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# ROLE OF MICRORNAS IN T-CELL ACTIVATION AND TRANSFORMATION BY HUMAN T-CELL LEUKEMIA VIRUS TYPE 1

**Direttore della Scuola: Ch.ma Prof.ssa Paola Zanovello**

**Supervisore: Dott. ssa Donna D'Agostino**

**DOTTORANDA: Dott.ssa Katia Ruggero**

Ai miei genitori

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# ABSTRACT

Human T-Lymphotropic virus type 1 (HTLV-1) is the causative agent of two distinct pathologies, adult T-cell leukemia/lymphoma (ATLL), an aggressive malignancy of mature CD4+ T-cells, and tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM), a demyelinating neurodegenerative disease. Despite intense study, many aspects of HTLV-1 replication, persistence and pathogenesis remain to be understood. The work described in the present thesis was aimed at defining the role of microRNAs (miRNAs) in HTLV-1 infection and ATLL pathogenesis.

We generated small RNA libraries from normal CD4+ cells (resting and stimulated) and two T-cell lines chronically infected with HTLV-1 (MT-2 and C91PL). Libraries were analyzed by 454 mass sequencing and data were processed through a series of computational steps to identify known and candidate new miRNAs for each library. Comparison of frequencies of known miRNAs in the different libraries led to the identification of 14 downregulated miRNAs and 4 upregulated species in infected cell lines vs. resting CD4+ cells, while 21 miRNAs were differentially expressed (16 downregulated, 5 upregulated) in stimulated compared to resting CD4+ cells. We validated the expression of some new miRNA candidates identified by bioinformatic analysis of the libraries through end point and quantitative RT-PCR. Two sequences mapped to the HTLV-1 genome, suggesting that the virus may produce its own miRNAs under certain conditions.

We examined the profiles of known miRNA expression in ATLL cells and normal resting and activated T CD4+ lymphocytes using microarrays. On the basis of miRNA expression, cluster analysis of ATLL samples and CD4+ controls showed that the resting controls were highly related to each other, while the tumor samples exhibited some heterogeneity. Statistical analysis revealed 6 upregulated and 21 downregulated miRNAs in ATLL cells compared to CD4+ T-cell controls. Several of the differentially expressed miRNAs identified in the libraries and by microarray analysis were validated by real time RT-PCR.

Since miRNA-mRNA interactions often result in degradation of the target mRNA, integration of results from target prediction programs with expression profiles for miRNAs and mRNAs can aid in identifying genuine mRNA targets. This approach

was applied to miRNA and mRNA microarray data obtained for our ATLL and resting CD4+ samples. Potential targets for 12 miRNAs differentially expressed in ATLL cells were identified by integrating miRNA and mRNA expression profiles. Functional enrichment analysis of predicted targets revealed the presence of several genes belonging to the cAMP signalling pathway, which is known to be activated upon HTLV-1 transformation. We also investigated the role of miR-34a, consistently upregulated in ATLL samples and HTLV-1 infected cells lines. Knockdown of miR-34a in infected cell lines determined an increased in cell death, suggesting that miR-34a could play an important role in the expansion of HTLV-1 infected cells and thereby in ATLL development.

# RIASSUNTO

Il virus T-linfotropico umano di tipo 1 (HTLV-1) è l'agente eziologico della leucemia/linfoma a cellule T dell'adulto (ATLL, adult T-cell leukemia/lymphoma) e della paraparesi spastica tropicale/mielopatia associata ad HTLV (TSP/HAM, Tropical spastic paraparesis/HTLV-associated myelopathy), una patologia degenerativa del sistema nervoso centrale.

Recenti evidenze suggeriscono che i microRNA (miRNA) contribuiscano a questo processo di trasformazione mediata da HTLV-1. Le ricerche condotte nel corso del mio dottorato sono state mirate ad approfondire il ruolo dei microRNA (miRNA) nell'infezione di cellule T da parte di HTLV-1 e nella patogenesi dell'ATLL.

Sono state realizzate librerie di cDNA di piccoli RNA, a partire da linfociti T CD4+ normali (resting e attivati) e da due linee cellulari cronicamente infettate con HTLV-1 (C91PL e MT-2). Le librerie sono state analizzate attraverso il sequenziamento di massa 454 e l'analisi bioinformatica delle sequenze ottenute ha permesso l'identificazione dei miRNA noti e nuovi miRNA candidati presenti in ciascuna libreria. Il confronto delle frequenze dei miRNA noti nelle diverse librerie ha evidenziato la presenza di 14 e 4 miRNA rispettivamente downregolati e upregolati nelle linee cellulari infettare rispetto ai linofociti T CD4+ resting, mentre 21 miRNA sono risultati differenzialmente espressi in linfociti T CD4+ stimolati in confronto ai linfociti T CD4+ resting (16 downregolati, 5 upregolati). L'espressione di diversi nuovi miRNA, individuati dall'analisi bioinformatica delle librerie, è stata validata attraverso RT-PCR end-point o RT-PCR quantitativa. Inoltre la nostra analisi ha rivelato nelle librerie da cellule infettate 2 sequenze che mappano in regioni trascritte del genoma di HTLV-1 e che potrebbero rappresentare dei miRNA virali.

Attraverso l'impiego di microarray il profilo di espressione dei miRNA noti è stato analizzato in pazienti ATLL e in linfociti T CD4+ resting e stimolati. In base ai profili di espressione di miRNA ottenuti i campioni sono stati raggruppati in cluster che indicano una forte similitudine all'interno dei campioni di linfocititi T CD4+ resting, mentre i campioni di ATLL hanno profili di espressione di miRNA più eterogenei. L'analisi statistica ha evidenziato 21 miRNA downregolati e 6 upregolati nei pazienti ATLL vs linfociti T CD4+ resting. Diversi miRNA differenzialmente

espressi identificati attraverso l'analisi delle librerie e dei microarray sono stati validati tramite RT-PCR quantitativa.

Dal momento che l'interazione miRNA-mRNA spesso comporta la degradazione del messaggero bersaglio, l'analisi integrata dei risultati dei programmi di predizione di bersagli con i profili di espressione di miRNA e geni può aiutare nell'identificazione di target. Abbiamo applicato questo approccio ai dati di espressione di miRNA e geni ottenuti per i nostri campioni di ATLL e linfociti T CD4+ resting. Dall'integrazione dei profili di espressione di miRNA e mRNA sono stati identificati i target putativi per 12 miRNA differenzialmente espressi nei pazienti ATLL. L'arricchimento funzionale dei geni bersaglio predetti ha evidenziato la presenza di diversi geni coinvolti nella via di segnale di cAMP, noto per essere presente ad alti livelli in cellule trasformate da HTLV-1.

Infine abbiamo indagato il significato funzionale di miR-34a, che risulta essere consistentemente upregolato in pazienti ATLL e linee cellulari infettate. Il silenziamento di miR-34a in linee cellulari infettate determina un aumento della morte cellulare, suggerendo che la deregolazione di questo miRNA possa svolgere un ruolo importante nell'espansione della popolazione di cellule infettate da HTLV-1 e quindi nello sviluppo dell'ATLL.

# 1. INTRODUCTION

## 1.1 HUMAN T-LYMPHOTROPIC VIRUS TYPE 1: TAXONOMY, EPIDEMIOLOGY AND PATHOGENESIS

Human T-lymphotropic virus type 1 (HTLV-1) was the first human retrovirus to be identified and is the only one with established oncogenic properties (Poiesz et al., 1980; Hinuma et al., 1981).

HTLV-1 belongs to the Retroviridae family, Oncovirinae sub-family, Deltaretrovirus genus, which also includes HTLV-2, -3, -4, the simian T-lymphotropic viruses (STLVs), and bovine leukemia virus (BLV). STLV and BLV infections are associated with neoplastic diseases, while the pathogenicity of HTLV-2, -3, -4 has not been clearly established (Araujo and Hall, 2004; Feuer and Green, 2005; Mahieux and Gessain, 2009). Deltaretroviruses are referred as "complex" retroviruses. In fact, they present at the 3' end of the genome the so called "X region" that encodes the regulatory proteins Tax and Rex and different accessory proteins. HTLV-1 infects about 20 million people worldwide and is endemic in South-western Japan, Central Africa, the Caribbean Basin, Central and South America and the Melanesian Islands. Sporadic infection occurs in Europe and North America. Transmission of the virus may occur in a "vertical" manner from mother to newborn (e.g. mainly through breastfeeding and in few cases during gestation or peripartum), or "horizontally" through exchange of biological fluids (e.g. sexual contact and parenteral transmission) (Proietti et al., 2005).

HTLV-1 is the causative agent of two pathologies, adult T-cell leukemia/lymphoma (ATLL), an aggressive malignancy of mature CD4+ T-cells that is extremely refractory to current therapies (Uchiyama et al., 1977; Tsukasaki et al., 2009), and tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM), a demyelinating neurodegenerative disease (Gessain et al., 1985; Osame et al., 1986). In addition, accumulating evidence supports an association between HTLV-1 infection and a number of chronic inflammatory diseases such as uveitis (Mochizuki et al., 1992; Pinheiro, 1995), arthropathy (Murphy et al., 2004; Yakova et al., 2005) and infective dermatitis (LaGrenade et al., 1990).

The majority of HTLV-1-infected individuals remain asymptomatic throughout life; only 2-5% develop ATLL or TSP/HAM after a latency period of decades or several years, respectively (Kawano et al., 1985). In spite of over 30 years of study, the molecular mechanisms determining ATLL or TSP/HAM have not been fully clarified.

ATLL is classified into four clinical forms: acute, chronic, smouldering and lymphoma (Tsukasaki et al., 2009). In some cases the acute phase of ATLL is preceded by peripheral lymphocytosis characterized by poly- or oligoclonal integration of the viral genome. Acute ATLL is characterized by the presence of a dominant proliferating leukemic clone with monoclonal integration of the provirus. These cells possess multi-lobulated nuclei and are called "flower cells". ATLL cells are usually CD3+ CD4+ CD8- CD25+ and frequently accumulate in peripheral blood as well as in lymphoid organs and skin. HTLV-1 infection is accompanied by a high frequency of T-cells expressing the surface marker Forkhead Box P3 (Foxp3) (Chen et al., 2006; Kohno et al., 2005), whose expression is characteristic of regulatory T cells (Treg cells), which play a critical role in suppressing the immune response. However, in ATLL, the Foxp3 positive (Foxp3+) leukemic T cell clones are distinct from the functional Treg population (Abe et al., 2008; Toulza et al., 2009). The increase in Foxp3+ cell frequency in HTLV-1 infection results from the upregulation of CCL22 chemokine production by HTLV-1-infected cells that express Tax. CCL22 engages the CCR4 receptor expressed on the functional Treg cell population, resulting in an enhancement of the migration and survival of Foxp3+ cells *in vitro*. These Foxp3+ cells may both retard the progression of ATLL and HTLV-1-associated inflammatory diseases and contribute to the immune suppression seen in HTLV-1 infection, especially in ATLL (Toulza et al., 2010).

One of the most common characteristics of ATLL is hypercalcemia, which results from the transcriptional activation of a parathormone-like peptide induced by the viral protein Tax. The prognosis of acute ATLL is extremely poor with an overall survival of a few months.

TSP/HAM is characterized by a slowly progressive spastic paraparesis, associated with bladder dysfunction and sensory disorders (Rodgers, 1965). Parenchymal and perivascular infiltration of mononuclear cells occurs in the white and gray matter of the spinal cord, resulting in demyelization and fibrosis (Iwasaki, 1990). The presence

of infiltrating T-cells in the spinal cord lesions and of Tax-specific cytotoxic T-lymphocytes (CTL) in the cerebrospinal fluid and in the peripheral blood mononuclear cells (PBMCs) suggests that TSP/HAM might have an autoimmune base (Osame, 2002; Verdonck et al., 2007). This hypothesis is consistent with the association between HLA haplotype and the risk of developing TSP/HAM (Barmak et al., 2003).

HTLV-1 infection of PBMCs leads to cell immortalization. After several months of culture it is usually possible to detect a mono- or oligoclonal profile of provirus integration as a result of a process that selects for one or few clones that carry several genetic alterations and acquire the capability to grow in an interleukin-2 (IL-2)-independent manner. These cells usually show a CD3+ CD4+ IL-2Rα+ (interleukin-2 receptor) phenotype, or, rarely, CD3+ CD8+ IL-2R+ (Green and Chen, 2001). Silencing of viral gene expression was shown to be due to viral promoter methylation, or accumulation of mutations in the viral tax gene, or deletions of the proviral 5' LTR (Takeda et al., 2004; Miyazaki et al., 2007). The dynamics of infection and immortalization observed *in vitro* recapitulate at least part of the natural history of ATLL *in vivo*. Indeed, ATLL cells also express very little, if any, viral proteins and frequently carry defective proviral copies integrated in the host genome. The propagation and persistence of the infected cells in the host relies on both *de novo* infection of new host cells and, mainly, on "mitotic transmission" of the integrated viral genome to daughter cells (Overbaugh and Bangham, 2001).

### 1.1.1 INFECTION AND VIRUS PROPAGATION

The HTLV-1 virion consists of a viral core that contains the viral-encoded enzymes (reverse transcriptase, integrase and protease) and the single-stranded diploid RNA genome surrounded by capsid and matrix proteins. A lipoproteic envelope, composed of a plasma membrane-derived lipid bilayer and the gp21 and gp46 envelope glycoproteins, surrounds the viral core (Figure 1) (Manel et al., 2005; Lairmore and Franchini, 2007). HTLV-1 presents a broad cell tropism *in vitro* (monocytes, microglial cells, epithelial cells, B- and T- lymphocytes), but it is mainly detected in CD4+ T-lymphocytes in ATLL and TSP/HAM patients and in asymptomatic carriers (Manel et al., 2005). Viral spread is mediated through the interaction between the viral envelope protein gp46 and the glucose transporter GLUT-1 (Manel et al., 2003).

Consequently, glucose consumption of the infected target cell is inhibited and extracellular milieau acidification is reduced, possibly causing metabolic alterations in the infected cells (Manel et al., 2003; Manel et al., 2005). The gp46-GLUT-1 interaction allows the envelope protein gp21 to mediate cellular membrane fusion and formation of the “virological synapse”. The virological synapse is an organized contact area whose assembly results from the polarization of the cytoskeleton of the infected cell and the accumulation of HTLV-1 core complexes and the HTLV-1 genome at the cell junction; the virion components are then transferred to the uninfected cell as enveloped particles (Igakura et al., 2003; Majorovits et al., 2008). Polarization of the microtubule-organizing centre (MTOC) is induced by the engagement of intracellular adhesion molecule-1 (ICAM-1) (Barnard et al., 2005) and activation of the Ras-MEK-ERK pathway (Nejmeddine et al., 2009). Tax is involved in the formation of the virological synapse: it localizes in the contact region between infected and target cells (Nejmeddine et al., 2005) and enhances MTOC formation by stimulating the CREB pathway (Nejmeddine et al., 2005; Nejmeddine et al., 2009). Proteins that mediate antigen recognition and cell adhesion (e.g. hDlg, neuropilin-1, heparan sulphate proteoglycans) also contribute to HTLV-1 binding and entry into the target cell and are part of the "virological synapse" (Pinon et al., 2003; Blot et al., 2004; Ghez et al., 2006). Another mode of HTLV-1 transmission is mediated through an extracellular biofilm-like structure that stores viral particles, facilitating virus spread after cell-to-cell contact (Pais-Correia et al., 2010). Both the virological synapse and biofilm-mediated transmission are consistent with the fact that cell-free HTLV-1 particles are usually undetectable in the serum of HTLV-1 infected subjects and cell-free blood products are not infectious (Fan et al., 1992; Derse et al., 2001); efficient viral spread instead requires direct cell-to-cell contact. More recently, dendritic cells have been shown to play a central role in HTLV-1 transmission as they can be infected by HTLV-1 cell-free virions and efficiently transfer virus to CD4+ T cells (Jones et al., 2008). In addition, recent studies demonstrated that HTLV-1 can also be transmitted to uninfected T cells by cellular conduits (membrane structures that contain F-actin, favor cell to-cell communication, and transport proteins, organelles, and viruses). The viral accessory protein p8 augments the number and the length of cellular conduits among T cells thus

enhancing the envelope-dependent transmission of HTLV-1(van Prooyen et al., 2010).

After virus entry into the target cell, the viral genome is reverse-transcribed by viral reverse transcriptase (RT), producing an RNA-DNA hybrid. The ribonuclease H (RNAse H) component of viral RT degrades the RNA strand, while the DNA strand is used as a template by RT, which also has DNA-polymerase-DNA-dependent activity, to synthesize a complementary DNA strand. The double-stranded DNA circularizes and transfers to the nucleus, where it integrates randomly in the host genome. Integration is mediated by the viral enzyme integrase and by the long terminal repeats (LTRs) located at both ends of the viral genome. Viral genes are then transcribed and translated by the cellular machinery. Virion assembly occurs in the cytoplasm, through the interaction between the viral nucleocapsid and the plasma membrane, and incorporation of two copies of the single stranded RNA genome along with tRNA, RT, protease and integrase.

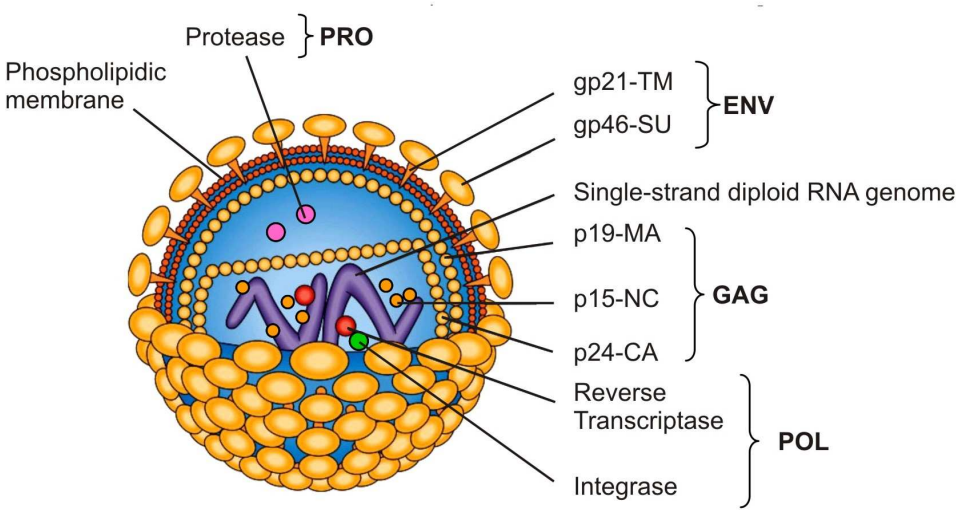


**Figure 1. Schematic representation of the HTLV-1 virion** (Le Blanc et al., 2001).

### 1.1.2 HTLV-1 GENETIC ORGANIZATION AND GENE EXPRESSION

The genome of HTLV-1 reflects the basic structure of the Deltaretrovirus genus (Figure 2): at the 5' and 3' ends are located the LTRs which flank the partially overlapping open reading frames (ORFs) of the gag, pro, pol and env genes that code for enzymes and structural proteins of mature virus particles. The region between the end of the env gene and the 3' LTR is termed the X region, and contains at least four partially overlapping ORFs, termed x-I through x-IV, coding for regulatory and accessory proteins (Figure 2A). The negative strand of HTLV-1 also contains an ORF located in the pX region (antisense orientation) (Larocca et al., 1989) which codes for the HBZ protein (HTLV-1 bZIP factor) (Figure 2B) (Gaudray et al., 2002).
The HTLV-1 transcripts can be grouped in 4 major classes (Figure 2A and B):
a) unspliced (US) mRNA, coding for Gag-Pro-Pol, and used as genomic RNA. The gag gene codes for the 19 kDa matrix (MA), 24 kDa capsid (CA) and 15 kDa nucleocapsid (NC) structural proteins. The pro gene encodes the viral protease. The 5' portion of the pol gene encodes the reverse transcriptase (RT) protein, while sequences downstream code for Integrase. These genes are translated as polyproteic precursors (Gag, Gag-Pro and Gag-Pro-Pol) generated through ribosomal frameshifting at the gag-pro and/or gag-pro-pol junction. The precursors are post-translationally modified by myristylation at the N-terminus, an essential step for their insertion in the internal side of the plasma membrane of the infected cell. After anchoring to the plasma membrane the precursors are cleaved by the viral protease to generate the single mature polypeptides.
b) singly-spliced (SS) mRNAs, coding for the envelope glycoproteins (Env) and for the accessory proteins p21rex, p12 and p13. The env gene encodes a 68 kDa precursor which is post-translationally modified by glycosylation and cleavage into two proteins named gp46-SU, localized at the surface of virions and responsible for the binding to the GLUT-1 receptor, and gp21-TM, the transmembrane protein that mediates membrane fusion and formation of the virological synapse. p12 and p13 are produced from mRNAs containing exon 1 (non coding) and exon B (p12) or exon C (p13) (mRNA 1-B; x-I ORF codes for p12; mRNA 1-C; x-II ORF for p13) (Ciminale et al., 1992; Koralnik et al., 1992). p12 is proteolytically cleaved within the endoplasmic reticulum (ER) to generate p8 (Fukumoto et al., 2009). p13 is a 87-

amino acid protein that corresponds to the C-terminal portion of p30tof (Koralnik et al., 1992). p21rex is coded by the x-III ORF and it is expressed from a singly-spliced mRNA which contains exons 1 and 3 (mRNA 1-3).

c) doubly-spliced (DS) mRNAs, coding for the regulatory proteins p40 (Tax) and p27 (Rex), and for the regulatory/accessory protein p30tof. Tax and Rex are coded by a dicistronic mRNA which contains exons 1, 2 and 3 (mRNA 1-2-3): Tax is expressed from the second start codon of exon 2 that is in frame with the x-IV ORF (located in exon 3), and Rex from the first start codon of exon 2 that is in frame with the x-III ORF (located in the exon 3). p30tof is coded in a mRNA containing exons 1, 2 and B (mRNA 1-2-B), starting from the second start codon of exon 2 that is in frame with the x-II ORF (located in the exon B).

d) mRNAs generated from the negative strand, coding for HBZ protein. The HBZ gene is coded by at least 2 different transcripts, one spliced (hbz sp1) and the other unspliced (hbz us) (Cavanagh et al., 2006; Murata et al., 2006; Satou et al., 2006).

