a 19b-2 92-2 20b 363	Key regulators of monocytopoiesis; expression induced under c-Myc and N-Myc regulation	Overexpressed in ~50% of T-cell leukemias; high expression levels noted in multiple solid tumors; downregulated in CML; overexpression enhances blast proliferation	Implicated in inducing apoptosis	AML1, M-CSFR, Rb, Mylip, Rbp1-like and HIPK3[a]	Fontana *et al.* (2007); Hossain *et al.* (2006); Landais *et al.* (2007); Matsubara *et al.* (2007); Sylvestre *et al.* (2007); Volinia *et al.* (2006)
miR-17-92 cluster					
17-5p 18 19a 20a 92	Shown to inhibit differentiation of lung tissue; c-Myc inducible	Limits tumor growth in breast cancer but promotes proliferation in lung epithelial progenitor cells; increased expression in B-cell lymphoma and facilitated cancer progression in a lymphoma murine model	Inhibition of miRNA cluster leads to an increase in apoptosis	AIB1, AML1, M-CSFR and Rb2	Dews *et al.* (2006); Hayashita *et al.* (2005); He *et al.* (2005b); Landais *et al.* (2007); Lu *et al.* (2007b); Matsubara *et al.* (2007); O'Donnell *et al.* (2005); Venturini *et al.* (2007); Zanette *et al.* (2007)

Abbreviations: ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia; ERK, extracellular signal-regulated kinase; HCC, hepatocellular carcinoma; IL, interleukin; JNK, Jun N-terminal kinase; miRNA, micro RNA; NSCLC, non-small-cell lung cancer.

[a] Predicted target.

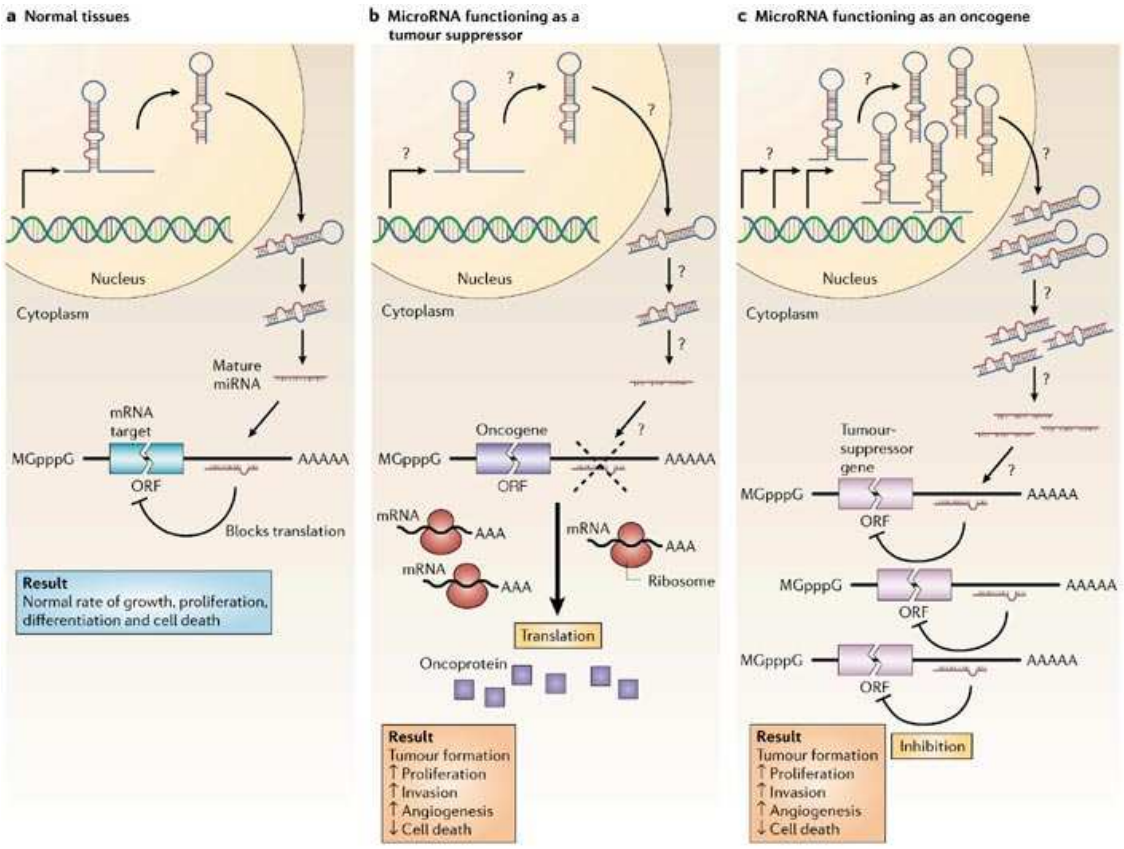

Figure 1.6 ***MicroRNAs can function either as tumour suppressors or oncogenes***

a. *In normal tissues, microRNA bound to complementary sequences on the target mRNA results in the repression of target-gene expression through a block in protein translation or altered mRNA stability (not shown). The overall result is normal rates of cell growth, proliferation, differentiation and death.* ***b.*** *The expression reduction or deletion of a miRNA that functions as a tumour suppressor leads to tumour formation. A reduction in or elimination of mature miRNA levels can occur because of defects at any stage of miRNA biogenesis (indicated by question marks) and ultimately leads to the inappropriate expression of the miRNA-target oncoprotein (purple squares). The overall outcome might involve increased proliferation, invasiveness or angiogenesis, decreased levels of apoptosis, or undifferentiated or de-differentiated tissue, ultimately leading to tumour formation.* ***c.*** *The amplification or over-expression of a miRNA that has an oncogenic role would also result in tumour formation. In this situation, increased amounts of the miRNA, which might be produced at inappropriate times or in the wrong tissues, would eliminate the expression of a miRNA-target tumour-suppressor gene (pink) and lead to cancer progression. Increased levels of mature miRNA might occur because of amplification of the miRNA gene, a constitutively active promoter, increased efficiency in miRNA processing or increased stability of the miRNA (indicated by question marks). ORF, open reading frame. (Esquela-Kerscher and Slack, 2006).*

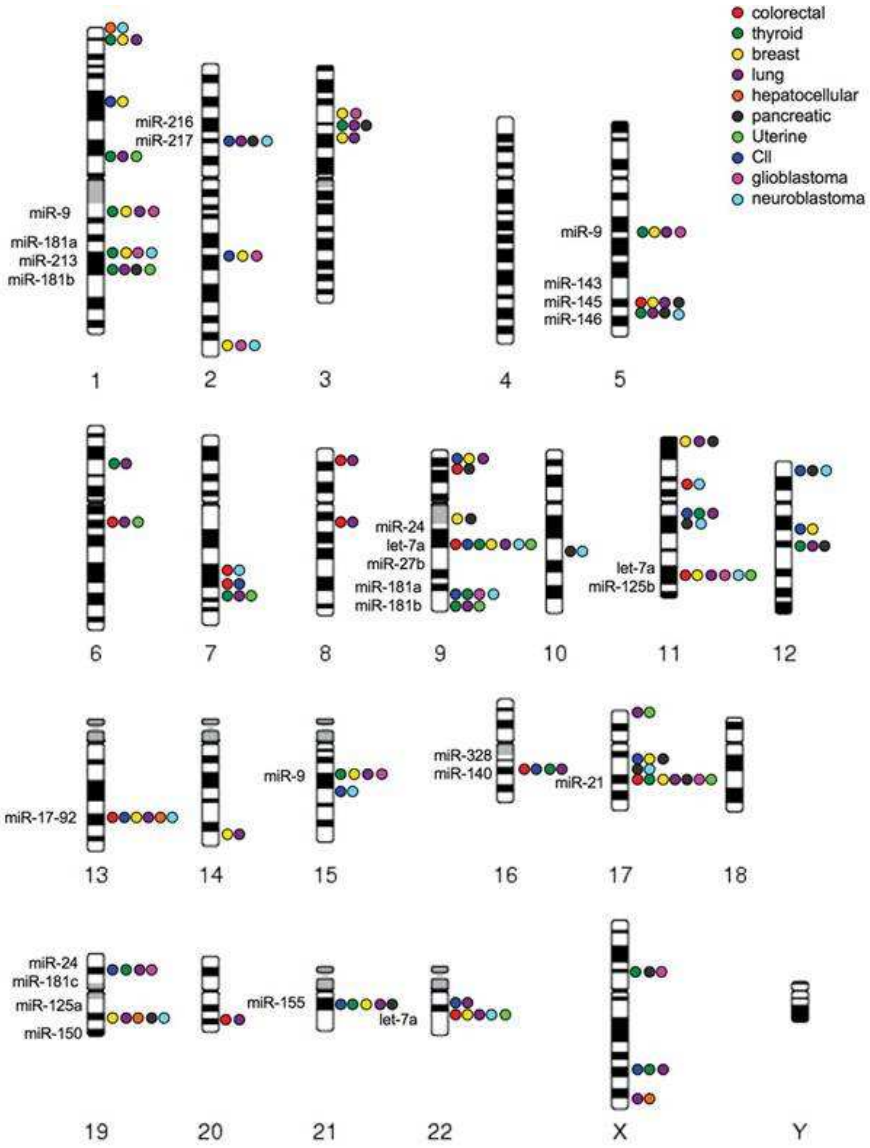

Figure 1.7 *A miRNA cancer map*

Chromosome position of miRNAs implicated in human cancer are shown as coloured dots. Each dot represents a single miRNA or a miRNA cluster. Colour refer to tumour tissue type, as indicated. Only miRNAs, whose expression levels were found to be significantly altered in tumours versus normal tissue in at least two tissue types are shown. MiRNA identified in at least four different tumour types are also indicated by their name. Data were collated from the studies of primary human tumours of the colorectum, thyroid, breast, lung, liver, pancreas, uterus and chronic lymphocytic leukemia, glioblastoma and neuroblastoma (Blenkiron and Miska, 2007).

A key regulator in the activation of the intrinsic apoptosis pathway in response to DNA damage, cellular stress and/or improper mitotic stimulation is the p53 protein. Several studies (Bommer et al., 2007; Chang et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007) identified *miR-34* as a target for p53. He et al. (2007) described that *miR-34* expression correlates with p53 expression. Using a p53-inducible system, they reported that the *miR-34* family of miRNAs is directly regulated by p53, and that *miR-34* mediates growth arrest in multiple cell lines via direct 3'-UTR regulation of cell cycle regulatory factors, such as cyclin-E2 (CCNE2), cyclin-dependent kinase 4(CDK4) and the hepatocyte growth factor receptor (c-Met). Furthermore, *miR-34* was shown to directly target E2F3. In addition, *miR-34* resulted in an increase in caspase-dependent death when introduced into two cell lines (Welch et al., 2007) and contributed to an increase in p53-mediated apoptosis (Chang et al., 2007; Raver-Shapira et al., 2007). *miR-34* was therefore described as a general sensitizer to apoptosis mainly through its link to p53, although its targets are still speculative. On the other hand, TP53INP1 was supposed to be direct target of *miR-155*: TP53INP1 is dramatically reduced in its expression in pancreatic ductal adenocarcinoma (Gironella et al., 2007) whereas *miR-155* is over-expressed in pancreatic cancer (Lee et al., 2007). Other miRNAs functionally connected to p53 are *miR-372* and *miR-373*, which were identified as oncogenes in testicular germ cell tumours, target the tumour suppressor LATS2 and neutralize p53-mediated CDK inhibition (Voorhoeve et al., 2006).

However the number of microRNA implicated in human cancer by expression profiling will still increase substantially for two reasons: first, the recent availability of commercial microRNA profiling platforms will wide the access to these tools and, second, the number of known human microRNA is still increasing. Some general themes are emerging from the expression profiles published up today. For example, a study of 334 human primary tumours and tissue interrogating the expression of 217 miRNAs pointed out that many microRNAs are down-regulated in primary tumours when compared with normal tissues and that microRNA expression profiles provide lineage-specific information and may classify even poorly differentiated tumours (Lu et al., 2005). The notion that a set of microRNA may be deregulated in many tumour types is also supported by the finding that a large number of microRNAs can be implicated, with different functions, in distinct tumour types. For example, *miR-21* may act as a tumour suppressor regulating PTEN in cholangiocytes (Meng et al., 2006) or an oncogene regulating BCL2 in breast cancer line MCF-7 (Si et al., 2007) depending

on cellular context. In the same way, *let-7*, a marker for differentiated tissues, could have a set of targets that have tumour-suppressing functions (Brueckner et al., 2007) whereas in other contexts (in certain tissues under certain conditions) it may not (Meng et al., 2007). It is down regulated in a number of human cancers such as lung, colon, or ovarian cancer, and is useful as a prognostic marker for disease outcome (Akao et al., 2007; Johnson et al., 2005; Shell et al., 2007; Takamizawa et al., 2004; Yanaihara et al., 2006). However, it cannot be viewed as a classical tumour suppressor gene because it consists of 12 individual genes transcribed from 8 chromosomal loci (Park et al., 2007) and, although all 12 miRNAs are predicted to have a similar set of targets, it is not clear whether all the twelve have the same function. Another example of miRNA with no clearly defined activity with respect to tumorigenesis is *miR-24*, which, depending on the cell type, can either promote or inhibit cell growth (Cheng et al., 2005). As such, a miRNA can only be considered a tumour suppressor in the particular tissue where its expression inhibits the expression of genes considered to have oncogenic properties. It is therefore most appropriate to refer to miRNAs that are involved in cancer development in more general term as oncomiRs.

microRNAs have also been found directly linked to metastasis formation. *miR-10b* was found to have elevated expression in metastatic breast cancer (Ma et al., 2007). Exogenous *miR-10b* increased invasion of established breast cancer cells together with increased proliferation both *in vitro* and *in vivo*, whereas *miR-10b* inhibition led to a 10-fold reduction of invasion (Ma et al., 2007). Additional miRNAs such as *miR-335* and *miR-126* were identified *in vivo* as key metastasis suppressors (Tavazoie et al., 2008). Recently, the *miR-200* family (which includes *miR-200a*, *b*, *c*, *miR-141* and *miR-429*) was identified as a powerful master regulator of epithelial to mesenchymal transition (EMT), which is viewed as an essential early step in tumour metastasis (Gregory et al., 2008; Park et al., 2008).

1.4.5 MicroRNAs as human cancer markers

In many studies (Landgraf et al., 2007) the microRNAs are described as potentially useful for the molecular diagnosis of tumours.

The expression of many miRNAs is highly specific for tissue and cell type, and this specificity is often retained in the corresponding tumour tissues. Identification of cell origin by miRNA profiling is more efficient compared with global analysis of mRNAs, because miRNAs are not confounded by such a large pool of irrelevant genes and are represented by a relatively small number of species. Therefore, miRNAs could

facilitate the accurate diagnosis of tumours hard to be classified with respect to the tissue origin by conventional means, for example metastatic lesion of unknown primary origin (Jeffrey, 2008), a highly aggressive malignancy that poses diagnostic and management difficulties (Varadhachary et al., 2004). Deregulation of miRNAs occurs frequently during tumorigenesis (Zhang et al., 2007), making them attractive candidates for molecular detection of malignancy. A subset of miRNAs can often be found up or down-regulated in tumours compared with the normal tissues. The differential expression of miRNAs between tumour and normal tissue may be exploited in the diagnosis on samples with scant or poorly preserved cells, where the malignancy diagnosis through traditional methods could be difficult (Mitchell et al., 2008). Moreover, in the non-invasive screening for cancer, alterations in specific miRNAs can be similarly detected in uninvolved body fluids such as peripheral blood (Mitchell et al., 2008).

miRNAs may also be useful in sub classifying tumour of a particular tissue origin (Rosenfeld et al., 2008). Expression of specific miRNAs has been shown to correlate with histological subtypes of certain types of cancer, for example, ductal and lobular breast carcinoma (Iorio et al., 2005), mucinous and non-mucinous carcinoma of the lung (Garfield, 2008; Yanaihara et al., 2006), and papillary, follicular and anaplastic thyroid carcinomas (He et al., 2005a; Visone et al., 2007; Weber et al., 2006). Indeed, miRNAs play a role in the distinct pathogenetic pathways leading to the different histological subtypes, and may be useful as a diagnostic tool in unusual difficult cases.

There is increasing evidence that miRNAs can be invaluable as a biomarker for patient prognosis. For example, a set of differentially expressed miRNAs was identified in chronic lymphocytic leukemia that can separate it into prognostic categories (Calin et al., 2005). Reduced expression of *let-7* and high expression of *miR-155* were associated with poor survival in human lung cancer (Takamizawa et al., 2004). High levels of *miR-21* are associated with poor survival and poor therapeutic outcome in colon cancer (Schetter et al., 2008). In addition, several miRNAs, including *miR-10b*, *miR-126*, *miR-335*, *miR-373* and *miR-520c* were recently found to promote or suppress invasion and metastasis in breast cancer cells (Huang et al., 2008; Ma et al., 2007; Tavazoie et al., 2008). Some of these miRNAs were also shown to correlate with clinical outcome and disease progression in breast cancer. Thus, levels of these miRNAs in primary breast tumours could be useful as predictive biomarkers of their metastatic potential (Foekens et al., 2008).

1.5 Aim of the present work

The aim of this study was to evaluate the potential of microRNAs as biomarkers to add to the already identified genes responsible of sarcoma tumours and add a new wedge in the understanding the genes or molecules influencing the process of extravasation of sarcoma cells.

The research was conducted by multidisciplinary approaches described in the following objectives:

- to verify miRNA as possible tumour markers in patients affected by osteosarcomas of different grades;
- to determine the specific intrinsic capability of some sarcoma cell types to *in vitro* transmigrate;
- to generate microRNA libraries in osteosarcoma cell lines with different migration capability;
- to better describe some microRNAs both for their expression and functional role inside cell;
- to devise an intravital microscopy system useful to study cells' capability to spontaneously invade the lymphatic vessels of the rat pleural cavity.

MATERIALS AND METHODS

2.1 Cell lines

Different human cell lines were cultured in humidified atmosphere (5% CO_2) using D-MEM medium 10%FBS. In particular, MG-63 (ATCC n° CRL-8303), 143B (ATCC n° CRL-1427) and Sa-Os-2 (ATCC n° HTB-85) ost eosarcoma cell lines, A-204 (ATCC n° HTB-8) rhabdomyosarcoma cell line, SW982 (ATCC n° HTB-93) synovial sarcoma cell line, SK-Ut-1 (ATCC n° HTB-114) sarcom a cell line, SK-LMS-1 (ATCC n° HTB-88) leiomyosarcoma cell line, HT1080 (ATCC n° C CL-121) fibrosarcoma cell line, He-La (ATCC n° CCL-2) cervix adenocarcinoma cell li ne were tested. Mesenchymal cells (ATCC n° CRL-1486) and osteoblast cells (ATCC n° CRL-11372) were used as reference lines.

The murine tumour cell lines, UMR-106 (ATCC n° CRL- 1661) rat osteosarcoma cell line, C6 (ATCC n° CCL-107) rat gliosarcoma cel l line, and B-16 (ATCC n° CRL-6322) mouse melanoma cell line- wild type and containing the over-expression of NG2 were cultured in humidified atmosphere (5% CO_2) using D-MEM medium 10%FBS.

2.2 FATIMA system

For motility assay, Human Umbilical Vein Endothelial Cells (HUVEC) coming from umbilical cords kindly given from Arcispedale Santa Maria Nuova of Reggio Emilia (Italy), were used.

The HUVEC were extracted (Figure 2.1), after 30 minute of incubation with 0.25% collagenase A (Roche Diagnostic GmbH – Mannheim, Germany). These haematic endothelial cells were seeded on a gelatin 1% layer and cultured in humidified atmosphere of 5% CO_2 using M199 Endothelial medium (Cambrex - Rockland, USA) and 20% FBS (Figure 2.1).

According to Fatima (Spessotto et al., 2002; Spessotto et al., 2000), a day before the experiment, $5x10^4$ HUVEC cells were seeded on a transwell insert (Figure 2.2) provided with a permeable membrane (8µm pore size). These insert (HTS Fluoro BLOK tm Insert, Falcon) were previously covered with 1% gelatine. 24 hours later the different sarcoma cells tested were collected and stained with 10µg/ml of red dye Fast Dil (Molecular Probe – D-3899) in DMEM with 10% FBS for 5 min at 37°C. Then $15x10^4$ cells were seeded in 100µl of serum free medium. The kinetics of transmigration of the different sarcoma cells was determined using Tecan Spectra Fluor Instrument, a fluorescent microplate reader, 1.5 ,4.5, 6, 20.5, 26 and 45 hours later (Figure 2.2).

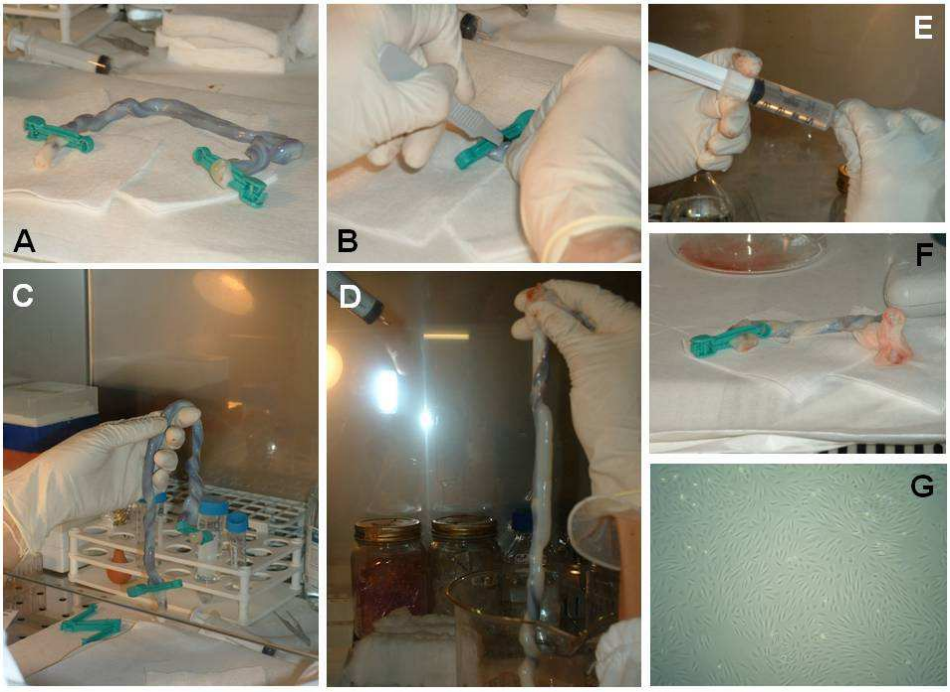

Figure 2.1 *HUVEC extraction*

*The umbilical vein endothelial cells were conserved at 4℃ using two clamps at the end (**A**). To collect the HUVEC cells, the umbilical cord was washed at least three times with 0.9% NaCl solution (**B,C,D**). and it was filled with collagenase solution (**E**). The time of collagenase reaction was almost 30 minutes (**F**) and soon afterwards the cells were collected and seeded on gelatin substrate (**G**).*

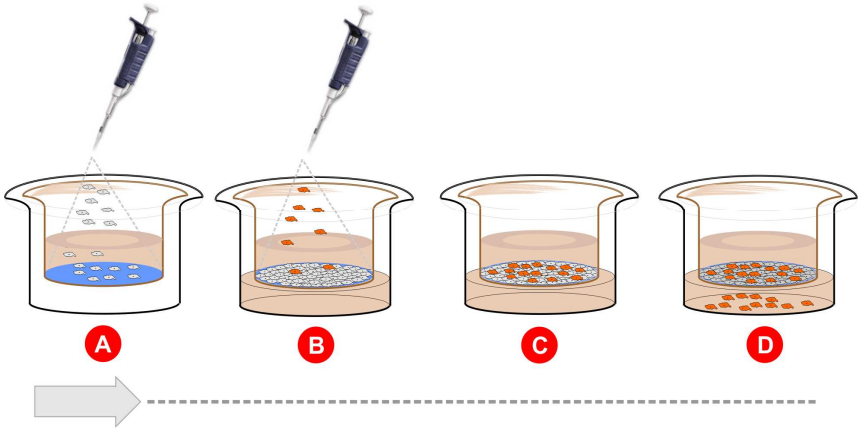

Figure 2.2 *FATIMA assay*

***A.** Endothelial haematic cells (HUVEC) were seeded on gelatin substrate on 0.8µm pore membrane (blue). **B.C.** Tumoral cells (orange,) previously colored with Fast Dil, were seeded on endothelial monolayer. **D.** The kinetics of migration was evaluated using a microplate reader, six reading in 2 day of experiment.*

2.3 RNAs extraction and quantification

Total RNAs were extracted from cells with a confluent density of 75% using Trizol® total RNA isolation reagent (Gibco BRL, Life Technologies, Gaitherburg, MD, USA), according to the manufacturer's instruction. The concentration and the purity of the samples were measured reading absorbance at 260 and 280 nm using BioPhotometr (Eppendorf), while integrity was determined using an electrophoretic run on 1% agarose gel.

2.4 Small-RNA library construction

20-100 µg of MG-63 and 143B osteosarcoma cell lines RNAs were run on an 8% denaturing polyacrylamide gel (Figure 2.3). The areas between 15 and 30 bp were cut and purified with phenol/chloroform standard procedure (Sambrook and Russell, 2001) and precipitated with 10 µg of glycogen. This small-RNAs fraction was dephosphorylated using Calf Intestine Phosphatase (CIP-New England Biolabs, Ipswich, MA) followed by ligation of a linker (adaptor) to the 3' end of the RNA using T4 RNA ligase (New England Biolabs)[1]. Subsequently a new run on 4% poliacrilamide gel and a new purification with phenol/chloroform standard procedure (Sambrook and Russell, 2001) were performed and then the two samples were phosphorilated using Poly Nucleotide Kinase (PNK) followed by ligation of a linker to the 5' end of the RNA using T4 RNA ligase[2]. First strand cDNA was synthesized using oligo-linker miRclon rev primer and AMV-reverse transcriptase (Finnzymes). The resulting cDNA was then PCR-amplified for 25 cycles using miRclon fwd[3] and miRclon rev[4] primer and Phusion polymerase (Finnzymes) following program A[5] in Gen Amp® PCR System 2700 (Applied Biosystems) (figure 2.3). After the purification on 8% polyacrylamide gel, the fraction of the cDNA in the range of 80–100 bp was reamplified using two times the

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[1] 3' adapter pUUUaaccgcatccttctc

[2] 5' adapter tactaatacgactcactAAA

[3] miRclon rev [5'] GAC TAG CTG GAA TTC AAG GAT GCG GTT AAA [3'] (Elbashir et. al., 2001)

[4] miRclon fwd [5'] CAG CCA ACG GAA TTC ATA CGA CTC ACT AAA [3'] (Elbashir et. al., 2001)

[5] program A: 98℃ 30" (98℃ 10", 43℃ 20", 72℃ 15") x25, 72℃ 7', ∞16℃

same reagents and conditions described above. The products were, then, digested with EcoRI enzyme whose sequence was in primers (underlined nucleotides) to generate 5' overhangs and the concatemerisation was carried out using T4 DNA ligase (New England Biolabs). The purified products, between 300 and 1000 bp, run on low melting temperature agarose gel, were ligated in EcoRI sites of TopoTA cloning vector (Invitrogen). Ligations were electroporated into DH5α electrocompetent cells (Invitrogen) resulting in 10^6 recombinant clones (Figure 2.3).