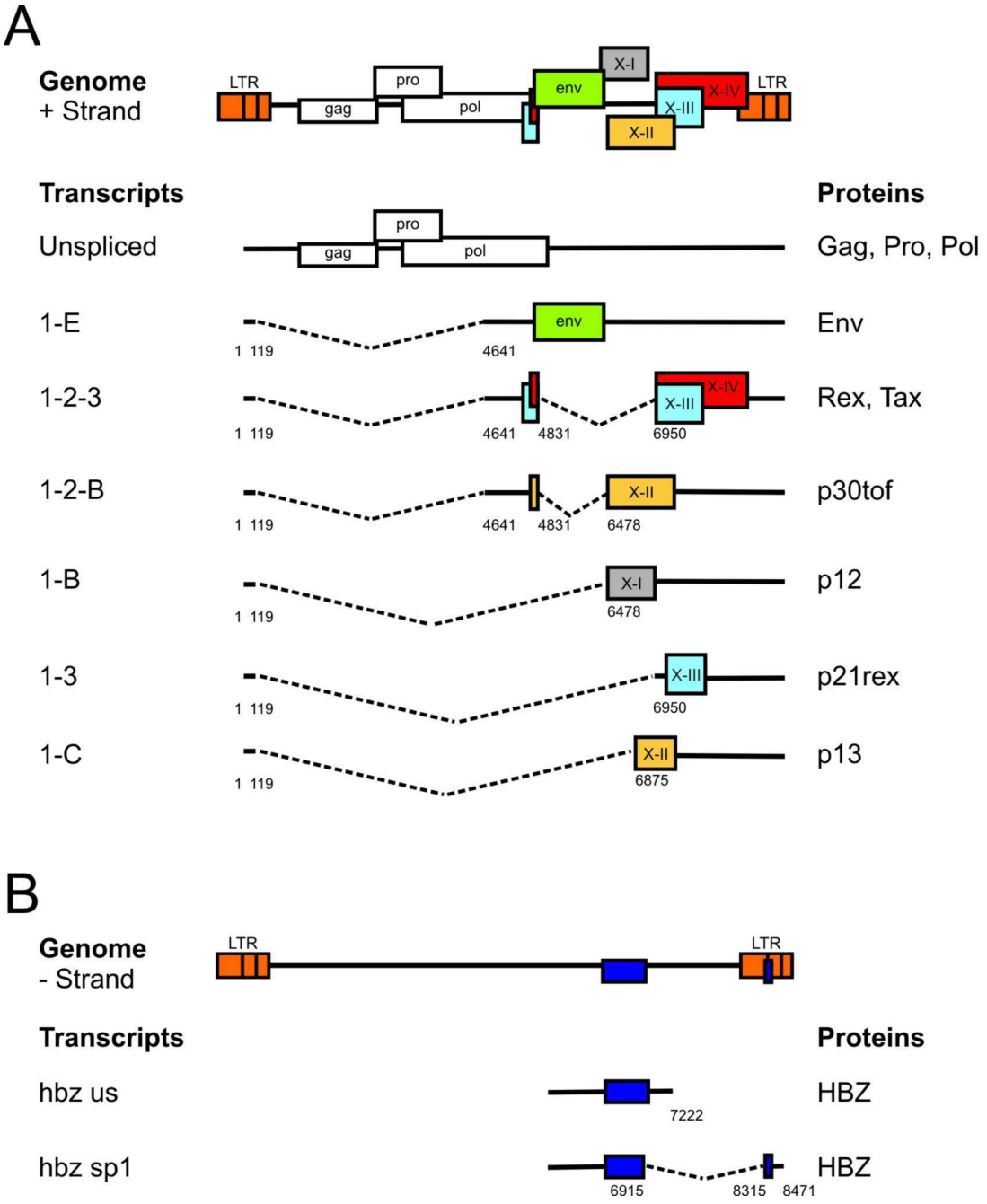


**Figure 2. Organization and expression of the HTLV-1 genome. A:** Plus-strand ORFs, transcriptional map and proteins coded by each mRNA are shown. The numbering indicates splicing sites used for the generation of the mature mRNAs. Resulting exons are: 1 (1-119), 2 (4641-4831), 3 (6950-8493), B (6478-8493), C (6875-8493) and E (4641-8493). mRNAs are named according to their exonic composition**. B:** For the minus-strand, the ORF, transcriptional map and proteins coded by each mRNA are shown. The numbering indicates the start sites used for the generation of the mature mRNAs. Resulting exons are: hbz us (7222-4834) and hbz sp1 (8471-8315 and 6915-4834) (Rende et al., 2011).

### 1.1.3 FUNCTIONS OF THE PX REGION PROTEINS

#### 1.1.3.1 Tax

Tax is a 353-amino acid (40-kDa), mainly nuclear, phosphoprotein that transcriptionally controls the expression of plus strand viral genes and a large number of cellular genes. Different functional domains have been mapped in the Tax sequence: the hydrophobic N-terminal domain contains the nuclear localization signal (NLS) that overlaps with a zinc finger region that is crucial for Tax's interaction with the CRE-binding/activating transcription factor (CREB/ATF) and the serum responsive factor (SRF); the central region of Tax encompasses a kinase-inducible domain (KID) that mediates the interaction with the kinase-inducible exchange (KIX) domain of the transcriptional co-activators CREB binding protein (CBP) and p300, and the dimerization domain; The C-terminal region contains a domain involved in the interaction of Tax with the transcriptional co-activator p300/CBP-associated factor (P/CAF). Tax was initially described as an activator of LTR-directed transcription (Felber et al., 1985). Three imperfectly conserved 21 base-pair (bp) repeat sequences called Tax responsive elements (TRE) located in the U3 region of the LTR are necessary and sufficient to confer Tax responsiveness (Brady et al., 1987). The TRE element shares homology with the consensus cAMP-responsive element (CRE) (5'-TGACGTCA-3') (Jeang et al., 1988). Tax exhibits poor affinity for DNA and does not bind directly to the TRE element (Giam and Xu, 1989) but interacts with members of the CREB/ATF family: CREB, CREM, ATF1, ATF2, ATF3, ATF4 (CREB2) and X-box-binding protein 1 (XBP1). These proteins share a cluster of basic residues allowing DNA binding and a leucine zipper (b-Zip) domain involved in homo- and heterodimerization. Dimer formation modulates their DNA binding specificity and transcriptional activity. Tax promotes formation of a Tax-CREB/ATF-TRE ternary complex by interacting with the b-Zip domain of CREB/ATF factors (Perini et al., 1995; Wagner and Green, 1993; Anderson and Dynan, 1994; Lundblad et al., 1998). Tax then recruits coactivators (CBP/p300 and P/CAF) to facilitate chromatin remodelling and transcriptional activation. Normally, the CREB-CBP/p300 interaction is controlled by CREB phosphorylation in response to different signal transduction pathways. In contrast, Tax, by directly interacting with the co-activator, physically links CREB and CBP/p300, making CREB

phosphorylation dispensable. This renders transcription of viral genome less dependent on cellular signals.

Tax also binds to TORC proteins (transducers of regulated CREB activity), a family of CREB co-activators (Koga et al., 2004; Siu et al., 2006). TORCs are thought to associate with the Tax ternary complex and participate in transcriptional activation of the LTR promoter.

Tax also stimulates transcription of a large number of cellular genes through interactions with the cellular transcription factors CREB/ATF, NF-κB and SRF. Tax activates a variety of cellular genes through its interactions with CREB/ATF proteins, for example those encoding interleukin 17 (IL-17) and c-fos (Dodon et al., 2004; Alexandre and Verrier, 1991). Conversely, Tax also represses expression of cyclin A, p53 and c-myb by targeting CREB/ATF factors (Kibler and Jeang, 2001; Mulloy et al., 1998; Nicot et al.,2000). Tax associates with the NF-κB factors, such as RelA, c-Rel, p50 and p52 (Suzuki et al., 1993; Murakami et al., 1995; Suzuki et al., 1994), and increases their dimerization which is essential for binding to target promoters (Suzuki et al., 1993; Suzuki et al., 1994; Petropoulos et al., 1996). In the canonical pathway of NF-κB, Tax associates with the IKKγ/NEMO subunit (Harhaj and Sun, 1999; Jin et al., 1999) as well as with activating upstream kinases such as MAPK/ERK kinase kinase 1 (MEKK1) and TGF-β activating kinase 1 (TAK1) (Wu and Sun, 2007). Tax thus connects activated kinases to the IKK complex and forces the phosphorylation of IKKα and IKKβ leading to degradation of IκBα and IκBβ (Harhaj and Sun, 1999; Jin et al., 1999). In addition, Tax binds directly to the IKKα and IKKβ subunits and activates their kinase activity independently of the upstream kinases (Chu et al., 1998). Binding of Tax to IκBs promotes their degradation independently of IKK phosphorylation (Hirai et al., 1994; Suzuki et al., 1995). Tax further interacts with two subunits of the 20S proteasome, causes anchorage of p105 which accelerates its proteolysis (Rousset et al., 1996). Tax thus leads to IκB degradation at multiple levels, thereby allowing nuclear translocation of NF-κB independently of external stimuli.

Tax increases the expression of the transcriptional factors AP-1 (activator protein -1) a homo- or heterodimeric complex of Fos (c-Fos, FosB, Fra1 and Fra2) and Jun (c-Jun, JunB and JunD) (Fujii et al., 1991; Fujii et al., 2000). Fos and Jun are under the transcriptional control of the serum responsive factor (SRF) that binds to SRF

responsive elements (SRE) in response to various stimuli such as cytokines, growth factors, stress signals and oncogenes. Tax interacts with transcription factors associated with the SRF pathway (Alexandre and Verrier, 1991; Fujii et al., 1991; Alexandre et al., 1991) and increases binding of SRF to the SRE (Dittmer et al., 1997). Furthermore Tax is able to interact with proteins stimulating the G1-S phase transition of the cell cycle. Tax interacts with cyclins-D1, -D2 and -D3 as well as with cyclin-dependent kinases (CDKs) 4 and 6 (Haller et al., 2002; Haller et al., 2000; Fraedrich et al., 2005; Neuveut et al., 1998). Through these interactions, Tax stabilizes the cyclin D2/CDK4 complex and enhances its kinase activity, leading to hyperphosphorylation of retinoblastoma protein (Rb). Tax also associates with the CDK inhibitors (CDKI) p15INK4b and p16INK4a and counteracts their inhibitory activity against CDK4 (Suzuki et al., 1996; Suzuki and Yoshida, 1997; Low et al., 1997; Suzuki et al., 1999). Finally, Tax binds to Rb and enhances its proteosomal degradation (Kehn et al., 2005).

Tax also inactivates the p53 tumor suppressor, thus impinging on the major pathway controlling genome integrity and favouring the emergence of a large spectrum of molecular alterations in infected cells (Tabakin-Fix et al., 2005).

Tax shows a strong anti-apoptotic activity through NF-κB activation (Kawakami et al., 1999), transcriptional activation of anti-apoptotic factors Bcl-XL, Bfl1 and HIAP-1 (Tsukahara et al., 1999; Nicot et al., 2000; De La Fuente et al., 2003) and downregulation of the pro-apoptotic protein Bax (Brauweiler et al., 1997).

Tax targets multiple components of the DNA damage repair pathway and promotes DNA abnormalities. Tax subverts mechanisms monitoring chromosomal segregation during mitosis; in fact, one of the hallmarks of Tax-expressing cells is chromosomal instability and aneuploidy (Marriott et al., 2002). Tax interacts with different proteins involved in centrosome amplification and in the mitotic spindle assembly checkpoint (SAC), for example, the anaphase promoting complex (APC), which controls the metaphase-anaphase transition and correct execution of mitosis, resulting in incorrect activation of separase and unequal chromosomal separation between cells (Liu et al., 2005). Tax was also reported to modulate the transforming growth factor β (TGFβ) pathway and the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (Hall et al., 2005).

Tax is the major target of cytotoxic T lymphocytes (CTLs). To escape from CTLs, ATLL cells frequently lose the expression of Tax by several mechanisms (loss of the viral promoter, nonsense or missense mutation of the tax gene, epigenetic change by hypermethylation). Thus, it is speculated that Tax plays an important role in the persistent proliferation of HTLV-1-infected cells during the carrier state, with the mutator phenotype conferred by Tax promoting accumulation of genetic and epigenetic changes that finally lead to Tax-independent proliferation and escape from the host immune system following silencing of Tax (Yasunaga and Matsuoka, 2007). Transgenic expression of Tax in T-cells of mice leads to T-cell leukemias and lymphomas (Hasegawa et al., 2006).

#### 1.1.3.2 Rex

Rex is a 189-amino acid, 27 kDa nuclear/nucleolar phosphoprotein that is able to shuttle between the nucleus and the cytoplasm (Palmeri and Malim, 1996; Narayan et al., 2003), allowing the nucleo-cytoplasmic export of incompletely spliced viral RNA, controlling in this way viral gene expression at the post-transcriptional level. This function is mediated through direct interaction with a 254-nucleotide stem-loop cis-acting RNA element termed the Rex-responsive element (RxRE) (Grone et al., 1994), present in the U3/R region of the 3' LTR of all HTLV-1 transcripts and predicted to form a stem-bulge-stem structure (Ahmed at al., 1990; Bogerd et al., 1991; Bogerd et al., 1992). Different functional domains have been mapped in the Rex protein. The N-terminal arginin-rich region serves as nuclear localization signal (NLS) (Siomi et al., 1998; Nosaka et al., 1989) and as RNA binding domain (RBD) which mediates Rex binding to the RxRE in the viral RNAs (Grassman et al., 1991; Bogerd et al., 1992). A leucine-rich sequence located near the middle of the protein functions as activation domain (AD) (Weichselbraun et al, 1992) and contains the nuclear export signal (NES) (Palmeri and Malim, 1996; Kim et al., 1996). The NES interacts with the protein chromosome region maintenance interacting protein 1 (CRM1/exporting 1) and allows export of the Rex-viral mRNA complex from the nucleus to the cytoplasm (Bogerd et al., 1995). CRM1 belongs to the importin-β family, whose members act as RNA transporters between nuclear and cytoplasmic compartments (Bogerd et al., 1998). The two NES flanking regions (Bogerd et al., 1995) constitute the Rex multimerization domain and are required for the assembly

of Rex into multimeric structures upon binding to the RxRE, which is critical for the nuclear export of viral mRNA (Bogerd et Greene, 1993). Although Rex is not required for cellular immortalization *in vitro*, it is necessary for infectivity and viral persistence *in vivo* (Ye et al., 2003), since expression of the US and SS viral RNAs encoding structural proteins is necessary for the assembly of virions. The fact that these mRNAs depend on Rex for expression suggests that the Rex-RxRE interaction may function as a molecular switch controlling the transition between productive and latent phases of HTLV-1 infection.

Furthermore Rex has been shown to affect cellular gene expression. Rex augments Tax-mediated upregulation of IL-2 (McGuire et al., 1993), of vascular cell adhesion molecule-1 (VCAM-1) and lymphocyte function-associated antigen-3 (LFA-3) (Valentin et al., 2001), important proteins in T-cell adhesion. In this manner, Rex may also contribute to the proliferation of infected cells and virus spread.

Interestingly, Rex has been shown to interact with Dicer (see Section 1.2.1), inhibiting its activity and thereby reducing the efficiency of the conversion of shRNA to siRNA (Abe et al., 2010). This finding opens the possibility that HTLV-1 could control miRNA biogenesis through the action of Rex.

#### 1.1.3.3 Accessory proteins

p21rex is a truncated isoform of Rex lacking the N-terminal arginine-rich domain of the full-length protein. It was hypothesized that it might act as a repressor of full-length Rex, thereby inhibiting the expression of transcripts coding for structural proteins, enzymes and accessory proteins (Heger et al., 1999) and playing a role as a latency-inducing factor in the HTLV-1 life cycle.

p30tof is a 241-amino acid nucleolar-nuclear non-shuttling protein (Ciminale et al., 1992; D'Agostino et al., 1997), presenting two arginine-rich domains as nucleolar retention signal (NoRS) and NLS (D'Agostino et al., 1997; Ghorbel et al., 2006), and two additional NLS in the N-terminal and C-terminal regions (Ghorbel et al., 2006). An HTLV-1 molecular clone containing a mutation in the x-II ORF is still able to produce infective virions and immortalize human T-lymphocytes (Robek et al., 1998), but shows a drastic reduction in *in vivo* infectivity in animal models (Bartoe et al., 2000). p30tof functions at the post-transcriptional level by inhibiting the nuclear

export of the tax/rex mRNA; this effect results in a global inhibition of viral gene expression, suggesting that p30tof might act as a latency factor (Nicot et al., 2004). In addition, p30tof was found to interact with the RNA-binding domain of Rex and thereby prevent Rex from interacting with the RxRE (Baydoun et al., 2008). p30tof also affects transcription from promoters with cellular CRE and viral TRE sequences by interacting with the co-activator CBP/p300 (Zhang et al., 2000; Zhang et al., 2001), and can disrupt the assembly of the CREB–Tax–p300/CBP complex on TREs. Taken together, these data suggest that p30tof might decrease transcription of the viral genome, thereby facilitating viral latency. By recruiting the co-activator Tat-interacting protein 60 (TIP60) p30tof promotes the formation of the Myc/TIP60 transcription complex on Myc-response E-box elements and thereby transactivates transcription (Awasthi et al., 2005). Due to the importance of Myc as a proto-oncogene, p30tof may contribute to the transformation of HTLV-1-infected cells. Recent studies have shown that p30tof expression results in alteration of the cell cycle events that would promote early viral spread and T cell survival (Datta et al., 2007).

p13 is integral membrane protein that localizes in the mitochondria mainly in the inner mitochondrial membrane and induces specific alterations in mitochondrial morphology (Ciminale et al., 1999; D'Agostino et al., 2002; D'Agostino et al., 2005). Studies of a p13-knockout virus showed that although the protein is dispensable for viral replication in cultured cells (Derse et al., 1997; Robek et al., 1998), it is required for establishing a persistent infection in a rabbit experimental model (Hiraragi et al., 2006). Functional studies of p13 revealed that it inhibits proliferation of HeLa cells and Jurkat T cells and sensitizes Jurkat T cells to apoptosis triggered by ceramide and Fas ligand (Silic-Benussi et al., 2004; Hiraragi et al., 2005). p13 also interferes with the ability of HeLa cells and Ras/Myc-transformed primary fibroblasts to grow as tumors in nude mice, suggesting that it may exert tumor-suppressor-like activity (Silic-Benussi et al., 2004).

p12 localizes in the endoplasmic reticulum (ER) and in the Golgi apparatus (Koralnik et al., 1993; Ding et al., 2001; Johnson et al., 2001). While this protein is not required for HTLV-1 replication *in vitro*, it plays a key role in the stabilization of a productive viral infection *in vivo* (Albrecht et al., 2000; Collins et al., 1996; Derse

et al., 1997; Robek et al., 1998). p12 interacts with the β and $\gamma_c$ chains of the interleukin-2 receptor (IL-2R), resulting in reduced surface expression (Mulloy et al., 1996). The binding to the cytoplasmic domain of the β chain, involved in the recruitment of Jak1 and Jak3, determines an increase in the transcriptional activity of STAT-5, providing a proliferative advantage to T cells (Nicot et al., 2001). p12 was also shown to sequester free MHC class I heavy chains (MHC-I-Hc), preventing their binding to β2-microglobulin, favouring escape from CTL recognition and clearance by the immune system of infected cells (Johnson et al., 2001). Furthermore, p12 causes a reduction in the expression of ICAM-1 and ICAM-2, which mediate adhesion of natural killer (NK) cells to the infected cells, resulting in the protection from NK cell-mediated cytotoxicity (Banerjee et al., 2007). These effects are particularly relevant in the context of HTLV-1 infection, which is able to induce a strong humoral and cellular immune response (Bangham, 2003). p12 interacts with calreticulin and calnexin (Ding et al., 2001), two ER-resident proteins that regulate $Ca^{2+}$ storage and release, suggesting a p12-mediated $Ca^{2+}$ leakage from the ER (Ding et al., 2002). Moreover, p12 stimulates nuclear factor of activated T-cells (NFAT) (Albrecht et al., 2002), by interacting with calcineurin, a $Ca^{2+}$-responsive protein phosphatase that controls NFAT activity (Kim et al., 2003). Taken together these effects decrease the threshold for T-cell activation (Nicot et al., 2005).

The HBZ protein contains an N-terminal transcriptional activation domain (AD), a central domain (CD) and a C-terminal basic ZIP domain (bZIP) and three NLS (Gaudray et al., 2002; Hivin et al., 2005). HBZ is not necessary for viral replication or immortalization *in vitro*, but increases infectivity and viral persistence *in vivo* (Arnold et al., 2006). HBZ localizes in the nucleus with a speckled pattern and interacts with a number of transcription factors, including CREB-2, p300/CBP, Jun family members, and NF-κB (Matsuoka and Green, 2009). Binding of HBZ to JunB and c-Jun decreases their DNA binding activity by preventing their interaction with Fos, leading to repression of the AP-1 complex; on the contrary the interaction of HBZ with Jun-D stimulates its transcriptional activity (Thebault et al., 2004), and results in the activation of JunD-dependent cellular genes including human telomerase reverse transcriptase (hTERT) (Kuhlmann et al., 2007). HBZ inhibits the activation of the classical NF-κB pathway by two different mechanisms: by

inhibiting the DNA binding of the NF-κB subunit p65 and by increasing the expression of PDLIM2, the E3 ubiquitin ligase of p65, leading to enhanced ubiquitination and degradation of p65 (Zhao et al., 2009). HBZ expression is associated with proliferation of ATLL cells *in vivo* and *in vitro* (Satou et al., 2006; Arnold et al., 2008). Mutational analyses of the hbz gene showed that hbz mRNA, rather than HBZ protein, has a growth-promoting effect on T-cells (Satou et al., 2006) possibly by up-regulating transcription of the E2F1 gene and its downstream targets. Transgenic expression of HBZ in mice leads to the development of T-cell lymphomas and systemic inflammatory diseases (Satou et al., 2011).

## 1.2 THE MIRNAS

The miRNAs belong to a class of small non-coding RNAs that regulate mRNA expression at the post-transcriptional level by hybridizing to complementary sequences on target transcripts, leading to the silencing of the mRNA target expression. miRNAs are single-stranded RNAs of 16-27 nt in length generated from endogenous transcripts containing hairpin structure (Kim 2005). Most miRNA-mRNA interactions involve base-pairing of nucleotides ~2-8 at the 5' end of the miRNA (the 'seed sequence') with a perfect or nearly-perfect complementary sequence usually in the 3' untranslated region (3'UTR) of the target mRNA. For many miRNA, the critical seed interaction is reinforced by additional base-pairing of the rest of miRNA sequence, which contributes to determine the target specificity. MiRNA binding typically leads to translational repression and exonucleolytic decay of the target mRNA. However, miRNAs can also bind with high complementary to their mRNA targets which are therefore cleaved endonucleolytically. In particular contexts, translational activation (Filipowicz et al., 2008) and heterochromatin formation (Kim et al., 2008) have also been described as possible miRNA regulatory mechanisms.

Many of the animal miRNAs are phylogenetically conserved, indicating that miRNAs have had important roles throughout evolution (Ibanez-Ventoso et al., 2008). Plant miRNAs instead seem to have evolved separately from those in animals because their sequences, precursor structure and biogenesis mechanisms are distinct

(Chapman et al., 2007; Millar et al., 2005). Most mammalian miRNAs are encoded by multiple genes (paralogues) with distinct genomic positions, probably the result of gene duplications. As an example, the let-7 family miRNAs are coded by 12 different loci in the human genome. Paralogues often have identical seed sequences and are thought to target the same mRNAs. However, because the 3′ sequences of miRNAs also contribute to target binding and because paralogue miRNAs often present distinct expression patterns, members of the same seed family might have distinct roles (Ventura et al., 2008). Approximately 50% of mammalian miRNA loci are found in clusters in the genome. Such clusters are generally transcribed as a single polycistronic transcription unit (TU) (Lee et al., 2002), although there may be exceptional cases in which individual miRNAs are transcribed from separate promoters. Approximately 40% and 10% of miRNA loci are located in introns or in exons of non-coding transcripts, respectively. miRNAs can be also found in protein-coding TUs, in exons or more commonly in intronic regions, which account for ~40% of all miRNA loci.