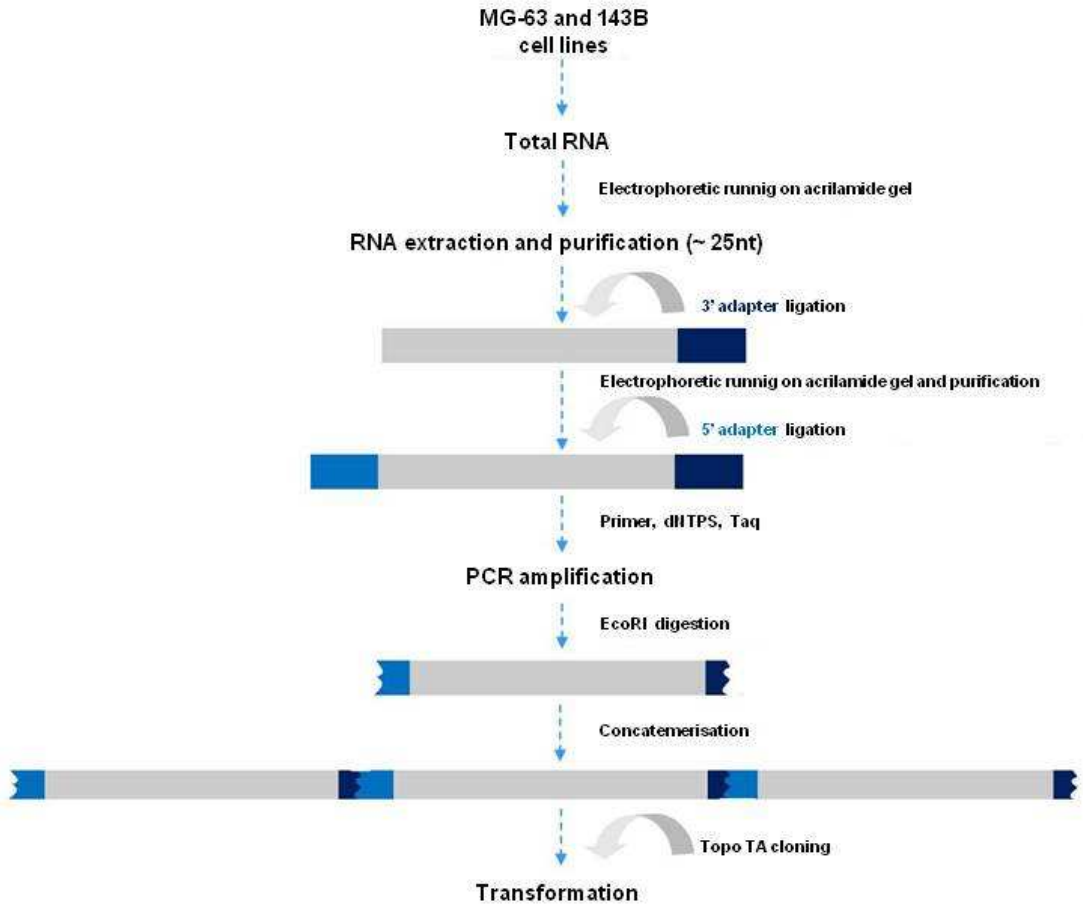

Figure 2.3 ***MicroRNA library preparation***

From total RNAs 25 nucleotides small RNA were extracted after electrophoretic run. Consecutively two adapter (3' and 5') were connected and after EcoRI digestion, the concatemer was bound and cloned in TOPO TA vector. The different plasmid products were insert in DH5α cells.

2.5 Sequencing of small-RNA cDNA libraries

Both libraries were plated on Luria-Bertani (LB) ampicilline and kanamicine plates and selected colonies, after re-growth were picked up and put in 20µl of water. Cells were lysed by heating in PCR machine (Applied Biosystem 7800) which follows the program B[6] using Go Taq Polimerase (Promega) and the primers seq fwd[7] and seq rev[8] in Gen Amp® PCR System 2700 (Applied Biosystems)[9]. The PCR products were run on 1% agarose gel. The longest amplification products were reamplified, purified using Qiaquick Gel extraction Kit (Qiagen GmbH, Hilden-Germany) and 30ng of purified DNAs, resuspended in 16µl of H_2O, were transferred in a new tube with 4µl of DTCS Quick Start Master Mix of Genome Lab Dye Terminator Cycle Sequencing kit (Beckmann) and 2µl of M13 forward primer[10]. Termocycling was performed with program C[11] on 2700 Applied Biosystems machine. Final products were purified with standard procedure using ethanol precipitation and analyzed in Beckman coulter CEQTM 2000 DNA Analysis System.

2.6 Sequence analysis

Base calling and quality timing of sequence chromatogram was done by Chromas Lite Sofware. Every sequence was analysed using megablast software (http://www.ncbi.nlm.nih.gov/BLAST). After masking of the vector and of the adapter sequence and after removing redundancy, inserts of length between 20-23 bases were compared with the small RNAs present in miRNA registry (http://microrna.sanger.ac.uk/sequences) and mapped to human genome using BLAST human genome: http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html. Genomic

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[6] program B: 95℃ 5' (95℃ 30", 61℃ 30", 72℃ 1') x3 0, 72℃ 7', ∞16℃

[7] Seq fwd 5' GTT TTC CCA GTC ACG ACG TTG TA 3'

[8] Seq rev 5' CAC AGG AAA CAG CTA TGA CC 3'

[9] buffer di annealing 10X: 100mM Tris pH 6.9, 400mM KCl, 5mM EDTA)

[10] M13 forward primer 5' TGT AAA ACG ACG GCC AGT 3'

[11] program C: (96℃ 20", 50℃ 20", 60℃ 4') x30, ∞16℃

regions containing inserts with 100nt flanks were retrieved from ENSEMBLE: http://www.ensembl.org/Homo_sapiens/ and sliding window of 100 nt was used to calculate RNA secondary structures by RNAfold (http://www.bioinfo.rpi.edu/applications/mfold/old/DNA/form1.cgi). Only the regions that folded into hairpin and contained an insert in one of the hairpin arms were used in further analysis. Since every non-redundant insert produced independent hits at this stage, hairpins with overlapping genomic coordinates were merged into one region, tracing locations of matching inserts which were used in downstream calculations as a mature sequence. Next, gene and repeated annotations for hairpin genomic regions were retrieved from Ensembl, and repetitive regions as well as ribosomal RNAs, tRNAs and snoRNAs were discarded. To find homologous hairpins in other genomes, mature regions were blasted against different organisms, in particular human, macaca, chimpanzee, ape, mouse, rat, cow, tetraodon, xenopus and fugu genomes. Homologs from different organisms were aligned with the original hairpin by clustalw to produce a final multiple alignment of the hairpin region. For new sequences that produce hairpins, values of thermodynamic were calculated.

2.7 m*iR-93* and *miR-210* expression analysis in tissue and sarcoma cell lines

Different human adult and fetal tissues[12] from Cell Applications Inc. (San Diego, CA) and the tumour cell lines described above were used to analised *miR-93* and *miR-210* expression. Reverse transcription and RT-Real Time PCR were carried out following TaqMan MicroRNA Assay Protocol (Applied Biosystems) and the expression of *miR-93* and *miR-210* was quantified using $2^{-\Delta\Delta CT}$ comparative method (Applied Biosystems, User Bulletin N°2 (P/N 4303859). The data were presented as $\log_{10}$ of relative quantity of target miRNAs, normalized respect U6 as endogenous reference (RNU6B 001093-PE Applied Biosystems, Foster City, CA). As relative calibrators we used cDNAs, from Mesenchimal Stem Cell for the different tumour cell lines and osteoblast cells for osteosarcoma cells. The threshold cycle (Ct) was determined using default threshold setting and transcript quantification was performed in duplicate for each samples.

[12] Bone marrow, Brain Frontal Cortex, Brain Occipital Cortex, Stomach, Spleen, Sketal muscle, Lung, Intestine, Pancreas, Heart, Kidney, Prostate, Liver, Fetal eye and Fetal Brain.

2.8 Drosha and Dicer analysis in sarcoma cell lines

The expressions of Drosha and Dicer were analysed using qRT-PCR TaqMan in some of the cells described above with specific primers[13,14]. The expression was quantified using $2^{-\Delta\Delta CT}$ comparative method (Applied Biosystems).

Briefly, RNA was initially reverse transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems, CA, USA) and then 10ng were amplified using master mix: FluoCycle SYBR (Euroclone) in a 20 μL PCR reaction following the protocol and amplification steps: denaturation at 95℃ for 5 min , followed by 45 cycles of denaturation at 95℃ for 15 sec and then annealing at 56℃ for 30 sec and extension at 72℃ for 30 sec. The melting curve was performed fr om 55℃ to 95℃. All reactions were carried out on the Chromo4 Continuous Fluorescence Detector (MJ Research). Relative quantitation was carried out, versus GAPDH[15] as an endogenous control, using the ΔΔCt method. Transcript quantification was performed in triplicate for each sample.

2.9 Cloning and transduction of the osteosarcoma cells

The gateway system of cloning was used to insert the studied *miR-93* and *miR-7 new* in pCDBGW-*miR* vector (Figure 2.4), kindly provided by Prof. Paolo Malatesta (Istituto Nazionale per la Ricerca sul Cancro, Genova – Italy). In brief, two complementary strands of 60 nt containing *miR-93* or *miR-7 new* were annealed for 4' at 95℃ then ligated over night in a pENTRY vector. After checking the transformation in DH5α subcloning cells and the ligation with PvuII (Promega Corporation, Madison, WI, USA) and BsrgI (New England Biolabs) enzymes, a clonase reaction with LR Clonase (Invitrogen) was performed. The final ligation in pCDBGW-*miR* was tested with MscI restriction enzyme (New England Biolabs).

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[13] Drosha fwd 5' GCT CTG TCC GTA TCG ATC AAC T 3'

Drosha rev 5' AAG TGG ACG ATA ATC GGA AAA GT 3'

[14] Dicer fwd 5' GGC CCC AAT CCT GGA CTT A 3'

Dicer rev 5' AAG CCG CTC CAG GTT AAA TC 3'

[15] GAPDH fwd 5' CTC TCT GCT CCT CCT GTT CGA C 3'

GAPDH rev 5' TGA GCG ATG TGG CTC GGC T 3'

The vector containing the studied miRNA, was transfected in MG-63, together with a vector containing G418 resistence in the ratio of 5:1, using Metafectene reagent (Biontex, Munchen - Germany) and following the manufacturer's protocol.

For 143B insertion, pCDBGW-*miR* retrovirus was prepared using $CaPO_4$ and HEPES solution following standard procedures. Infection of retrovirus was performed in the same cell type after transfection of a plasmid containing ecotropic receptor.

After two days the green colonies were picked up and put in a single well of a 96 multiwell. After few days, the colonies of each green well were picked up again and splited in a new multiwell 96 (one cell every three wells) (Figure 2.5). The real expression of *miR-93* was tested as described in paragraph 2.7.

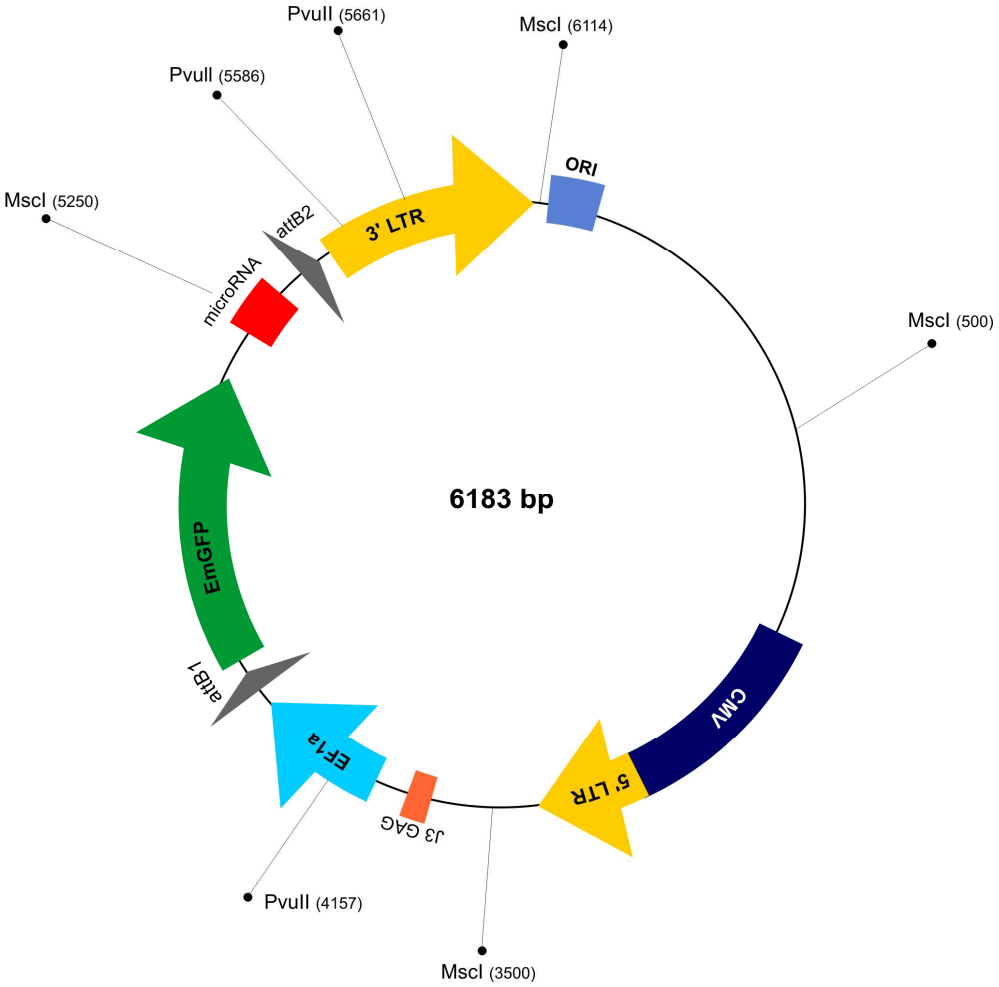

Figure 2.4 *PCDBGW-miR vector*

pCDBGW-miR vector is composed of 6183 bp. It is an Ampicillin resistance vector and it was modified from pCEG (cutting the IRES-GFP sequence and binding the gateway box) in the laboratory of Prof. Malatesta. It contains Cito Megalovirus (CMV) promoter and EmGFP as reported gene. The microRNAs were inserted using the gateway system.

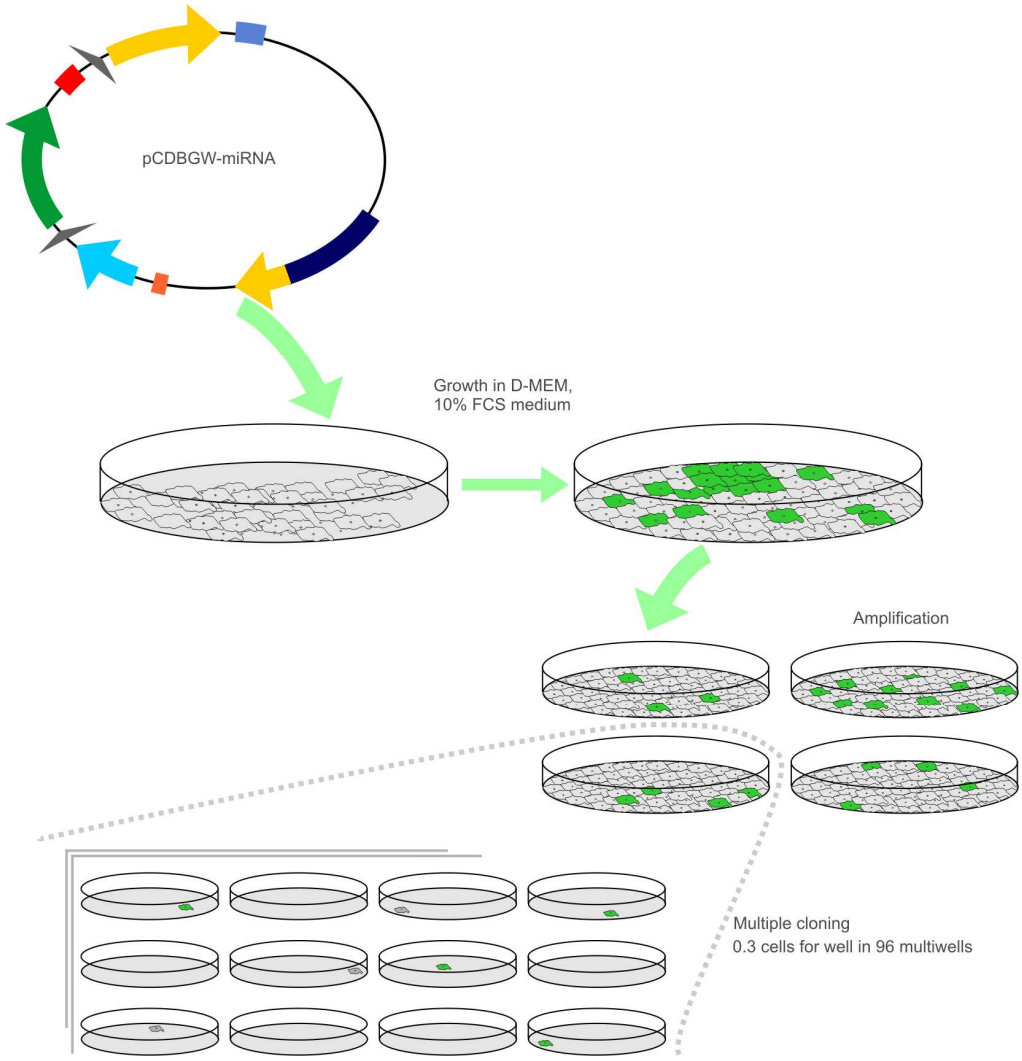

Figure 2.5 *Multiple cloning procedure*

pCDBGW-miR vector was infected or transfected in osteosarcoma cells. The green cells where selected, amplified and seeded in 96 wells plate to create a single clone.

2.10 Proliferation assay

The proliferation assay was performed in a 96-well format using CellTitre 96 One solution Cell proliferation assay (Promega Corporation, Madison, WI, USA). In an individual experiment, proliferation under each condition (0%, 0.1%, 1%, 10% FBS) was studied in triplicate and the overall experiment was repeated at least twice.

2.11 Search of microRNA targets

To predict the probable target genes of studied microRNA the results of PicTar (http://pictar.mdc-berlin.de/), TargetScan (http://www.targetscan.org/) and Miranda (http://microrna.sanger.ac.uk/targets/v5/) bioinformatic softwares were compared. Four possible targets of *miR-93* (ARID4B[16], TXNIP[17] ZNFX1[18], ANK2[19]) were tested[20] by PCR Real-Time, using SyBr Green (Lonza, Milan-Italy).

To detect the functional mRNA targets of *miR-93* a method described by Vatolin (Vatolin et al., 2006) was performed. In brief, a cDNA was synthesized using miRNAs as endogenous primer a mRNA template, purified hybrid cDNA-miRNA molecules were purified using PCR purification kit (Qiagen) and ligated with a 5' adapter using T4 RNA ligase. A pre-amplification was performed with primers on adapter and miRNA; the new synthesized products were then run on 1% agarose gel, purified and sequenced on Applied Biosystem machine using Big Dye Terminator kit (Applied Biosystems), following the manufacturer's protocol.

2.12 Global microRNA expression analysis

miRNA expression levels were examined using the Applied Biosystems TaqMan® Low Density Array (cod. 4384792) consisting of 365 well characterized miRNAs and 2 nucleolar RNAs (snoRNAs) as endogenous controls for data normalization. Single stranded cDNA was generated from total RNA sample by reverse

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[16] ARID4 B fwd: 5' GAG GAG AGG AAT ATA ATA CCA A 3'

ARID4 B rev: 5' CCT GAT CAG AAT GTG TAG GT 3'

[17] TXNIP fwd: 5' GTG TGA AGT TAC TCG TGT CA 3'

TXNIP rev: 5' CTT CCA GAA GAA GCG TGT CT 3'

[18] ZNFX1 fwd: 5' CTC CTA ACC AAC GAG TCT GT 3'

ZNFX1 rev: 5' GCT GGT CTT CTG ACA ATT GT 3'

[19] ANK2 fwd: 5' TGC ATC TTT GGC TGG ACA AG 3'

ANK2 rev: 5' CAG TGC TCT GAT TAG CTC CA 3'

[20] program D: 95℃ 5' (95℃ 30", 56-60℃ gradient 30 ", 72℃ 30") x30, 72℃ 7', ∞16℃

transcription using the Applied Biosystems TaqMan MicroRNA Multi Reverse Transcription Human pool set (cod. 4384791), following manufacturer's protocol. Briefly, RT reactions contained 80 ng of total RNA, 1X RT buffer, 0.20 mM each of dNTPs, 0,01 U Reverse Transcriptase and 0.25 U RNase Inhibitor and 1 µl of specific primer pool. PCR amplification was carried out on the Applied Biosystems 7900 Real-Time PCR system. Arrays were incubated at 50℃ for 2 min then 94.5℃ for 10 min, followed by 40 cycles of 97℃ for 30 s and 59.7℃ f or 1 min. The array was run in duplicate for each case to allow the assessment of technical variability, the relative quantitation was carried out using the ΔΔCT method. Expression of miRNA was normalized versus an endogenous control RNU48 and normal osteoblast cells were used as calibrators.

2.13 DNA microarray analysis

The infected/transfected *miR-93* and *miR-7 new* MG-63 and 143B osteosarcoma cell lines RNAs (extracted using Rnase kit -Qiagen GmbH, Hilden – Germany- according to manufacturing protocols) were analysed on 1% agarose gel and quantified using Nanodrop ND1000 (NanoDrop Technologies, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was employed to confirm the quality of the samples.

For the study of gene expression, an Agilent high density slide containing 4 X 41,000 60-mer oligonucleotide probes was used and the two colours technology was applied

500 µg of each infected/transfected RNA sample were reverse transcripted to cDNA, amplified and then transcripted in cRNA and labelled with Cy5 dye following Agilent's Two-Color Microarray-Based Gene Expression Analysis protocol. Each sample was mixed with the same amount of Cy3-labeled product from a MG-63 or 143B used as RNA reference. Spike-in RNA (Agilent Technologies, Palo Alto, CA, USA) was used as internal control. The post labelling efficiency was quantified both by Nanodrop to check the amplification and the dye incorporation, and by Bioanalyzer to check the cRNA integrity. The two different labelled cDNA were then hybridized together on slides for 16 hour at 37℃.

After washing, slides were scanned with an Agilent's G2565AA Microarray Scanner System. Dye-normalized, background-subtracted log-ratios of sample to reference expression were calculated using Agilent's Feature Extraction Software v9.5. Hybridization quality was checked using the software's quality report.

2.14 Intravital microscopy

UMR-106 and B16 cells ($2X10^6$) were colored with green viable dye (Cell Tracker Green - Molecular Probes) and introduced in pleural cavity of a female rat (Figure 2.6). After 12, 24, 30 and 48 hours the rats were sacrificed, diaphragms were documented using a 3D camera (Figure 2.7) and cryo-prepared for histological analysis.

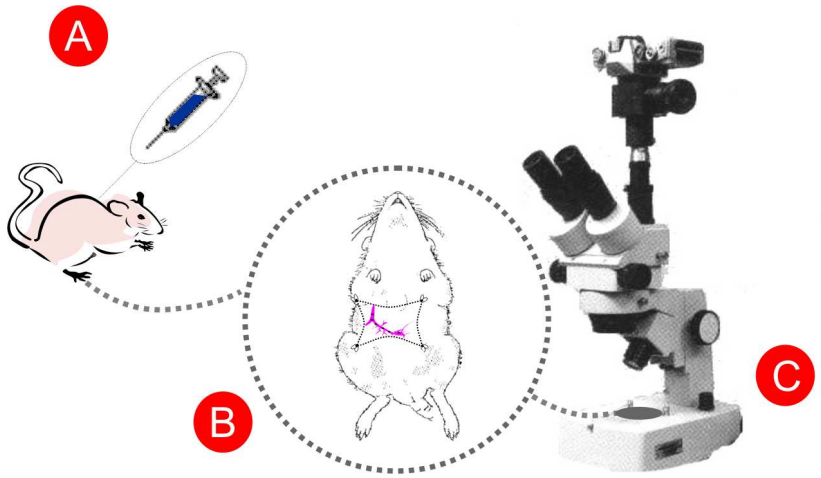


Figura 2.6 ***Intravital microscopy procedure***

$2X10^6$ tumour cells previously coloured with a vital staining were introduced in the pleural cavity of a female rat. The spreading of the tumoral cells were evaluated in the mesenteric duct of diaphragm. This evaluation was performed using stereomicroscopy and 3D camera.

Figura 2.7 ***Analysis using 3D camera***

The diaphragm was analysed and documented using 3D camera. The tumour cells appeared as a white spot.

RESULTS AND DISCUSSION

3.1 microRNA expression in surgical samples of osteosarcoma samples

Preliminary analyses were carried out on three osteosarcoma cell lines to identify microRNAs expressed by these tumour cells. Their over-expression and down-regulation (Table 3.1 and Table 3.2) were detected by using the pre-cast microRNA platform cards.

Table 3.1 *microRNA up-expressed by three osteosarcoma cell lines*

143B		MG-63		U2Os	
microRNA	**Expression**	**microRNA**	**Expression**	**microRNA**	**Expression**
miR-9	10.130	*miR-9*	150.640	*miR-7*	4.150
miR-183	10.180	*miR-24*	3.190	*miR-9*	49.860
miR-196a	2.600	*miR-31*	6.570	*miR-28*	4.590
miR-330	14.120	*miR-151*	2.530	*miR-30d*	2.670
miR-326	3.270	*miR-152*	3.170	*miR-99b*	3.810
miR-342	2.950	*miR-155*	2.770	*miR-126*	5.980
miR-449	4.140	*miR-183*	17.210	*miR-148*	3,780
miR-484	3.250	*miR-196a*	6.170	*miR-151*	3.450
miR-550	2.440	*miR-484*	4.890	*miR-183*	39.950
				miR-194	5.330
				miR-196a	3.200
				miR-200c	12.340
				miR-203	24.000
				miR-330	3.160
				miR-362	4.720
				miR-484	1.960
				miR-550	2.600

Table 3.2 *microRNA down-expressed by three osteosarcoma cell lines*

Tumour Cell Line	microRNA	Expression
143B	*miR-125b*	0.035
	miR-214	0.047
MG-63	*miR-27b*	0.052
	miR-135b	0.041
	miR-200c	0.014
	miR-133b	0.094
U2Os	*miR-146a*	0.082
	miR-155	0.015
	miR-134	0.011

To further examine the microRNAs expressed in patients affected by osteosarcomas of different grades, the four microRNAs found to be over-expressed in the three cell lines (Table 3.3) were analysed in surgical specimens of patients with low and high grade osteosarcomas.

Table 3.3 *microRNA up-regulated in all osteosarcoma cell lines*

Tumour Cell line	*miR-9*	*miR-183*	*miR-196a*	*miR-484*
143B	10.126	34.178	2.602	3.249
MG-63	150.644	17.208	6.169	4.890
U2OS	49.864	39.948	3.204	1.693

We chose to analyse those also considering that three of four microRNAs detected in cell lines have already been described as important in various cellular pathways.

For istance, *miR-9* is expressed at high levels during human pancreatic islet development (Joglekar et al., 2009). It is important as transcriptoma guardian showing a down-regulation during oligodendrocyte differentiation (Lau et al., 2008). Moreover, it promotes cell growth arrest and apoptosis in medulloblastoma (Ferretti et al., 2009). *miR-9* up-regulation in osteoarthritic cartilage and bone tissue (Jones et al., 2008) suggests this microRNA as a mediator in inflammatory function and pathways.

miR-183 has been identified as a potential metastasis-inhibitor in lung cancer regulating the expression of other genes involved in migration and invasion (Wang et al., 2008a). Another study (Loscher et al., 2008) suggests a relationship between *miR-183* down-regulation and retinal degeneration.

miR-196a is reported to decrease human adipose tissue-derived mesenchymal stem cell proliferation and enhance osteogenic differentiation (Kim et al., 2008). In chronic pancreatitis and pancreatic cancer, *miR-196a* permits to discriminate normal from altered pancreas tissues (Szafranska et al., 2007) and its over-expression is considered to play a role in poor survival prediction (Bloomston et al., 2007).

Low grade osteosarcoma - The four microRNAs resulted differently expressed in the surgical samples of low grade osteosarcoma patients (Figure 3.1), but none of these samples seemed to be characteristic of this tumour type. *miR-9* and *miR-183* expression in patients was low, if we compare to the three cell lines previously investigated. Only one of the samples showed a relatively high expression of *miR-196a*, with levels equivalent in the cell lines (Table 3.2). A certain uniformity of *miR-484* expression was found in all the subjects but the lack of a healthy control tissue did not permit to establish if microRNA could be a tumour marker in this osteosarcoma grade (up-or down- regulation?). Further studies on a wider patient cohort have to be performed for a better definition of the significance of the expression pattern of these microRNAs.

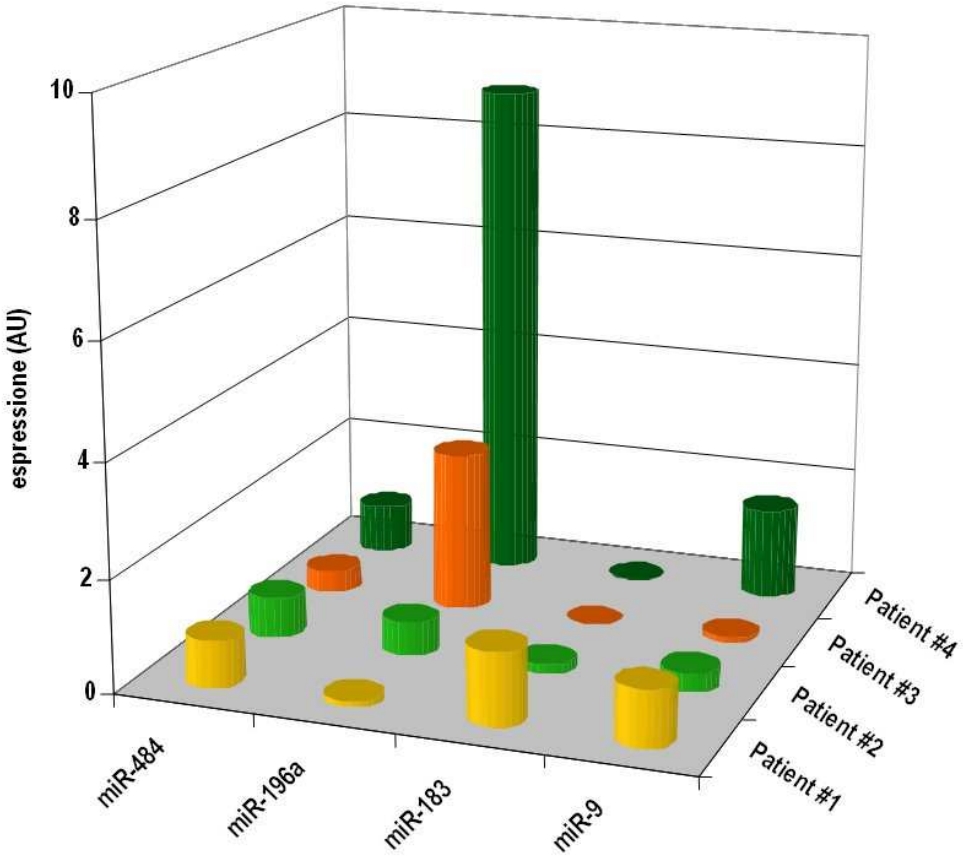


Figure 3.1 ***Analysis of pre-selected microRNA on surgical samples of low osteosarcoma grade***

Expression analysis of miR-9, miR-183, miR-196a and miR-484.

High grade osteosarcoma – A comparison of microRNA expression in tumoral and normal tissues was carried out in surgical samples from patients with high grade osteosarcoma (Figure 3.2). The normal tissue came from the surgical lesion edge of the same patient.

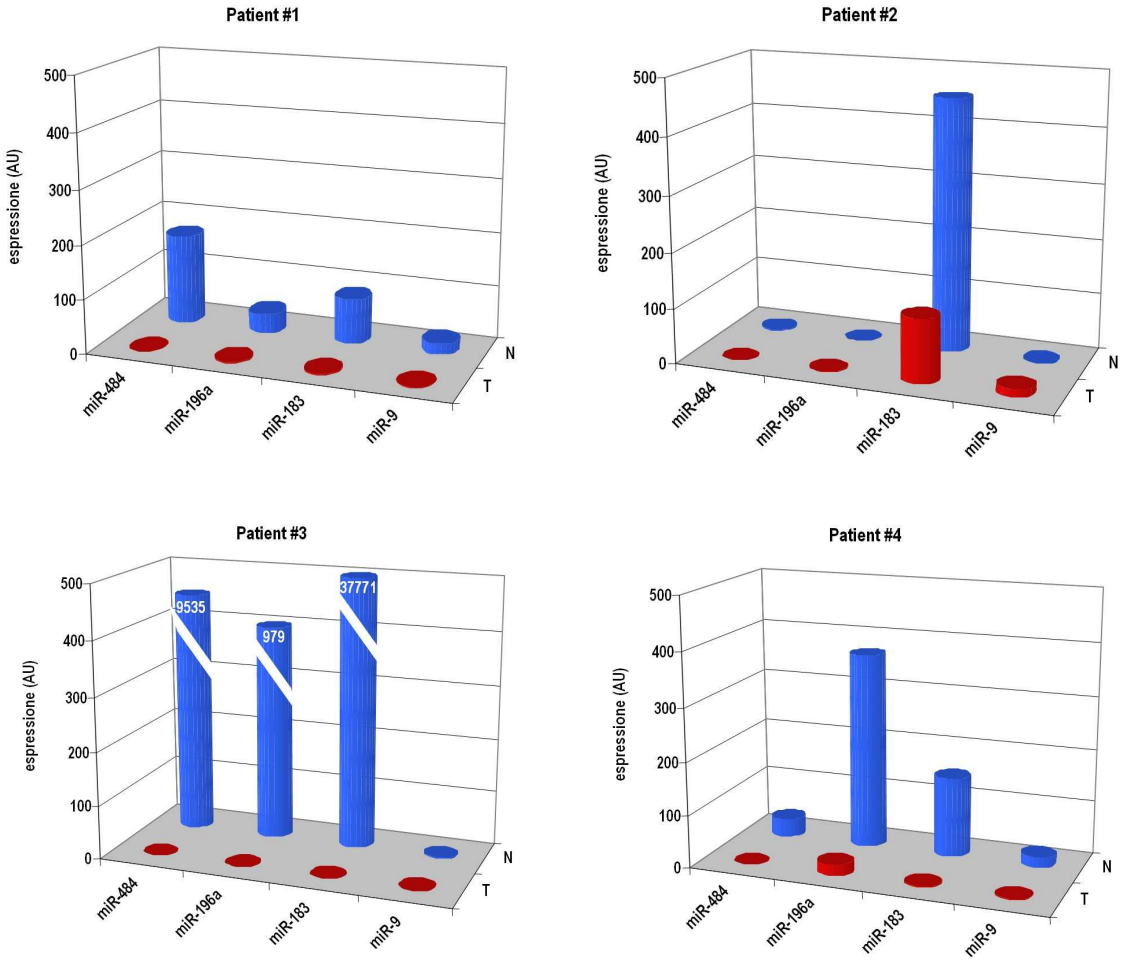


Figure 3.2 ***Pre-selected microRNA on surgical samples of high osteosarcoma grade***

The analysis was carried out on normal (N) and tumour (T) tissue samples of the same patient.

Data analysis pointed out a great heterogeneity of microRNA expression among patient normal tissues (Figure 3.2; Table 3.4). In particular: (i) *miR-9* resulted like osteoblast control expression[21] in one sample, down-regulated in another, and over-expressed, in a similar manner, in two patients; (ii) *miR-196a* was weakly down-

[21] *Osteoblast cells were used as control since classical osteosarcoma originates from the bone made up of mesenchymal cells, which tend to produce bone substance that is to be differentiated in a osteoblastic sense (Campanacci, 1990).*

regulated in one sample and up-regulated in the others, even if with completely different levels; (iii) both *miR-484* and *miR-183* were up-regulated in comparison to primary osteoblasts. When considering the patient with the lowest expression (≈ 3) of *miR-484* in its normal tissue, all samples of both low (Figure 3.1) and high (Figure 3.2) grade osteosarcoma had down-regulated *miR-484*. Also *miR-183* resulted down-regulated in neoplastic lesion with respect to the normal tissue of the lesion edge (Figures 3.1 and 3.2).

Although in all samples of high osteosarcoma grade we observed a microRNA down modulation, only *miR-484* and *miR-183* could be considered as possible biomarkers for these tumours. These results, obtained in collaboration with the group of Dr. Maria Serena Benassi (Istituti Ortopedici Rizzoli, Bologna, Italy), need to be widen to encompass a larger number of cases to better understand the neoplastic role of these microRNAs and the correlation between their different expression pattern and prognosis of these diseases.

Table 3.4 *microRNA expression in tumour samples of high grade osteosarcoma patients (1-4)*

	1	2	3	4
miR-484	168.664	2.596	9535.000	34.488
miR-196a	35.770	0.492	979.109	369.967
miR-183	82.711	455.087	37771.000	151.167
miR-9	19.835	0.311	1.010	19.835

3.2 Transmigration of tumour cells

To increase the knowledge of molecules important in metastasis formation and in particular the process of extravasation, spreading, we set up an assay using HUVEC and a Transwell system as described in Materials and Methods. The kinetics of transmigration of different sarcoma and other tumour cell lines obtained by using a fluorescent microplate reader is reported in Figure 3.3.

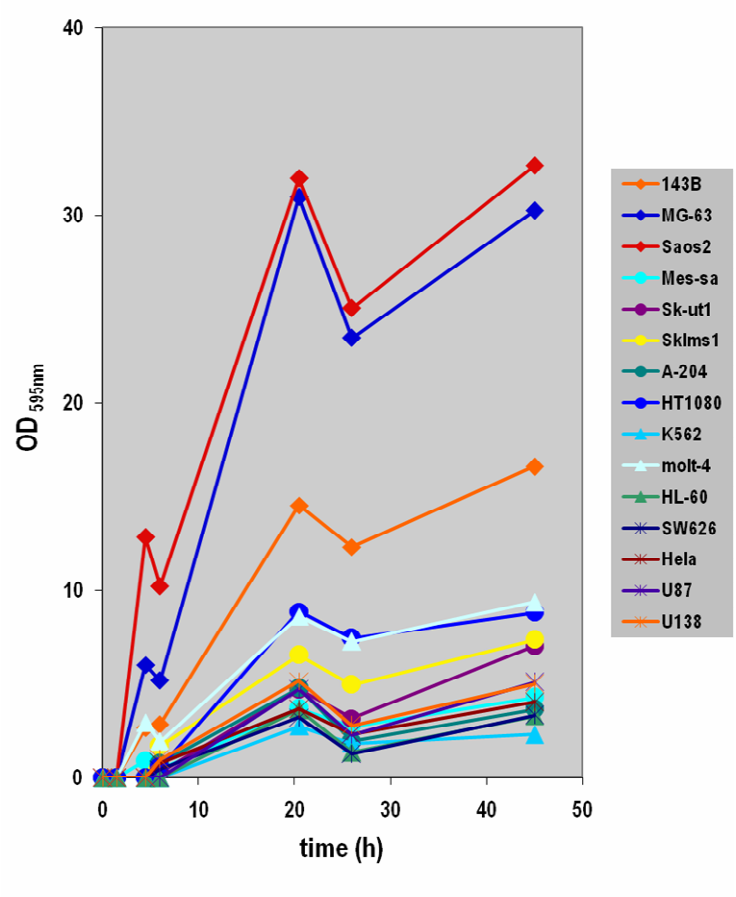


Figure 3.3 ***Kinetics of transendothelial migration of a panel of tumour cells***

Graphic representation of the migration kinetics of different tumour cell types. Rhomboid marks: osteosarcoma cell lines; circle marks: sarcoma cell lines; triangle marks: haematological tumour cell lines; star marks: different types of carcinoma.

In this type of assay, the three osteosarcoma cell lines showed a migration capability different from that of other sarcoma and non-sarcoma cell lines. From a regression analysis (Figure 3.4), a very similar migration behaviour was observed for MG-63 and Saos-2 osteosarcoma cell lines. Both showed an identical transmigration movement for time unit (≈0.75), whereas, the 143B cell line migrated with about half the speed in comparison with the others two osteosarcoma lines.

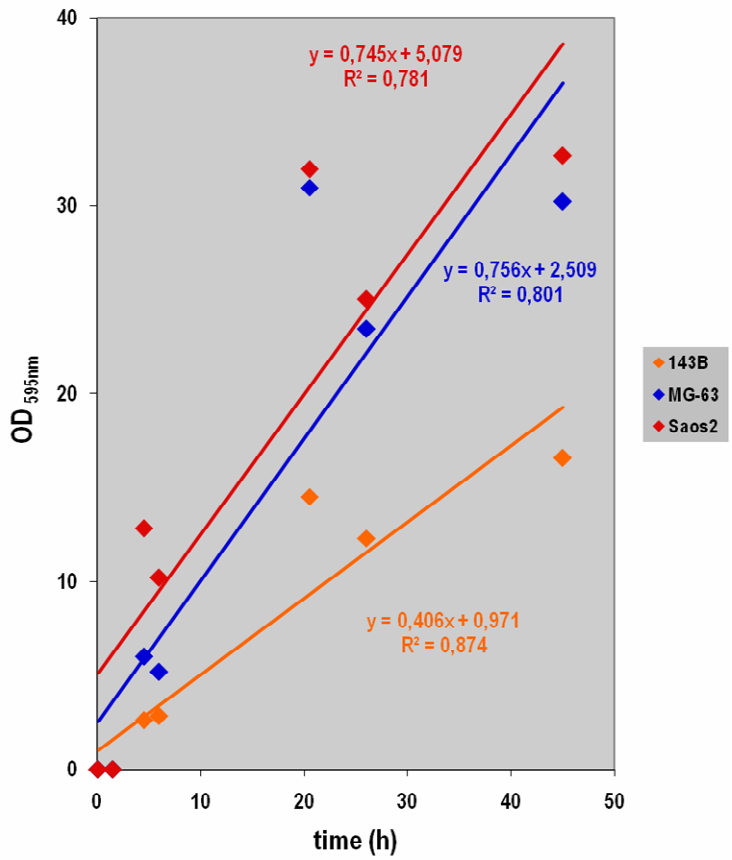


Figure 3.4 *Regression analysis of the transendothelial migration kinetics of osteosarcoma cell lines*

Saos-2 and MG-63 show a similar migration behaviour whereas 143B shows an half regression coefficient.

Having observed this different behaviour in the studied osteosarcoma cell lines, we performed further investigations on MG-63 and 143B cell lines. Furthermore, we consider the expression analysis of some oncosuppressor genes. MG-63 and Saos-2 are both p21 positive (Li et al., 1999; Merli et al., 1999) and p53 negative (Fogal et al., 2000; Lauricella et al., 2001). Saos-2 expresses p14 and p16 (Munro et al., 1999) but lacks of Rb functional product (Lauricella et al., 2003; Li et al., 1999) whereas MG-63 shows the Rb expression (Merli et al., 1999) but lacks of both p14 and p16 (Park et al., 2002). Expression analysis on 143B sarcoma cell lines shows the presence of p53 (Rajkumar and Yamuna, 2008) and Rb (Benassi personal communication). For our further investigations we referred to p53 different expression in the osteosarcoma cell lines considered (143B and MG-63) maintaining the presence of Rb expression to reduce their difference.

3.3 Screening of microRNA libraries

microRNA library was prepared using RNA with good quality (Figure 3.5A) on the two chosen cell lines (143B and MG-63) in agreement with standard procedures as described in Mat & Met. The bacterial colonies used for PCR amplification were grown on LB agar plate (Figure 3.5B).

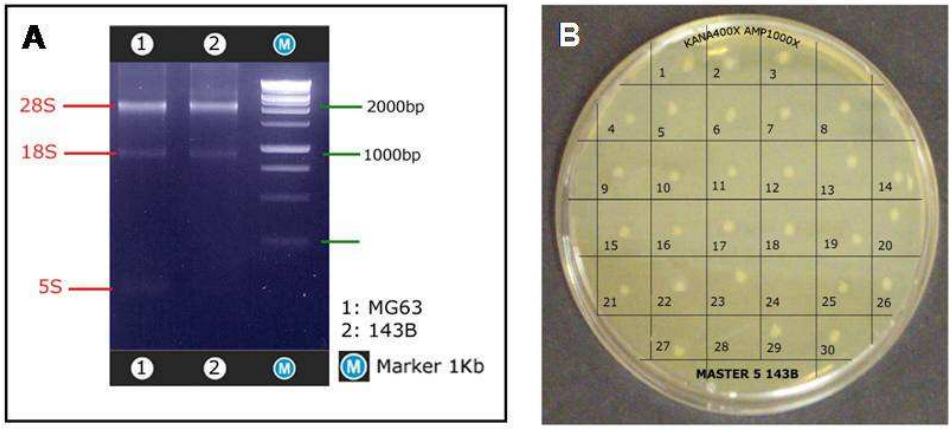


Figure 3.5 *Libraries preparation*

A *.Electrophoretic run of MG-63 and 143B RNAs.* ***B.*** *Bacterial clone growth on master LB plate.*

The PCR sample run was performed on 1% agarose gel (Figure 3.6) and selected products were then sequenced.

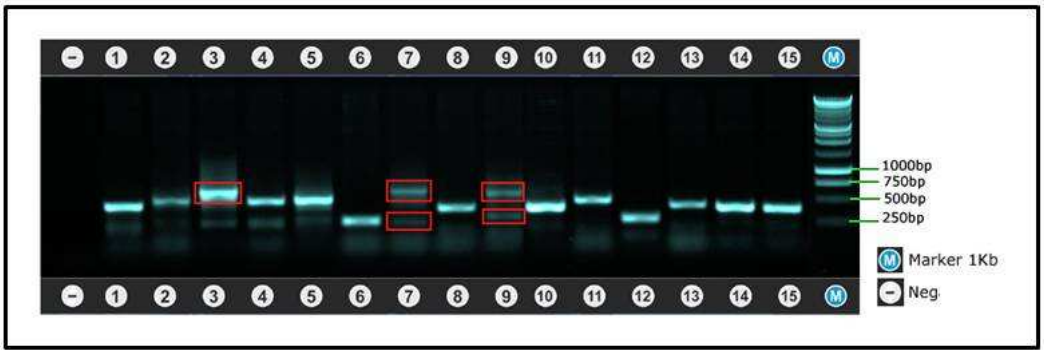


Figure 3.6 *Screening preparation*

Electrophoretic run of PCR products. In red square are highlighted the fragments chose for the following sequencing.

The sequencing analysis of concatamer with 500-600 bp identified the following microRNAs (Table 3.5).

Table 3.5 *microRNAs expressed in MG-63 and 143B osteosarcoma cell lines*

miRNA	sequence	size	localization	MG-63	143B
miR-16-1	UAGCAGCACGUAAAUAUUGGCG	22 nt	13q14.2	x	x
miR-16-2	UAGCAGCACGUAAAUAUUGGCG	22 nt	3q25.33	x	x
miR-17-5p*	CAAAGUGCUUACAGUGCAGGUAG	23 nt	13q31.3	x	x
miR-20a*	UAAAGUGCUUAUAGUGCAGGUAG	23 nt	13q31.3	x	
miR-20b*	CAAAGUGCUCAUAGUGCAGGUAG	22 nt	Xq26.3	x	
miR-21	UAGCUUAUCAGACUGAUGUUGA	22 nt	17q23.2	x	
miR-24-1	UGGCUCAGUUCAGCAGGAACAG	22 nt	19 p13.12		x
miR-24-2	UGGCUCAGUUCAGCAGGAACAG	22 nt	19 p13.12		x
miR-93*	CAAAGUGCUGUUCGUGCAGGUAG	23 nt	7q22.1	x	x
miR-103	AGCAGCAUUGUACAGGGCUAUGA	23 nt	20p13	x	
miR-106a*	AAAAGUGCUUACAGUGCAGGUAG	23 nt	Xq26.2	x	
miR-106b*	UAAAGUGCUGACAGUGCAGAU	21 nt	7q22.1	x	
miR-107	AGCAGCAUUGUACAGGGCUAUCA	23 nt	10q23.31	x	
miR-130a	CAGUGCAAUGUUAAAAGGGCAU	22 nt	11q12.1	x	x
miR-130b	CAGUGCAAUGAUGAAAGGGCAU	22 nt	22q11.21	x	x
miR-139	UCUACAGUGCACGUGUCUCCAG	22 nt	11q13.4	x	
miR-195	UAGCAGCACAGAAAUAUUGGC	21 nt	17p13.1	x	
miR-210	CUGUGCGUGUGACAGCGGCUGA	22 nt	11p15.5	x	
miR-323	CACAUUACACGGUCGACCUCU	21 nt	14q32.31		x

** microRNAs of miR-17 family that belongs to miR-17-92 cluster*

3.3.1 Hsa[22] *miR16-1* and Hsa *miR-16-2*

These two microRNAs (Figure 3.7) have the same sequence but localize in two different chromosomes, probably as a result of gene duplication. Only *miR-16-1* is well known because it resides within the same cluster of *miR-15*, cluster that seems to control the anti-apoptotic gene BCL2 (Cimmino et al., 2005). Deletion or down-regulation of this cluster is associated with poor prognosis in chronic lymphocytic leukemia (Calin et al., 2008; Calin et al., 2005). *miR-16-1* and *miR-16-2* were found down regulated in pituitary adenomas (Bottoni et al., 2005). Recent studies report that *miR-16-1* acts as tumour suppressor gene by targeting both CCND1 (encoding cyclin D1) (Chen et al., 2008b) and WNT3A (in prostate cancer), and by controlling cell survival, proliferation and invasion (Bonci et al., 2008).

[22] *Hsa: Homo sapiens*

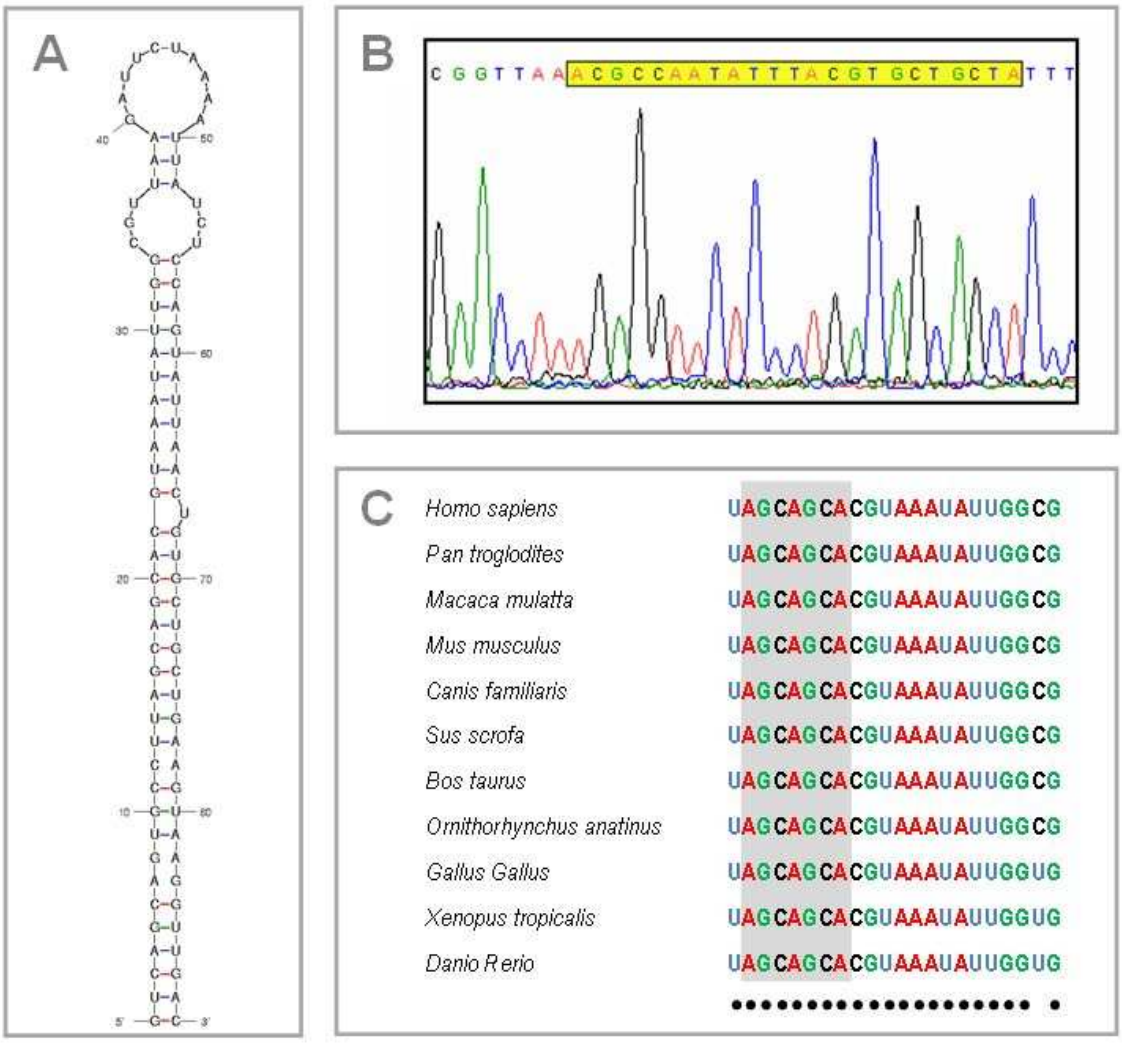


Figure 3.7 ***miR-16-1 and miR-16-2***

miR-16-1 and miR-16-2 are localized respectively in 13q14.2 and 3q25.33 of the human genome. They are a part of miR-15 family. ***A.*** *Secondary structure prediction of pre-miRNA, ΔG = -36.70.* ***B.*** *Electropherogram of a sequenced fragment, in yellow, miR-16.* ***C.*** *Phylogenetic conservation of miR-16s, in grey the seed region.*

3.3.2 Hsa *miR-17-5p*

miR-17-5p (Figure 3.8) was characterized as microRNA associated to different cancer types (Volinia et al., 2006), able to regulate cancer cell proliferation (Hossain et al., 2006). In particular its expression level was significantly up-regulated in bladder cancer compared to normal bladder mucosa (Gottardo et al., 2007). It was already found stably expressed in osteosarcoma cell line SOPS-9607 and osteosarcoma tissues (Gao et al., 2007). Moreover *miR-17-5p* was over-expressed in ectopic and eutopic endometrium compared to normal endometrium having, indeed, direct implications in the pathogenesis of endometriosis (Toloubeydokhti et al., 2008).