Since the discovery in nematodes in 1993 of the first small RNA lin-4 (Lee et al., 1993; Wightman et al., 1993), the number of known miRNAs has exponentially expanded, mainly as a result of the cloning and sequencing of size-fractionated RNA (Lau et al., 2001; Lee et al., 2001; Lagos-Quintana et al., 2001). The recent development of deep sequencing technologies (Lu et al., 2005; Margulies et al., 2005) and computational prediction methods (Lai et al., 2003; Nam et al., 2005; Li et al., 2006; Huang et al., 2007) has accelerated the discovery of new small RNA sequences. miRNAs have been identified in protozoa, plants, metazoan animals and viruses. The sequences and genomic locations of the known miRNAs are catalogued in the Sanger miRBase at http://www.mirbase.org/ (Griffiths-Jones et al., 2008). The current miRBase (version 18, released in November 2011) contains 1921 human miRNAs. Global miRNA profiling studies (Basso et al., 2009; Ghisi et al., 2011; Landgraf et al., 2007; Morin et al., 2008) indicate that some miRNAs are specific for a particular cell lineage or differentiation stage, while others are expressed in many cell types and thus probably play broader roles in fine-tuning gene expression.

A single miRNA has the potential to regulate hundreds of different target genes and a gene generally contains several target sites for different miRNAs, thus leading the generation of a extremely complex miRNA regulatory network. Bioinformatic

predictions estimated that 60% of all 3'UTRs of human protein-coding genes contain perfect binding sites for miRNA seed sequences (Friedman et al., 2009). Consequently, the unique combination of miRNAs in each cell type regulates the expression of thousands of mRNAs. miRNAs are thus likely to regulate most normal biological processes, including developmental timing, cell differentiation, cell proliferation, cell death, metabolic control, transposon silencing and antimicrobial defense. Not surprisingly, aberrant miRNA expression or function contributes to the pathogenesis of many diseases, including cancer (Sayed and Abdellatif, 2011). The first direct evidence for the importance of miRNAs in human cancer came from a study of chronic lymphocytic leukemia (CLL), which revealed a tumor suppressor function for miR-15a and miR-16-1 (Calin et al., 2002). Subsequent studies have identified many additional miRNAs with oncogenic or tumor suppressor activities in the context of solid and hematopoietic tumors (Croce, 2009).

All viruses rely on the host gene expression machinery for their replication and may therefore be affected by the host miRNA network at some level. In turn, viruses have evolved mechanisms that exploit the miRNA network to impinge on host cell turnover and immune defenses to promote expansion and persistence of infected cells (Umbach and Cullen, 2009). It is therefore not surprising that some of the miRNAs shown thus far to be exploited by viruses are also involved in neoplastic transformation. One notable example is miR-155, a miRNA that is overexpressed in several solid tumors and haematological malignancies; miR-155 is upregulated by Epstein-Barr virus and expressed as a viral ortholog by human herpesvirus 8 (Lin and Flemington, 2011). In addition, some viruses produce RNAs or proteins that suppress the RNAi pathway and thereby may have general effects on miRNA expression (Strebel et al., 2009).

### 1.2.1 MIRNA BIOGENESIS

Most miRNA genes are transcribed by RNA polymerase II (Pol II) (Lee et al., 2004; Cai et al., 2004); some miRNAs that are associated with Alu repeats can be transcribed by Polymerase III (Pol III). A range of Pol II-associated transcription factors elaborately regulate the transcription of miRNA genes in accord to specific conditions and cell types (Lee et al., 2008).

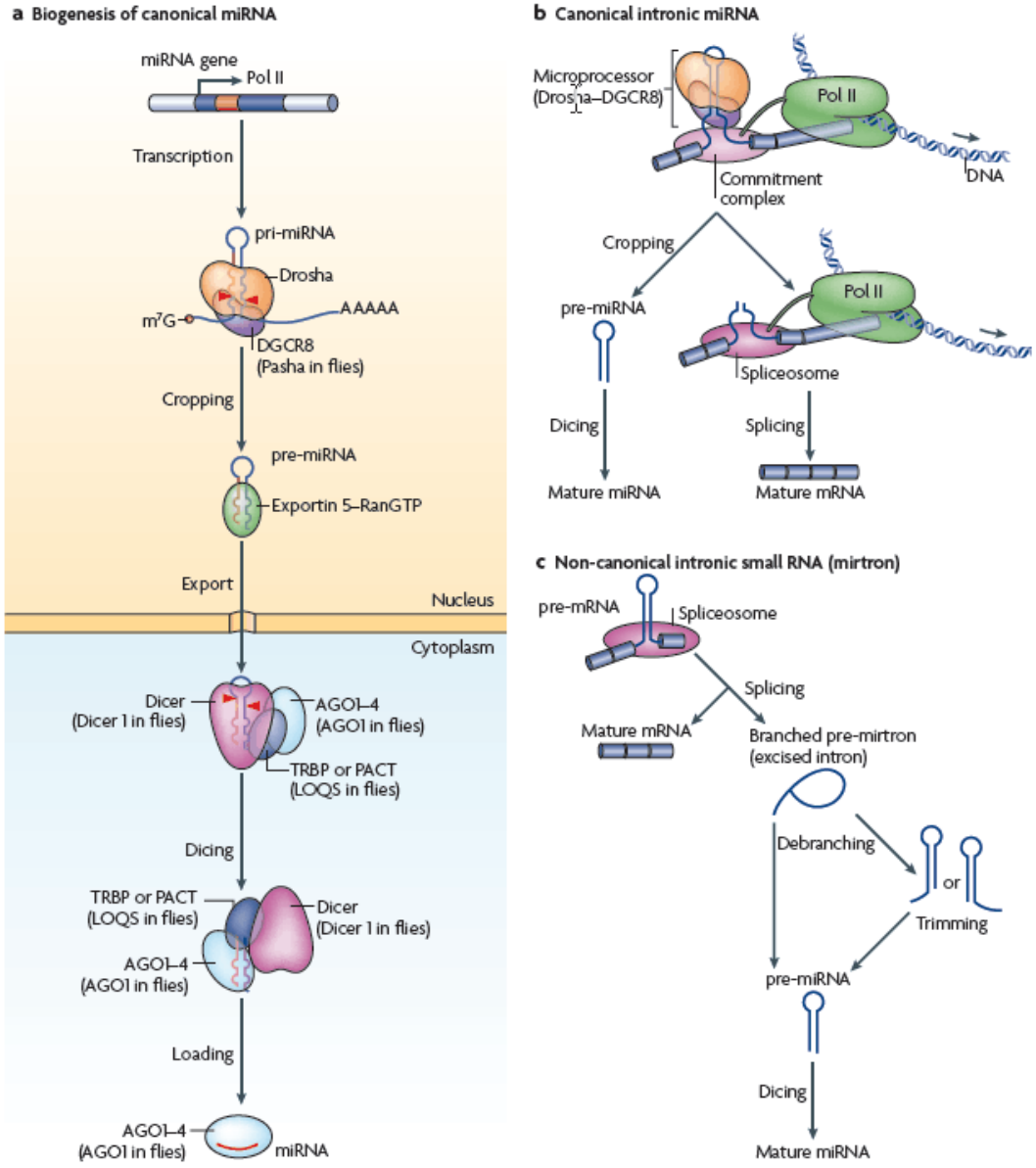


**Figure 4. miRNA biogenesis.** a) RNA polymerase II (Pol II) transcribes the canonical miRNA genes as a primary transcript called pri-miRNA. The Drosha–DGCR8 complex (the Microprocessor complex) mediates the processing (cropping)

of the pri-miRNA into the ~65 nt miRNA precursor (pre-miRNA). The pre-miRNA structure (a short stem with a ~2-nt 3′ overhang) is recognized by the nuclear export factor exportin 5 (EXP5). After nuclear export, the cytoplasmic RNase III Dicer is responsible for the second processing step (dicing) that produces the miRNA duplex. In humans, Dicer, in association with TRBP or PACT, interacts with Argonaute (AGO)1–4 to mediate the processing of pre-miRNA and the assembly of the RISC (RNA-induced silencing complex). One strand of the duplex is retained by the RISC as the mature miRNA, whereas the other strand is degraded. D. melanogaster homologues, Dicer 1, Loquacious (LOQS) and AGO1, responsible for the same process are indicated. b) Canonical intronic miRNAs are processed before splicing in a co-transcriptional event. The splicing commitment complex is thought to bind the Microprocessor compex which cleaves the intronic miRNA. The hairpin-shaped pre-miRNA can enter the canonical miRNA pathway, whereas the rest of the transcript undergoes splicing. c) Non-canonical intronic small RNAs (mirtrons) are produced from spliced and debranched introns able to fold in a hairpin pre-miRNA. Mirtrons bypass the Drosha-processing step and a trimming step eliminates 5′ or 3′ extra tails before pre-miRNA export. m7G, 7-methylguanosine. (Kim et al., 2009).

*Nuclear processing by Drosha.* RNA Pol II generates a primary transcript (pri-miRNA), That is usually several kilobases long and contains a local hairpin structure. A typical pri-miRNA consists of a stem of ~33 nt, a terminal loop of ~10 bp and flanking single-stranded tails (Figure 4a). The first step of miRNA maturation (termed “cropping”) occurs in the nucleus and consists of the cleavage at the base of the stem of the hairpin structure, which releases a ~65 nt hairpin termed pre-miRNA (Lee et al., 2002). The cropping process seems to be co-transcriptional (Kim et al., 2007; Morlando et al., 2008; Pawlicki et al., 2008). The pre-miRNA is formed by a short stem with a ~2-nt 3′ overhang, which is specifically recognized by the nuclear export factor exportin 5 (EXP5) (See below). This reaction is carried out by the nuclear RNase III-type enzyme Drosha (Lee et al., 2003) which associates with a cofactor, the DiGeorge syndrome critical region gene 8 (DGCR8) protein (Pasha in D. melanogaster and C. elegans) to form a large protein complex known as the Microprocessor complex, which is ~500 kDa in D. melanogaster and ~650 kDa in humans (Han et al., 2004; Denli et al., 2004; Gregory et al., 2004; Landthaler et al., 2004). DGCR8 interacts with the ssRNA tails and the stem of the pri-miRNA, while Drosha cleaves the stem ~11 bp from its base (Han et al., 2006; Zeng et al., 2005). Canonical intronic miRNAs are processed before splicing in a co-transcriptional event (Kim et al., 2007) (Figure 4b). The splicing commitment complex is thought to bind the intron and interact with the Microprocessor complex which cleaves the

intronic miRNA before the intron is excised. For transcripts harboring intronic miRNAs, cropping and splicing might therefore be highly coordinated co-transcriptional processes. The hairpin-shaped pre-miRNA can enter the canonical miRNA pathway, whereas the rest of the transcript undergoes splicing.

A small group of miRNAs identified in flies and mammals are embedded in short introns and do not require Drosha processing for their biogenesis (Ruby et al., 2007; Okamura et al., 2007; Berezikov et al., 2007) ("mirtrons"; Figure 4c). Following splicing, the lariat-shaped intron is debranched and folds into a hairpin structure that resembles pre-miRNA. Some mirtrons contain extended tails at either the 5′ or 3′ end and undergo exonucleolytic trimming in order to become a substrate for nuclear export. In addition, small RNAs can also derive from other non-coding RNAs, such as tRNA (Babiarz et al., 2008) or small nucleolar RNA (snoRNA) (Ender et al., 2008). Multiple non-canonical pathways can therefore generate, through Drosha-independent processes, miRNA precursors that finally enter the common miRNA pathway (Kim et al., 2009).

*Nuclear export by exportin 5.* Pre-miRNAs are exported to the cytoplasm by exportin 5 (EXP5), a member of the nuclear transport receptor family (Figure 4a) (Kim 2004; Lund et al., 2004; Yi et al., 2005; Bohnsack et al., 2004). EXP5 binds cooperatively to the pre-miRNA and the GTP-bound form of the cofactor Ran in the nucleus, and releases the pre-miRNA in the cytoplasm following the hydrolysis of GTP to GDP. EXP5 is able to specifically recognize and bind the characteristic pre-miRNA structure, presenting a >14 bp dsRNA stem with a short 3′ overhang (1–8 nt) (Lund et al., 2004; Gwizdek et al., 2003; Basyuk et al., 2003; Zeng et al., 2004).

*Cytoplasmic processing by Dicer.* Following nuclear export, in the second processing step (dicing) the RNase III Dicer cleaves the pre-miRNAs near the terminal loop, releasing a ~22 nt miRNA duplex (Bernstein et al., 2001; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight et al., 2001) (Figure 4a). the mature miRNA sequence is defined both by Drosha, which generates one end of the mature miRNA, and by Dicer, which measures ~22 nt from the pre-existing terminus of the pre-miRNA and generates the second end of mature miRNAs. Dicer is a highly conserved protein in almost all eukaryotic organisms; some organisms contain multiple Dicer homologues which can present distinct activities (Lee et al., 2004; Xie et al., 2004). In D. melanogaster, as an example, Dicer 1 is responsible for miRNA

biogenesis and Dicer 2 for siRNA production (Lee et al., 2004). Similar to Drosha in the nucleus, during the pre-miRNA processing Dicer acts in association with dsRNA-binding proteins: human Dicer binds to two closely related proteins, TRBP (TAR RNA-binding protein; also known as TARBP2) (Chendrimada et al., 2005; Haase et al., 2005) and PACT (also known as PRKRA) (Lee et al., 2006), which seem to contribute to the formation of the RNA-induced silencing complex (RISC; see below), although they are not necessary for processing activity itself. In D. melanogaster Dicer 1 binds to loquacious (loQS; also known as R3D1), which contains three dsRNA-binding domains (Forstemann et al., 2005; Jiang et al., 2005; Saito et al., 2005).

*Argonaute loading.* The ~22 nt RNA duplex generated by the action of Dicer is loaded onto the effector complex, named RNA-induced silencing complex (RISC). The RISC core is composed by proteins of the Argonaute (AGO) family. The AGO family is composed by the AGO and the PIWI subfamilies. The human AGO family, which interacts with miRNAs or small siRNAs, includes 4 members: AGO 1-4, also known as EIf2C1-4. AGO proteins are composed of four domains: the amino-terminal domain; the PAZ domain, which binds the 3′-end of miRNAs; the MID domain, which binds the 5′-phosphate of miRNAs; and the PIWI domain, which adopts an RNase H-like fold and has endonucleolytic activity in some AGOs (Jinek et al., 2009). One strand of the ~22 nt RNA duplex is bound by AGO and is retained in the RISC as a mature miRNA (the guide strand), whereas the other strand (the passenger strand) is degraded. For the siRNA duplexes, the relative thermodynamic stability of the two ends of the duplex determines which strand is retained: the strand with more unstable base pairs at the 5′ end typically is selected as guide strand (Schwarz et al., 2003; Khvorova et al., 2003). Studies on thermodynamic stability of miRNA duplexes confirm that the same mechanism of strand selection might be applied to most miRNAs; nevertheless some precursors have been demonstrated to produce miRNAs from both strands at comparable frequencies since strand selection is not a stringent process (Han et al., 2006; Khvorova et al., 2003). Together with AGO proteins, in humans Dicer and TRBP (and/or PACT) contribute to effector complex assembly by forming the so called RISC loading complex (RlC). In flies, the corresponding RlC is formed by Dicer 1, loQS and AGO1 (Chendrimada et al., 2005; Maniataki et al., 2005; MacRae et al., 2008; Tomari et al., 2004). RlC seems to

bind to RNA duplexes and facilitates mature miRNA loading on AGO. After cleavage, Dicer releases the miRNA duplex and the more stable end of the duplex is retained by TRBP in the RlC, while the other end corresponding to the guide strand is bound by the AGO protein (Tomari et al., 2004; Preall et al., 2005). The endonucleolytic enzymatic activity (called slicer activity) of AGO2 in humans is responsible for the removal of the passenger strand of siRNA duplexes and some miRNA duplexes with perfect sequence complementarities (Matranga et al., 2005; Miyoshi et al., 2005; Leuschner et al., 2006; Diederichs et al., 2007). However, AGO1, AGO3 and AGO4 lack slicer activity, which prevents the cleavage of the passenger strand, and most miRNA duplexes (unlike siRNA duplexes) contain mismatches in the middle. It has been proposed that the passenger strand is unwound by an RNA helicase activity and degraded after removal (Kawamata et al., 2009; Yoda et al., 2010).

miRNA sequences can be subjected to editing events carried out by ADARs (adenosine deaminases that act on RNA), which catalyse the conversion of adenosine to inosine in dsRNA, thereby altering the base-pairing and structural properties of target RNA. Both pri-miRNAs and pre-miRNAs can be targeted by ADARs at different stages in miRNA biogenesis, and the modifications can affect Drosha and Dicer activity and also prevent the export of pre-miRNAs. A differential editing that affects the biogenesis process may in part be responsible for tissue- specific miRNA expression. On the other hand, editing events leading to seed sequence changes can have an important impact on the target specificity of the miRNA (Heale et al., 2009; Kawahara et al., 2007). miRNA sequences often show heterogeneous ends due to addition or deletion of 1-2 nt (Azuma-Mukai et al., 2008; Seitz et al., 2008). The 3′ ends tend to be much more variable than the 5′ ends because changes in the 5′ terminus result in shifts of the seed sequences, which alter the target specificity of the miRNA. The mechanisms of the variations are unknown but they might be explained by imprecise or alternative processing by RNase III enzymes or by deletions due to exonucleolytic activities. The 3′ ends of miRNA can often present untemplated nucleotides (mostly uracil and adenine), likely added by unknown terminal uridyl/adenyl transferases (Kim et al., 2009).

### 1.2.2 MIRNA FUNCTION

Most animal miRNAs bind to imperfect complementary sequences in the 3′-UTR of target mRNAs, leading to either translation repression or mRNA deadenylation and decay promotion. The relative contributions of these two different ways of target regulation by miRNAs remain unclear and several controversies surround the topic (Filipowicz et al., 2008; Huntzinger et al., 2011).

Deadenylation of miRNA targets is mediated by GW182 (glycine-tryptophan protein of 182 kDa) proteins, which interact with AGO proteins and represent the other core components of RISC (Figure 5a). Vertebrates present three GW182 isoformes, TNRC6A (also known as GW182), TNRC6B and TNRC6C, while D. melanogaster contains one (GW182) and fungi and plants lack orthologue proteins (Behm-Anmant et al., 2006). GW182 proteins are formed by two globular domains, an ubiquitin-associated-like domain (UBA) and an RNA-recognition motif (RRM), contained in the N-terminal (N-term) and carboxy-terminal (C-term) unstructured regions, respectively. The N-terminal domain also contains a variable number of glycine–tryptophan repeats (GW-repeats). The mid and C-terminal regions constitute a bipartite silencing domain, which includes a PABPC-interacting motif 2 (PAM2 motif) and the M1 and M2 regions (upstream and downstream of the PAM2 motif, respectively). A region rich in glutamine (Q-rich) is located between the N-term and the mid domain (Huntzinger et al., 2011). GW182 interacts with the AGO MID/PIWI domain through the GW repeats present in its N-terminal part (Till et al., 2007), while the GW182 C-terminal part interacts with the cytoplasmic poly(A)-binding protein (PABPC) that in turn recruits the deadenylase complex. The PAM2 motif of GW182 proteins directly binds to PABPC (Fabian et al., 2009; Huntzinger et al., 2010; Kozlov et al., 2010; Jinek et al., 2010) while a less-defined sequence comprising the M2 and C-terminal regions interacts indirectly with PABPC, probably through additional proteins (Huntzinger et al., 2010). Through PABPC binding miRNAs direct their targets to the cellular 5′-to-3′ mRNA decay pathway (Figure 5b) (Rehwinkel et al., 2005; Behm-Ansmant et al., 2006; Wu et al., 2006; Eulaio et al., 2007; Eulaio et al., 2009; Piao et al., 2010). mRNAs are first deadenylated by the CAF1–CCR4–NOT deadenylase complex, and then decapped

by the decapping complex DCP1-DCP2. Finally, decapped mRNAs are degraded by the major cytoplasmic 5′-to-3′ exonuclease XRN1.

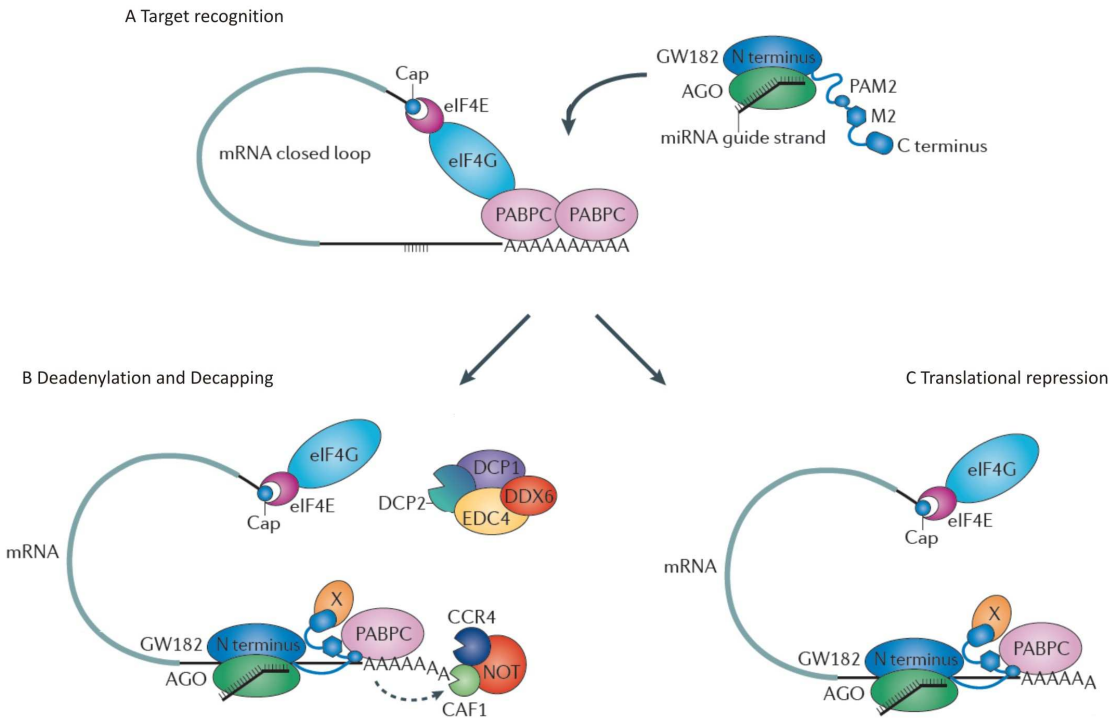


**Figure 5: Mechanisms of miRNA-mediated gene silencing in animals**. A) The cytoplasmic poly(A)-binding protein (PABPC), associated with the mRNA poly(A) tail, interacts with eukaryotic translation-initiation factor 4G (eIF4G), which in turn is associated with the cap structure through interaction with the cap-binding protein eIF4e. This interaction gives rise to a closed loop conformation of mRNAs that are efficiently translated and protected from degradation. RISC-associated miRNAs recognize their mRNA targets by base-pairing to partially complementary binding sites, predominantly in the mRNA 3′ UTR. AGO proteins bind to GW182, which interacts with PABPC. B) The AGO–GW182 complex directs the mRNA to deadenylation by recruiting components of the major deadenylase complex (CAF1, CCR4 and the NOT complex). Deadenylated mRNAs are then decapped by the decapping enzyme DCP2 and several decapping activators (for example, DCP1, EDC4 and DDX6) and then rapidly degraded by the major 5′-to-3′ exonuclease XRN1. C) The interaction of AGO-GW182 with PABPC blocks the formation of the eIF4F complex and the closed conformation of the mRNA and inhibits translation initiation. (modified from Huntzinger et al., 2011).