Although this microRNA is normally expressed within the mesoderm of gastrulating embryos during mammalian development (Foshay and Gallicano, 2008), a tight correlation was found between *miR-17-5p* and amyloid precursor protein during brain development and neuron differentiation (Hebert et al., 2008).

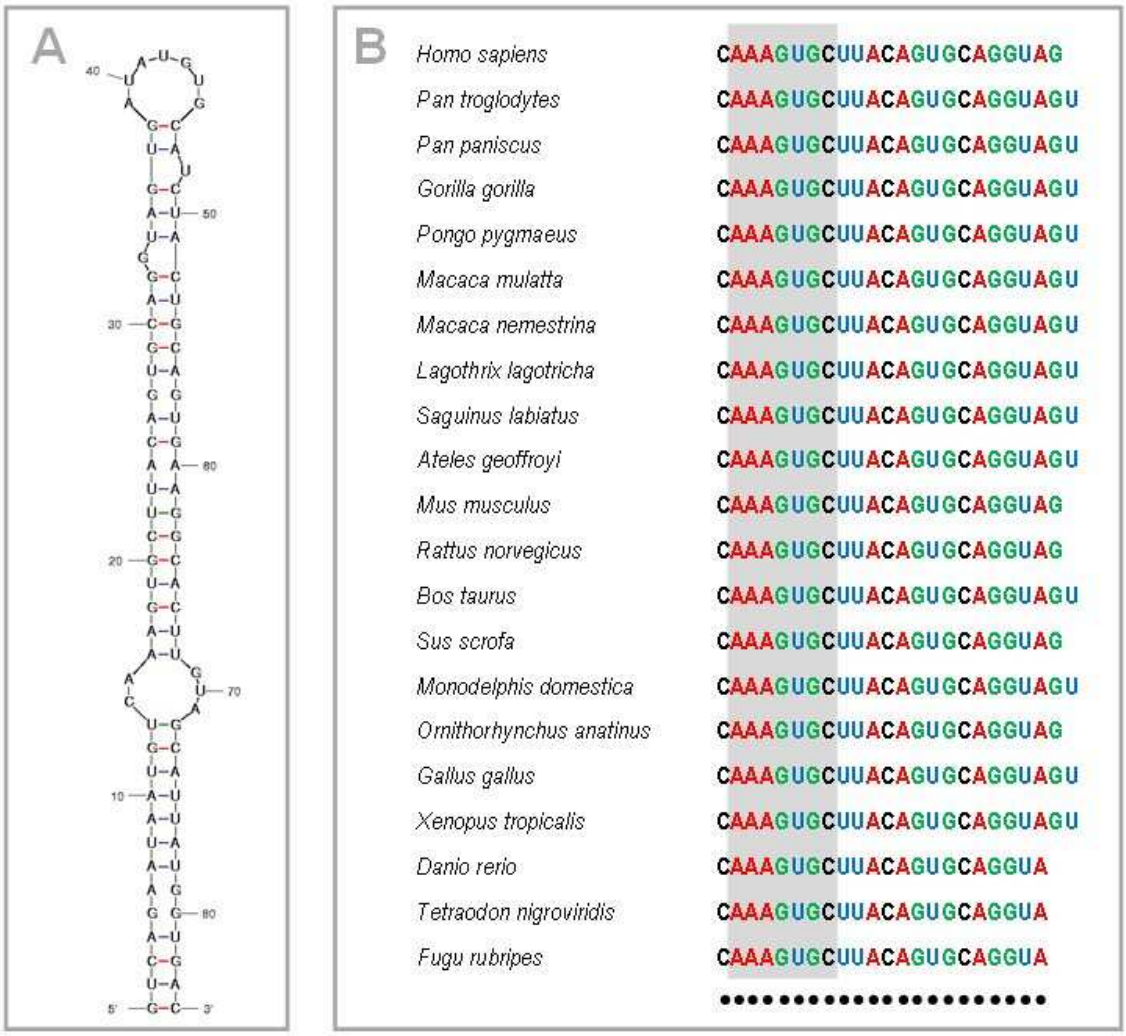


Figure 3.8 *miR-17-5p*

miR-17-5p is a part of the miR-17 family and it localized in cluster on the human chromosome 13 (see Introduction). ***A.*** *Secondary structure prediction of stable miR-17-5p, ΔG = -34.30.* ***B.*** *Phylogenetic conservation of miR-17-5p, in grey the seed region.*

3.3.3 Hsa *miR-20a*

Over-expression of this microRNA (Figure 3.9) was found in different tumour types (Volinia et al., 2006) and seems to modulate cell cycle progression (Pickering et al., 2009). Recently Poliseno and colleagues (Poliseno et al., 2008) revaluated *miR-20a* as a tumour suppressor and found out its role in inducing senescence.

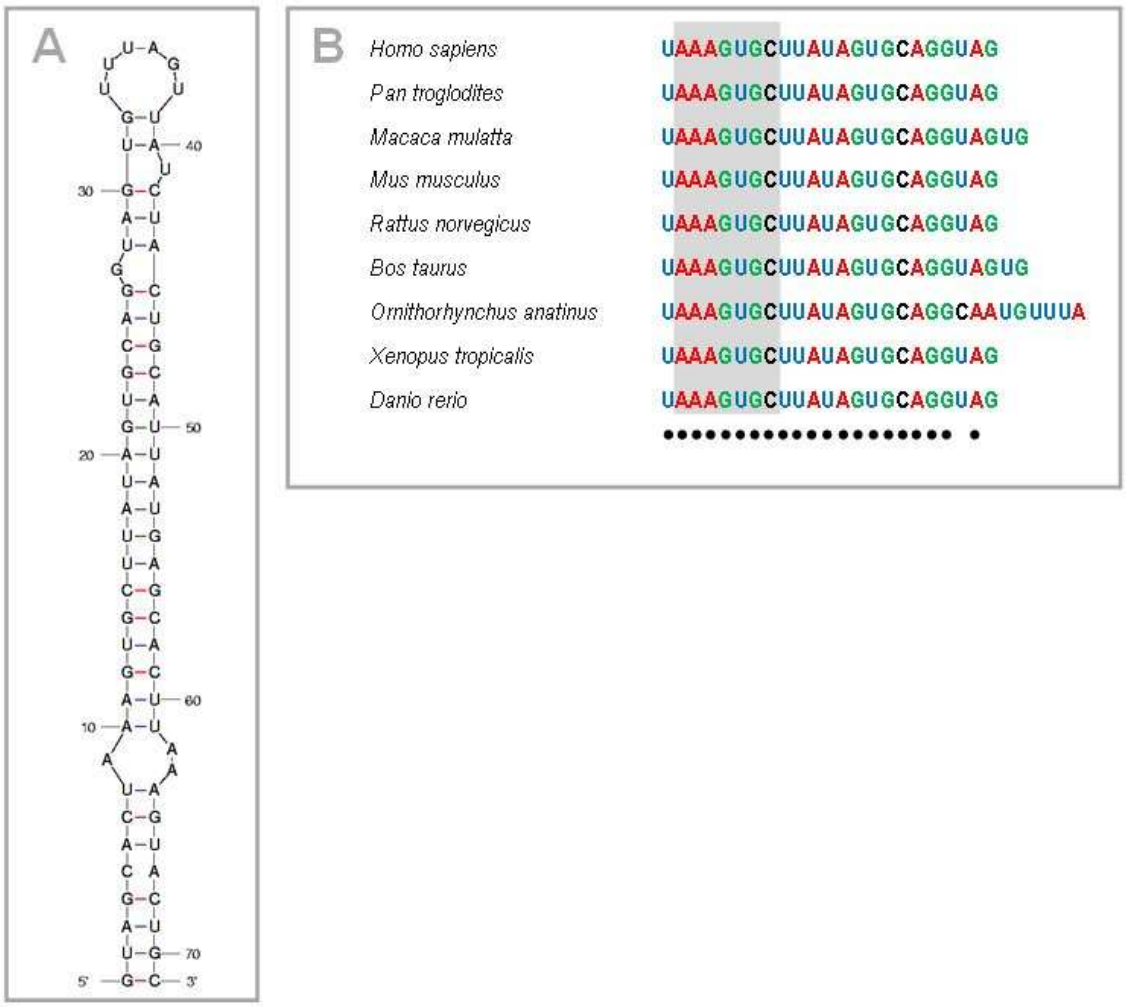


Figure 3.9 *miR-20a*

miR-20a is closely clustered to miR-17-5p on the chromosome 13. ***A.*** *Secondary structure prediction of pre-miRNA, ΔG = -31.00.* ***B.*** *Phylogenetic conservation of this microRNA, in grey the seed region.*

3.3.4 Hsa *miR-20b*

miR-20b (Figure 3.10) is over-expressed in human T-cell leukemias (Landais et al., 2007) as well as in c-Myc positive mammary tumours (Sun et al., 2008). In a recent study investigating the translation regulation in osteoblasts exposed to porous polyethylene (Medpor), *miR-20b* was found to be one of the microRNAs significantly up-regulated in Medpor-treated MG-63 osteosarcoma cells (Palmieri et al., 2008).

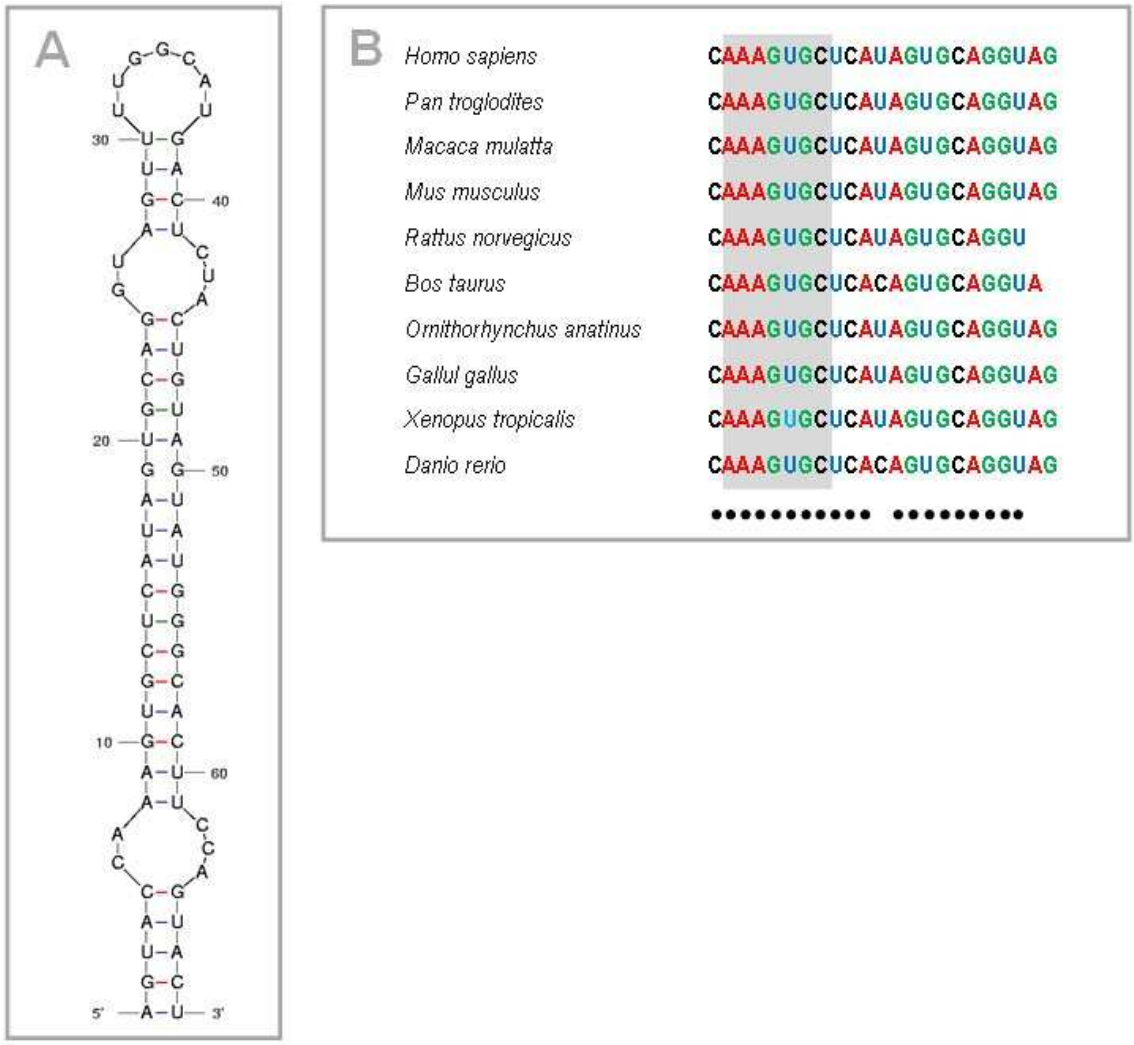


Figure 3.10 ***miR-20b***

Mature microRNA has the same sequence of miR-20a but it localized on Xq26.3 of the human genome. It belong to miR-17 family. ***A.*** *Secondary structure prediction of pre-miRNA, ΔG = -29.80.* ***B.*** *Phylogenetic conservation of miR-20b, in grey the seed region.*

3.3.5 Hsa *miR-21*

miR-21 (Figure 3.11) appears to function as an anti-apoptosis factor in glioblastomas by regulating the tumour suppressor gene PDCD4 (Chen et al., 2008c). Indeed, it is considered an onco-microRNA that acts by promoting tumour cell growth, invasion and metastasis. In particular, in breast cancer, high *miR-21* expression is associated with features of aggressive disease, including high tumour grade, negative hormone receptor status and ductal carcinoma. Moreover, even through *miR-21* was correlated with a specific breast cancer biopathologic features (Yan et al., 2008), its high expression was associated with poor disease-free survival in early stage patients (Qian et al., 2008). These data suggest *miR-21* as possible marker in breast cancer.

Other studies showed that its high expression significantly enhances cell proliferation and invasion in a gastric cancer cell line suggesting the importance of this microRNA in this tumour type too (Zhang et al., 2008). On the other hand, in non-small cell lung cancer, miR-21 over-expression directly correlates with overall survival of the patients (Markou et al., 2008).

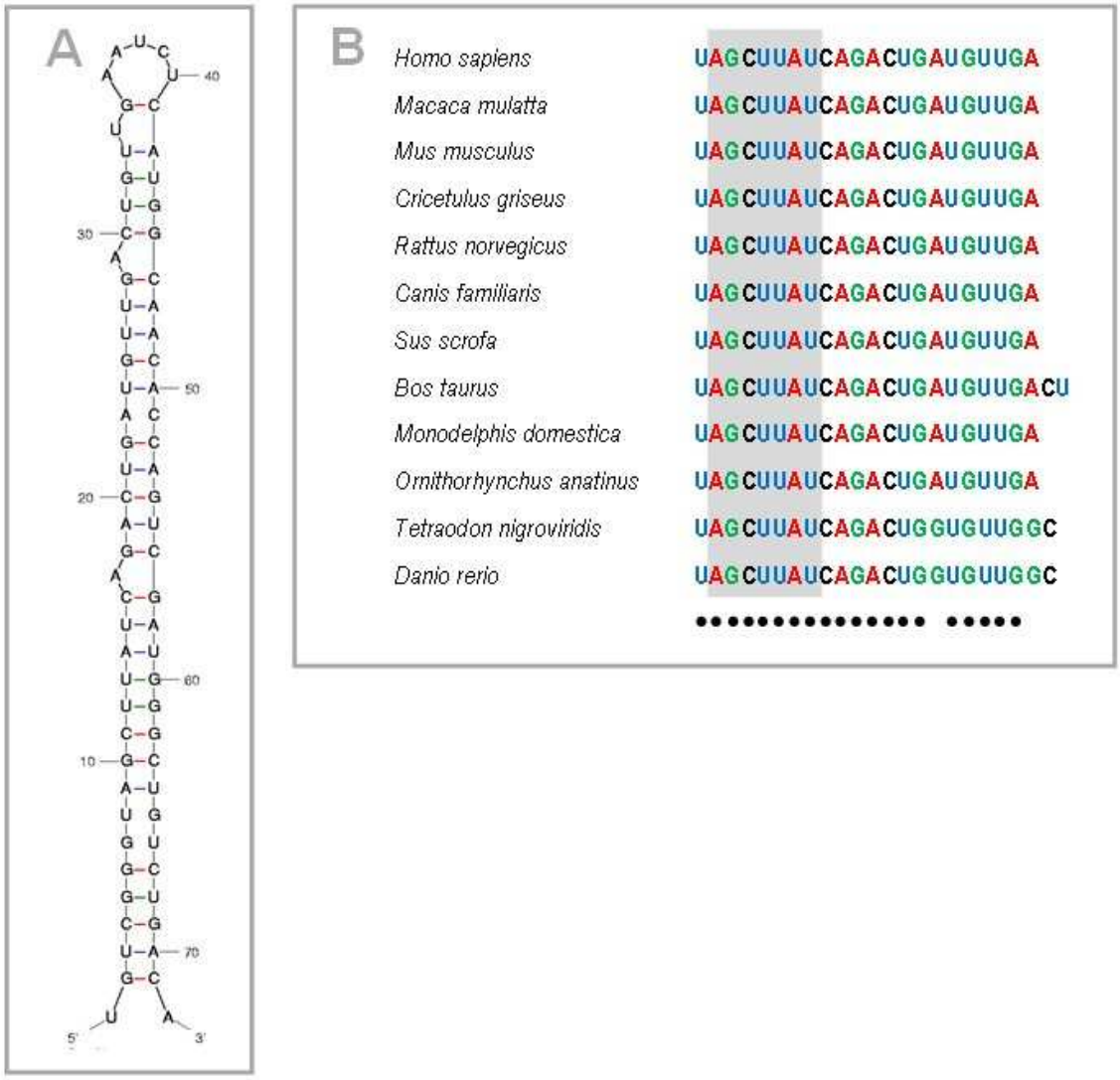


Figure 3.11 *miR-21*

miR-21 is a part of the miR-21 family and it is localized on the 17q23.2 human chromosome. ***A.*** *Secondary structure prediction of stable miR-21, ΔG = -35.80.* ***B.*** *Phylogenetic conservation of this microRNA, in grey the seed region.*

3.3.6 Hsa *miR-24-1* Hsa *miR-24-2*

They are two microRNAs (Figure 3.12) located in tandem on chromosome 19. Probably they are the result of gene duplication and no one polimorfism could distinguish them. They are part of *miR-24* family and they were identified in human fetal liver (Fu et al., 2005) and in different cell lines and tissue types (Landgraf et al., 2007) and, after the modulation induced by 12-O-tetradecanoylphorbol-13-acetate (TPA), in HL-60 cells (Kasashima et al., 2004).

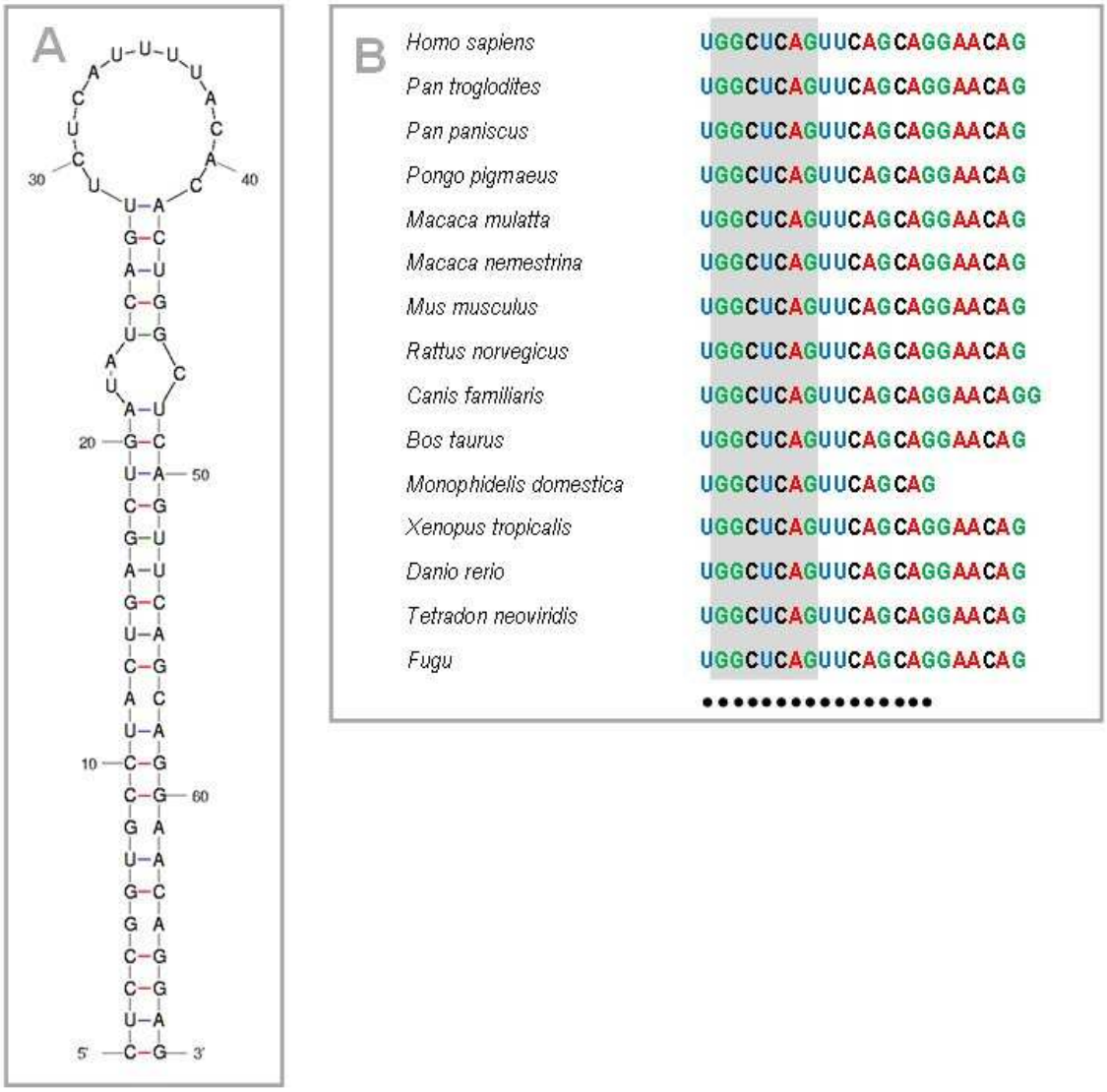


Figure 3.12 *miR-24-1 and miR-24-2*

miR-24-1 and miR-24-2 belong to the miR-24 family. ***A.*** *Stable secondary structure prediction of pre-miRNA, ΔG = -26.30.* ***B.*** *Phylogenetic conservation of miR-24-1 and miR-24-2, in grey the seed region.*

3.3.7 Hsa *miR-93*

miR-93 (Figure 3.13) is transcribed in the same RNA together *miR-106b* and *miR-25* giving origin to a *miR*-cluster (see Introduction). It is highly expressed in primary peripheral blood mononuclear cell from adult T-cell leukemia patients where it seems to regulate TP53INP1 (Yeung et al., 2008). In gastric cancer it seems to be involved in E2F1 post-transcriptional regulation and may play a key role in the modulation of TGF-beta signaling in this tumour type (Petrocca et al., 2008). It is a potential biomarker in ovarian cancer since it was found over-expressed in the serum of cancer patients when compared to the controls (Resnick et al., 2009).

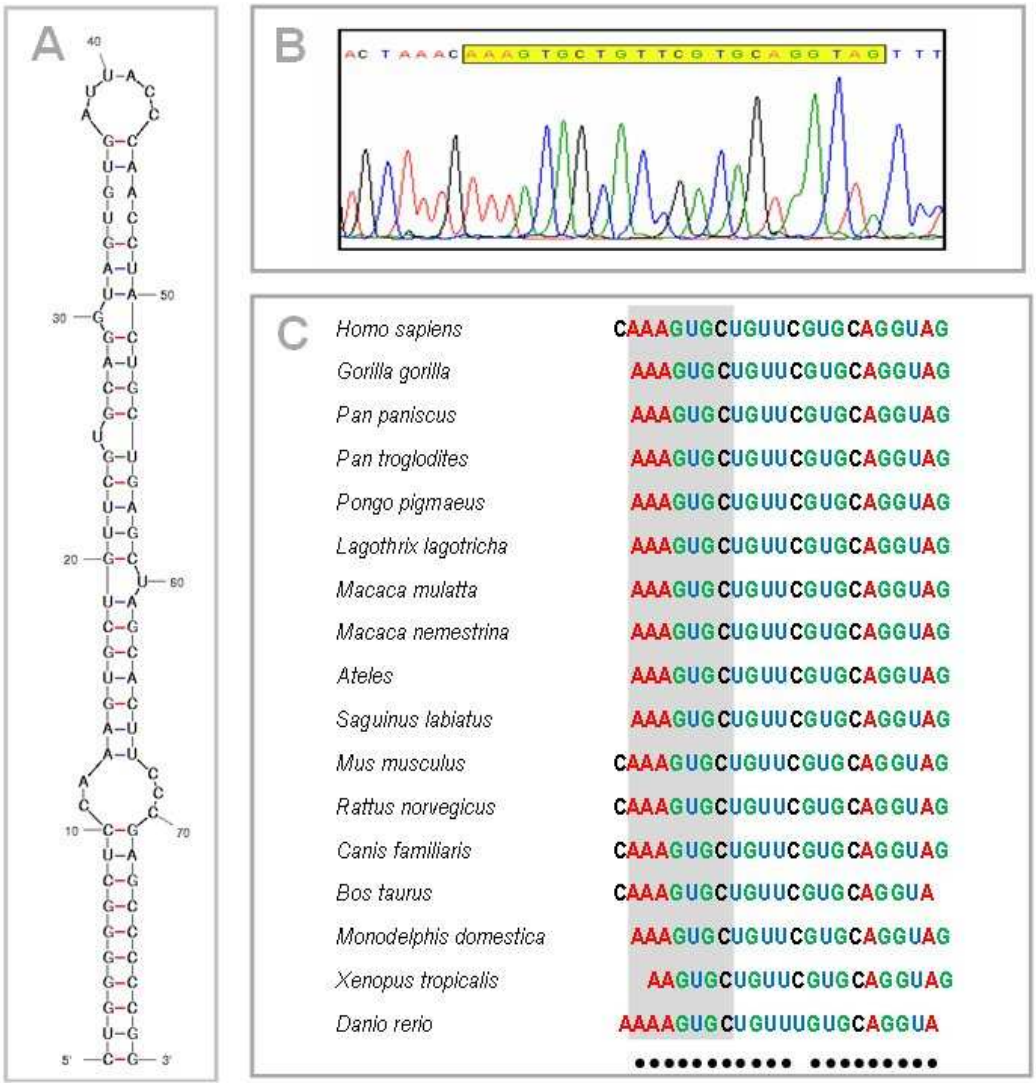


Figure 3.13 *miR-93*

mR-93, together with other cluster microRNAs of the miR-17 family localized on 7q22.1 chromosome of the human genome. ***A.*** *Secondary microRNA structure prediction, ΔG = -44.80.* ***B.*** *Electropherogram of a sequenced fragment, in yellow miR-93.* ***C.*** *Phylogenetic conservation of this microRNA, in grey the seed region.*

3.3.8 Hsa *miR-103*

mir-103 (Figure 3.14) is significantly up-regulated in bladder cancer compared to normal bladder (Gottardo et al., 2007). Its high expression correlates with poor survival in esophageal cancer (Guo et al., 2008). In a multifactorial disease as osteoarthritis, its relationship with BMI (body mass index) could be useful for the development of new therapies (Iliopoulos et al., 2008).

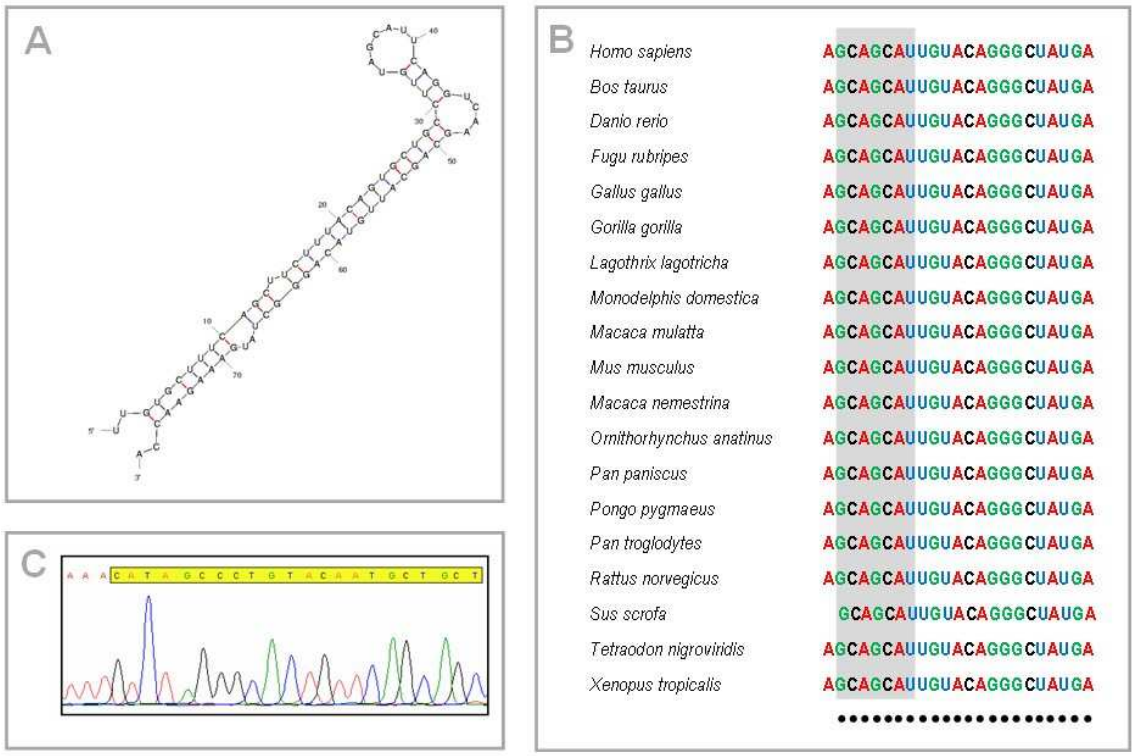


Figure 3.14 *miR-103*

miR-103 is a part of the miR-103 family, it localizes on the human chromosome 20p13. ***A.*** *Stable microRNA secondary structure prediction, ΔG = -28.10.* ***B.*** *Graphic sequence of sequenced fragment, in yellow miR-103.* ***C.*** *Phylogenetic conservation of miR-103, in grey the seed region.*

3.3.9 Hsa *miR-106a*

miR-106a (Figure 3.15) was widely studied in different tumour types. In particular in gastric carcinoma (Xiao et al., 2009) its expression was significantly higher than in non-tumour tissue and it was associated with tumour stage, size and differentiation. In colon cancer patients (Diaz et al., 2008) the deregulation of *miR-106a* was considered as a marker of disease–free survival and overall survival independently

from tumour stage. Moreover, the inverse correlation with E2F1 supports the hypothesis that *miR-106a* targets this transcription factor.

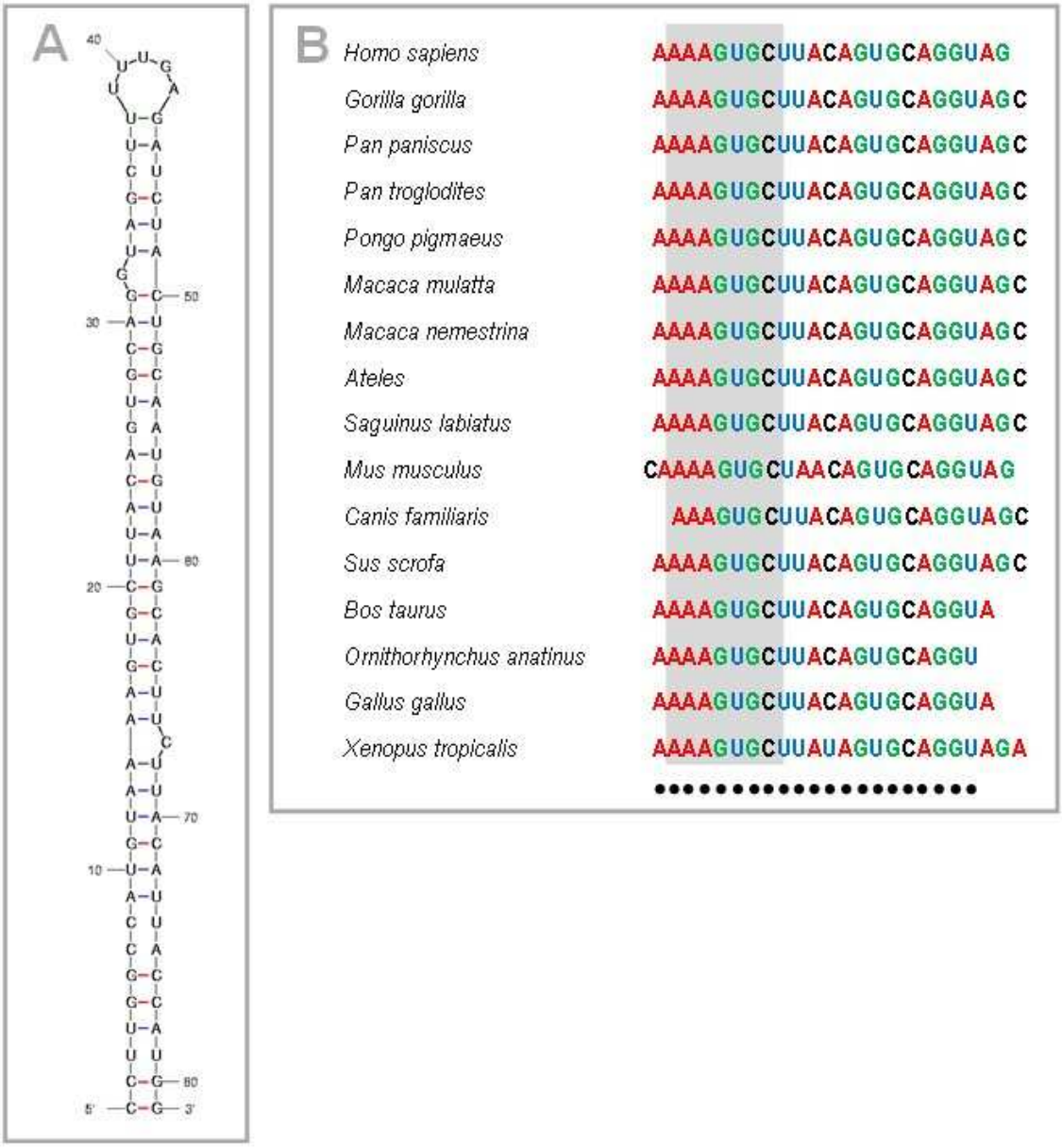


Figure 3.15 ***miR-106a***

miR-106 is a part of the miR-17 family and it localizes in the Xq26.2 human chromosome position. ***A.*** *Stable secondary structure of pre-miRNA predicted, ΔG = -34.70.* ***B.*** *Electropherogram of a sequence, in yellow miR-106a.* ***C.*** *Phylogenetic analysis conservation of this microRNA, in grey the seed region.*

3.3.10 Hsa *miR-106b*

Together with its family (*miR-17* family), *mir-106b* (Figure 3.16) was found involved in the cell cycle progression by modulating checkpoint function (Ivanovska et al., 2008). It was over-expressed in multiple myeloma pathogenesis (Pichiorri et al., 2008) and in prostate cancer (Ambs et al., 2008). Alterations of its nucleotide sequence were found in hepatocellular carcinomas (Yang et al., 2008).

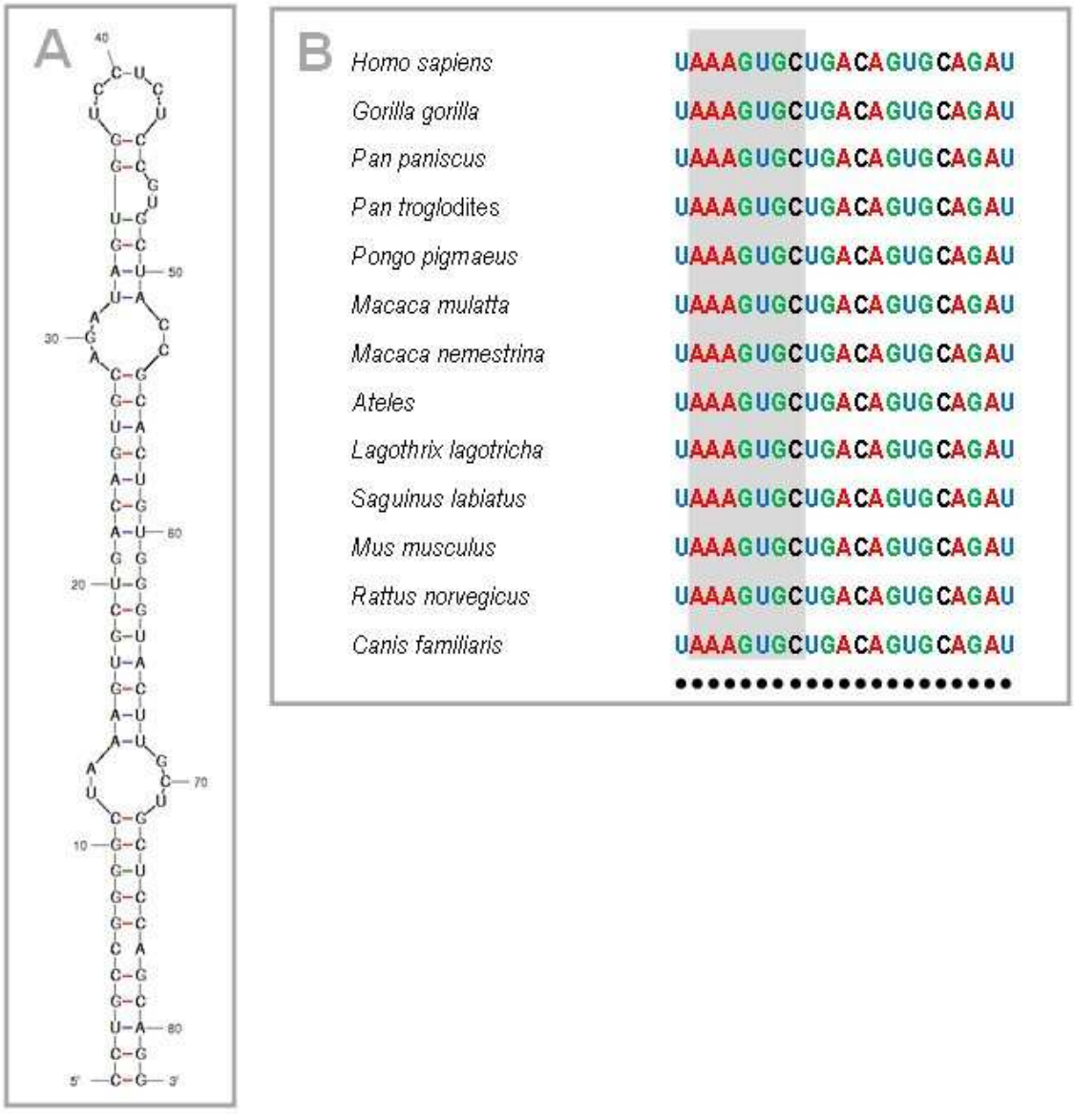


Figure 3.16 *miR-106b*

miR-106b has very similar mature sequence to miR-106a, but it localizes on chromosome 7q22.1 of the human genome. It belongs to the miR-17 family. ***A.*** *Secondary structure prediction of pre-miR-106b, ΔG = -43.90.* ***B.*** *Phylogenetic conservation of this microRNA, in grey the seed region.*

3.3.11 Hsa *miR-107*

miR-107 (Figure 3.17) is localized on chromosome 10 and seems associated with traumatic brain injury (Redell et al., 2008). In the brain of Alzheimer's disease patients *miR-107* levels are significantly decreased even in the earliest stages of the pathology, resulting involved in accelerated disease progression through regulation of beta-site amyloid precursor protein-cleaving enzyme 1 (BACE1) (Wang et al., 2008b). In tumoural field, *miR-107* down-regulation was found in pancreatic and acinar tumours (Roldo et al., 2006) and in hepatocellular tumours specifically associated with Hepatocyte Nuclear Factor 1α (Ladeiro et al., 2008).

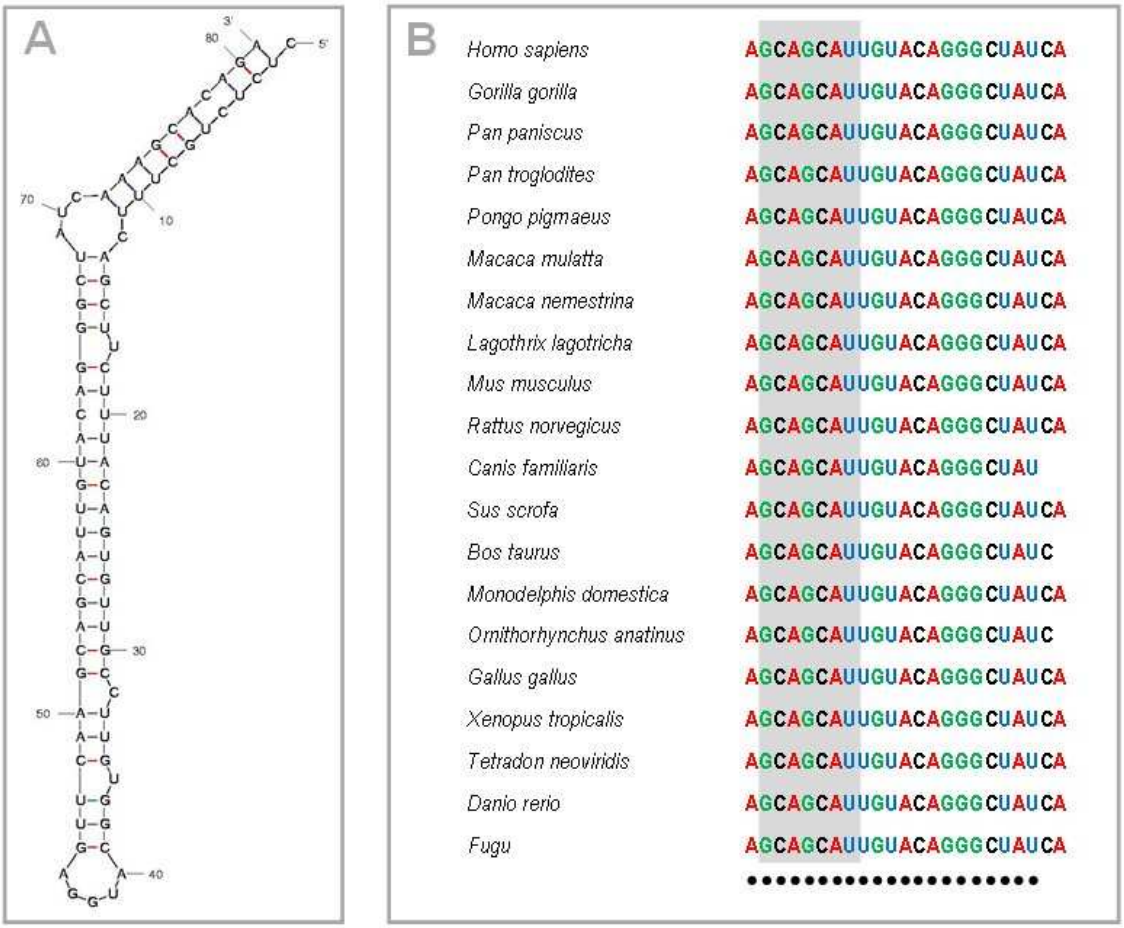


Figure 3.17 *miR-107*

miR-107 is one of the member of the miR-103 family and has a very similar sequence to miR-103. It is located on chromosome 10q23.31 of the human genome. ***A.*** *Stable secondary structure predictied, ΔG = -29.60.* ***B.*** *Sequence fragment electropherogram, in yellow microRNA 107.* ***C.*** *Phylogenetic analysis of this microRNA, in grey the seed region.*

3.3.12 Hsa *miR-130a*

MicroRNA 130a (Figure 3.18) was identified in recent studies aimed to the identification of factors that regulates the expression of homeoboxes GAX and HOXA5, molecules that inhibit the angiogenesis in vascular endothelial cells (Chen and Gorski, 2008; Urbich et al., 2008). It was down-regulated in ovarian cancer cell lines resistant to the chemotherapy. This down-regulation was linked to the translation activation of M-CSF gene, a well-known resistance factor for ovarian cancer (Sorrentino et al., 2008).

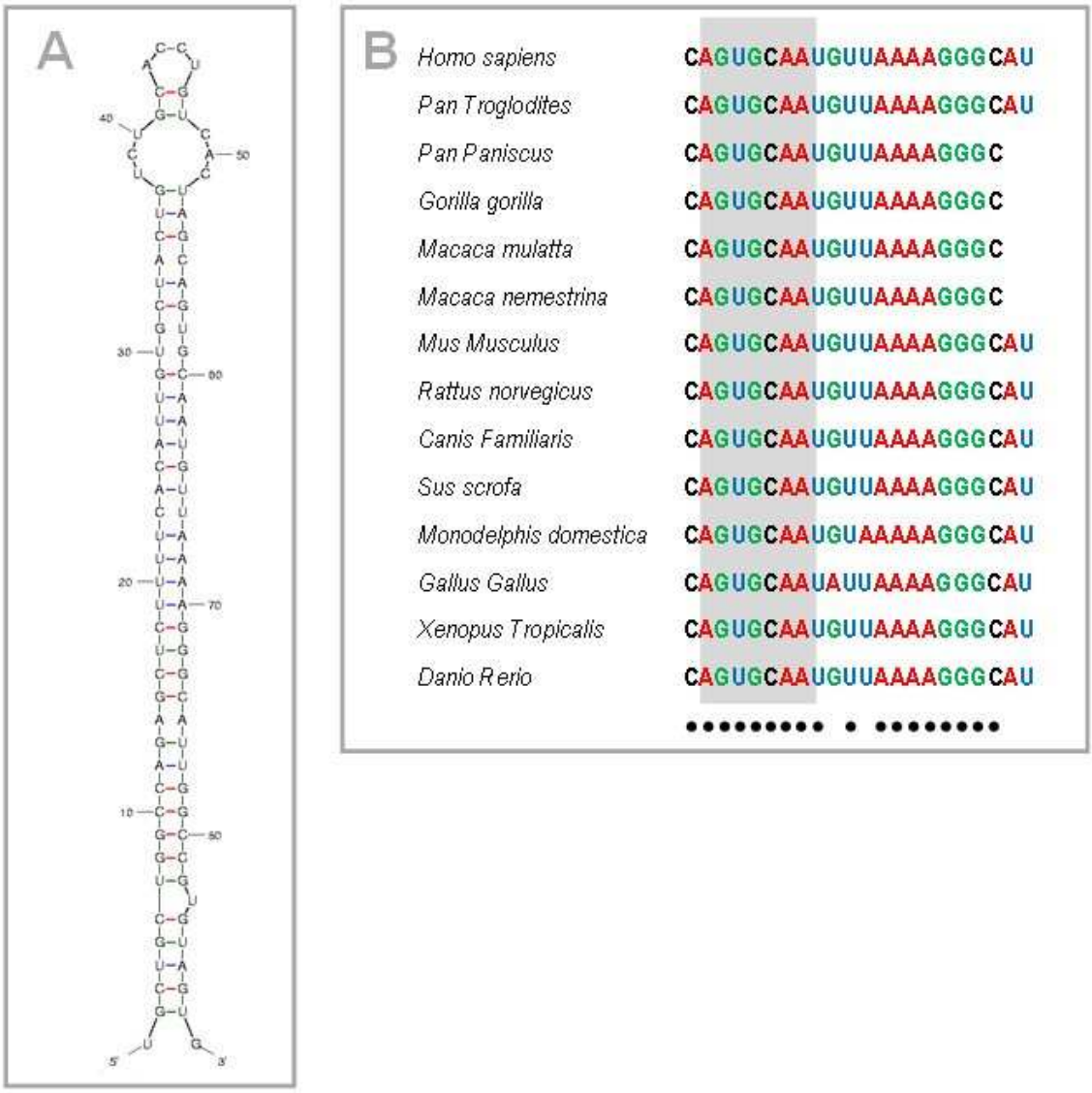


Figure 3.18 *miR-130a*

miR-130 belongs to the miR-130 family and it is located on human chromosome 11q12.1. ***A.*** *Secondary structure predicted, ΔG = -42.60.* ***B.*** *Graphical sequence of a cloned fragment, in yellow miR-130a.* ***C.*** *Analysis of genetic conservation of this microRNA, in grey the seed region.*

3.3.13 Hsa *miR-130b*

miR-130b (Figure 3.19) was studied in the brain of schizophrenia patients (Burmistrova et al., 2007) but no statically association was found. Recently it was studied in HTLV-1 cells and it seemed implicated in the increasing of their apoptosis (Yeung et al., 2008).

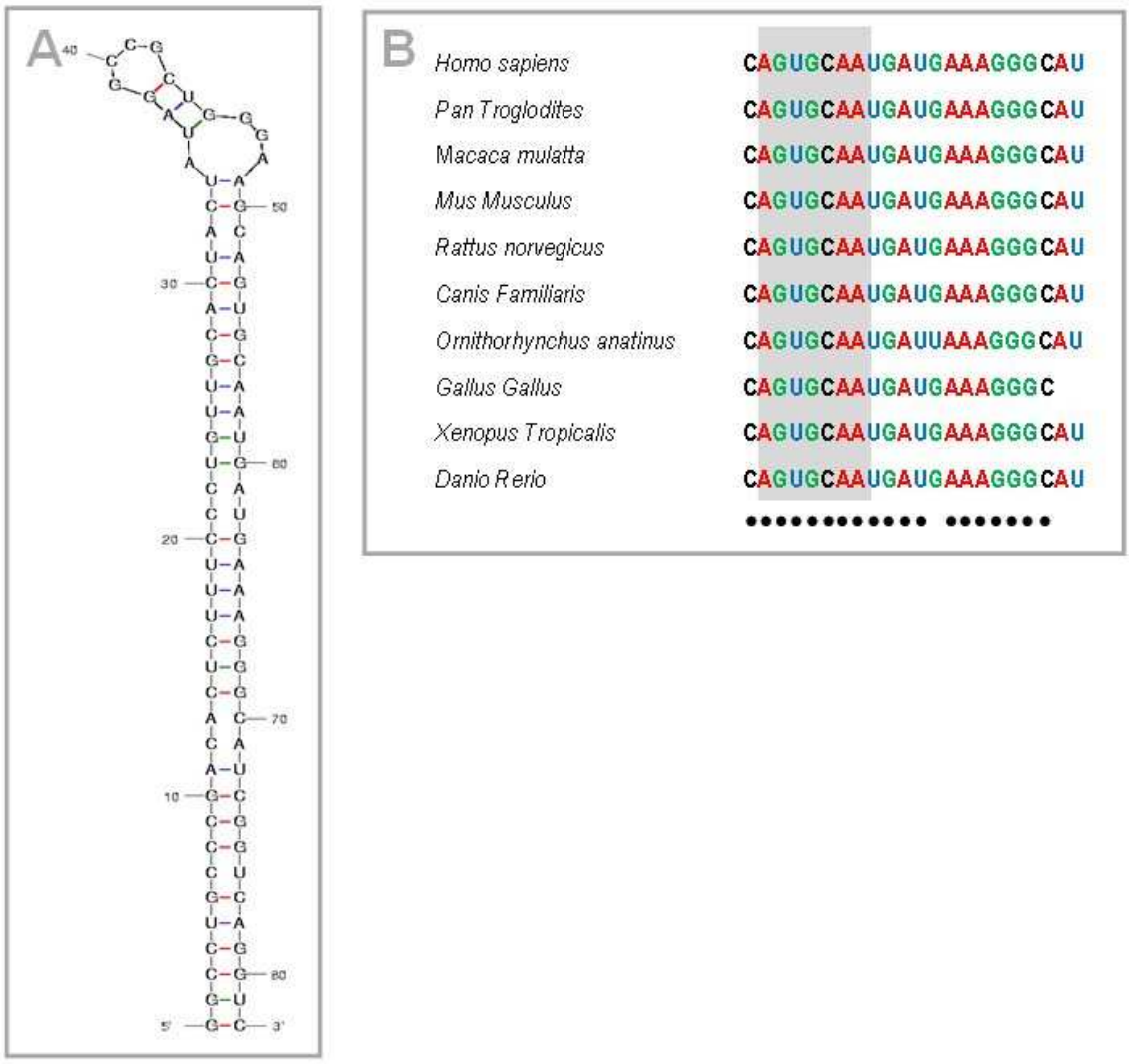


Figure 3.19 *miR-130b*

miR-130b is located on chromosome 22q11.21 of the human genome. It belongs to the miR-130 family and it distinguished from 130a for one nucleotide. ***A.*** *Stable secondary structure predicted, ΔG = -35.30.* ***B.*** *Phylogenetic conservation analysis of this microRNA, in grey the seed region.*

3.3.14 Hsa *miR-139*

miR-139 (Figure 3.20) was found up-regulated in human bronchial squamous carcinoma (Mascaux et al., 2008) resulting an useful tool for early detection of lung cancer.