Four distinct mechanisms for translational repression have been proposed in animals: inhibition of translation initiation; inhibition of translation elongation; co-translational protein degradation; and premature termination of translation (Carthew

et al., 2009; Wu et al., 2008; Eulalio et al., 2008; Filipowicz et al., 2008). Increasing evidence suggests that translational repression occurs predominantly at initiation (Huntzinger et al., 2011).

In the initiation step PABPC, associated with the mRNA poly(A) tail, interacts with eukaryotic translation-initiation factor 4G (eIF4G), which is associated with the cap structure through interaction with the cap-binding protein eIF4e, forming the eIF4F complex (Figure 5a). This interaction determines the formation of circular mRNAs that are efficiently translated and protected from degradation (Derry et al., 2006). miRNAs interfere with the function of the eIF4F complex and PABPC during translation (Zdanowicz et al., 2009; Ding et al., 2009) (Figure 5c).

When miRNAs bind to target mRNA with nearly perfect sequence complementarity, the mRNA can be cleaved endonucleolytically and degraded by RISC containing AGO 2 (Bartel 2009; Carthew et al., 2009; Chekulaeva et al., 2009; Fabian et al., 2010). This is the common way of miRNA action in plants but is instead rare in animals. However, miRNAs in plants may also imperfectly recognize mRNA targets and repress their translation (Voinnet 2009). In the case of fully perfect or nearly miRNA-mRNA complementarity, target mRNA cleavage occurs between nucleotides 10 and 11, opposite the miRNA strand, and is catalysed by AGOs. The resulting mRNA fragments are degraded from the newly generated 3′ and 5′ ends.

### 1.2.3 MIRNAS IN NORMAL CD4+ T-CELLS

*miRNA profiles in T-cell development*. The first strong experimental evidence for a role of miRNAs in T-cell development and homeostasis came from experiments performed in mice demonstrating that conditional deletion of Dicer at the stage of DN (double negative) thymocytes resulted in a loss of both CD4+ and CD8+ peripheral T-cell populations (Cobb et al., 2006). Deletion of Dicer during the DN/DP (double positive) transition greatly reduced the number of peripheral CD8+ cells and impaired the ability of peripheral CD4+ T cells to differentiate into mature helper cells (Muljo et al., 2005). In a recent analysis of the global expression profile of miRNAs in human DP, SP CD4+ and SP CD8+ T cells, the miRNA profile in DP cells was found to be distinct from those of CD4 SP and CD8 SP cells, while the SP

populations showed important similarities; a general upregulation of miRNAs from the DP to the SP stage was noted (Ghisi et al., 2011). Comparison of miRNA profiles in DP, SP and peripheral SP CD4 and CD8 cells indicated a progressive upregulation of miR-150, miR-146a, and miR-146b and downregulation of miR-128; miR-181 was less abundant in mature peripheral T lymphocytes compared with DP thymocytes (Ghisi, et al., 2011).

*miRNAs in activated T cells.* In an early microarray-based comparison of murine CD4+ T cells before and after stimulation with anti-TCR and anti-CD28, Cobb et al. reported 20 upregulated and 20 downregulated miRNAs (Cobb et al., 2006). A recent study by Grigoryev et al. that profiled miRNA expression in human CD2+ T lymphocytes following stimulation with CD3/CD28 beads indicated a trend toward upregulation of miRNA expression upon activation (Grigoryev, et al., 2011). The top 5 upregulated miRNAs were miR-221, miR-210, miR-98, miR-29b and miR-155, and the top 5 downregulated miRNAs were miR-181a, miR-199a, miR-223, miR-224 and miR-127-3p, most of which had not been previously described as being involved in the regulation of immune activation. Knock-down of miR-155 or miR-221 with locked nucleic acid (LNA) anti-miRNAs increased proliferation of CD4+ T cells. This effect was accompanied by increased expression of PIK3R1 (85-kDa regulatory subunit of phosphoinositide-3-kinase) and IRS2 (insulin receptor substrate-2, an insulin receptor docking protein) induced by anti-miR-155, and the transcription factor FOS, induced by both anti-miRs. The authors proposed that these effects provide a negative-feedback loop inhibiting cell proliferation and regulating survival in response to activation (Grigoryev, et al., 2011).

*miRNAs in Tregs.* The early studies of mice with deletion of Dicer at the stage of thymocytes also implicated the miRNA pathway in Treg development, with a reduction in their numbers in both the thymus and periphery (Cobb et al., 2006); deletion of Dicer after Treg lineage commitment (at the time of Foxp3 induction) resulted in a profound impairment of Treg suppressor function and fatal systemic autoimmune disease (Liston et al., 2008; Zhou et al., 2008). A comparison of miRNA profiles in murine Tregs (CD4+CD25+GITR+) vs. non- Treg CD4+ naive T cells yielded many miRNAs that were differentially expressed in the Treg population. In vitro stimulation of the naïve T cells resulted in a transient shift in their miRNA profile toward that of Tregs. When compared to naïve cells, both Tregs

and activated T cells exhibited an upregulation of miR-155, miR-214, miR-23b, miR-22, miR-21, miR-23a, miR-24, miR-27a, miR-103 and downregulation of miR-29c, miR-142-5p, miR-142-3p, let-7 family members, miR-30b, miR-30c, miR-25a, miR-26b, and miR-150 (Cobb et al., 2006). A comparison of human Tregs (CD4+, CD25+, Foxp3+) vs naïve T-cells (CD4+CD25-) isolated from umbilical cord blood indicated that human Tregs can be distinguished on the basis of their increased levels of miR-21, miR-181c and miR-374 and reduced levels of miR-31 and miR-125a (Rouas et al., 2009). This study also demonstrated the regulation of Foxp3 by two of the miRNAs in this signature: miR-31 downregulated expression of Foxp3 through direct targeting of its 3'UTR, while miR-21 upregulated its expression through an indirect mechanism (Rouas et al., 2009). Rossi et al. recently carried out a thorough investigation of the miRNA signatures of 17 lymphocyte subsets purified from human peripheral blood (Rossi et al., 2011). Results of this analysis revealed many miRNAs that were not previously reported as differentially expressed in human T cells. The authors showed that 20 miRNAs were upregulated and 5 miRNAs were downregulated in naïve CD4+ T cells compared to CD4+ memory cells. Among these, 4 miRNAs appeared to be specific for TH1 memory cells, 2 for TH17, and 4 were upregulated in TH2 memory cells. In vitro stimulation with anti-CD3 and anti-CD28 antibodies resulted in the downregulation of 19 miRNAs that were expressed at high levels in naive CD4+ T cells. Moreover, the study showed that miR-125b controls a network of target genes involved in CD4+ T cell ontogenesis; forced expression of miR-125b resulted in a block in differentiation and favored a naive phenotype of CD4+ T cells.

### 1.2.4 CELLULAR MIRNA EXPRESSION IN HTLV-1-INFECTED CELL LINES AND ATLL SAMPLES

Figure 6 summarizes results of 3 recent studies that examined changes in cellular miRNA expression in HTLV-1-infected cell lines and ATLL samples. Although it is striking how little the results of these studies overlap with each other, each yielded one or more miRNAs with established roles in other tumors.

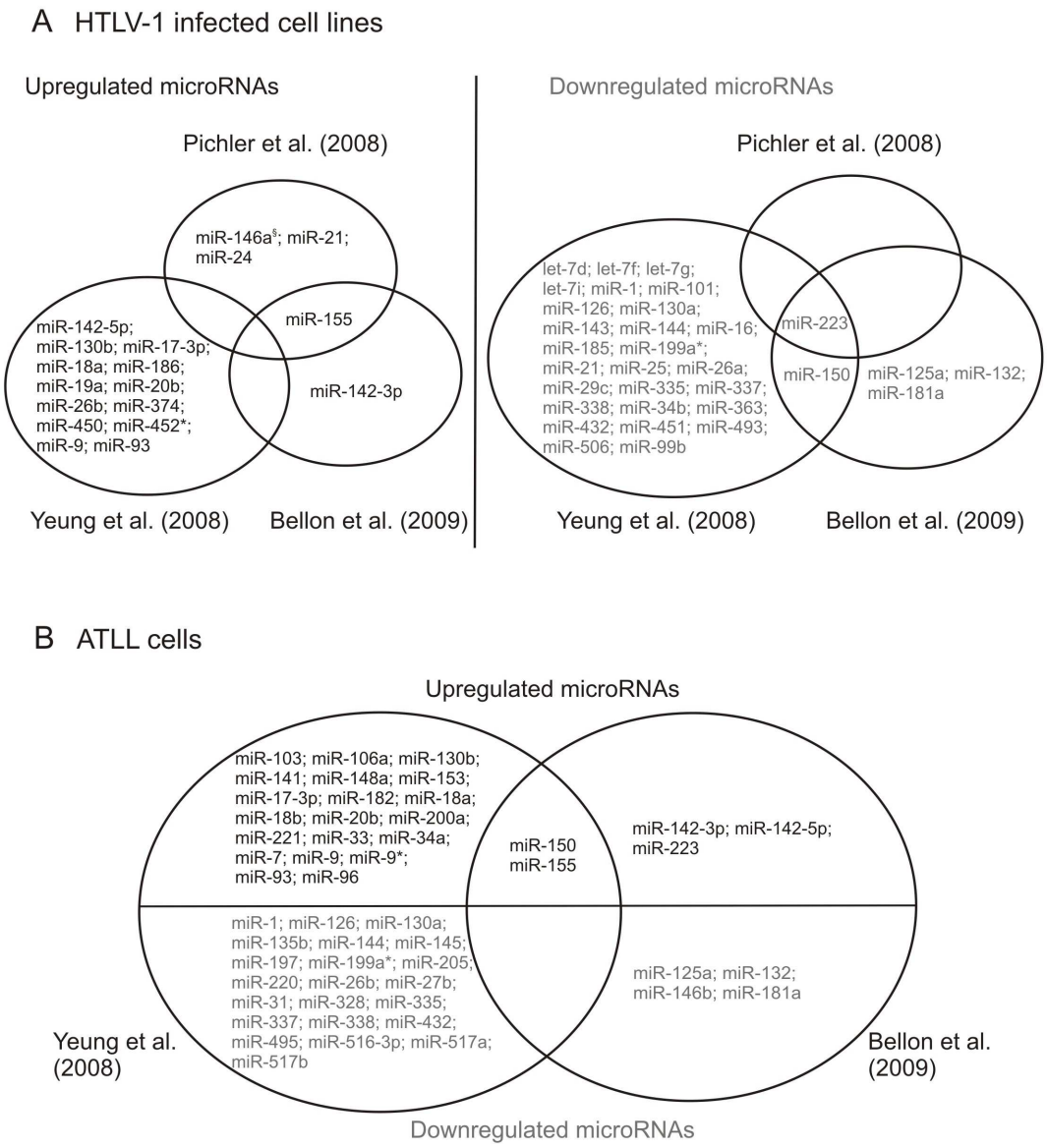


**Figure 6: Cellular miRNA expression in HTLV-1-infected cell lines and ATLL samples.** Data are from the indicated references. §Upregulation of miR-146a in cell lines was verified by Tomita et al., 2009. (Ruggero et al., 2010).

Pichler et al. employed quantitative RT-PCR to study a selected set of miRNAs in HTLV-1-transformed cell lines (Pichler et al., 2008). Given that HTLV-1-transformed cells express some of the phenotypic markers of Tregs, the investigators focused on 7 miRNAs that are upregulated in this T-cell subset compared to resting CD4+ T-cells (Cobb et al., 2006) and that had already been implicated in oncogenic

transformation, i.e., miR-21, miR-24, miR-146a, miR-155, miR-191, miR-214 and miR-223. The study analyzed cell lines derived from ATLL patients and TSP/HAM patients, cell lines generated by cocultivating umbilical cord blood cells with ATLL cells, and a T-cell line that expresses Tax in repressible manner; controls included uninfected PBMC, CD4+ T-cells and uninfected T-cell lines. Results demonstrated that miR-21, miR-24, miR-146a and miR-155 were significantly upregulated in the HTLV-1-transformed cell lines, while miR-223 was downregulated.

Two studies investigated miRNA expression profiles in the context of HTLV-1 using miRNA-arrays (Bellon et al., 2009; Yeung et al., 2008). Yeung et al. examined PBMC from patients with acute ATLL compared to pooled control PBMC, as well as HTLV-1-transformed cell lines compared to umbilical cord blood cells. Although the ATLL samples and infected cell lines yielded somewhat different expression profiles, they shared 6 upregulated miRNAs (i.e., miR-18a, 9, 17-3p, 130b, 20b, and 93) and 9 downregulated miRNAs (i.e., miR-1, 130a, 199a*, 126, 144, 335, 337, 338, 432). The investigators also examined PBMC exposed to the tumor-promoting agent phorbol-12-myristate 13-acetate (PMA) compared to untreated PBMC, and identified 3 miRNAs that were upregulated in ATLL cells, HTLV-1-infected cell lines and PMA-treated cells, namely miR-93, miR-130b, and miR-18a. Bellon et al. (Bellon et al., 2009) used arrays to compare miRNA expression in ATLL cells versus control PBMC and CD4+ T-cells. miRNAs with altered expression levels included miR-150, miR-155, miR-223, miR-142-3p and miR142-5p (upregulated) and miR-181a, miR-132, miR-125a and miR-146b (downregulated). Examination of these miRNAs in HTLV-1-infected cells yielded similar data, with the exception that miR-150 and miR-223 were downregulated instead of upregulated. The authors also showed that treatment of HTLV-1-infected cell lines with an inhibitor of NF-κB (pathenolide) or JNK (JNK II) resulted in reduced levels of the miR-155 precursor.

#### 1.2.4.1 Upregulated miRNAs

*miR-146a*. Among the 4 miRNAs upregulated in HTLV-1-infected cell lines examined by Pichler et al. (miR-21, miR-24, miR-146a, miR-155), miR-146a was found to be activated by Tax in an NF-κB-dependent manner (Pichler et al., 2008). An independent study confirmed upregulation of miR-146a in the context of HTLV-1 and verified the ability of Tax to induce its expression through NF-κB (Tomita et

al., 2009). The miR-146a promoter was highly bound by NF-κB complexes in HTLV-1-infected cells, while treatment with a NF-κB inhibitor reduced binding and interfered with expression of the miRNA. In addition, treatment of infected cell lines with an anti-miR-146a interfered with their growth without affecting uninfected T-cell lines. On the other hand, a growth-enhancing effect was observed in infected cell lines forced to overexpress miR-146a. Upregulation of miR-146a expression therefore appears to be one of the strategies used by HTLV-1 to favor proliferation of infected cells. It is noteworthy that all 4 miRNAs upregulated in HTLV-1-infected cell lines examined by Pichler et al. (miR-21, miR-24, miR-146a, miR-155) are also upregulated in EBV-infected B-cells during latency III, the viral growth program that drives B-cell proliferation (Cameron et al., 2008). miR-146a is overexpressed in various solid cancers (He et al., 2005; Volinia et al., 2006) and in pediatric AML and B-ALL (Zhang et al., 2009). It was first identified as a miRNA that fine-tunes the innate immune response, as its expression is induced by NF-κB after Toll-like and IL-1 receptor (TIR) engagement, and exerts a negative feedback control on TIR signalling by targeting the adaptor proteins IRAK1 and TRAF6 (Taganov et al., 2006). miR-146a is upregulated in CD4+ T-cells after activation through the T-cell receptor (TCR) (Cobb et al., 2006), resulting in a reduction of IL-2 production by impairing AP-1 transcriptional activity, leading to an attenuation of the IL-2 signal (Curtale et al., 2010). miR-146a also targets FADD (Fas-Associated Death Domain) and thereby protects T-cells from AICD (Activation Induced Cell Death), an apoptotic response that follows TCR stimulation and is crucial for the termination of the immune response and for peripheral tolerance to self-antigens (Curtale et al., 2010). Although in some cell contexts miR-146a can downregulate NF-κB activity (Bhaumik et al., 2009) and therefore silence its own expression, this feedback mechanism appears to be blocked in HTLV-1-infected cells, likely through the action of Tax.

*miR-24.* miR-24 is expressed in many normal tissues and is upregulated in differentiated cells. The upregulation of miR-24 in tumor cell lines and primary cells causes arrest in G1, while its silencing induces cell proliferation, by targeting of several genes involved in cell cycle control including Myc and E2F2 (Lal et al., 2009). There is also evidence that miR-24 can exert oncogenic rather that growth-suppressive properties: miR-24 exerts an anti-apoptotic effect in several cell lines by

targeting the pro-apoptotic factor FAF-1 (Fas-associated factor 1) through binding to its coding sequence (Qin et al., 2010), and inhibits apoptosis of cardiomyocytes by targeting Bim (Qian et al., 2011). Studies in leukemic cell lines demonstrated that miR-24 also targets H2AX, resulting in a defect in the DNA damage repair response (Fernandez-Capetillo et al., 2004) which could lead to the accumulation of transforming mutations.

*miR-155.* High levels of miR-155 are evident in Tregs and activated T cells as well as in activated B cells, activated macrophages and dendritic cells (Faraoni et al., 2009). Mice deficient in miR-155 show impairments in T-, B- and dendritic cell functions (Rodriguez et al., 2007). CD4+ T-cells from miR-155-deficient mice tend to differentiate into Th2 cells and show reduced IL-2 and IFN-γ production in response to antigen stimulation (Thai et al., 2007). miR-155 expression in Tregs depends on the activity of Foxp3; miR-155 in turn blocks expression of SOCS1, a negative regulator of IL-2R signalling, thus maintaining Tregs highly sensitive to IL-2 (Lu et al., 2009). Overexpression of miR-155 in CD4+ T-cells renders them resistant to Treg -mediated suppression (Stahl et al., 2009). In addition to ATLL, Overexpression of miR-155 has been documented in several other hematological malignancies and solid tumors (e.g., lung, thyroid, pancreas, breast, colon, cervix) (Faraoni, et al., 2009). In mice, forced expression of miR-155 induces polyclonal pre-B-cell tumors (Costinean et al., 2006). miR-155 may also be important in the mechanisms of B-cell transformation driven by EBV, which induces miR-155 expression, and HHV-8, which codes for a miR-155 orthologue with target specificity similar to that of the cellular miRNA (Gottwein et al., 2007; Skalsky et al., 2007). The EBV LMP-1 protein upregulates cellular miR-155 through NF-κB (Gatto et al., 2008; Lu et al., 2008; Rahadiani et al., 2008). miR-155 in turn targets IKKε (Lu et al., 2008), a transcriptional target of the NF-κB pathway that is involved in the interferon antiviral response, suggesting that The NF-κB pathway might be responsible for high levels of miR-155 in HTLV-1-infected cells. The oncogenic properties of miR-155 can in part be explained by its ability to block expression of tumor protein 53-induced nuclear protein 1 (TP53INP1), a nuclear protein that promotes cell cycle arrest and apoptosis (Gironella et al., 2007).

*miR-93.* miR-93 is a member of the miR-106b-25 cluster, which also includes miR-25 and miR-106b, located on chromosome 7q22.1 within an intron of the MCM7

gene. These miRNAs share sequence similarity with those encoded in the oncogenic miR-17-92 cluster on chromosome 13 (Mendell, 2008). miR-93 and its relatives are highly abundant in hematopoietic progenitor cells and promote their expansion when ectopically expressed (Meenhuis et al., 2011). miR-93 is upregulated in several tumors of epithelial derivation (Ambs et al., 2008; Blenkiron et al., 2007; Kan et al., 2009; Li et al., 2009; Nam et al., 2008; Petrocca et al., 2008b; Yanaihara et al., 2006). Silencing of miR-93 or miR-106b interferes with the growth of an esophageal adenocarcinoma cell line in nude mice, indicating an oncogenic role (Kan et al., 2009). Studies performed in gastric, esophageal and mammary epithelial cell lines (Ivanovska et al., 2008; Kan et al., 2009; Petrocca et al., 2008a; Petrocca, et al., 2008b) showed that, through targeting of E2F1 and p21, the miR-106b-25 and miR-17-92 clusters play an important role in fine-tuning the TGF-β pathway, which is often functionally inactivated in epithelial tumors. miR-93 also targets MICB (major histocompatibility complex class I chain-related B), a ligand for a receptor present on NK, CD8+ and γδ T-cells that is upregulated by stress signals such as viral infection; it has been proposed that overexpression of miR-93 in tumor cells leads to loss of MICB and immune evasion (Stern-Ginossar et al., 2008). Interestingly, miR-93 along with miR-155 are included in a panel of 49 miRNAs shown to be upregulated in skin biopsies of mycosis fungoides (van Kester, et al., 2011).

*miR-130b*. In addition to ATLL, miR-130b is upregulated in pediatric ALL and in multiple myeloma during progression of gliomas to glioblastomas (Malzkorn et al., 2009) and, along with miR-93 and other miRNAs, is able to interfere with Ras-induced senescence (Borgdorff et al., 2010) suggesting an oncogenic role. Analysis of the miR-130b predicted promoter region revealed potential binding sites for several transcription factors, including NF-κB; results of promoter-reporter assays verified that the promoter sequence was responsive to Tax (Yeung et al., 2008). Yeung et al. identified the tumor suppressor TP53INP1 as a target for miR-93 and miR-130b (Yeung, et al., 2008); it is noteworthy that TP53INP1 was also indicated as a potential mRNA target of all 4 miRNAs identified by Pichler et al. as upregulated in the HTLV-1- cell lines (miR-21, miR-24, miR-146a and miR-155) (Pichler, et al., 2008). Accordingly, knockdown of miR-93 and miR-130b with antagomirs in an HTLV-1 cell line resulted in increased expression of TP53INP1 accompanied by an increase in apoptotic cell death. Loss of TP53INP1 expression

has been documented during the progression of gastric cancer (Jiang et al., 2006) and early in the development of pancreatic adenocarcinoma, where it is targeted by overexpressed miR-155 (Gironella, et al., 2007). Reintroduction of TP53INP1 in a pancreatic adenocarcinoma cell line interfered with its growth in nude mice (Gironella, et al., 2007). TP53INP1 is upregulated by p53 and p73 in response to stress signals and contributes to the cell cycle arrest and apoptosis induced by these tumor suppressors (Tomasini et al., 2003; Tomasini et al., 2005). ATLL is characterized by inactivation of p53 either as a result of inactivating mutations (Yamada and Kamihira, 2005), or more often through other mechanisms that lead to accumulation of the protein in an inactive state (Tabakin-Fix et al., 2005). Targeting of TP53INP1 by miRNAs provides a further mechanism by which p53/p73 function could be blocked in ATLL cells.

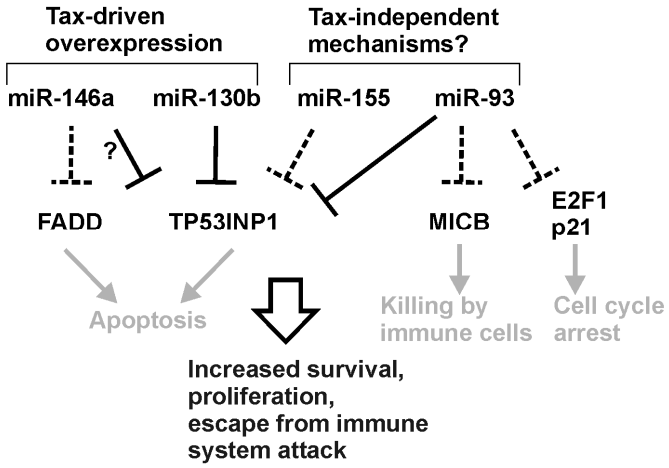


**Figure 7: Functional impact of miRNAs induced by HTLV-1 on viral persistence and transformation.** miRNAs upregulated during HTLV-1 infection/transformation include several miRNAs that may control expression of the tumor suppressor TP53INP1: miR-130b and miR-93 (interactions confirmed in HTLV-1-infected cells), miR-146a (predicted interaction), and miR-155 (interaction confirmed in other cell systems). Downregulation of TP53INP1 and additional potential targets for these miRNAs including FADD, MICB, E2F1 and p21 (confirmed in other cell systems; indicated by dotted lines) could protect HTLV-1-infected cells from apoptosis, drive their proliferation, and interfere with their recognition by the immune system attack. (Ruggero et al., 2010).

As depicted in Figure 7, the multiplicity of HTLV-1-upregulated miRNAs capable (or potentially capable) of targeting TP53INP1, including miRNAs that are driven by

Tax (miR-146a, miR-130b) as well as others that are apparently Tax-independent (e.g., miR-93, miR-155), provides a mechanism by which p53/p73 function could be blocked in ATLL cells. Data from studies of miR-93, miR-155, miR-146a and miR-130b in other cell systems reveal further important targets with roles in apoptosis (i.e., FADD, targeted by miR-146a), recognition by the immune system (i.e., MICB, targeted by miR-93) and the cell cycle (E2F1 and p21, targeted by miR-93) whose miRNA-mediated silencing could favor persistence and transformation of infected cells.

*miR-142-5p/3p.* The miR-142-5p/3p locus was originally identified as a rearrangement partner of c-Myc in prolymphocytic B-cell leukemia (Gauwerky et al., 1989). miR-142 is specifically expressed in hematopoietic tissues and is considered a lymphoid marker (Chen et al., 2004; Merkerova et al., 2008; Ramkissoon et al., 2006). miR-142-5p and miR-142-3p are either up- or downregulated in many haematological malignancies (Schotte et al., 2009; Navarro et al., 2009; Zhao et al., 2010; Zhang et al., 2009; Merkel et al., 2010; Flamant et al., 2010; Ju et al., 2009; Chi et al., 2011; Cammarata et a., 2010). A study aimed at understanding how murine Tregs maintain elevated levels of cAMP revealed that Foxp3 downregulates expression of miR-142-3p, which would otherwise inhibit the expression of adenylate cyclase-9, the enzyme responsible for cAMP production.

#### 1.2.4.2 Downregulated miRNAs

*miR-223.* miR-223, consistently downregulated in HTLV-1-infected cell lines in the 3 studies described above, is instead upregulated in murine Tregs (Cobb et al., 2006). miR-223 is a key modulator of promeylocyte-to-granulocyte differentiation (Fazi et al., 2005). miR-223-deficient mice exhibit an increase in granulocytic progenitors and neutrophils with an unusual morphology and hypersensitivity to activating stimuli, accompanied by a spontaneous lung inflammation with extensive neutrophil infiltration (Johnnidis et al., 2008). miR-223 is upregulated in bladder cancer (Gottardo et al., 2007), esophageal adenocarcinoma (Mathe et al., 2009), and in recurrent ovarian cancer (Laios et al., 2008), but is downregulated in hepatocellular carcinoma (Wong et al., 2008). miR-223 is also significantly down-regulated in acute lymphoblastic leukemia compared with acute myeloid leukemia and together with

other miRNAs may represent a differential diagnostic signature for these tumors (Mi et al., 2007). miR-223 downregulation is also included in miRNA signatures predicting poor prognosis in CLL (Calin et al., 2004; Fulci et al., 2007; Stamatopoulos et al., 2009). miR-223 therefore appears to exert either oncogenic or tumor suppressor properties depending on the cell context.

*miR-181a.* miR-181a, downregulated in the ATLL samples and HTLV-1-infected cell lines examined by Bellon et al. (2008) (Figure 6), is highly expressed in thymocytes and is temporally regulated during T-cell development, with very low levels in mature T-cells (Chen and Lodish, 2005; Li et al., 2007; Neilson et al., 2007). Increasing the expression of miR-181a in mature T-cells augments both the strength and sensitivity of TCR signaling by targeting several tyrosine phosphatases that act as negative feedback modulators in TCR signaling (Li et al., 2007). miR-181a is considered a marker of B-cells, and its forced expression in hematopoietic stem cells results in increased production of B-cells (Chen et al., 2004). miR-181a is downregulated in glioblastoma (Ciafre et al., 2005; Shi et al., 2008) and in CLL (Marton et al., 2008) Visone et al., 2009) and controls the expression levels of the oncogenic transcription factor PLAG1 (Pallasch et al., 2009). miR-181a expression levels correlate with morphological subclasses of acute myeloid leukemia (Debernardi et al., 2007). Conversely, upregulation of miR-181a is observed in multiple myeloma, and inhibition of miR-181a expression in multiple myeloma cell lines leads to a significant suppression of their growth as tumors in nude mice (Pichiorri et al., 2008).

*miR-150.* miR-150, consistently upregulated in ATLL samples (Bellon et al., 2009; Yeung et al., 2008), is gradually upregulated during T-cell development and downregulated in activated CD4+ T-cells, and is downregulated in Tregs through the action of Foxp3 (Cobb et al., 2006). Overexpression of miR-150 was shown to inhibit the growth of B-lymphoma cell lines, indicating its possible function as a tumor suppressor (Chang et al., 2008). Recent studies indicated that miR-150 is also downregulated in Sezary syndrome (Ballabio, et al., 2010), in NK/T-cell lymphomas (Watanabe et al., 2011), and in several other hematological tumors (Zhang et al., 2009; Merkel et al., 2010; Roehle et al., 2011; Zhao et al., 2010; Di Lisio et al., 2010; Flamant et al., 2010; Machova Polakova et al., 2011); furthermore, ectopic expression of miR-150 in NK cell lines reduces proliferation and induces apoptosis

(Watanabe et al., 2011). One of the cellular genes known to be targeted by miR-150 is c-Myb, a transcription factor that is overexpressed in some human leukemias and in tumors of the breast and colon (Ramsay and Gonda, 2008) Ghisi et al. observed that forced expression of miR-150 in T-ALL cell lines (which were found to constitutively express very low levels of this miRNA) triggered a reduction in proliferation and apoptotic death, and identified Notch3 as a direct target of miR-150 in T-cells (Ghisi, et al., 2011). The interaction of miR-150 with a component of the Notch pathway is particularly interesting, as this pathway plays a key role in the development of the T-cell compartment (Sultana et al., 2010). It would be of interest to investigate the influence of miR-150 on the Notch pathway in ATLL cells given the recent finding of a high rate of activating Notch mutations and constitutive activation of the Notch pathway in ATLL patients (Pancewicz et al., 2010).

# 2. AIMS OF THE STUDY

Dysregulation of miRNA expression plays an important role in development and progression of human cancers, where miRNAs have been demonstrated to act as tumor suppressors or oncogenes. Recent studies showed that HTLV-1 mediated transformation leads to major changes in cellular miRNA expression profiles. The viral transactivator Tax can drive the overexpression of cellular miRNAs leading to an increased in proliferation of infected cells, and a tumor suppressor gene has been identified as a target for 2 miRNAs altered in ATLL samples (i.e. TP53INP1, targeted by miR-93 and miR-130b). However, miRNA expression profiles identified so far in HTLV-1 infected cell lines and ATLL patients show little overlap among the different studies. Moreover, the function and mechanisms controlling the expression of the majority of dysregulated miRNAs identified remain to be investigated.

The work described in the present thesis was aimed at defining the role of miRNA in lymphocytes that are “resting”, activated or transformed by HTLV-1. First of all we wanted to gain a profile of the pattern of miRNA expression in control CD4+ cells and infected cell lines through analysis of small RNA cDNA libraries. The generation of miRNA libraries also permitted us to discover new miRNA candidates with a possible role in HTLV-1 tranformation; we validated some new miRNA sequences through the use of alternative techniques. Expression data for known miRNAs in control CD4+ cells and ATLL samples obtained with microarrays were further confirmed by quantitative RT-PCR. We intended to characterize the functional role and the mRNA targets in the context of HTLV-1 infection of the differentially expressed miRNA. To this purpose, we developed an integrative analysis of miRNA and gene expression data that led to the generation of a network of miRNA-mRNA targets supported by expression data and in silico prediction. Functional enrichment analysis of predicted targets permitted the identification of pathways involved. We investigated the role of miR-34a, significantly upregulated in ATLL samples and HTLV-1 infected cells lines, through a knockdown assay in order to evaluate the effect of miR-34a silencing on cell death of infected cell lines. In

addition, we investigated the possibility that Tax or Hbz could influence miR-34a expression.

# 3. MATERIALS AND METHODS

## 3.1 CELL CULTURES

Cell lines C91PL, HUT-102, MT-2, and C8166, chronically infected with HTLV-1, and T-ALL cell lines Jurkat, Cem, Tall, and Molt-3, and Burkitt lymphoma cell line BL41 were maintained in RPMI (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM glutamine (Invitrogen), 100 units/ml penicillin and 20 units/ml streptomycin (complete RPMI). Cell lines 293T, HeLa, Panc1, and U87 were maintained in DMEM (Dulbecco modified Eagle medium; Sigma-Aldrich) supplemented with 10% FBS, 100 units/mL penicillin, and 20 units/mL streptomycin.

Unstimulated and stimulated CD4+ T-cells were isolated from peripheral blood mononuclear cells (PBMC) of healthy donors obtained by separation of the buffy coat fraction through Ficoll-Hypaque (GE Healthcare). CD4+ cells were isolated by negative selection or positive selection. For negative selection, half of the PBMC sample was immediately processed using the MACS CD4+ T cell Isolation Kit II (Miltenyi Biotec); resulting CD4+ cells were harvested for total RNA using TRIzol (Invitrogen). The remaining PBMC were placed in complete RPMI ($1x10^6$ cells/ml) and stimulated with 100 μg/ml phytohemagglutinin (PHA, Sigma-Aldrich) for 48 hours and then with 50 U/ml interleukin-2 (IL-2, Proleukin-Chiron) for 48 hours. CD4+ cells were then isolated with the MACS CD4+ T cell Isolation Kit II and lysed in TRIzol. For positive selection, the entire PBMC sample was immediately processed using MACS CD4 Microbeads (Miltenyi); half of the resulting CD4+ cell sample was immediately lysed in TRIzol, and the other half was placed in culture, stimulated with PHA followed by IL-2, and then lysed in TRIzol. Aliquots of the separated cells were stained with anti-CD4 antibody and analyzed with a FACSCalibur apparatus (BD Coulter) and CellQuest software (BD Biosciences), which revealed >90% CD4+ cells in the samples. The quality of the RNA was assessed by electrophoresis using the RNA 6000 Nano Assay LabChip Kit and Agilent 2100 Bioanalyzer, and RNA concentration was measured using a Nanodrop spectrophotometer.

## 3.2 PATIENT SAMPLES

Total RNA was isolated from PBMC of 10 ATLL patients (9 with leukemia, 1 with lymphoma; see Table I). Details regarding the patients are provided in (Pise-Masison et al., 2009). Samples were collected in the context of National Cancer Institute Institutional Review Board-approved studies with informed consent obtained from all subjects in accordance with the Declaration of Helsinki. RNA from patients highlighted in grey in Table I was analyzed by both miRNA microarrays and quantitative RT-PCR; remaining samples were analyzed by quantitative RT-PCR. Abbreviations: M, male; F, female; WBC, white blood count (normal range, 3300-9600 cells/μl); RIT, radioimmunotherapy; Zenapax, humanized monoclonal antibody against IL-2Rα; CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone; CVP, cyclophosphamide, vincristine, and prednisone.

| Patient | Gender/age (years) | Diagnosis | WBC (cells/μl) | CD4+CD25+ (cells/μl) | % CD4+CD25+ | Therapy |
|---|---|---|---|---|---|---|
| ATL1 | M/48 | leukemia | 40,500 | 31,646 | 78 | RIT |
| ATL2 | M/41 | leukemia | 37,300 | 29,780 | 80 | Zenapax |
| ATL3 | M/53 | leukemia | 39,700 | 30,211 | 76 | Zenapax |
| ATL5a | F/50 | leukemia | 49,300 | 40,640 | 82 | CHOP |
| ATL12 | M/41 | leukemia | 10,500 | 4,958 | 47 | CVP |
| ATL14 | M/34 | leukemia | 90,600 | 84,715 | 94 | None |
| ATL20 | F/62 | leukemia | 62,700 | 49,056 | 78 | None |
| ATL24 | F/36 | lymphoma | 6,680 | 1,486 | 22 | CHOP |
| ATL31 | M/45 | leukemia | 271,000 | 249,510 | 92 | None |
| ATL32 | M/54 | leukemia | 34,400 | 26,595 | 77 | None |

**Table I. ATLL patients**

## 3.3 GENERATION OF SMALL RNA LIBRARIES

Small RNA libraries were generated according to Lau et al. (2001) as follows. A 10 μg aliquot of each total RNA preparation was spiked with a $^{32}$P-labelled 23-nt RNA tracer and then subjected to polyacrylamide gel electrophoresis (PAGE) through a 15% denaturing gel along with a $^{32}$P-labelled aliquot of Decade RNA ladder

(Ambion); the presence of the RNA tracer permitted visualization of each modification and purification step after exposure of the gel to a phosphorimaging screen (Storm, GE Healthcare). Species migrating in the 18-25 nucleotide size range were excised from the gel, eluted and ethanol-precipitated. The RNA was then modified by addition of an 17-nt oligonucleotide linker (miRNA Cloning Linker 1, IDT) at the 3' end with RNA ligase (GE Healthcare), PAGE-purified through a 12% denaturing gel, modified with a second 17-nt oligonucleotide linker at the 5' end, and PAGE-purified again through a 10% denaturing gel. Resulting modified RNA was reverse-transcribed and PCR-amplified in preparation for 454 sequencing according to a protocol provided by Dr. G. Hannon (Cold Spring Harbor Laboratory) with minor modifications as follows. RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) and an anti-sense primer complementary to the 3' linker. Resulting cDNA was PCR-amplified with Platinum Taq DNA polymerase (Invitrogen) with primers specific for the 5' and 3' linkers. PCR products were purified through a 12% nondenaturing polyacrylamide gel and digested with PacI (New England Biolabs) to eliminate the tracer cDNA, which contains a PacI site. The digestion mixture was subjected to PAGE through a 12% nondenaturing gel to eliminate the digested tracer molecules. This DNA was subjected to a second round of PCR using primers specific for the tags and containing additional identifier sequences. The PCR product was PAGE-purified, digested again with PacI, and gel-purified again as described above. The resulting sample was subjected to 454 mass sequencing in the laboratory of G. De Bellis (CNR, Milano) as described (Margulies et al., 2005).

## 3.4 IDENTIFICATION OF SMALL RNA SEQUENCES BY BIOINFORMATICS ANALYSIS

In collaboration with A. Guffanti (CNR, Milano), sequence reads were subjected to a series of computational analyses to identify known and potential new miRNAs (Soldà et al., 2009; Basso et al., 2009). In the first step, sequence reads were 'trimmed' of their 5' and 3' tags and sized to keep those of 17-25 nt. These sequences were mapped to the human genome (hg18, March 2006), with 1 mismatch or 1 gap

allowed. Sequences with more than 50 distinct locations on the genome and sequences of low complexity (i.e., likely from repetitive elements) were discarded. The genomic region (90 nt) 5' and 3' to each sequence was added to construct a 'pre-miRNA' which was tested for the probability of forming a hairpin-loop structure (Basso et al., 2009). Sequences that passed the secondary-structure test were then filtered to exclude those mapping to the genomic locations of known non-coding RNAs to exclude sno-RNAs, snRNAs, rRNAs and tRNAs. The sequences that passed this test ('cleaned sequences') were analyzed against known human miRNAs using the UCSC database of miRNA hairpins and the Sanger miRBASE; 2 gaps and 2 mismatches were allowed in order to account for possible RNA editing events. This step divided the sequences into known miRNAs and candidate new miRNAs. Sequences in the second category were compared to known miRNAs of other species present in the Sanger miRBASE, with 2 gaps and 2 mismatches allowed to distinguish between 'homologues' and 'new miRNA candidates'. Sequences in the latter group were classified according to their conservation across 17 species considered by PHAST software (Homo sapiens, various other mammals, chicken, vertebrate fishes, Xenopus) and genomic location (outside existing genes, i.e., intergenic, or within existing genes, i.e., intragenic). Intragenic sequences were further classified according to their position within within exons or introns of coding or noncoding genes. To identify viral miRNAs, the 'trimmed' sequence sets were compared to sequences in the HTLV-1 genome that were previously identified as having the potential to form a pre-miRNA-like hairpin structure (Li et al., 2008; downloaded from the Vir-Mir database at http://alk.ibms.sinica.edu.tw). The trimmed sequences were also mapped against the entire HTLV-1 genome (clone ATK, Genbank accession no. J02029 M33896). In both searches 2 gaps and 2 mismatches were allowed.

### 3.5 MIRNA MICROARRAYS

This analysis was performed on total RNA from 7 ATLL samples (PT 1, 3, 5a, 12, 14, 24, 32), 3 sets of negatively sorted resting and stimulated CD4+ cells (samples A, B and D in Figure 11), and one set of positively sorted resting and stimulated CD4+ cells (sample P in Figure 11). A 100-ng aliquot of total RNA was CY-3 labelled at

the 3' end using T4 RNA ligase and hybridized to Agilent miRNA microarrays (V1) according to the manufacturer's instructions. Hybridization signals were detected using an Agilent microarray scanner and processed with Agilent Feature Extraction software. Signal intensity values were normalized, using quantile-quantile normalization, after substituting negative value with the lowest positive value obtained for the miRNA probe set. The distribution of signal intensities was then plotted to distinguish miRNAs of possible biological relevance from those with overall very weak signals. 137 miRNAs with at least two samples in the upper 75th percentile (corresponding to a signal intensity of >5.91) were further analyzed. Hierarchical clustering of samples was performed with SPSS software, using Pearson correlation as metric and average clustering as linking method. Differentially expressed miRNAs were identified using the samr package for R software from Bioconductor, considering a false discovery rate threshold < 0.01. Tumor sample 24 (a lymphoma) was not included in this analysis.

### 3.6 GENE EXPRESSION ARRAYS

Gene expression data for ATLL samples were obtained using Affymetrix Human Genome U133A 2.0 Arrays as described (Pise-Masison et al., 2009). Resting and stimulated CD4+ cells (sample sets A, B, D, P) were examined using Affymetrix Human Genome U133 Plus 2.0 Arrays. The raw intensity signals of genes were extracted from CEL files and normalized using the default settings of the affy package for Bioconductor and re-annotated using Manhong Dai custom cdf, HGU133A2_Hs_ENTREZG and HGU133Plus2_Hs_ENTREZG (available at http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/12.1.0/entrezg.asp). Differentially expressed genes were identified using the samr package for R software from Bioconductor, considering a false discovery rate threshold < 0.01. The differential analysis was performed on the genes present in botht Affymetrix platforms.

## 3.7 IDENTIFICATION OF MIRNA TARGETS USING TARGET PREDICTION AND ARRAY DATA

Transcripts potentially targeted by miRNAs were identified by integrating miRNA expression data with mRNA expression data as described (Lionetti et al., 2009). In the first step of the analysis, potential targets for the 137 miRNAs were obtained using the TargetScan algorithm (release 5.2) available online (www.targetscan.org). Pearson correlation coefficients were then calculated for each potential miRNA-gene pair. Anticorrelation scores in the top 10% were collated as corresponding to targeting relations predicted by TargetScan analysis and supported by expression data. From these results, miRNAs and genes that were differentially regulated in ATLL samples compared to resting CD4+ cells were identified and elaborated into a putative miRNA/gene regulatory network using Cytoscape software (Shannon et al., 2003). Analysis of microarray data and target prediction studies were performed by M. Biasiolo and S. Bortoluzzi (Department of Biology, University of Padova).

## 3.8 QUANTITATIVE RT-PCR

Total RNA was subjected to reverse transcription and quantitative RT-PCR to detect known miRNAs using Applied Biosystems Taqman microRNA assays and a 7900HT Fast Real-Time PCR System according the manufacturer's protocol; RNU44 was used as an endogenous control. New miRNA candidates were detected by quantitative RT-PCR with SYBR green using the miR-Q method as described by Sharbati-Tehrani et al. (2008) and a LightCycler 480 Real-Time PCR System (Roche) according the manufacturer's protocol; U6 snRNA was used as an endogenous control. tRF-1001 and tRF-1006 were detected by SYBR green quantitative RT-PCR based on method described by Fulci et a. (2007) and a 7900HT Fast Real-Time PCR System according the manufacturer's protocol; U6 snRNA was used as an endogenous control.

For quantitative RT-PCR of ADCY6 and RAC1, total RNA was reverse transcribed using SuperScriptII reverse transcriptase (Invitrogen) and random hexamers according to the manufacturer's protocol, and amplified using specific primers and

Universal ProbeLibrary (UPL) probe and a LightCycler 480 Real-Time PCR System (Roche) according the manufacturer's protocol; PBGD was used as an endogenous control.

### 3.9 PLASMIDS

Plasmid LcXL codes for HTLV-1 Tax under the control of the viral LTR promoter (Ciminale et al., 1992). Plasmid for pLTR-mRFP, which expresses monomeric red fluorescent protein under the control of the LTR promoter, served as a reporter for Tax activity (D'Agostino, unpublished). Plasmid HBZ, encoding HBZ, was kindly provided by Professor M. Matsuoka (Satou et al., 2006). The plasmids used for miR-34a knockdown, pGFP-anti-miR-34a and pGFP-control, were constructed by subcloning fragments from retroviral vectors kindly provided by D. Rao (Rao et al., 2010) into pcDNA3.1 (Invitrogen), and express GFP alone or with a synthetic 3'UTR containing 4 binding sites for miR-34a.