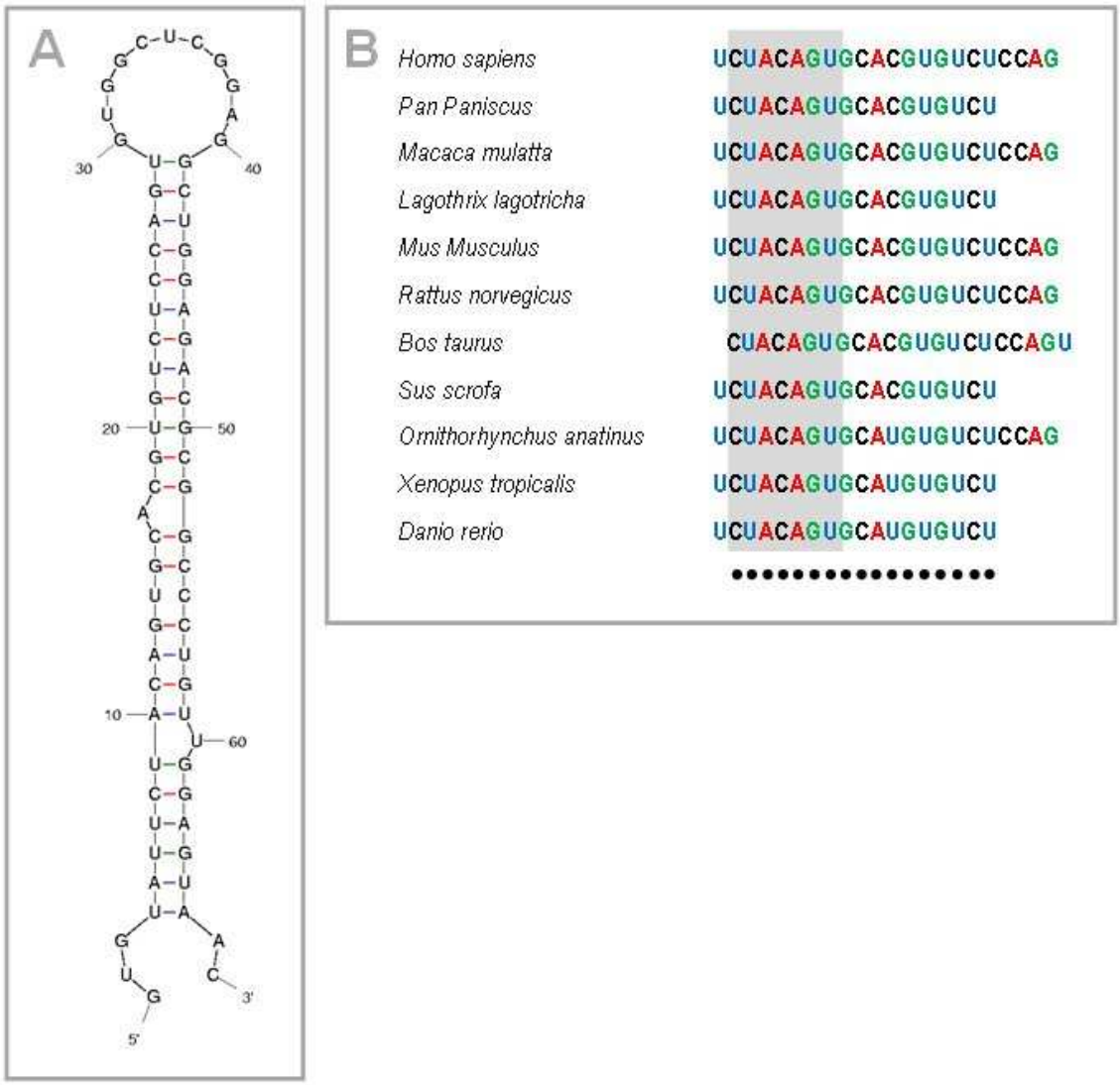


Figure 3.20 *miR-139*

miR-139 is on the same human chromosome of miR-130a but localizes in 11q13.4 position. miR-139 is a part of the miR-139 family. ***A.*** *Predicted secondary structure, ΔG = -33.40.* ***B.*** *Phylogenetic conservation of this microRNA, in grey the seed region.*

3.3.15 Hsa *miR-195*

In a recent research (Flavin et al., 2008), *miR-195* (Figure 3.21) may play a potential role as a tumour suppressor gene in primary peritoneal carcinoma in patients with ovarian serous carcinoma. It seems to be an important element in starting to determine the stress-response that can evoke cardiac hypertrophy and heart failure in transgenic mice (van Rooij et al., 2006).

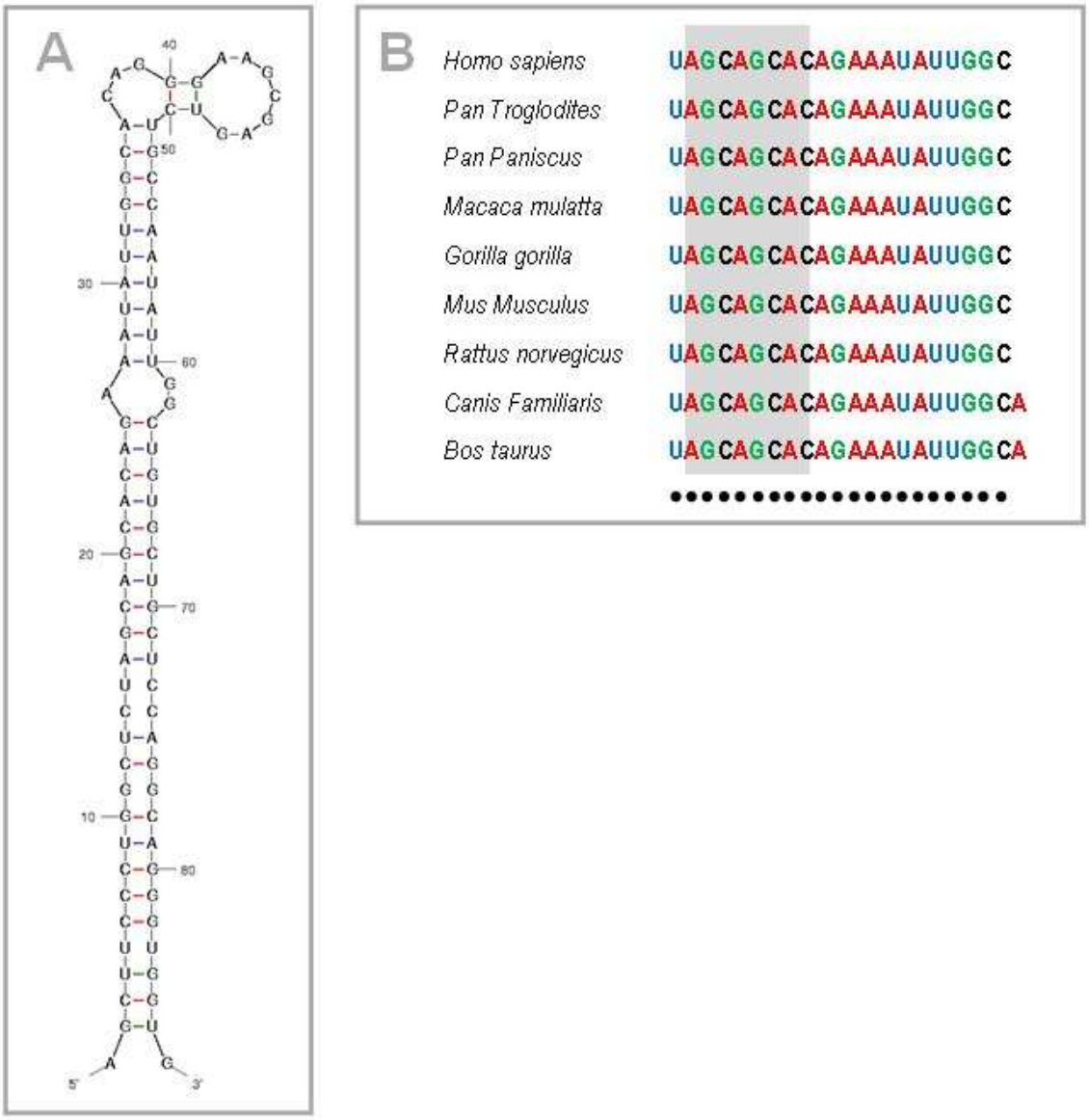


Figure 3.21 *miR-195*

Together with miR-15 and miR-16 belongs to the miR-15 family. It is located on 17p13.1 human chromosome. ***A.*** *Secondary structure predicted, ΔG = -47.50.* ***B.*** *Analysis of phylogenetic conservation of this microRNA, in grey the seed region.*

3.3.16 Hsa *miR-210*

In ovarian cancer (Giannakakis et al., 2008) *miR-210* (Figure 3.22) was the most prominent microRNA consistently stimulated under hypoxic conditions and it seemed to play a crucial role in tumour onset by regulating the E2F3 transcription factor, a key protein in cell cycle (Giannakakis et al., 2008). Moreover *miR-210* over-expression in normo-oxigenated endothelial cells stimulated the formation of capillary-like structures on Matrigel and the vascular migration of endothelial growth factor-driven cell. In this framework it seems to control the expression of Ephrin-A3, an inhibitor molecule of tubulogenesis and chemotaxis (Fasanaro et al., 2008).

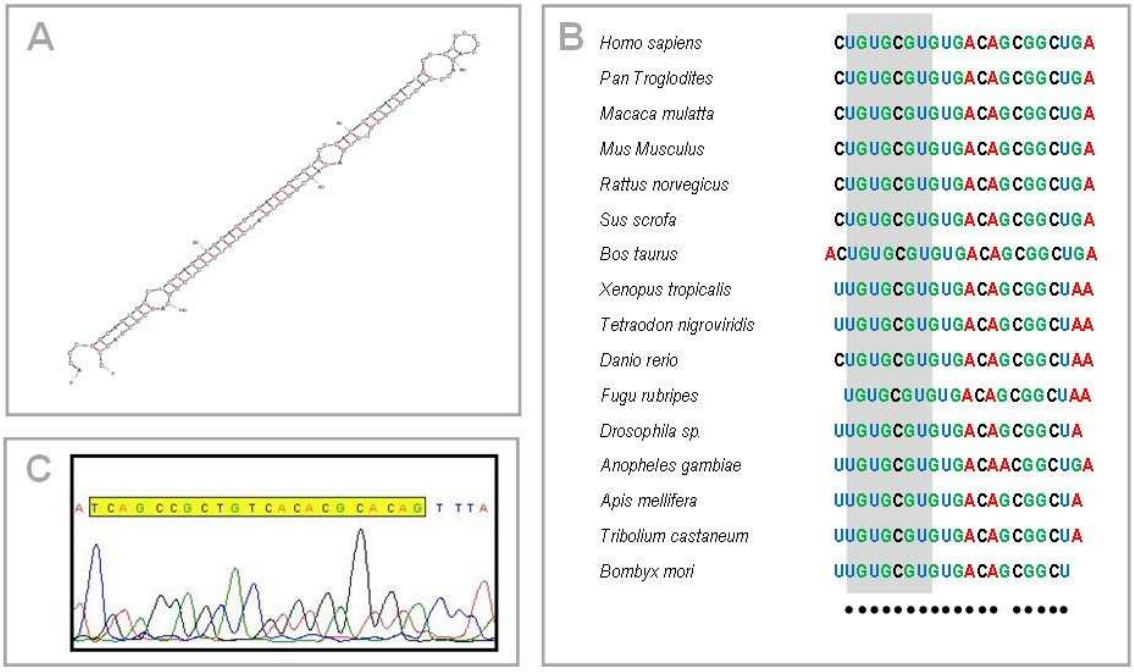


Figure 3.22 *miR-210*

miR-210 is located on 11p15.5 human chromosome, it belongs to the miR-210 family. ***A.*** *Predicted secondary structure of pre-miRNA, ΔG = 59.60.* ***B.*** *Electropherogram of a sequenced fragment, in yellow, miR-210.* ***C.*** *Phylogenetic analysis of miR-210, in grey the seed region.*

3.3.17 Hsa *miR-323*

In acute myeloid leukaemia up-regulation of *miR-323* (Figure 3.23) could be a marker that could sub-classify the disease and determine its etiology (Dixon-McIver et al., 2008). It is highly expressed in human leiomyoma compared to normal myometrium and could play a role in the uterine pathogenesis (Marsh et al., 2008).

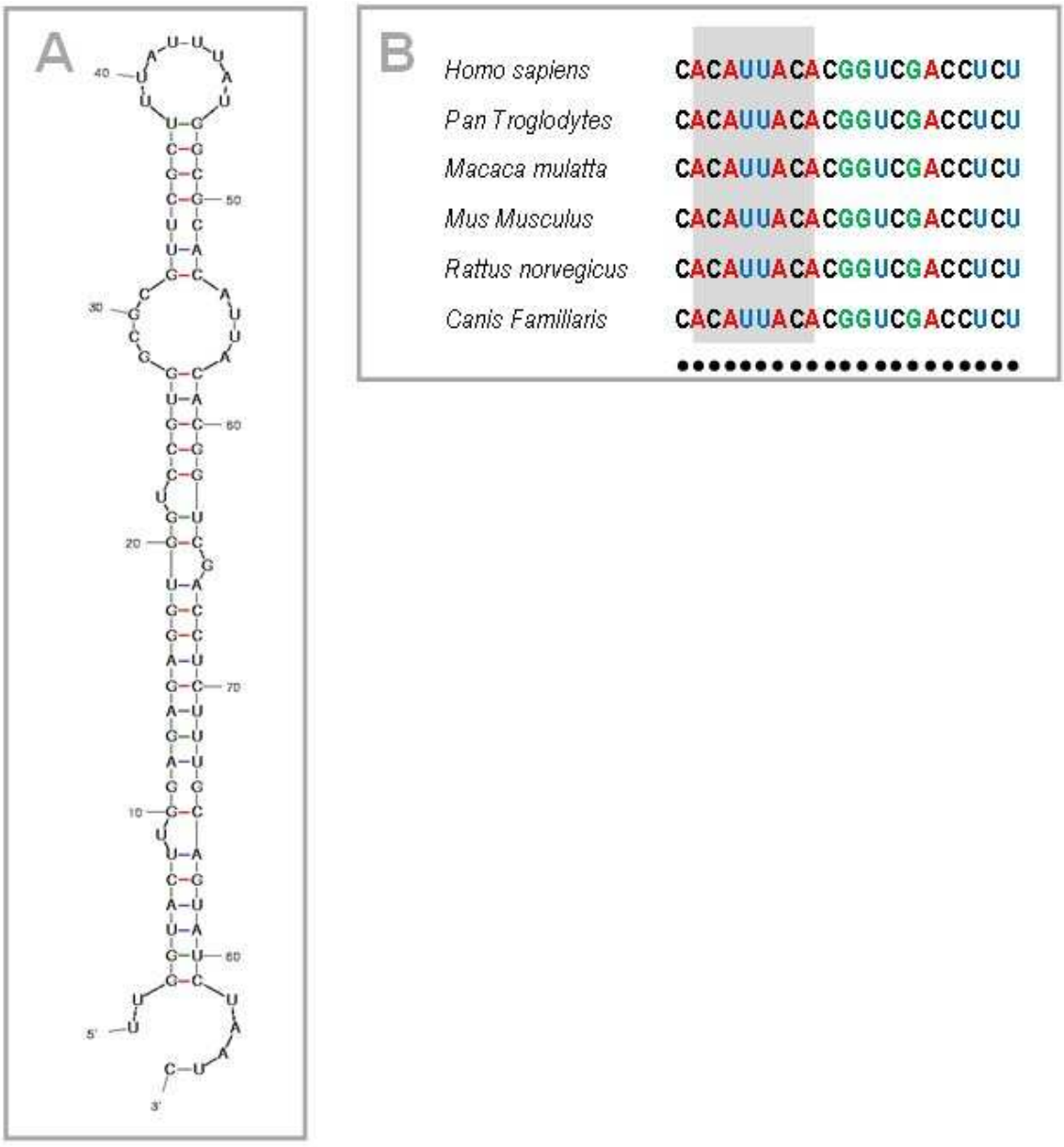


Figure 3.23 *miR-323*

miR-323 is localized on the human chromosome 14, in the position 14q32.31, it belongs to the miR-154 family. ***A.*** *Predicted secondary structure visualization, ΔG = -34.50.* ***B.*** *Analysis of phylogenetic conservation of this microRNA, in grey the seed region.*

3.4 Identification of a new microRNA

An unknown sequence, not yet reported in microRNA database, was found expressed in MG-63 cell line. This putative microRNA, localized in 7p15.2 human chromosome, was called *miR-7 new* (Figure 3.24A). From a structural analysis of pre-miRNA, i.e. harping stability, the sequence of *miR-7 new* showed a ΔG = -45.50 that represents an acceptable stability value (Figure 3.24B). Moreover, a phylogenetic analysis through data bases revealed the sequence conservation (Figure 3.24C).

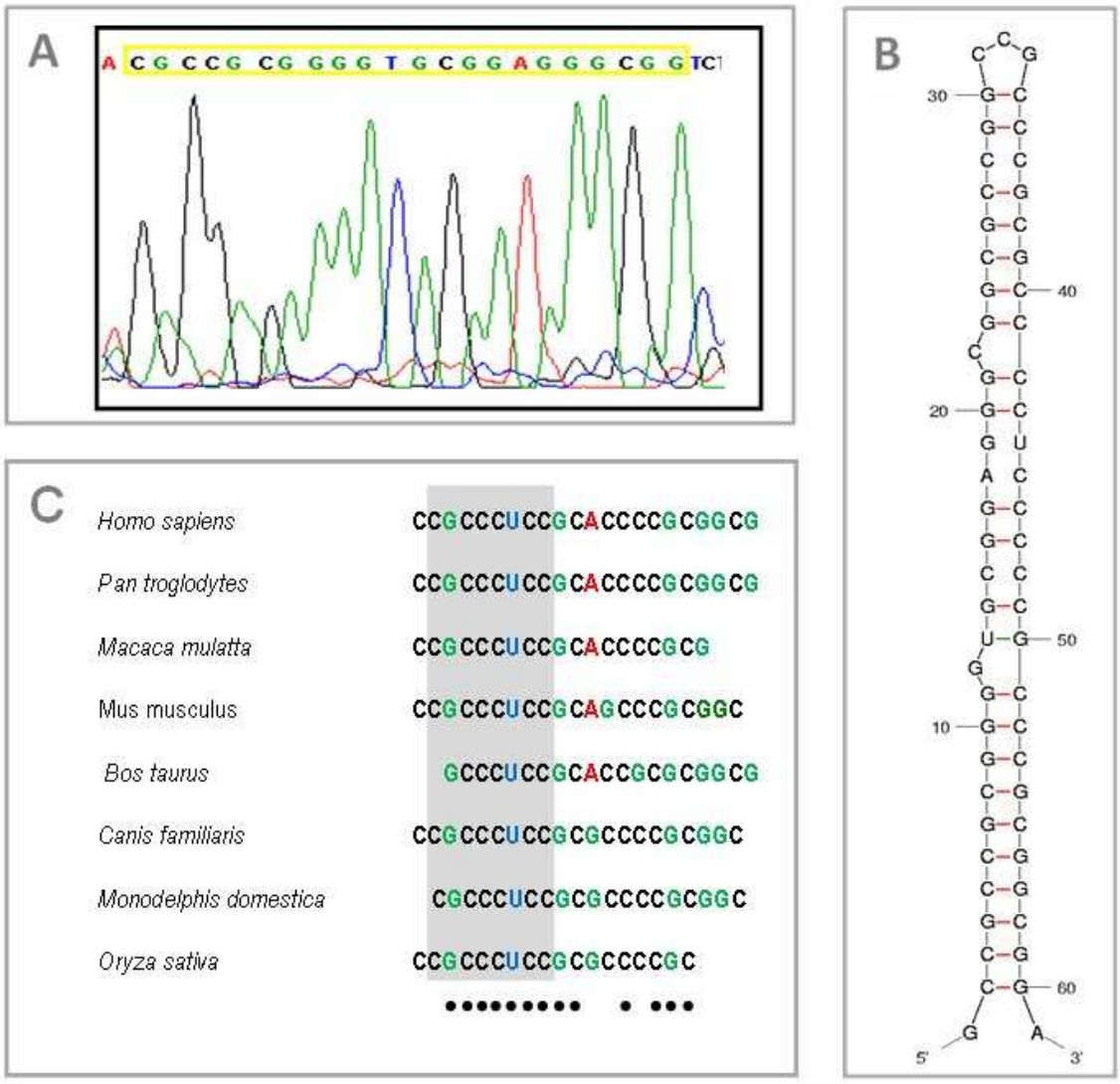


Figure 3.24 *MicroRNA 7 new*

***A**. Electropherogram of a miR-7 new (yellow rectangle). **B.** Predicted secondary structure. **C.** Analysis of phylogenetic conservation of this new putative microRNA.*

3.5 Drosha and Dicer analysis

MicroRNA maturation is reported up to date as being mediated by two enzymes, Drosha in the nucleus and Dicer in the cytoplasm (Hammond, 2005; Lee et al., 2003). A study on the expression of both enzymes was carried out in collaboration with Dr. Claudio Casoli (Dipartimento di Scienze Biomediche – Ospedale "Luigi Sacco" (Milano), Università degli Studi di Milano, Italy). It was found that Drosha was over-expressed in two of the osteosarcoma cell lines (MG-63 and Saos-2) whereas Dicer was found similarly up- regulated in the same two cell lines and down-regulated in 143B osteosarcoma cell line (Figure 3.25).

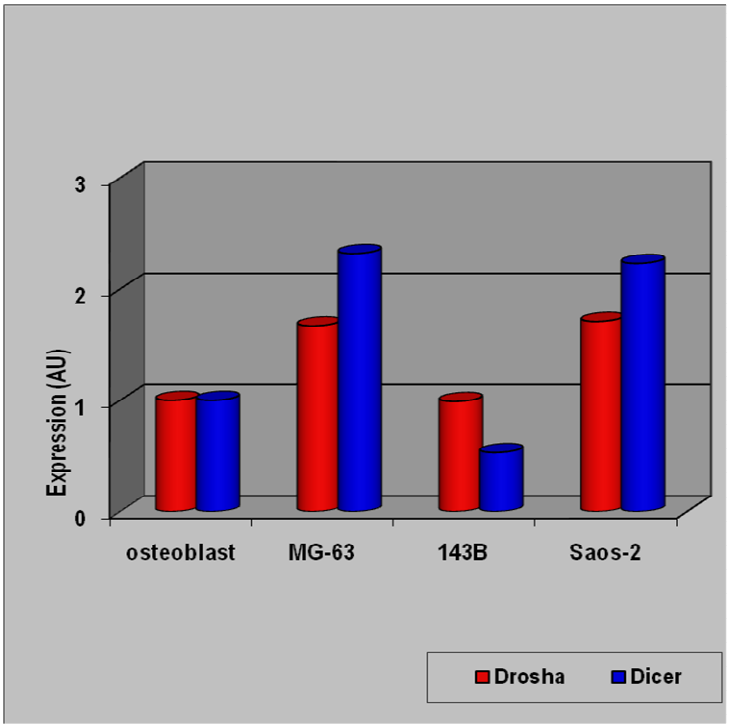


Figure 3.25 ***Drosha and Dicer expression***

Different expression of Drosha and Dicer in osteosarcoma cell line. Osteoblasts were used as reference.

It is interesting to recall the different transendothelial migration kinetics (Figure 3.4) of 143B cells with respect to the other two osteosarcoma lines. There seems to be an apparent relationship between Drosha and Dicer expression and cell migration capability of the cells that needs further investigation.

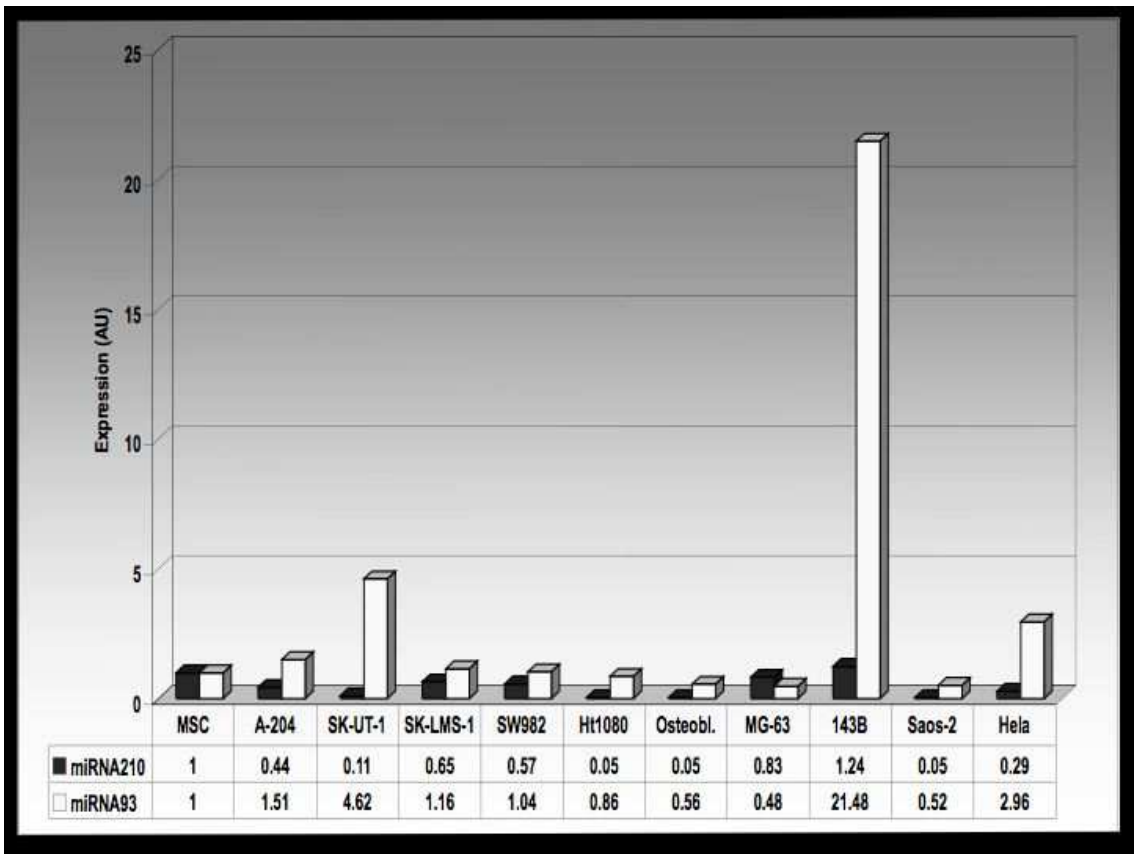
3.6 Characterization of microRNA 93 and microRNA 210

Two microRNAs (*miR-93* and *miR-210*) were further characterized for their expression, cellular function and physiological role.

miR-93 belongs to the oncogenic cluster 17-92, a prototypical example of polycistronic miRNA gene widely previously described (Introduction, 1.4.2.1). MicroRNA 93 is considered as a possible tumoral marker in adult T-cell leukemia (Yeung et al., 2008), in gastric cancer (Petrocca et al., 2008) and in ovarian cancer (Resnick et al., 2009) but is still poorly described in sarcomas.

miR-210 is particularly interesting since it localizes on chromosome 11 in a region regulated by imprinting mechanisms. Furthermore, it has been recently found a link between *miR-210* and breast cancer aggressiveness and metastatic capability (Foekens et al., 2008). *miR-210* is also proposed to have a diagnostic utility in large B-cell lymphoma (Lawrie, 2008) and to play a crucial role in tumour onset as a key regulator of the hypoxia response in epithelial ovarian cancer (Giannakakis et al., 2008).

Cell lines – The expression of the microRNAs was investigated in various tumoral cell lines and in mesenchymal stem cells (MSC) and osteoblasts, as reference cells. The analysis of data reported in Figure 3.26, where MSC was the reference, showed that *miR-210* is generally down-expressed; MG-63 and 143B are quite similar to MSC for this microRNA expression (0.83 and 1.24 respectively) whereas Saos-2 behaviour is like that of the osteoblasts. More heterogeneity was observed for *miR-93* with a maximum of expression for 143B and a minimum for MG-63 and Saos-2, with values similar to that found in osteoblasts.



	MSC	A-204	SK-UT-1	SK-LMS-1	SW982	Ht1080	Osteobl.	MG-63	143B	Saos-2	Hela
miRNA210	1	0.44	0.11	0.65	0.57	0.05	0.05	0.83	1.24	0.05	0.29
miRNA93	1	1.51	4.62	1.16	1.04	0.86	0.56	0.48	21.48	0.52	2.96

Figure 3.26 ***MicroRNA 93 and microRNA 210 expression in tumour cell lines***

Comparison of different levels of miR-93 and miR-210 in tumour cell lines. Mesenchymal Stem Cells (MSC) were used as calibrator.