### 3.10 MIR-34A KNOCKDOWN IN HTLV-1 INFECTED CELL LINES

Cell lines MT-2 and C91PL were electroporated using a Microporator (Digitalbio) with plasmid pGFP-anti-miR-34a or pGFP-control together with pMACS-LNGFR (Miltenyi Biotec; included to identify transfected cells) and pBlueScript KS+ (BS, Stratagene; included as carrier) and analyzed 3 days later by flow cytometry using an BD FACSCalibur. GFP and LNGFR (detected using an APC-conjugated anti-LNGFR antibody; Miltenyi Biotec) signals were analyzed by multiplying the % positive cells by the mean fluorescence intensity. Cells were labelled with Live/Dead Red (Invitrogen) and FITC-anti-LNGFR (Miltenyi Biotec) following the manufacturer's recommendations to evaluate cell death. The percent cell death was calculated by dividing the number of cells positive for both Live/Dead Red and LNGFR by the total number of LNGFR-positive cells.

### 3.11 TRANSFECTION OF TAX AND HBZ EXPRESSION PLASMIDS

To assess the effect of Tax effect on miR-34a expression, 293T cells were seeded in 60-mm cell culture dishes at 4 x $10^5$ cells/dish and transfected one day later with 0.6 µg of pLCXL or pBlueScript (BS), 0.4 µg of pLTRmRFP, 0.4 µg of pMACS-LNGFR, and BS up to 2 µg total DNA. pLTRmRFP was included as reporter for the activity of Tax. After 48h cells were labeled with FITC-anti-LNGFR and analyzed by flow cytometry to check the efficiency of transfection and induction of mRFP expression by Tax. Cells were harvested to quantify miR-34a by TaqMan quantitative RT-PCR. Cells were 39.4-44.7% positive for mRFP.

To analyze the effect of Hbz on miR-34a expression, 293T cells were seeded in 35-mm cell culture dishes at 2 x $10^5$ cells/dish and transfected one day later with 0.6 µg of pHBZ or pBlueScript (BS), 0.2 µg of pMACS-LNGFR, and BS up to 1 µg total DNA. After 24h the cells were labeled with FITC-anti-LNGFR and analyzed by flow cytometry to check the efficiency of transfection. Cells were harvested to isolate RNA; Hbz mRNA expression was confirmed by end point RT-PCR with primers described by Rende et al. (2011).

Transfections were carried out by using GeneJuice (Novagen) following the manufacturer's recommendations.

# 4. RESULTS

## 4.1 GENERATION AND ANALYSIS OF SMALL RNA LIBRARIES

Small RNA libraries from resting CD4+ lymphocytes, CD4+ lymphocytes stimulated in vitro with PHA and IL-2 (see Material and Methods) and from the HTLV-1-chronically infected cell lines C91PL and MT-2 were generated according to the method described by Lau et al. (2001) and analyzed with the 454 mass sequencing technique (Margulies et al., 2005). This approach permitted the identification of all the sequences of small RNA present in the samples, with the possibility to investigate the presence of polymorphisms or mutations in the sequences of known miRNA and to discover new miRNA candidates. The quantitative aspects of the mass sequencing technique permitted evaluation of expression levels and differential expression of known and new miRNAs in each library. Sequence reads from the 4 libraries were processed through a series of computational steps described by Soldà et al. (2009) performed by the bioinformatics team of G. De Bellis and A. Guffanti (CNR, Milano), designed to distinguish known miRNAs and candidate new miRNAs. Briefly, 17-25 nt sequences were first mapped on the human genome and the flanking genomic sequences were tested in silico for their ability to form an hairpin precursor. These sequences were filtered to eliminate other non coding RNAs (such as tRNA, rRNA) and identified as known miRNAs or putative new miRNA on the basis of sequence comparison with known miRNAs reported in the Sanger miRBase.

Table II shows the number of known miRNAs and new miRNA candidates found in each library. Total sequence reads were similar in the 4 libraries (6801 to 7818). The infected cell line C91PL yielded the highest number of known and candidate new cellular miRNAs (sum of known and new sequences = 174), and MT-2 was the least complex library (sum of known and new sequences = 116). Most of the new sequences (approximately 70%) were located within introns or noncoding exons of existing genes, and were not highly conserved among vertebrates.

| | Resting CD4+ | Activated CD4+ | HTLV-1 infected (C91PL) | HTLV-1 infected (MT-2) |
|---|---|---|---|---|
| Number of sequences | 7709 | 7818 | 7603 | 6801 |
| Known miRNAs (at least 1 read) | 99 | 91 | 125 | 73 |
| New miRNA candidates (≥2 reads) | 29 | 37 | 49 | 43 |

**Table II. Numbers of sequence reads, known miRNAs and new miRNA candidates identified in CD4 cells and HTLV-1-infected cell lines.**

Figure 8 shows analyses of the distribution of new miRNA candidates and known miRNAs. Of interest, only 12 of the 49 new sequences identified in C91PL were also identified in resting and/or stimulated CD4 cells, and only 6 of a total of 92 new miRNAs (6.5%) were found in all 3 libraries. Concerning the MT-2 cell line, 21 of the 43 new miRNA candidates were in common with normal CD4 cells, and 16 of a total of 67 new miRNAs (24%) were found in all 3 libraries. Many new sequences were specific for only one library (Figure 8A).

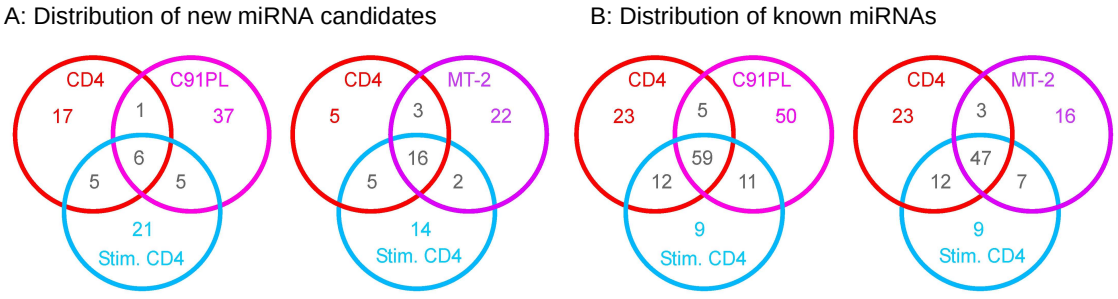


**Figure 8: Distribution of new miRNA candidates (A) and known miRNAs (b) in the different libraries.**

The majority of known sequences found in resting and stimulated CD4 were shared among the 2 libraries (72% for resting and 79% for stimulated cells). For C91PL, 59 of a total of 169 miRNAs (35%) were found in all 3 libraries, and 47 of 117 miRNAs (40%) were found in common between MT-2 and normal CD4 cells. C91PL differed substantially from resting and stimulated CD4, with 50 of its 125 known miRNAs

(40%) absent from both controls. Infected cells had more miRNAs in common with stimulated than with resting CD4+ cells.

In collaboration with A. Corradin (Department of Information Engineering, University of Padova), sequencing data from the library generated from C91PL cells were elaborated using a bootstrap technique designed to estimate the total number of miRNAs present (Basso et al., 2009). Results predicted 199 miRNAs. Comparison of this estimate with the identified numbers of known and potential new miRNAs found in the library (174) indicated that the sequences covered 87% of the total population of new and known miRNAs. This result suggested that the libraries provided a good semi-quantitative representation of the miRNA profile. We therefore used the sequencing data calculate relative abundances of individual miRNAs, i.e., the number of reads for that miRNA divided by the total number of reads for all known miRNAs in that library.

| miRNAs downregulated in infected cells vs resting CD4+ T-cells | | | miRNAs upregulated in infected cells vs resting CD4+ T-cells | | |
|---|---|---|---|---|---|
| miRNA | MT-2/CD4+ | C91PL/CD4+ | miRNA | MT-2/CD4+ | C91PL/CD4+ |
| let-7g | 0.08 | 0.22 | miR-21 | 2.73 | 5.84 |
| miR-16 | 0.42 | 0.09 | miR-34a | 98 | 172.11 |
| miR-26a | 0.09 | 0.01 | miR-103 | 2.13 | 2.73 |
| miR-26b | n.d. in MT-2 | 0.30 | miR-155 | 7.73 | 3.65 |
| miR-29b | 0.46 | 0.27 | Tot: 4 | | |
| miR-29c | 0.34 | 0.03 | | | |
| miR-30b | 0.16 | 0.19 | | | |
| miR-30c | n.d. in MT | 0.21 | | | |
| miR-101 | n.d. in MT | 0.14 | | | |
| miR-140 | 0.13 | 0.03 | | | |
| miR-146b-5p | n.d. in MT | n.d. in C91PL | | | |
| miR-150 | n.d. in MT | n.d. in C91PL | | | |
| miR-342-5p | n.d. in MT | n.d. in C91PL | | | |
| miR-342-3p | 0.05 | 0.11 | | | |
| Tot: 14 | | | | | |

**Table III: miRNAs down and up-regulated in infected cell lines compared to resting CD4 T cells.** The fold change is reported.

Differences in the frequencies of known miRNAs among infected cell lines versus resting CD4+ cells were then calculated, with the analysis restricted to miRNAs yielding at least 20 reads in one of the 2 libraries being compared. As shown in Table III, a total of 14 miRNAs were found to be downregulated by at least 2-fold in both C91PL and MT-2, while only 4 miRNAs were more abundant in the infected cells. miR-34a was highly upregulated in infected cells, as observed also by Yeung et al., 2008 in ATLL samples. miR-150 and miR-146b were not identified in the infected cell lines.

| miRNAs downregulated upon stimulation | | miRNAs upregulated upon stimulation | |
|---|---|---|---|
| miRNA | Stim. CD4+/CD4+ | miRNA | Stim. CD4+/CD4+ |
| hsa-let-7b | 0.25 | hsa-miR-20b | 7.46 |
| hsa-let-7g | 0.16 | hsa-miR-21 | 6.41 |
| hsa-let-7i | 0.34 | hsa-miR-25 | 2.40 |
| hsa-miR-16 | 0.45 | hsa-miR-93 | 4.75 |
| hsa-miR-26a | 0.16 | hsa-miR155 | 4.28 |
| hsa-miR-26b | 0.20 | Tot: 5 | |
| hsa-miR-29a | 0.17 | | |
| hsa-miR-29b | 0.42 | | |
| hsa-miR-30b | 0.12 | | |
| hsa-miR-30c | 0.15 | | |
| hsa-miR-101 | 0.09 | | |
| hsa-miR-140 | 0.12 | | |
| hsa-miR-142 | 0.38 | | |
| hsa-miR-150 | 0.04 | | |
| hsa-miR-342-5p | 0.20 | | |
| hsa-miR-342-3p | 0.04 | | |
| Tot: 16 | | | |

**Table IV: miRNAs down and up-regulated in stimulated compared to resting CD4 T cells.** The fold change is reported.

Comparison of stimulated versus resting CD4+ T cells revealed 16 downregulated and 5 upregulated miRNAs (Table IV). These miRNAs may be considered to represent markers of CD4 cell activation. Several miRNAs had already been described as expressed (i. e. miR-155, miR-21, miR-93), or downregulated upon stimulation (mir-26a, mir-150, miR-16, miR-30c, miR-30b, miR-142-3p, miR-26a,

let-7i/g) (Cobb et al., 2006; Curtale et al., 2010; Grigoryev, et al., 2011, Rossi et al., 2011).

### 4.1.1 VALIDATION AND EXPRESSION PROFILING OF NEW MIRNA CANDIDATES

The analysis of small cDNA libraries revealed many sequences of candidate new small RNAs. These sequences have been included in a patent request which is under processing.

The expression of 10 new miRNA candidates in PBMC and C91PL cells was validated with end point RT-PCR. In a second step, 6 of the new miRNA candidates were validated by quantitative RT-PCR (miR-Q method; Sharbati-Tehrani et al., 2008). We analysed a panel of infected and non-infected cell lines as well as 3 samples of resting and stimulated CD4+ T cells.

As shown in Figure 9, new miRNA #3 showed significantly higher expression levels in HTLV-1 infected cell lines (C91PL, MT-2, HUT-102, C8166) compared to non infected T cell lines (JURKAT, CEM, TALL, $p=0.003$) and to resting CD4+ controls ($p=0.012$). Small RNAs #1, #2, and #3 showed an increase upon stimulation.

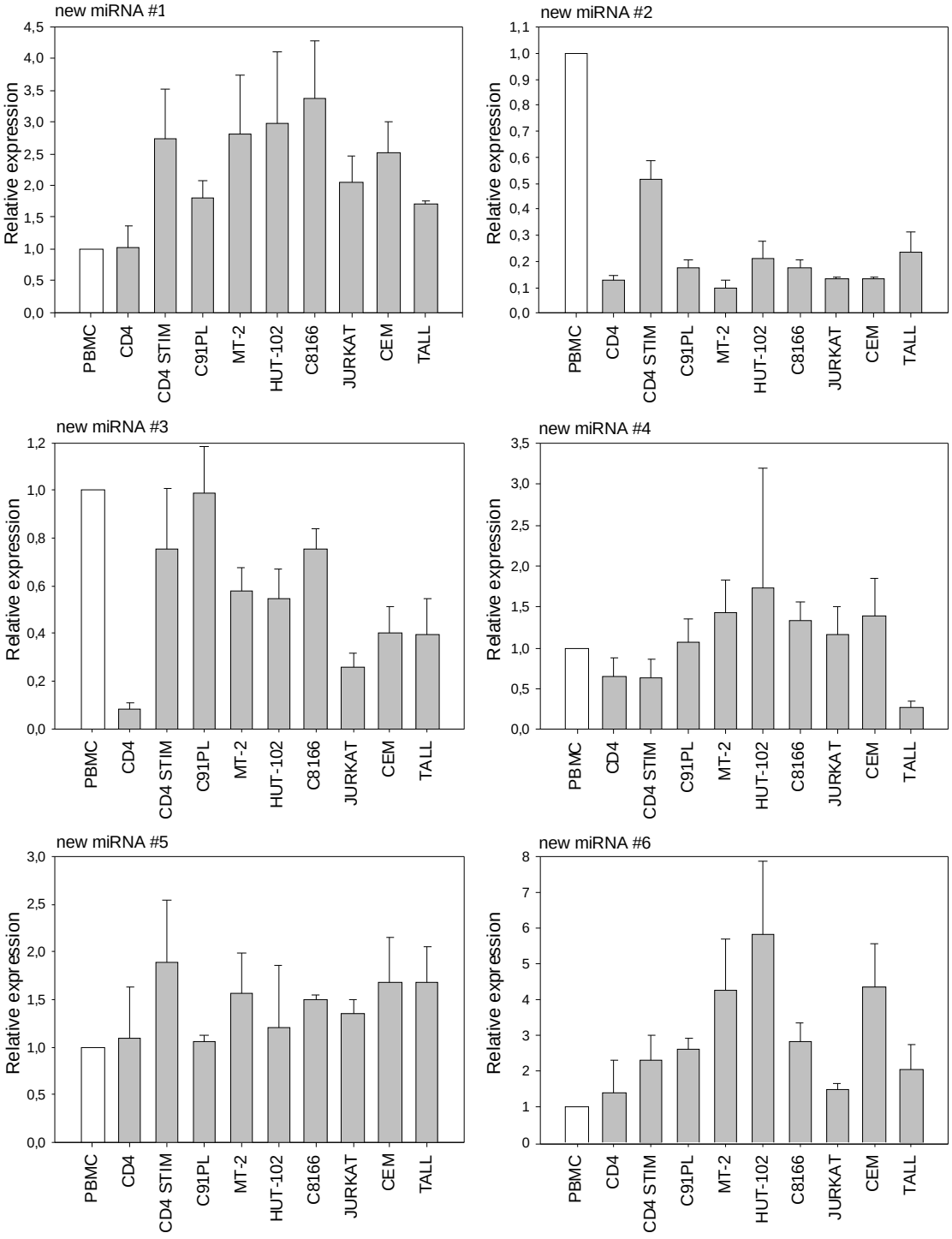


**Figure 9: Expression profiles of new miRNA candidates in T-cell lines and primary CD4+ lymphocytes.** New miRNAs were quantified by real time RT PCR with SYBR green (miR-Q method). A pool of PBMC isolated from 3 healthy donors was used as a calibrator sample and U6 was used as a housekeeping RNA. Means and error bars of 3 independent repeats are shown.

Some tRNAs undergo specific cleavage events to generate tRNA-derived fragments, or tRFs, which may represent a new class of small regulatory RNAs (Lee et al., 2009). Preliminary characterization of a 19-nt tRF named tRF-1001 (generated from the 3' end of a tRNA-Ser-TGA) indicated that it is highly expressed in transformed cell lines compared to most primary tissues examined; furthermore, treatment of cells with an siRNA to silence tRF-1001 resulted in a block in cell proliferation and arrest in G2 (Lee et al., 2009).

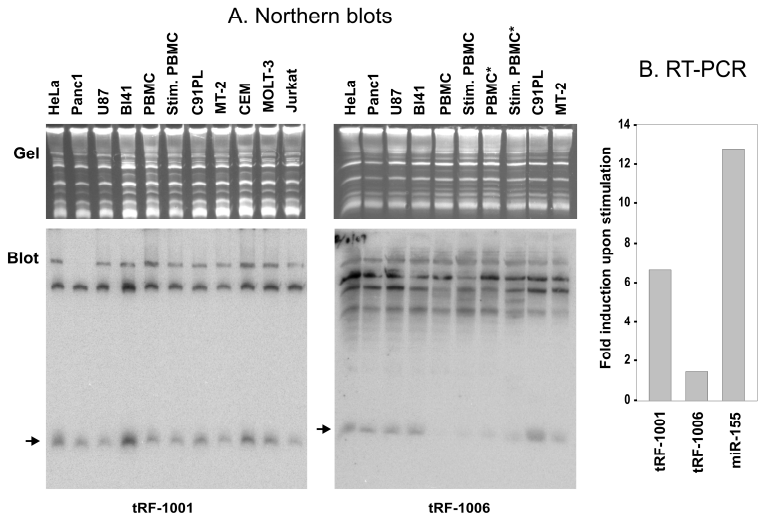


**Figure 10. Expression of tRFs in HTLV-1-infected cell lines and primary T-cells.** A) Northern blots to detect tRF-1001 and tRF-1006. Northern blots were prepared as described (Basso et al., 2009). Shown are the upper portions of the polyacrylamide gels (top panels) and entire blots after incubation with $^{32}$P-labelled oligonucleotides complementary to tRF-1001 and tRF-1006 (bottom panels). Radioactive signals were detected with a Molecular Dynamics Storm 820 phosphorimaging system. Arrows indicate tRF bands. HeLa (cervical carcinoma), Panc1 (pancreatic adenocarcinoma), U87 (glioblastoma), BL41 (an EBV-negative Burkitt lymphoma), C91PL and MT-2 (T-cell lines chronically infected with HTLV-1); CEM, MOLT-3 and Jurkat (T-ALL), PBMC and stimulated PBMC (2 preparations were analyzed for tRF-1006). B) Results of quantitative RT-PCR analysis of RNA from CD4+ T-cells. tRF-1001 and tRF-1006 were detected by SYBR Green RT-PCR (Fulci et al., 2007). miR-155 was analyzed by TaqMan RT-PCR (Applied Biosystems). The amount of each miRNA in the unstimulated samples was set at 1. (Ruggero et al., 2010).

tRF-1001 was one of the most abundant small RNA species identified in the 4 libraries. We investigated the expression of tRF-1001 and a second tRF named tRF-1006 by northern blot in a panel of cell lines, including MT-2 and C91PL, and PBMC, unstimulated and following stimulation with PHA and IL-2 (Figure 10. Ruggero et al., 2010). tRF-1001 and tRF-1006 were detected in all of the cell types examined, including the two HTLV-1-infected cell lines. Results of quantitative real time RT-PCR analysis to detect the two tRFs in purified CD4+ T-cells before and after PHA/IL-2 stimulation indicated that tRF-1001 is strongly upregulated upon stimulation (Figure 10B); miR-155 served as a positive control for a miRNA that is upregulated in stimulated T-cells. Given its ability to activate transcription driven by RNA polymerase III, which is responsible for tRNA synthesis, it will be interesting to determine whether Tax influences expression of these and other tRFs and to study their possible functional significance in the context of HTLV-1-infection and pathogenesis.

### 4.1.2 SEARCH FOR VIRAL MIRNAS

Production of viral miRNAs by RNA viruses was proposed to be unlikely, as the processing events that give rise to a mature miRNA would involve cleavage of the viral genome; furthermore, RNA viruses that replicate in the cytoplasm would produce miRNA precursors that are in the wrong compartment for Drosha processing (Cullen, 2009). In accordance with these ideas, small-RNA cloning studies of cells infected with HIV-1, HTLV-1 and 2 flaviviruses did not yield viral miRNAs (Lin and Cullen, 2007; Pfeffer et al., 2005). However, a study of another HIV-1-infected cell line identified an abundantly expressed viral miRNA derived from the nef region (Omoto et al., 2004) that downregulates Nef expression and interferes with transcription from the LTR promoter (Omoto and Fujii, 2005). Retroviruses thus appear to be capable of producing miRNAs, at least in certain cell contexts.

A computational analysis to identify segments of the HTLV-1 genome that could form stem-loop structures capable of being processed into miRNAs yielded 10 sequences (Li et al., 2008). We aligned these sequences on the HTLV-1 genome and found that 2 could be produced from plus-strand transcripts and 4 from minus-strand

transcripts. To search for production of miRNAs from these sequences, we performed a BLAST search of all the sequence reads obtained from libraries prepared from the HTLV-1-infected cell lines C91PL and MT-2 against the HTLV-1 genome. This analysis yielded 2 sequences mapping to transcribed regions, i.e., in exon 3 and in the LTRs of the plus strand transcript. Each of the two sequences was found only once among a total of more than 14000 sequence reads (see table II), and were found only in MT-2 cells (Ruggero et al., 2010). Interestingly, the sequence in the LTRs was also contained within one of the hairpins predicted by Li et al. (Li et al., 2008). Assuming that the 2 sequences indeed represent authentic viral miRNAs (a point still to be verified), the fact that they were exceedingly rare and present only in MT-2 cells suggests that they might be cell-line specific and that their production might not be favored in the context of chronic infection.

## 4.2 MIRNA PROFILING IN ATLL SAMPLES

We analysed the pattern of expression of known miRNA with the use of *Agilent miRNA Microarray System version 1.0* for 7 ATLL patients (total RNA from C. Pise-Masison, NCI) and 4 pairs of resting and in vitro-stimulated CD4+ T cells (A,B,D,P). Each array presents specific probes for 470 human miRNAs and 64 viral miRNAs (miRBASE version 9.1). Array data were elaborated in collaboration with S. Bortoluzzi and M. Biasiolo (Department of Biology, University of Padova)

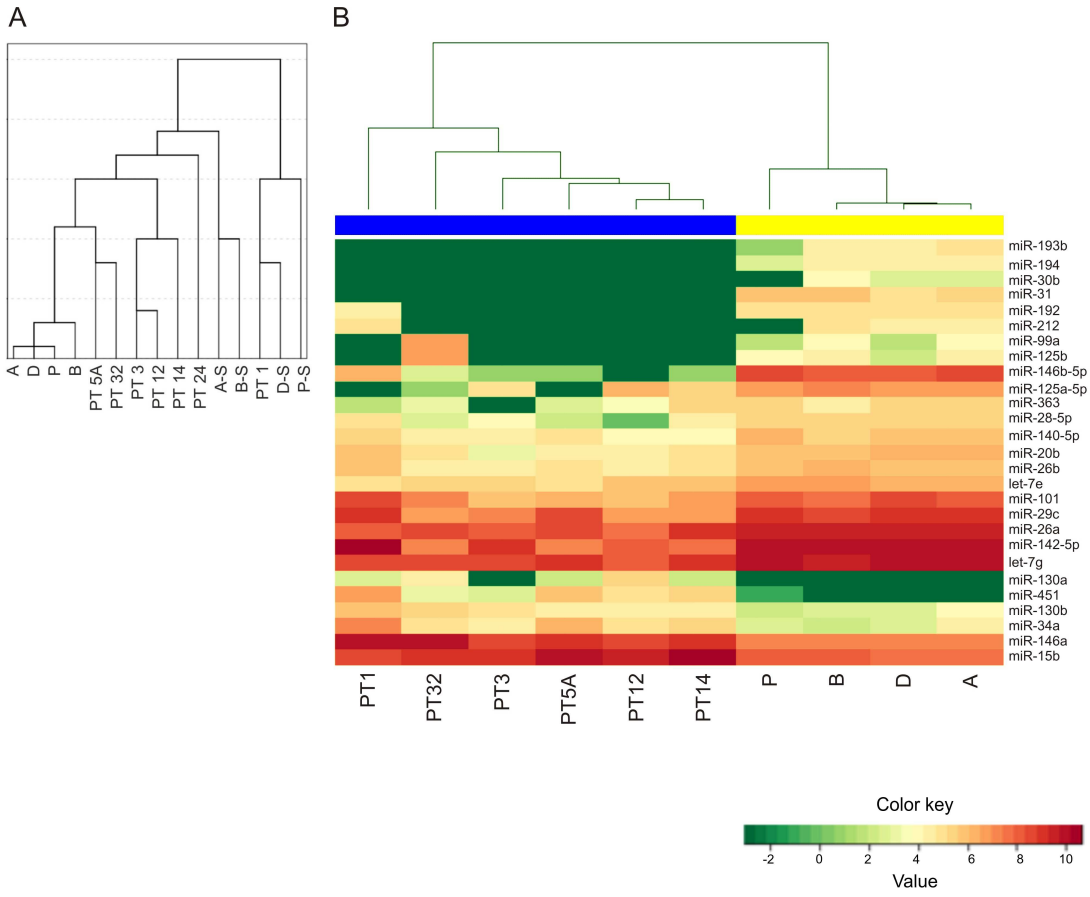


**Figure 11. Analysis of miRNA expression in ATLL vs. normal CD4+ T-cells with the use of microarrays.** A) Unsupervised cluster analysis of ATLL samples (PT) and CD4+ controls [A,B,D,P; resting and in vitro-stimulated (S)] performed using SPSS software, with Pearson correlation as metric and average clustering as linking method. B) Heat Map of miRNAs with a significant difference in expression in ATLL samples versus normal resting CD4+ cells. The analysis was performed on data from samples described in panel A (excluding PT24, a lymphoma) using the samr package for R software from Bioconductor, considering a false discovery rate

threshold < 0.01. Expression levels are represented by the colour key in which bright red corresponds to high expression levels and bright green to low levels.

Reasoning that miRNAs with very low signals were likely to be of marginal biological relevance, bioinformatic analysis of microarray data was carried out to identify miRNAs with signal intensities in the top 3 quartiles in at least two samples (corresponding to a signal intensity of >5.91). The resulting 137 miRNAs were further analyzed. Figure 11A shows a hierarchical cluster analysis of all the samples: the degree of correlation is represented on the y axis of the dendogram and samples that share similar overall miRNA expression profiles are grouped in clusters. While the resting CD4+ samples clustered together, thus exhibiting overlapping miRNA profiles, stimulated controls did not show great similarity. ATLL patients showed high heterogeneity, although all samples except PT1 were more similar to resting CD4+ cells than to their stimulated counterparts. The variability among ATLL samples did not reflect differences in the percentage of CD4+CD25+ cells (Table I). These observations suggest that ATLL cells are functionally distinct from mitogen-stimulated CD4+ T-cells, although they express CD25, commonly considered to represent a marker of activated T-cells.

Statistical analysis to identify differentially expressed miRNAs revealed 21 downregulated miRNAs and 6 upregulated miRNAs in ATLL samples compared to resting CD4+ cells, as illustrated in the heat map shown in Figure 11B. Six of the 27 differentially expressed miRNAs identified in our ATLL samples had also been reported in previous microarray-based studies of ATLL (Figure 6), miR-26b, miR-31, miR-125a and miR-146b (downregulated) and miR-34a and miR-130b (upregulated), although confirmatory RT-PCR were published for only 3 (miR-130b by Yeung et al., 2008, and miR-125a and miR-146b by Bellon et al., 2008).

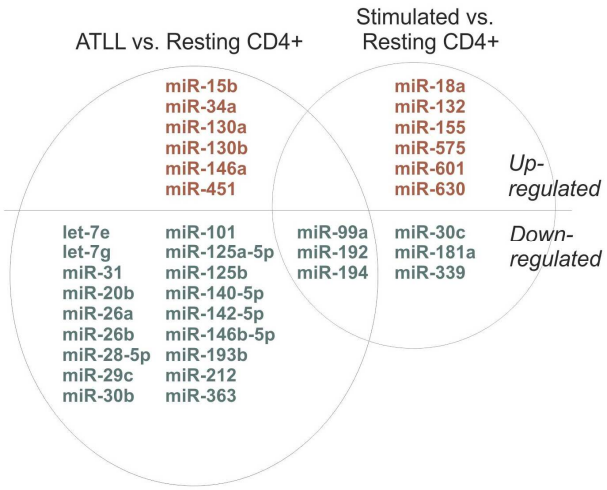


**Figure 12: Differentially expressed miRNAs in ATLL vs Resting CD4+ cells and in stimulated versus resting controls.**

Figure 12 shows a comparison of the miRNAs differentially expressed in ATLL samples and stimulated CD4+ T samples vs resting CD4+ T samples. Stimulated CD4+ T cells presented 6 upregulated miRNAs and 6 downregulated miRNAs. Only three miRNAs (miR-99a, miR-192 and miR-194) that were downregulated in ATLL cells were also less abundant in stimulated versus resting CD4+ cells. These observations suggest that the majority of miRNAs differentially expressed in ATLL cells are not simply the consequence of the acquisition of a phenotype of activated cells.

## 4.3 REAL TIME RT-PCR ANALYSIS OF THE DIFFERENTIALLY EXPRESSED MIRNAS

To extend observations made from the analysis of the microarray and the libraries, expression levels of selected miRNAs were investigated using quantitative RT-PCR, with a total of 10 ATLL samples and 11 resting CD4+ controls included in the analysis, and a pool of 3 PBMC samples from healthy donors used as a calibrator for calculation of relative quantities (RQ) (Figure 13). We confirmed significant upregulation of miR-34a in ATLL samples compared to resting T CD4+ cells and significant downregulation of miR-193b, miR-192, miR-125a-5p and miR-146-5p.

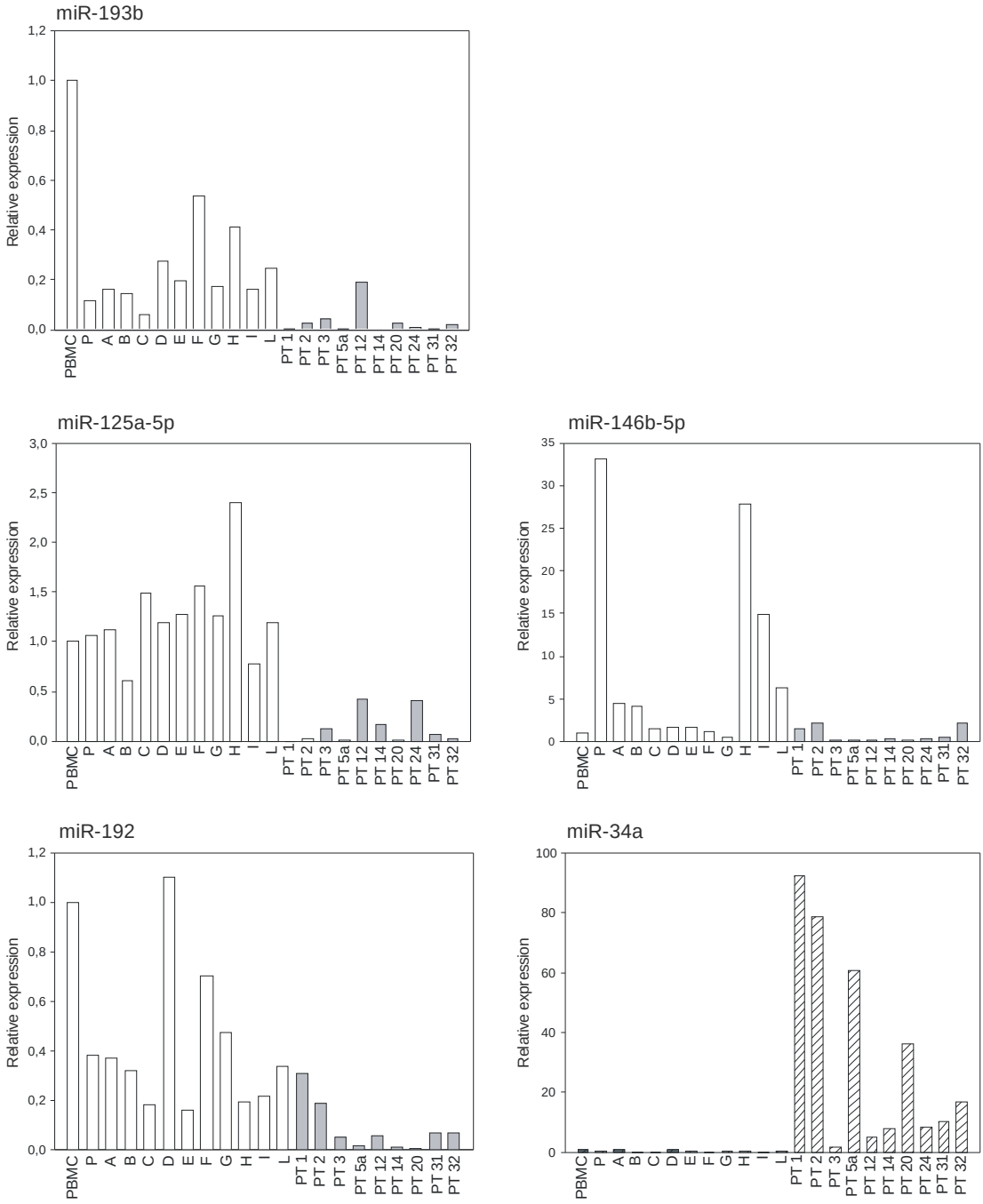


**Figure 13: Quantitative RT-PCR to detect miRNAs in ATLL samples and controls.** Pool of 3 PBMC samples is used as calibrator. p values were calculated using the Mann-Whitney rank-sum test for ATLL vs CD4+. miR-193b: $p < 0.001$; miR-125a-5p: $p < 0.001$; miR-146b-5: $p = 0.002$; miR-192: $p = 0.001$; miR-34a: $p < 0.001$.

A further analysis of the expression levels of the differentially expressed miRNAs in HTLV-1-infected cell lines compared to T cells lines (Figure 14) revealed a strong

and statistically significant ($p<0.001$) overexpression of miR-34a in all the infected cell lines (C91PL, MT-2, HUT-102) while its expression was almost absent in non infected control T cell lines (JURKAT, CEM, TALL). miR-125a-5p showed a significant overexpression ($p = <0.001$) in MT-2 and C91PL compared to non infected cell lines; on the contrary in HUT-102 (and also in CEM) the expression of miR-125a-5p was below the limit of detection. Similar results were obtained for miR-146b-5p, upregulated ($p = 0.002$) in infected cell lines vs non infected cell lines. For miR-192 and miR-193b the expression levels were not statistically different between infected and uninfected cell lines.

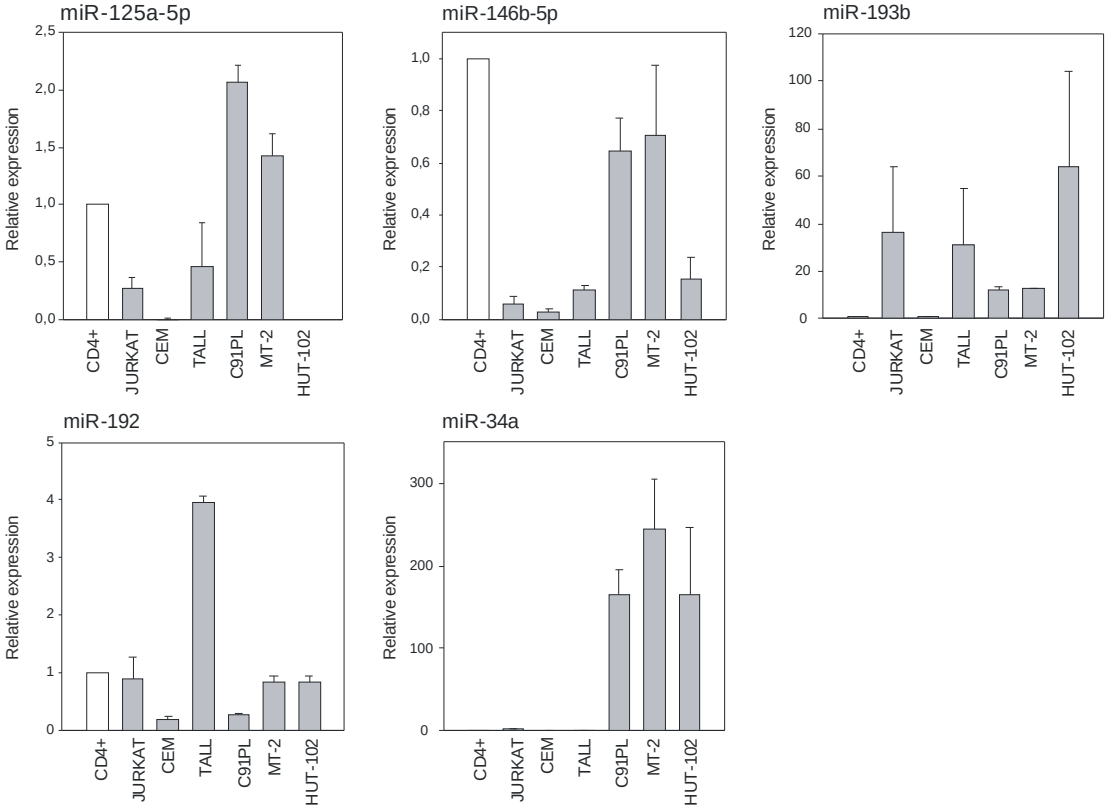


**Figure 14: Quantitative RT-PCR to detect miRNAs in HTLV-1 infected cell lines and uninfected T cell lines.** The mean of expression values of all CD4+ samples represented in Figure 13 was set a t 1. p values were calculated using the Mann-Whitney rank-sum test for infected cell lines (C91PL, MT-2, HUT-102) vs uninfected T cell lines (JURKAT, CEM, TALL). Standard errors are represented. miR-192, miR-193b: 2 repeats. miR-146b-5p; miR-34a; miR-125a- 5p: 3 to 8 repeats.

## 4.4 INTEGRATED ANALYSIS OF MIRNA AND GENE EXPRESSION

Since miRNA-mRNA target interactions often result in degradation of the mRNA (see Introduction), integration of results from target prediction programs with expression profiles for miRNAs and mRNAs can aid in identifying mRNA targets of possible biological relevance in a particular cell system (Lionetti et al., 2009; Sales et al., 2010).

Our study was centered on differentially expressed genes and miRNAs in ATLL samples compared to resting CD4+ (see Figure 11). We analyzed gene expression for the CD4+ samples (P, A, B, D) with microarrays (Affymetrix v. HGU133Plus2), while for 6 ATLL patients (PT 1, PT 3, PT 5a, PT 12, PT 14, PT 32) we used published data (Pise-Masison, 2009; Affimetrix v. HGU133A2.0). We identified 5251 genes differentially expressed in ATLL samples vs resting CD4+ samples (5126 upregulated and 125 downregulated) with the samr package for R software from Bioconductor, considering a false discovery rate threshold < 0.01.

Transcripts potentially targeted by miRNAs were identified by integrating miRNA expression data with mRNA expression data as described (Lionetti et al., 2009). Potential miRNA targets were obtained by TargetScan predictions (release 5.2) available online (www.targetscan.org). Pearson correlation coefficients were then calculated for each potential miRNA-gene pair. Anticorrelation scores in the top 10% were collated as corresponding to targeting relations predicted by sequence analysis and supported by expression data. From these results, miRNAs and genes that were differentially regulated in ATLL samples compared to resting CD4+ cells were identified and elaborated into a putative miRNA/gene regulatory network using Cytoscape software (Shannon et al., 2003) (Figure 15). We observed a prevalence of downregulated miRNAs and upregulated genes. Several genes were targeted by more than one miRNA; one example is RAB38, member of the Ras oncogene family, targeted by miR-194 and miR-30b (Table III).

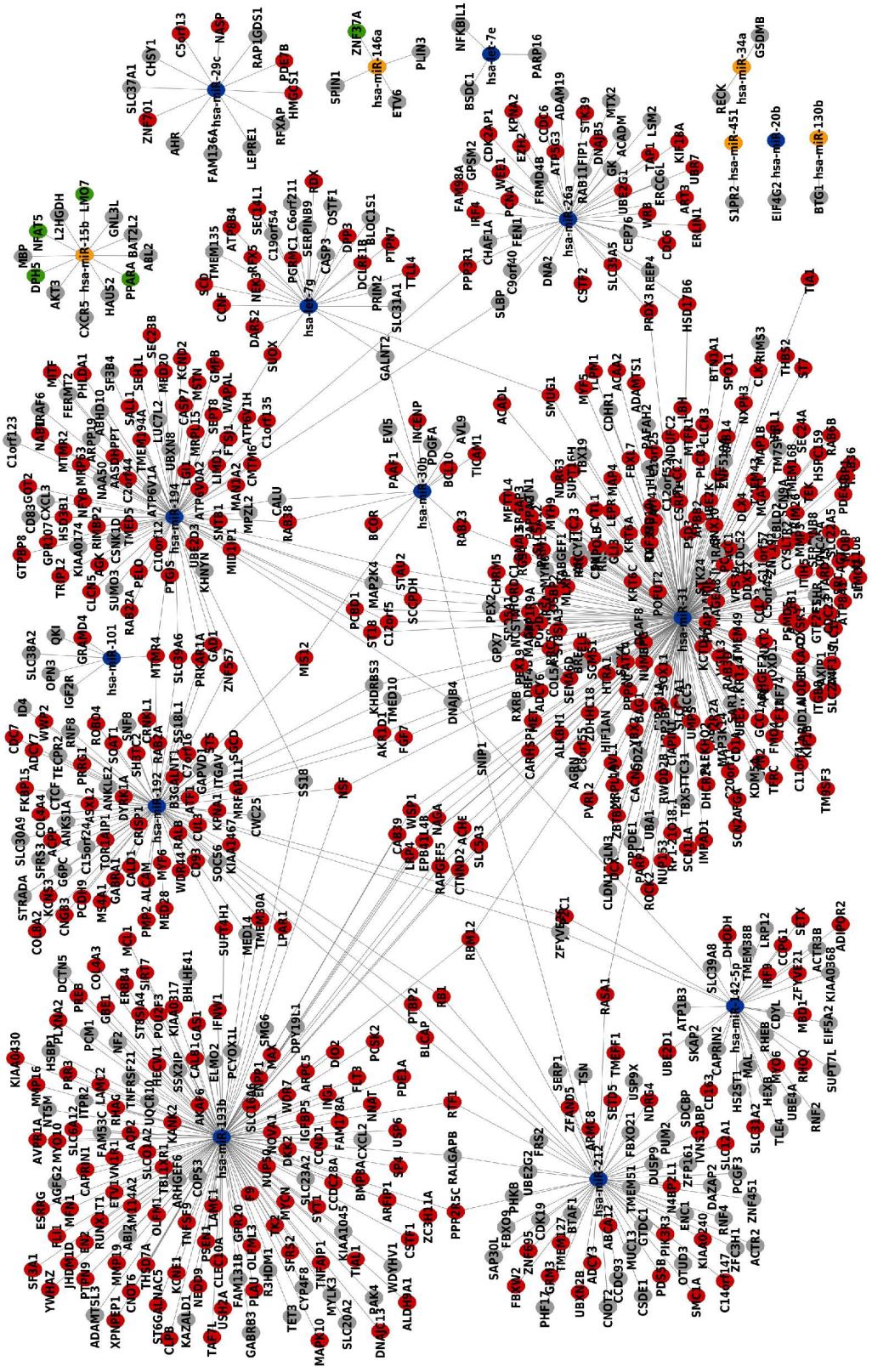

**Figure 15: Network of predicted targets for miRNAs differentially regulated in ATLL cells.** Shown are 18 miRNAs that were differentially regulated in ATLL cells compared to resting CD4+ controls with anticorrelated genes. MiRNAs indicated in

yellow and blue were up- and downregulated, respectively. Genes indicated in red and green were up- and downregulated, respectively. Genes in grey were not differentially expressed in ATLL vs CD4+ resting cells.

The GOrilla Gene Enrichment Analysis and Visualization Tool (Eden et al., 2009) was employed to discover functional categories that were significantly enriched in the differentially expressed target genes present in the network. We then used REVIGO (Supek et al., 2011) to find representative subsets of GO terms.

| GO:0009143: Nucleoside triphosphate catabolic process | |
|---|---|
| Gene | miRNA |
| ADCY3 - adenylate cyclase 3 | miR-212 |
| ADCY6 - adenylate cyclase 6 | miR-31 |
| ADCY7 - adenylate cyclase 7 | miR-192 |
| RAC1 - ras-related c3 botulinum toxin substrate 1 (rho family, small gtp binding protein rac1) | miR-31 |
| RAB38 - rab38, member ras oncogene family | miR-194; miR-30b |
| RAB14 - rab14, member ras oncogene family | miR-31 |
| RAB3D - rab3d, member ras oncogene family | miR-31 |
| RAB6B - rab6b, member ras oncogene family | miR-31 |
| RAB22A - rab22a, member ras oncogene family | miR-194 |
| RAB2A - rab2a, member ras oncogene family | miR-192 |
| RALB - v-ral simian leukemia viral oncogene homolog b (ras related; gtp binding protein) | miR-192 |
| RHOQ - ras homolog gene family, member q | miR-142-5p |
| ATP6V1H - atpase, h+ transporting, lysosomal 50/57kda, v1 subunit h | miR-194 |
| ENPP1 - ectonucleotide pyrophosphatase/phosphodiesterase 1 | miR-193b |
| GNAI3 - guanine nucleotide binding protein (g protein), alpha inhibiting activity polypeptide 3 | miR-31 |
| KIF1B - kinesin family member 1b | miR-31 |
| MFN1 - mitofusin 1 | miR-193b |
| MYH9 - myosin, heavy chain 9, non-muscle | miR-31 |

**Table III: Predicted target genes of the network enriched in the GO term nucleoside triphosphate catabolic process.** The genes enriched belonging to the GO term and the controlling miRNAs are reported. All genes present in the array were used as background.