The expression of the two microRNAs is down-regulated in normal osteoblasts when compared with the expression in MSC, especially for *miR-210*. Since osteoblasts are the more similar to osteosarcoma cells (Campanacci, 1990), the three osteosarcoma cell lines were also compared with osteoblasts for microRNA expression (Figure 3.27). *miR-93* is confirmed to be over-expressed in 143B. Whereas the analysis of microRNA libraries showed *miR-210* as present in 143B but not in MG-63 (Table 3.5), its expression resulted up-regulated in both cell lines in comparison with osteoblasts and Saos-2.

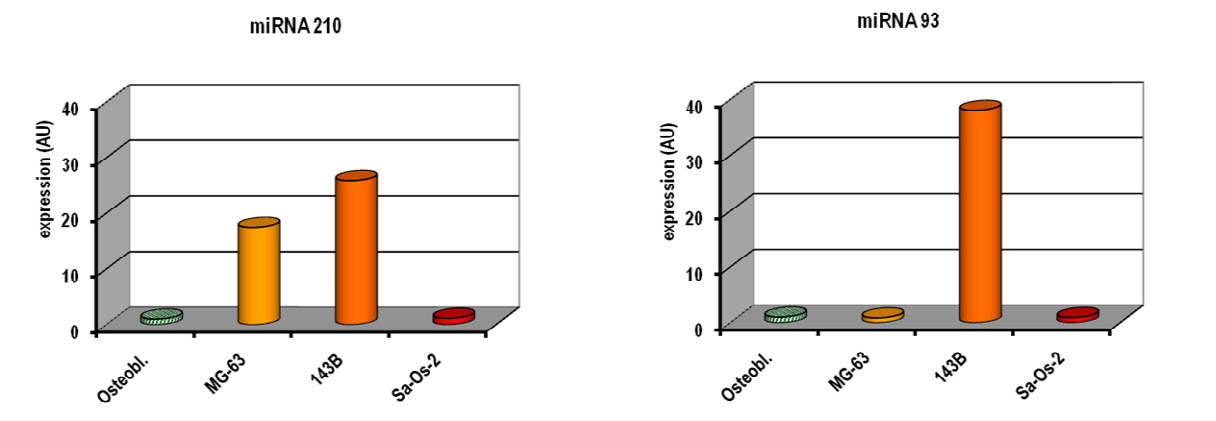


Figure 3.27 ***MicroRNA 210 and microRNA 93 expression in osteosacoma cell lines***

Comparison of the different levels of miR-210 and miR-93 in osteosarcoma cell lines. Osteoblasts were used as reference.

As previously reported, the expression of the two enzymes involved in microRNA maturation, Drosha and Dicer (Figure 3.25) was found to be lower in 143B than in MG-63 and Saos-2. Moreover, the expression of both *miR-93* and *miR-210* was up-regulated in 143B with respect to the other osteosarcoma cell lines. Some considerations can be made. For example, the high expression could be related to some compensatory mechanisms, or to enzyme specificity for these microRNAs, or to a "bias" of the data on enzyme expression resulting from the sampling in a turn-over moment. Further investigations need to be performed to clarify this apparent discrepancy. The findings on cell lines point out a methodological observation. The expression of *miR-210* was not found during the library sequencing in 143B. On the contrary, the same microRNA resulted up-regulated in this cell line when assayed by specific q-PCR. In fact, to consider only some of the longest PCR products for library sequencing could be responsible for the loss of some microRNA expression. Moreover this loss could be related to unusual conformations of the RNA that may reduce the cloning efficiency.

Normal tissues – The expression of the two microRNAs was also investigated in normal tissues from different districts (Table 3.6, Figure 3.28 and Figure 3.29) using the housekeeping gene *U6* as internal control. As expected, the data show wide value dispersion in relationship to the specific tissue for both the microRNAs with a higher expression of *miR-93*.

Table 3.6 *MicroRNA 93 and microRNA-210 expression in normal tissues*

Tissue	U6	miR-93	miR-210	miR-93 arbitrary expression	miR-210 arbitrary expression
Bone marrow	32.07	25.33	29.34	-6.74	-2.73
Skeletal muscle	26.55	0.96	23.14	-3.89	-3.41
Heart	23.05	0.88	21.48	-2.40	-1.57
Stomach	24.27	0.98	23.22	-1.31	-1.05
Intestin	0.93	0.90	23.59	-0.75	0.10
Spleen	1.01	20.56	26.23	-3.12	2.55
Liver	1.00	0.92	24.08	-2.01	0.41
Pancreas	22.56	20.47	24.10	-2.09	1.54
Kidney	23.05	0.88	0.97	-2.44	-0.26
Prostate	1.03	19.12	23.16	-4.86	-0.82
Lung	0.82	15.21	0.81	-3.73	-0.08
Frontal Cortex	25.35	18.22	24.89	-7.13	-0.46
Occipital cortex	27.74	19.32	24.92	-8.42	-2.82
Fetal brain	19.45	14.25	18.17	-5.20	-1.28
Fetal eye	0.97	0.80	20.07	-4.13	-2.74

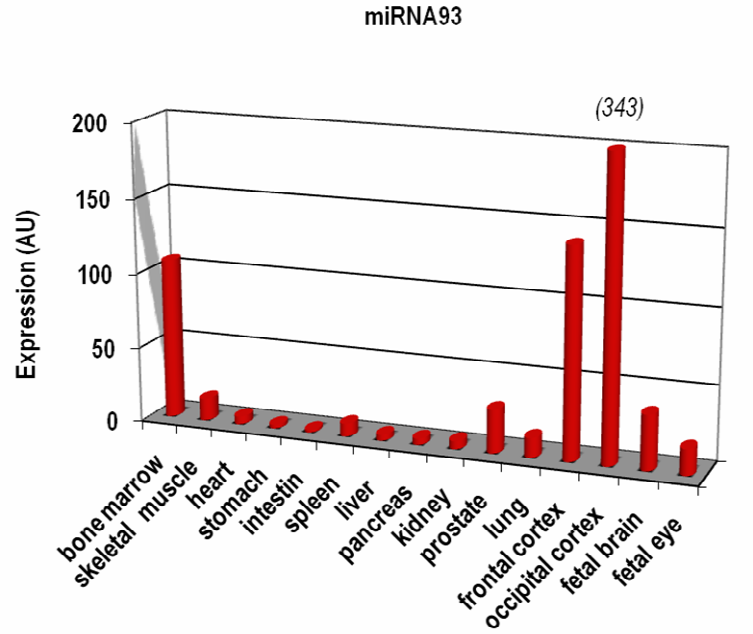


Figure 3.28 *MicroRNA 93 expression in different tissues*

The picture shows the relative expression of each sample not compared with a reference.

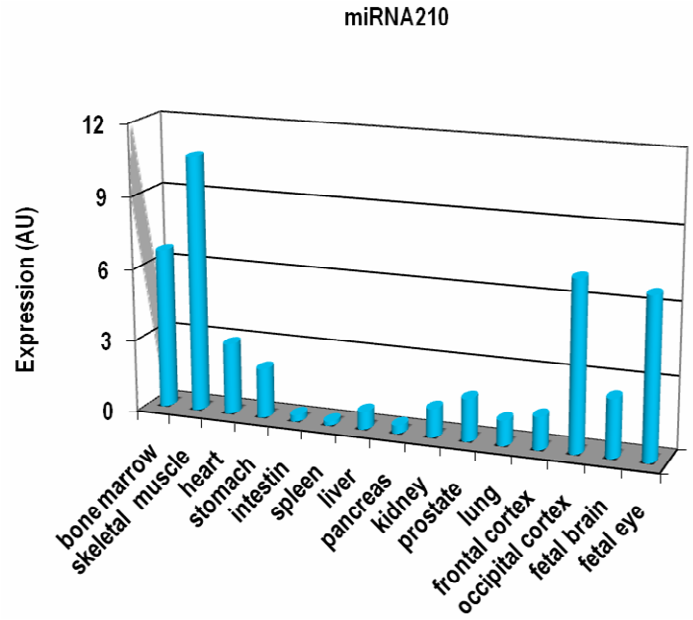


Figure 3.29 *MicroRNA 210 expression in different tissues*

The picture shows the relative expression of each sample not compared with a reference.

Since the supplying firm of analysis kit suggests as good quality samples those that present for *U6* reference a value in the range 22.5-24.5, the data are also reported (Figure 3.30) and analyzed in this context. Both the microRNAs were up-regulated in fetal eye tissue; *miR-93* was also over-expressed in prostate tissue. *miR-210* was weakly expressed in spleen and pancreas.

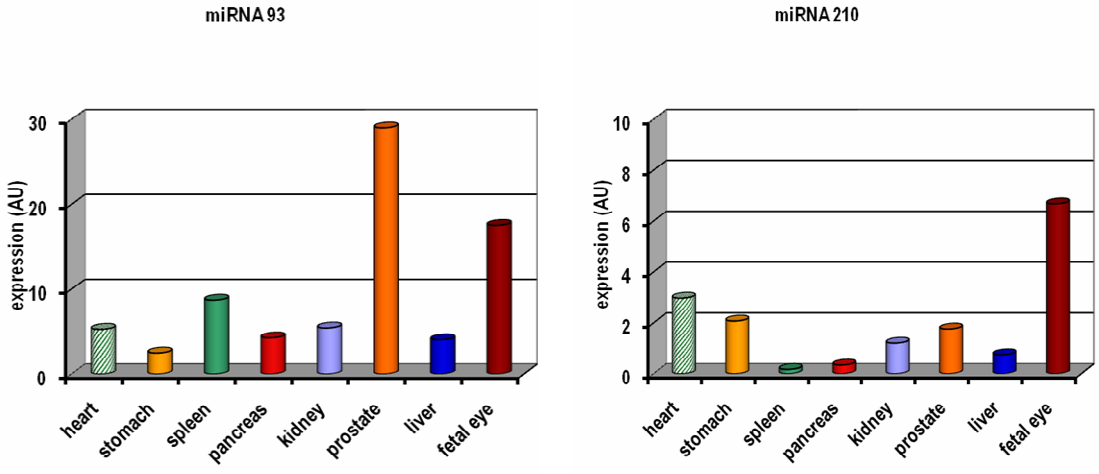


Figure 3.30 *MicroRNA 93 and microRNA 210 expression in different tissues*

The pictures report only the tissue sample with the same U6 thresold of the heart, considerated as a reference for manufactory producer.

3.7 Transduction of microRNAs in osteosarcoma cell lines

The importance of some microRNAs on cell behaviour was investigated on 143B and MG-63 cell lines after infection or transfection through vectors containing *miR-93* or *miR-7 new* (see Mat & Met).

Cell morphology – The infection of 143B cells with, and its consequent over-expression, was able to change cell morphology, depending on the different efficiency in relationship to the clone (Figure 3.31). In particular, the wider changes were observed in the 143B *miR-7 new* 2-3A clone: the cells changed from spindle-shaped to coerce. To understand the importance of this different morphology in relation to the expression, immunocytochemical (focal adhesion kinases) and molecular (target expression) approaches will be carried out.

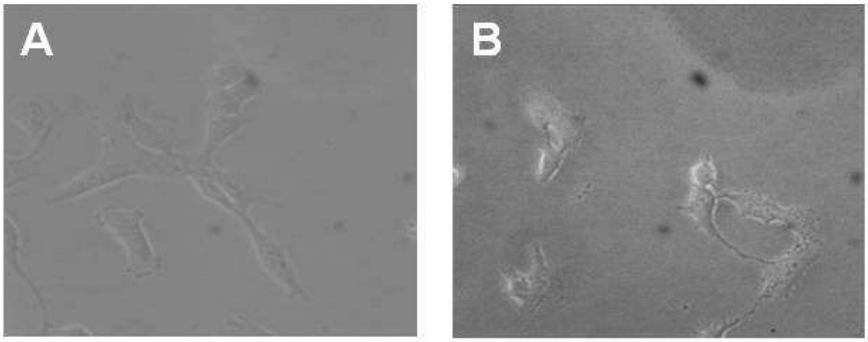


Figure 3.31 ***Morphological change in 143B***

The picture shows morphological changes after miR-7 new over-expression. ***A.*** *143B wild type;* ***B.*** *143B miR-7 new 2-3A clone.*

Proliferation of infected and transfected cell clones – To study the effects of miRNAs on cell proliferation, cell clones of 143B and MG-63 infected/transfected with *miR-93* or *miR-7 new* were tested. The best growing conditions were previously determined for both cell lines using different serum levels (Figure 3.32): previous starvation condition (24 hours at 0% serum) and later 10% serum (A); 1% serum (B); 10% serum (C). A higher proliferation level (about the double in all experimental conditions) was observed in 143B osteosarcoma cell line in comparison with MG-63. Cell growth was affected by serum content restriction (conditions A and B). For this reason, the proliferation assay was carried out at 10% serum condition.

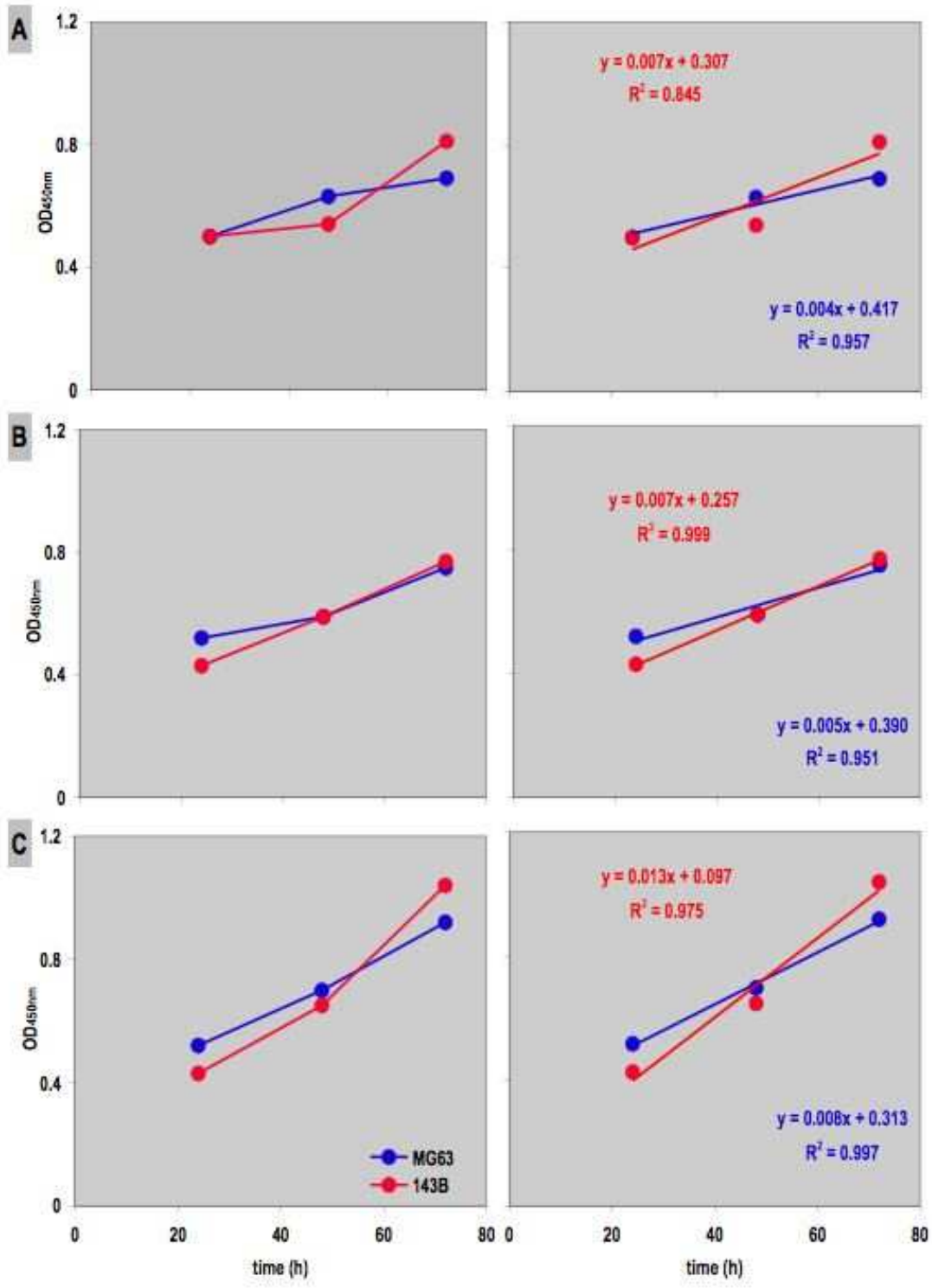


Figure 3.32 *Kinetics and regression analysis of osteosarcoma cells growth*

The panels on the left indicate the kinetics of growth. The right panels describe the regression analysis. ***A.*** *10% serum after 24 hours of starvation;* ***B.*** *1% serum;* ***C.*** *10% serum.*

In 143B, the two *miR-93* clones showed a proliferation index higher than the wild type cells (Figure 3.33A). A similar growth behaviour was displayed by *7 new* 2-3A

clone, the same already mentioned as the widely modified in cell morphology (Figure 3.31). The *7 new* 2-3B clone proliferation did not significantly differ from the wild type one.

For MG-63 the findings were less clear than for 143B (Figure 3.33B) even if it is possible to observe that both the *miR-7 new* clones seem to proliferate less than the wild type MG-63.

The two tumour lines appear to respond in a different manner to microRNA expression. In particular, the over-expression of *miR-93* in 143B (1-4A and 1-4B clones) induces a proliferation increase whereas the proliferation is not significantly modified in MG-63 (B new and DB clones). Considering together these findings with the fact that *miR-93* is over-expressed in 143B wild type cells, but not in MG-63, (Figure 3.27) and the fact that cell proliferation index is about the double in the first than in the last cell line (Figure 3.32), specific 143B molecular targets of *miR-93* for cell replication could be hypothesized. On the other hand, *miR-93* over-expression in MG-63 clones does not significantly alter cell proliferation for a possible lack of specific mRNA targets. The identification of these molecules could support our considerations.

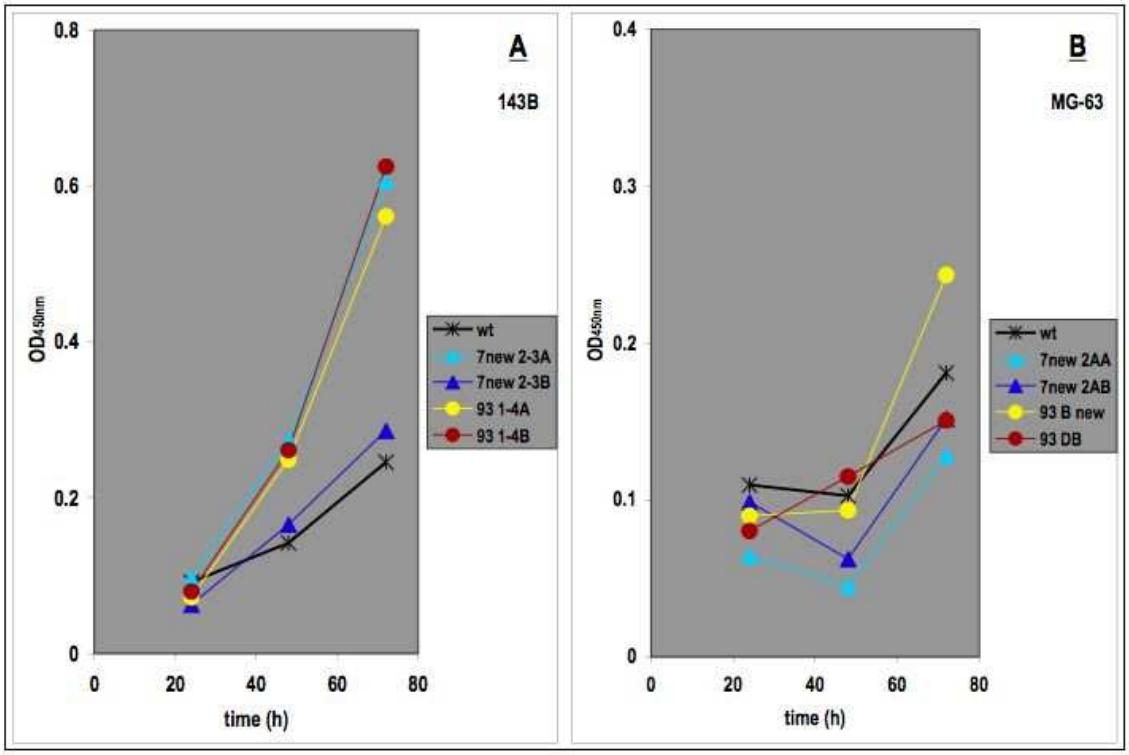


Figure 3.33 *Growth proliferation analysis*

Analysis of growth of different clones containing miR-7 new o miR-93 in comparison to wild type line. ***A.*** *143B;* ***B.*** *MG-63.*

3.8 Targets of *miR-93* and *miR-7 new* in osteosarcoma cell lines

Different approaches were carried out to determine the gene expression changes after insertion of *miR-93* and *miR-7 new* in 143B and MG-63 cells.

In silico analysis - The identification of putative different genes targets derived from the comparison of mRNAs as possible targets of *miR-93* found in different databases (see Mat & Met) and the presence of the same *miR-93*-regulated mRNAs both in human and mouse genome. For further investigations, four of them were chosen in relation to their function in cell physiology:

- ANK2 - Ankyrin 2 encodes a member of the ankyrin family of proteins that link the integral membrane proteins to the underlying spectrin-actin cytoskeleton. Ankyrins play key roles in activities such as cell motility, activation, proliferation, contact and in the maintenance of specialized membrane domains (Hortsch et al., 2002; Le Scouarnec et al., 2008; More et al., 2001; Ogawa et al., 2006).
- ARID4B - AT Rich Interactive Domain 4B (RBP1-like) encodes a protein with sequence similarity to retinoblastoma-binding protein-1. The encoded protein is a subunit of the histone deacetylase-dependant SIN3A transcriptional co-repressor complex, which is involved in several cellular processes including proliferation, differentiation, apoptosis, oncogenesis, and cell fate determination. The gene product is recognized by IgG antibody isolated from a breast cancer patient and appears to be a molecular marker associated with a broad range of human malignancies (Cui et al., 2004).
- TXN1P - Thioredoxin Interacting Protein is a new hypoxia marker in human microendothelial cells (Le Jan et al., 2006). It interacts with reduced thioredoxin modulating the reductive/oxidative cellular state and playing a role in stress-induced cellular apoptosis (Chen et al., 2008a; Pang et al., 2008).
- ZNFX1 - Zinc Finger, NFX1-type containing 1 is a new transcription factor, not much described in the literature. As previously reported for other transcription factor (Romania et al., 2008; Tazawa et al., 2007), it could be modulated by microRNAs.

q-PCR - These mRNA were investigated on 143B infected (Figure 3.34) and MG-63 transfected (Figure 3.35) clones.

The 143B mir-93 clones were tested (Figure 3.34) for mRNA expression (see Mat& Met). The comparison with the wild type points out a similar expression of clone mir-93-4A for ANK2 and mir-93-4B for ZNFX1. Very high expression of ARID4B and TXN1P was detected in mir-93-4B, whereas the same clone showed down-regulation for ANK2. The mir-93-4A clone over-expressed TXN1P and was down regulated for ARID4B and ZNFX1. The data, which showed great expression variability in both the clones and the genes, did not permit to establish the importance of the four mRNA as *miR-93* possible targets in this cellular type.

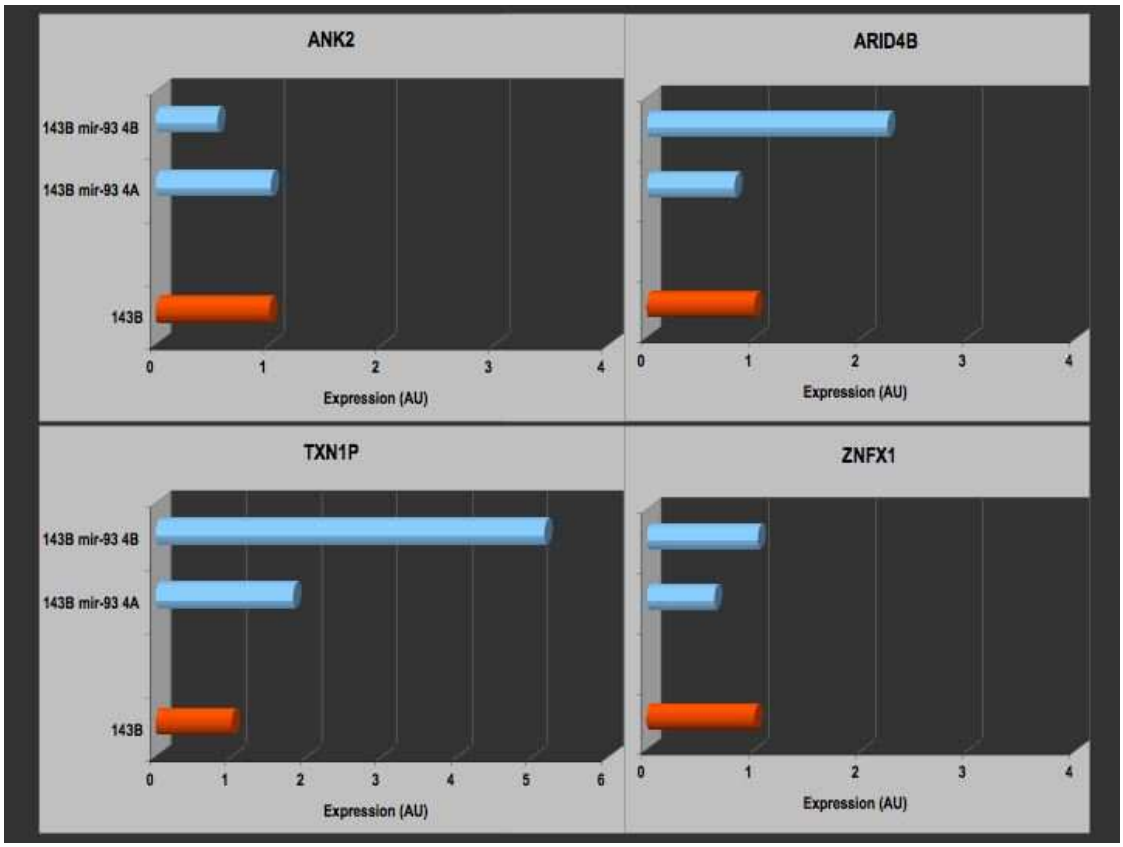


Figure 3.34 ***Analysis of candidate genes in 143B miR-93 clones***

Graphical representation of arbitrary expression of candidate genes in 143B miR-93 clones in comparison with 143B wild type osteosarcoma cells.

The analysis of data on MG-63 cell line (Figure 3.35) showed an “opposite” behaviour of the two clones with respect to the wild type: when a gene over-expression was present in one clone, a down regulation was found in the other, and viceversa. The

over-expression of *miR-93* caused a strong down-regulation of TXN1P in MG-63 mir-93 DB clone, which, on the other hand, showed an overamplification of ANK2, ARID4B and ZNFX1. In the MG-63 mir-93 B new clone, whereas high TXN1P expression was detected, the microRNA appeared to induce a more or less evident down-expression for ANK2, ARID4B and ZNFX1.

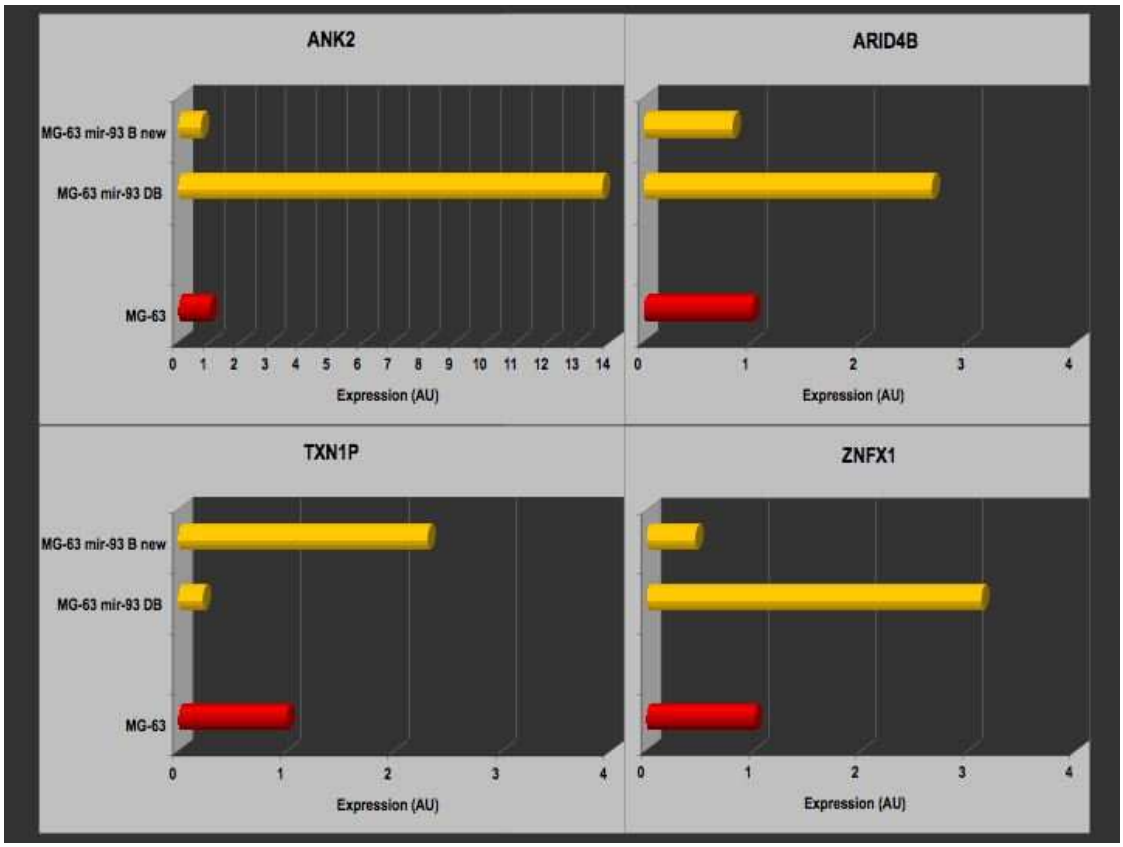


Figure 3.35 ***Analysis of candidate genes in MG-63 miR-93 clones***

Graphical representation of q-PCR of candidate genes in the different clones transfected with miR-93. MG-63 wild type line was used as a reference.

The relevance of *miR-93* on the expression of the considered gene appears controversial in MG-63 as well as in 143B cell line. The sampling "bias" during a RNA turn-over without microRNA 93 expression could explain these results. Further investigations, using siRNA and other analysis on more clones, must be performed to clearly determine if *miR-93* could target these different genes in the same tumour type context.

Microarray - To understand the involvement of *miR-93* and *miR-7 new* in physiological cell pathways, microarray analysis for every microRNA was carried out using the total RNA extracted from each stable microRNA clones and wild type cells. The RNA quality was determined as described in Mat & Met (Figure 3.36).

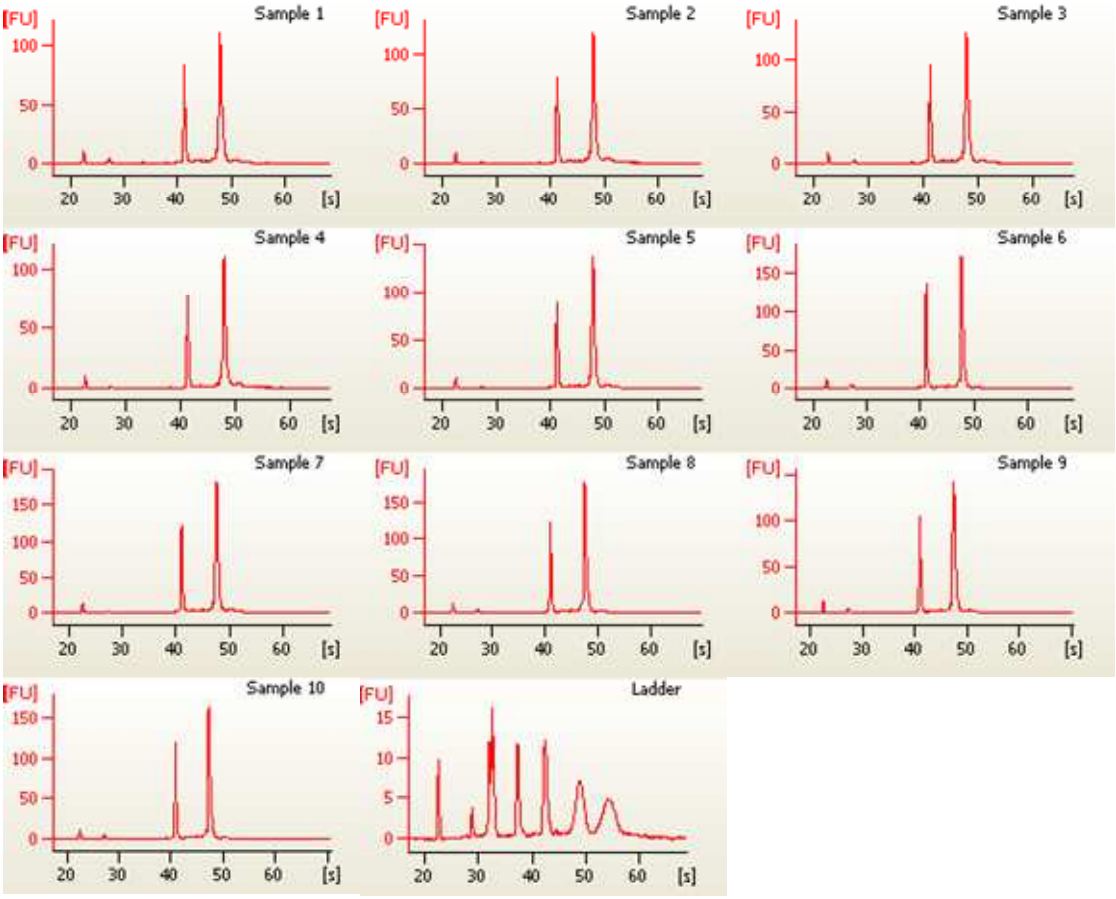


Figure 3.36 ***BioAnalyzer analysis***

Graphical representation of RNA test quality of analyzed samples. Every sample shows the two peaks typical of ribosomal RNA.

Microarray analysis suggests that both microRNAs could control different gene classes (Table 3.8, Table 3.9) pointing out the great relevance of *miR-93* and *miR-7 new* in cellular pathways.

Table 3.8 *Candidate target gene of miR-93*

Gene number	description
NM_024833	zinc finger protein 671 (ZNF671), mRNA
NM_001017372	solute carrier family 27 (fatty acid transporter), member 6 (SLC27A6), mRNA
NM_080672	phosphatase and actin regulator 3 (PHACTR3), mRNA
NM_020686	4-aminobutyrate aminotransferase (ABAT), mRNA
NM_001017424	potassium channel, subfamily K, member 2 (KCNK2), mRNA
THC2376418	voltage-gated calcium channel alpha(2)delta-3 subunit, (Q8IZS8), mRNA
NM_016358	iroquois homeobox protein 4 (IRX4), mRNA
NM_014867	kelch repeat and BTB (POZ) domain containing 11 (KBTBD11), mRNA
NM_014839	plasticity related gene 1 (LPPR4), mRNA
THC2311329	Hexaribonucleotide binding protein 3 (Q8VI61), mRNA
NM_182898	cAMP responsive element binding protein 5 (CREB5), mRNA
NM_006472	thioredoxin interacting protein (TXN1P), mRNA
NM_006125	Rho GTPase activating protein 6 (ARHGAP6), mRNA

Table 3.9 *Candidate target gene of miR-210*

Gene number	description
NM_001003395	tumor protein D-52-like1 (TPD52L), mRNA
NM_016358	iroquois homeobox protein 4 (IRX4), mRNA
NM_001546	inhibitor of DNA binding 4 (ID4), mRNA
NM_024016	homeobox B8 (HOXB8), mRNA
NM_017943	F-box protein 34 (FBXO34), mRNA
NM_004613	transglutaminase 2 (TGM2), mRNA
NM_183045	ring finger protein (C3H2C3 type) 6 (RNF6), mRNA
NM_002609	platelet-derived growth factor receptor (PDGFRB), mRNA
NM_198974	protein tyrosine kinase 9 (PTK9), mRNA

The data obtained with microarray permit to identify a lot of genes down regulated by the *miR-93*. TXN1P, a gene previously assayed by q-PCR in the two osteosarcoma cell lines 143B and MG63, appears within these targets. The PCR assay detected a down-regulation only in MG63 mir-93 DB clone but not in 143B clones. These findings suggest that *miR-93* could act with a cell type-dependent specificity.

The two microRNAs seem to control specific gene targets (Figure 3.37). In particular, *miR-93* appears to interact with genes relative to “ion channels” and “transporters” and *miR-7 new* with those relative to “receptors” and “oncogenes/tumour suppressors (OG/TS)”. Further investigations are needed to unravel the role of these microRNAs inside the cell.

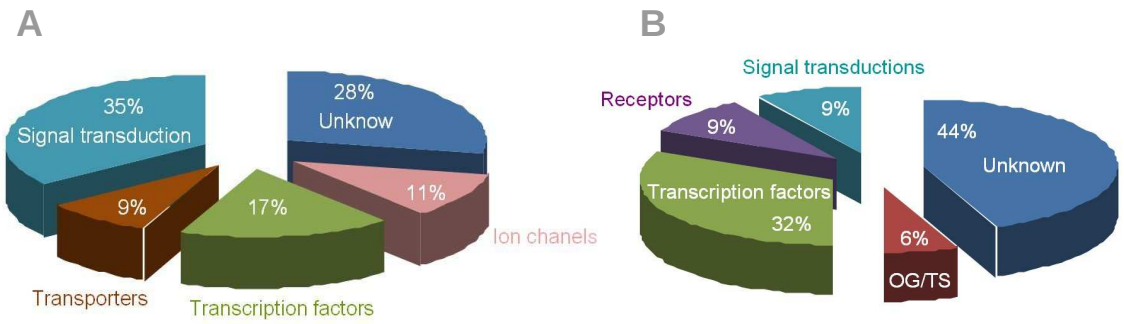


Figure 3.37 ***Microarray results***

*Graphical representation of different classes of regulatory gene probably modulated by **A.** miR-93 and **B.** miR-7 new.*

Detection of functional mRNA targets - The new method published by Vatolin and colleagues (Vatolin et al., 2006) was applied to identify the targets of *miR-93*. The data obtained by comparison with Blast database showed that all the amplified sequences of the purified fragments always aligned with mRNA of Tat, a protein involved in Acquired Immune Deficency Syndrome (AIDS), independently from their length. These data suggest that *miR-93* inside osteosarcoma cells has only a target gene. These results are unexpected because it is known that microRNA normally regulates the expression of hundreds of target genes. A possible reason for these findings could be due to the tight parameters used for PCR: in this way not only the seed region but a large portion of or the whole microRNA fully paired with a complementary no-target mRNA.

To unravel the question, new experiments with weaker PCR condition have to be carried out.

3.9 Towards the development of a new intravital microscopy assay for the analysis of lymphatic intravasation

Lymphatic invasion of tumour cells is a fundamental step in the metastatic process, but little is known about the mechanism of this phenomenon and the factors that may control it. Although several intravital microscopy systems are available for the real-time assessment of haematic extravasation no system is currently available for the analysis of lymphatic intravasation. In the effort to provide such system, an intravital microscopy approach was exploited in collaboration with the group of Prof. Daniela Negrini (Dipartimento Scienze Biomediche Sperimentali e Cliniche – Università degli Studi dell'Insubria, Varese – Italy). The technique allows to monitor cell capability to

spontaneously invade the lymphatic vessels of rat pleural diaphragmatic cavity (Cahalan et al., 2002). A first experiment was carried out using syngenic osteosarcoma cell line UMR-106 to avoid the immunological complications due to species differences between human and rat tumour cell lines. Diaphragm and lung were observed after two different times (24h and 30h) since sarcoma cell injection (Figure 3.38).

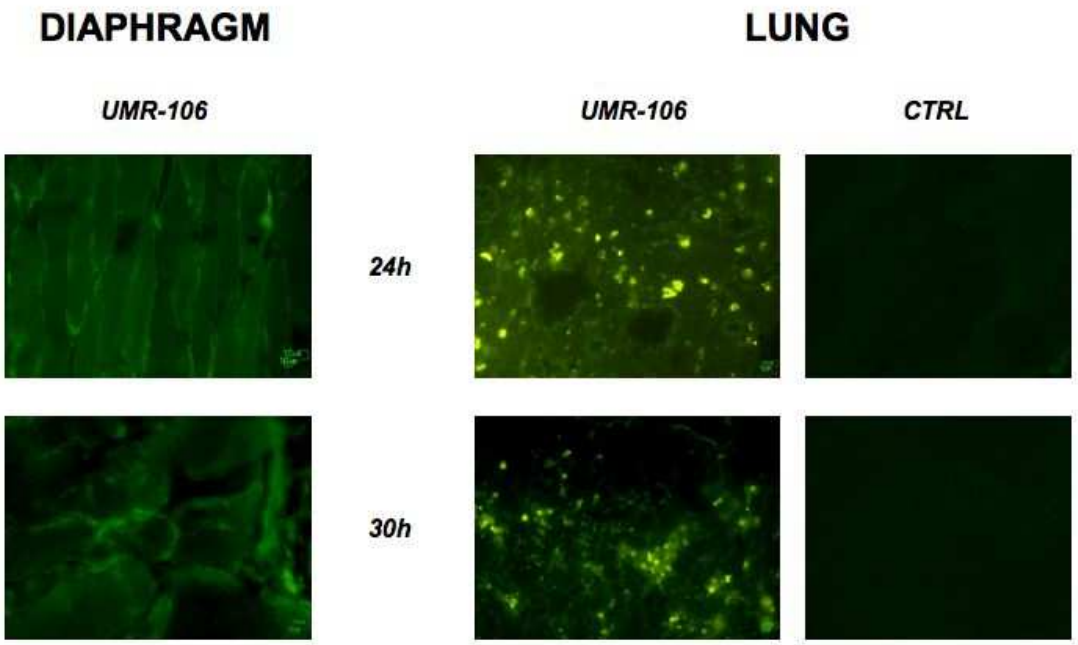


Figure 3.38 *UMR-106*

Diaphragm (left) and lung (right) analysis after UMR-106 injection.

As shown in Figure 3.38, the presence of sarcoma cells in the lung, but not in the diaphragm, after only 24 hours following cell inoculation, suggested that the cells crossed the diaphragm lymphatic vessels very quickly. Although care was taken to avoid to insert cells directly in to the lungs, this possibility could not entirely be excluded and the consistency of the results at different time intervals also suggested that this error was not likely.

To confirm the great capability of tumour cells to quickly spread through lymphatic vessels, the monitoring of injected gliosarcoma cells (C6), known as highly aggressive cells (Dexter et al., 1983), was also performed at shorter time (12 hours) in diaphragm (Figure 3.39) of other animals. Whereas it was possible to note the presence of tumour cells in the diaphragm at 12 hour post cell injection, the images after 24 and 48 hours suggested cell migration from diaphragm.

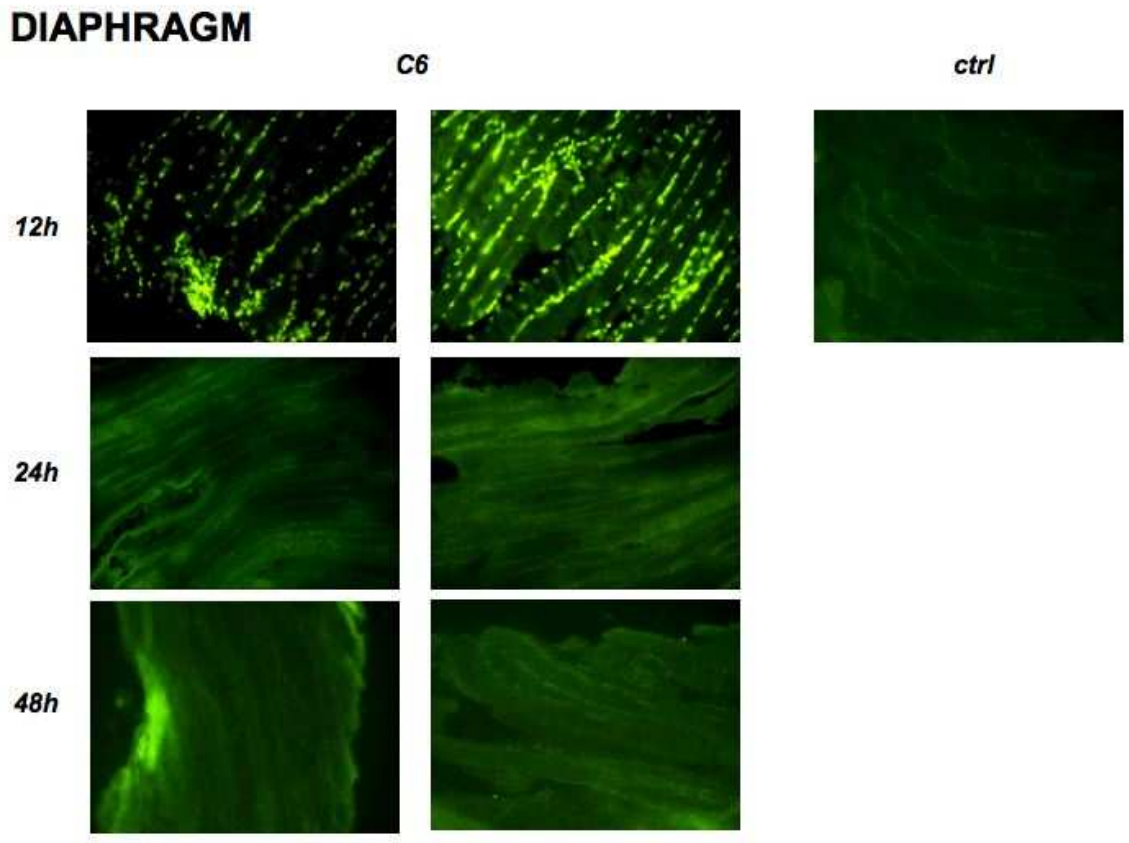


Figure 3.39 *C6*

Different time of analysis of different diaphragms Only into 12 hours after injection of C6 tumoural cells, the cells are detectable in the diaphragm.

These first results hinted to a quick spreading of tumoral cells using a diaphragmatic lymphatic loop able to supply a low-resistance pathway for fluid (and probably cell) drainage from the serous cavities (Moriondo et al., 2008).

To further demonstrate the applicability of the above intravital microscopy assay to monitor the tumour cell aggressiveness and their ability to invade lymphatic vessels, another experiment was carried out (Figure 3.40) using murine melanoma cells (B16) stably transfected with vector (B16-mock) or a vector containing NG2 proteoglycan (B16-NG2). B16 is a very aggressive cell line (Dexter et al., 1983; Maiorana et al., 1992), NG-2 is a membrane proteoglycan involved in cell motility (Burg et al., 1998; Burg et al., 1997; Eisenmann et al., 1999; Fang et al., 1999) and in cellular responses to growth factors (Goretzki et al., 1999; Grako et al., 1999; Grako and Stallcup, 1995; Nishiyama et al., 1996) that could alter cell spreading efficiency. After 12 hours from injection (Figure 3.23A) only B16-mock were still present inside the diaphragmatic wall whereas the B16 clone with stable expression of NG2 was less detectable in this area.

In 24 hour-samples (Figure 3.40B), green melanoma cells were not found in any of the two models.

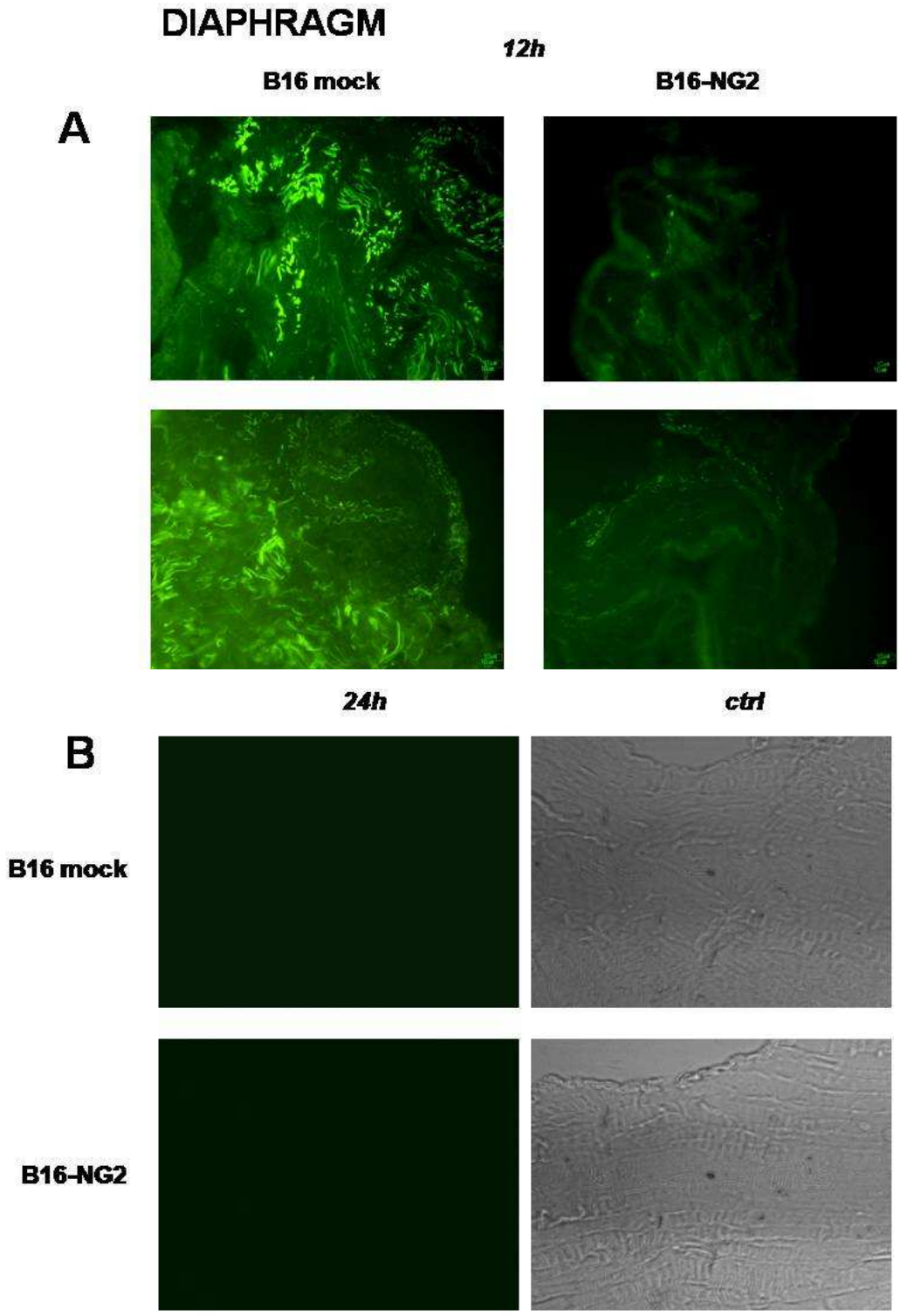


Figure 3.40 *B16-NG2 cells seem to be already poorly present in diaphragm after 12 hours of injection.*

Thus NG2 over-expression seemed to increase the spreading of tumour cells through the lymphatic vessels, recently emphasized as an exclusive stream for metastasis formation (Azzali, 2007), suggesting a putative role of NG2 in this process.

CONCLUSIONS

The importance of small RNAs in the control of biological processes is rapidly increasing (Aboobaker et al., 2005; Abrahante et al., 2003; Alvarez-Garcia and Miska, 2005; Chang et al., 2004; Chen et al., 2004; Fazi et al., 2005; Giraldez et al., 2006; Grosshans et al., 2005; Hornstein et al., 2005; Johnston and Hobert, 2003; Lin et al., 2003; Naguibneva et al., 2006; Poy et al., 2004; Schratt et al., 2006; Sokol and Ambros, 2005; Wienholds et al., 2005; Zhao et al., 2005). In particular microRNAs have been described as potential biomarkers in different diseases (Calin et al., 2005; Foekens et al., 2008; Huang et al., 2008; Ma et al., 2007; Schetter et al., 2008; Takamizawa et al., 2004; Tavazoie et al., 2008) because their over- or down-regulation may modulate the expression of important cellular factors involved in metabolism, apoptosis and cancer.

- Four microRNAs, found to be up regulated in three osteosarcoma cell lines were further analyzed in surgical samples of low and high grade osteosarcoma patients. *miR-484* in both osteosarcoma grade was down regulated and has the potential of being a marker of these diseases. In high grade osteosarcoma another microRNA, *miR-183*, was similarly down regulated. *miR-484* and *miR-183* together, could be putative biomarkers of high grade osteosarcoma and a wider number of cases is now under examination to establish a possible prognostic significance of this differential microRNA expression.
- In a transendothelial migration assay using HUVEC cells, the three osteosarcoma cell lines showed a distinct migration capability, different from that of other tumour cell lines tested. In particular Saos-2 and MG-63 cells showed the same behaviour, whereas 143B cells crossed the endothelial layer more slowly than the other two osteosarcoma lines. Moreover, 143B grew faster than MG-63. Using other endothelial systems, for example lymphatic endothelial cells monolayer, for transmigration assay may produce different results.
- Two microRNA libraries were created using RNAs from 143B and MG-63 osteosarcoma cell lines. microRNA sequencing allowed the identification of an unknown sequence, named *miR-7 new*, localized on chromosome 7 that shows the characteristic of a putative microRNA. Some microRNAs described in the databases were identified in both the two lines. Some other, for example *miR-210*, seemed to be characteristic of one of the two cell lines. Otherwise, the more careful analysis with qRT-PCR identified the presence of *miR-210* in both cell types. This finding points out a methodological observation: to consider only some of the longest PCR products for library sequencing could be responsible for the loss of some microRNA expression.

Moreover this loss could be related to unusual conformations assumed by RNA that may reduce the cloning efficiency.

- The presence of two microRNAs, *miR-93* and *miR-210*, was detected both in various tumoral cell lines and normal tissues. A specific expression was found in relation to the different cell lines and tissues. In particular *miR-93* is over-expressed in 143B cell line and in brain tissues.
- Several approaches (infection/tranfection, in silico analysis, qRT-PCR, DNA microarrays) have been undertaken to determine the importance of *miR-93* and *miR-7 new* in biological and molecular pathways. *miR-7 new* seemed to be involved in the control of cell morphology. Studies are in progress to understand their gene targets.
- To be able to address the possible role of microRNA in the control of metastasis formation and in particular the steps entailing entrance of the cells into the lymphatic circuits, an effort was made to devise a new intravital microscopy approach. Cells of several tumour types with different aggressiveness were used to define the usefulness of this system. The system seemed to be able to define times, factors and molecules potentially involved in the lymphatic intravasation process.

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