The analysis yielded the following major GO terms enriched in the ontology Biological process: embryonic digit morphogenesis (GO:0042733; p value: 7,02E-5); nucleoside triphosphate catabolic process (GO:0009143; p value: 4,52E-4); membrane lipid biosynthetic process (GO:0046467; p value: 5,79E-4). Table III lists 18 predicted target genes (out of 451 present in the network), and the corresponding controlling miRNA, belonging to the nucleoside triphosphate catabolic process GO term. Among them, adenylate cyclase 3, 6, and 7, GNAI3 [(guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3)], and RAC1 [(ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)] belong to the Chemokine Signaling Pathway, according to KEGG (Kyoto

Encyclopedia of Genes and Genomes) pathway analysis (http://www.genome.jp/kegg/). The presence of 3 adenylate cyclase genes and G proteins in the network was of particular interest, suggesting that the downregulation of miR-192, miR-212 and miR-31 may act in a cooperative way in cAMP signalling by determining the upregulation of several forms of adenylate cyclase and other members of the cAMP pathway (see Figure 15).

Resting CD4+ T cells present low levels of cAMP. This condition is needed for their activation; in fact, it is known that cAMP strongly inhibits T-cell growth and proliferation (Estes et al., 1971) by regulating the function of transcription factors, members of the mitogen-activated protein kinase pathway, phospholipases, Ras homolog (Rho)A, and proteins controlling cell cycle progression (Mosender et al., 2011). Tregs harbour high levels of cAMP and transfer cAMP into conventional T cells through gap junctions, thus suppressing their function (Bopp et al, 2007). Huang et al. (2009) showed that miR-142-3p regulates the level of cAMP by targeting adenylate cyclase 9 in normal CD4+ cells, where miR-142-3p is highly expressed, and in Tregs, where miR-142-3p is downregulated by Foxp3.

Consistent with the fact that ATLL cells harbour some phenotypic markers of Tregs, HTLV-1-transformed T-cells present elevated levels of cAMP (Kress et al., 2010). In transformed cells with conditional Tax expression, cAMP levels decreased when Tax was silenced, but overexpression of Tax alone was not able to induce an increase in cAMP levels. The authors observed a Tax-independent downregulation of phosphodiesterase 3B (PDE3B), an enzyme that degrades cAMP, in HTLV-1-transformed cells. Overexpression of PDE3B in HTLV-1-transformed cells resulted in a decrease in cAMP levels, thus revealing at least one mechanism responsible for the elevated cAMP levels presented in HTLV-1 transformed cells (Kress et al., 2010).

Poteat et al. (1989) demonstrated that the increase of cAMP in HTLV-1 infected cells determines an induction of the long terminal repeat (LTR), suggesting that, in contrast with the suppressive role in normal CD4+ cells, in HTLV-1 transformed cells high cAMP levels may contribute to stimulate replication, especially in ATLL cells, where the expression of Tax might be very low or absent.

The elevated expression of adenylate cyclases in ATLL samples revealed by microarray analysis is consistent with previous observations of elevated levels of

cAMP in HTLV-1 transformed cell lines (Kress et al., 2010). Moreover, the integrated analysis of miRNA and gene expression suggested that differentially expressed miRNAs may contribute to the regulation of the adenylate cylase/cAMP pathway in HTLV-1 transformation. We were able to confirm a significant upregulation of adenylate cyclase 6 in ATLL samples vs resting CD4+ cells by real time RT PCR (Figure 16).

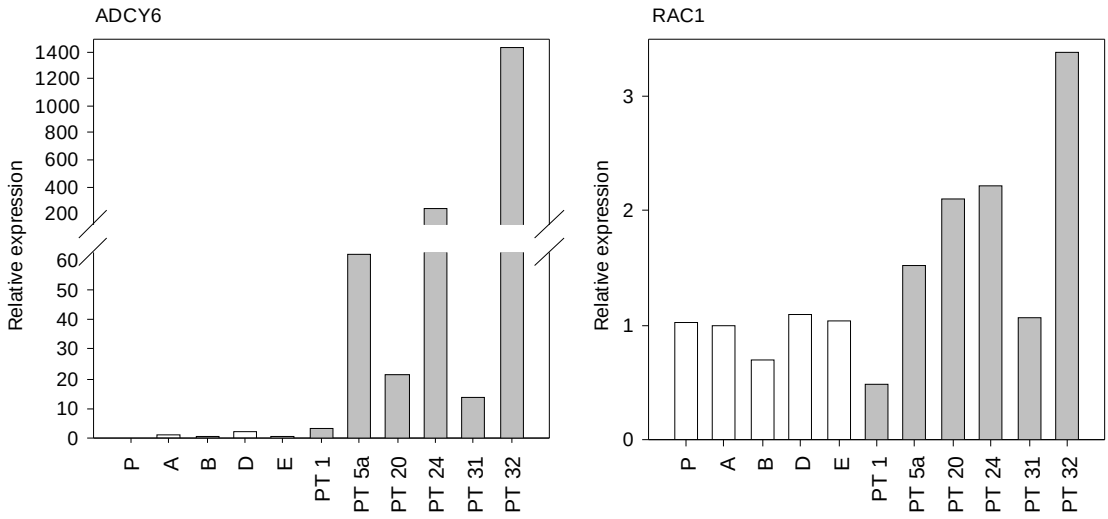


**Figure 16: Quantitative RT-PCR to detect adenylate cyclase 6 (ADCY6) and RAC1 in ATLL patients and resting CD4+ samples.** Results confirmed a significant upregulation of ADCY6 in ATLL vs CD4+ ($p = 0.004$, Mann-Whitney rank-sum test). RAC1 showed an increase in 4 out of 6 ATLL samples tested ($p = 0.016$ without PT1, Mann-Whitney rank-sum test). PBGD was used as housekeeping gene and CD4+ sample A as calibrator.

The enriched GO term nucleoside triphosphate catabolic process also included several members of the RAS superfamily of small GTPases: RAB14, RAB22A, RAB2A, RAB38, RAB3D, and RAB6B of the RAB family, RHOQ (Ras homolog gene family, member Q), and RALB (Ras-related protein Ral-B), RAC1. These genes were predicted to be targets of several miRNAs in the network (miR-31, miR-192, miR-194, miR-30b, miR-142-5p) (Table III and Figure 15). Small GTPase proteins have been associated with cancer cell migration and invasiveness (Subramani et al., 2010; Parise et al., 2000). Moreover, the RAS superfamily of small GTPases are activated by cAMP (Goto et al., 2011; Mosender et al., 2011). One

example is RAC1, which is known to be involved in control of cytoskeletal organization, transcription and cell growth (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998) and is activated by cAMP (Spindler et al. 2011, Goto et al., 2011). It is noteworthy that RAC1, as well as other small GTPase proteins, was found in protein complexes with Tax, an observation that suggests a way by which Tax can regulate migration, invasion, and adhesion of leukemic cells (Wu et al., 2004).
Quantification of RAC1 expression by real time RT-PCR (Figure 15) confirmed a slight increase in some ATLL samples which however did not reach statistical significance.

## 4.5 INVESTIGATION OF MIR-34A UPREGULATION

The consistent pattern of upregulated miR-34a expression in ATLL and chronically infected cell lines suggested that miR-34a could contribute to the growth or the survival of HTLV-1 infected cells. This possibility is in accordance with the finding that knockdown of miR-34a interfered with viability of a panel of solid tumor cell lines (Dutta et al., 2007), and by a subsequent study which showed that the breast cancer cell line MDA-MB-231 became more sensitive to death induced by gamma irradiation after miR-34a knockdown and less sensitive when its levels were increased (Kato et al., 2009).
To test the functional role of miR-34a in infected T-cells, we transfected MT-2 cells and C91PL cells with a plasmid expressing GFP with a synthetic 3'UTR containing 4 binding sites for miR-34a, previously shown to act as a 'sponge' for the miRNA (Rao et al., 2010), or with a control GFP-expressing plasmid. To allow detection of the transfected cells we included a plasmid expressing LNGFR; this was necessary since the interaction of miR-34a with the sponge sequence downregulated GFP production (Figure 17A). Seventy-two hours after transfection the cells were co-labelled with FITC-anti-LNGFR and Live/Dead Red, a dye that specifically labels dead cells. Results revealed a significant increase in dead cells in the cultures transfected with the miR-34a sponge compared to the control plasmid (Figure 17). These results suggest that high levels of miR-34a are beneficial for survival of HTLV-1-infected cells, and lead to the suggestion that drugs targeting miR-34a might be effective for

eliminating infected cells from the host either alone or in combination with other therapeutic agents.

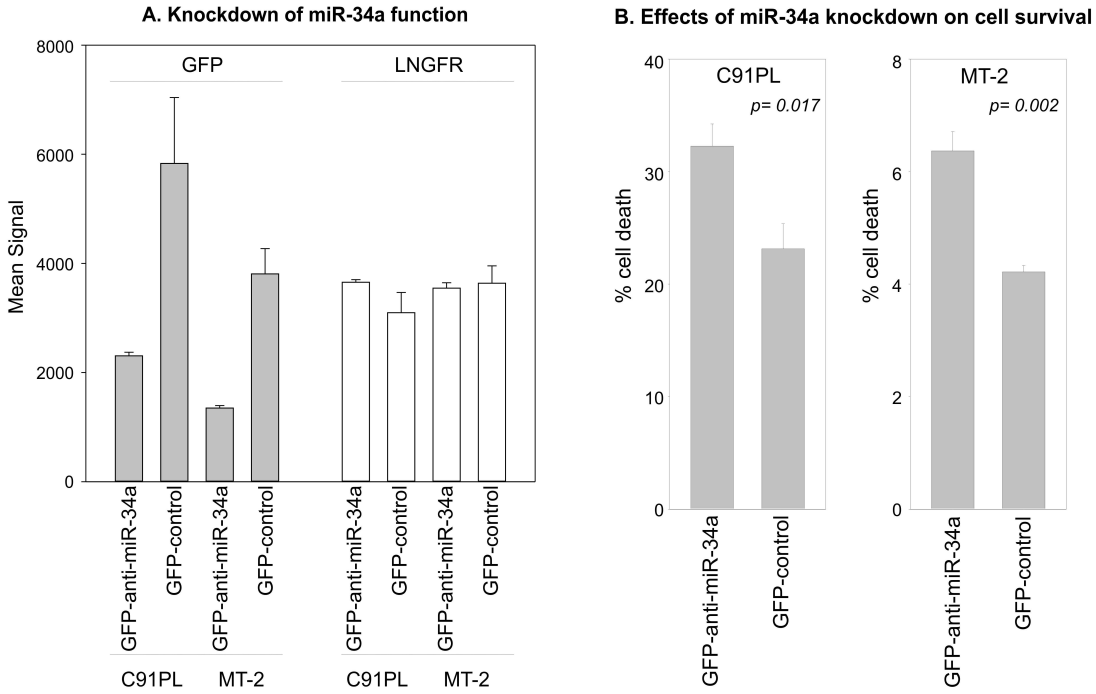


**Figure 17: miR-34a knockdown in HTLV-1 infected cell lines.** Cell lines MT-2 and C91PL were electroporated with plasmid pGFP-anti-miR-34a or pGFP-control (see Material and Methods) together with pMACS-LNGFR (Miltenyi Biotec; included to identify transfected cells). In Panel A, GFP and LNGFR (detected using an anti-LNGFR antibody) signals were analysed by flow cytometry and calculated by multiplying the % positive cells by the mean fluorescence intensity. Plotted are means from 3 transfections with standard error bars. In Panel B, cultures (6 for MT-2, 5 for C91PL) were labelled with Live/Dead Red (Invitrogen) to identify dead cells and with anti-LNGFR antibody to identify transfected cells. The percent cell death was calculated by dividing the number of cells positive for both Live/Dead Red and LNGFR by the total number of LNGFR-positive cells. Plotted are mean % cell death values with standard errors. P values were calculated using the Mann-Whitney rank sum test.

The mechanism driving upregulation of miR-34a in HTLV-1-infected cells remains to be determined. miR-34a might be a marker of persistent engagement of the DNA damage repair pathway. While miR-34a expression is induced by p53 in many cell types, most ATLL tumors and HTLV-1-transformed cell lines harbour functionally inactive p53 (Yamada and Kamihira, 2005). This suggests that miR-34a may be

regulated by p53-independent pathways in the context of HTLV-1. This possibility is supported by a study of HPV-infected keratinocyte raft cultures, which showed initial downregulation of miR-34a due to interaction of E6 with p53, followed by restoration of miR-34a expression despite continued p53 inactivation (Wang et al., 2009). It is also possible that Tax directly upregulates miR-34a, but the high levels of the miRNA found in the uncultured ATLL samples suggest that continued Tax expression might not be essential for miR-34a upregulation, since ATLL cells generally express Tax at very low levels (Takeda et al., 2004). In alternative, the viral HBZ protein might be responsible for the high expression of miR-34a in HTLV-1 transformed cells. In fact it is known that HBZ is able to drive the transcriptional expression of several cellular genes (see Introduction) and its expression is maintained in ATLL samples (Rende et al., 2011).

The influence of Tax and HBZ on miR-34a expression was tested by transfecting the 293T cell line with a plasmid expressing Tax or control plasmid (pBlueScript, BS). Figure 18 reports the results of the quantification by real time RT-PCR of the expression of miR-34a. We did not observed an increase in miR-34a levels in the presence of Tax or HBZ.

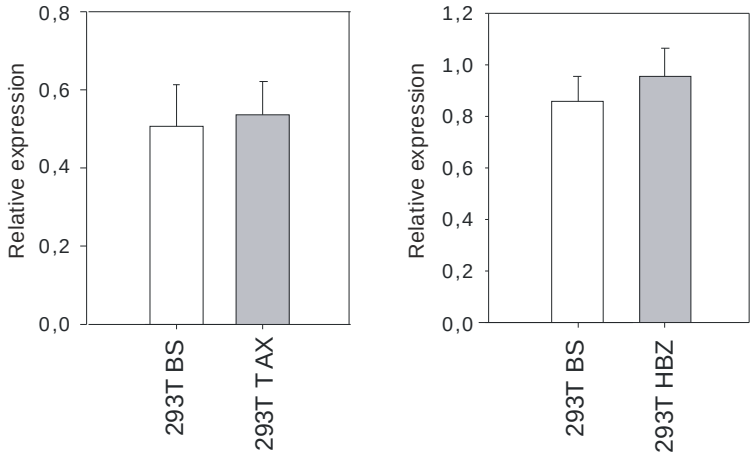


**Figure 18: Quantitative RT-PCR to detect miR-34a in 293T cells expressing Taz or HBZ.** 293T cells were transfected with control plasmid (pBlueScript, BS) or a plasmid expressiong Tax or HBZ. 48 or 24 hrs later, respectively, the cultures were harvested for RNA isolation, and expression of miR-34a was analysed by TaqMan quantitative RT-PCR together with the housekeeper RNA RNU44. One transfection with BS was used as calibrator sample. Plotted are means for 6 transfections with standard errors.

# 5. DISCUSSION

The published papers aimed at defining the miRNA expression profiles in HTLV-1 infected/transformed cells (Bellon et al., 2009; Pichler et al., 2008; Yeung et al., 2008) pioneered the analysis of the role of miRNAs in HTLV-1 biology and revealed an important tumor suppressor (TP53INP1) that is deregulated in ATLL cells through miRNA overexpression. Analyses based on deep sequencing of small RNA libraries from HTLV-1-infected cells described in the present thesis provided very useful information for completing the profile of miRNA deregulation in HTLV-1 infection. Unlike arrays, lists of sequence reads from small RNA libraries never become obsolete with the addition of new miRNAs to the Sanger miRBase. The small RNA libraries were generated to compare the expression of miRNA in infected cell lines and CD4 cells and to discover new miRNA candidates. The analysis revealed a strong upregulation of miR-34a in infected cell lines and the downregulation of several miRNAs, and in particular miR-146b-5p and miR-150, which were undetected in infected cell lines. Library-based studies also provide the unique possibility to identify the sequences of new miRNAs and other small RNA species. In the first instance, we were able to identify in each library several sequences that possess a distinguishing characteristic of miRNAs, i.e. their location in a precursor transcript able to fold in a hairpin structure (see Materials and Methods). Moreover, we were able to validate the expression of several new miRNAs and to describe by real time PCR analysis their pattern of expression in a panel of cell lines and primary samples. In particular, we identified new miRNAs differentially expressed in infected cell lines or upon stimulation. More work is needed to define the role of these new miRNAs in normal haematopoiesis and in HTLV-1 transformation. It will also be of interest to investigate their expression in a larger spectrum of cell types, to understand if we identified lymphocytes-specific or ubiquitously expressed miRNAs. The analysis of the sequences obtained from infected cell lines permitted us to search for the expression of viral miRNAs. This analysis yielded only 2 sequences with one count in the MT-2 library which mapped to transcribed regions of the HTLV-1 genome. Assuming that the 2 sequences indeed represent authentic viral miRNAs, the fact that they were exceedingly rare suggests

that they might be cell-line specific and that their production might not be favoured in the context of chronic infection. It will therefore be important to search for these and other potential viral miRNAs in different phases of viral infection. For example, one could envision the production of viral miRNAs from introns generated through splicing of the primary transcript during early phase of infection when Rex is not yet active.

Unfortunately, it was not possible to construct miRNA libraries from ATLL patient samples because of the large amount of starting total RNA required. Therefore we used microarrays to compare levels of known miRNAs. The analysis identified 21 downregulated miRNAs and 6 upregulated miRNAs in ATLL samples with respect to resting CD4+ T-cells. As shown in Figure 19, six miRNAs were common to our analysis of ATLL cells and one of two previous studies that examined miRNA expression in ATLL (Bellon et al., 2009; Yeung et al., 2008), i.e., miR-34a and miR-130b (upregulated) and miR-26b, miR-31, miR-125a-5p and miR-146b-5p (downregulated). The most striking result of this comparison is the limited agreement among the three studies, which is in part likely due to different array formats and data analysis methods as well as the characteristics of tumor samples and controls employed in the studies. These considerations underscore the need for validation of array data by another more standardized miRNA detection method such as quantitative RT-PCR. Our RT-PCR assays confirmed differential expression of miR-34a, miR-125a-5p, miR-146b-5p and miR-192 and miR-193b.

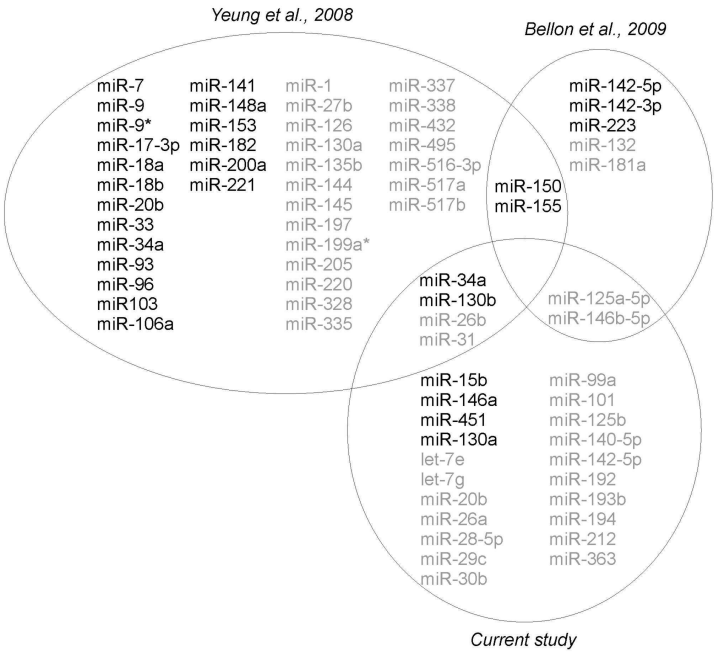


**Figure 19. Differentially expressed miRNAs in ATLL cells**. Circles indicate miRNAs with a significant difference in expression levels in ATLL samples versus resting CD4+ controls in the study reported in the present thesis and in reports that compared ATLL samples to PBMC or CD4+ T-cells (Bellon et al., 2009; Yeung et al., 2008). Upregulated miRNAs are shown in black and downregulated miRNAs are shown in grey.

While technical innovations over the past 5 years allow researchers to obtain in a short time massive amount of data concerning miRNA expression and discovery, the functional characterization and identification of miRNA target genes remains a scientific challenge. Target prediction algorithms (Lai et al., 2003; Nam et al., 2005; Li et al., 2006; Huang et al., 2007) generate lists of hundreds of possible target genes for each miRNA, and their validation with reporter assays is very laborious. To circumvent these difficulties, several strategies have been developed to shorten the starting number of possible target genes to be validated. One of these approaches involves the integration of miRNA and gene expression data with the target prediction algorithm output (Lionetti et al., 2009; Sales et al., 2010). It is in fact known that miRNA binding causes a decrease in mRNA levels of target genes (see Introduction), leading to the assumption that high miRNA expression is correlated to low target mRNA expression, and vice versa. The integrated analysis thus points out anticorrelated miRNA-mRNA profiles to generate a more restricted list of predicted

target genes. We performed this analysis on differentially expressed miRNAs and genes in ATLL vs resting CD4+ T cells and targets predicted by TargetScan. The resulting network of miRNAs and predicted targets genes altered in HTLV-1 transformation showed a prevalence in downregualted miRNA-upregulated target genes and revealed the shared targets of several differentially regulated miRNAs. In order to identify functional interactions with biological relevance in HTLV-1 transformation, we searched for the presence of pathways and functional categories enriched among the hundreds of differentially expressed genes present in the network. The Gene Ontology analysis allowed us to identify a enrichment for several enzymes and RAS superfamily small GTPases involved in the cAMP pathway, which is known to be activated in HTLV-1 transformed cells with a positive role on LTR activation. We confirmed by real time PCR the presence of very high levels of adenylate cyclase 6 mRNA in tumor cells from ATLL patients. The next steps will be the validation of a miRNA-based control of adenylate cyclase 6 expression (by miR-31, consistently downregulated in ATLL samples, as predicted by the network).

Our libraries, array-based and real time RT-PCR profiling revealed a strong upregulation of miR-34a both in ATLL samples as in chronically infected cell lines. Many studies have been focused on miR-34a due to its upregulation by p53 in response to a variety of stress agents and its downregulation in many solid tumor types, suggesting a tumor suppressor function (Hermeking, 2010). miR-34a targets mRNAs coding for genes affecting cell proliferation and survival, including Cyclins D1 and E2, Bcl-2, CDK4 and CDK6, c-Myc and N-Myc, E2F3, Met, and SIRT1. In turn, downregulation of SIRT1 enhances p53 activity (Hermeking, 2010; Yamakuchi and Lowenstein, 2009). Expression of miR-34a in hematological malignancies has not been studied extensively. It is downregulated in aggressive CLL with inactivated p53 (Mraz et al., 2009) but appears to be upregulated in AML blasts compared to normal bone marrow cells or CD34+ hematopoietic progenitor cells (Isken et al., 2008). It is noteworthy that upregulation of miR-34a was also indicated in an analysis of EBV-transformed B-cells (Mrazek et al., 2007), with a subsequent study indicating increased expression of pre-miR-34a as well as pre-miR-146b and other cellular miRNAs during latency type III (the growth program phase) compared to latency I (Cameron et al., 2008).

The upregulation of miR-34a in ATLL samples and HTLV-1-infected cell lines suggests that this miRNA might provide a survival advantage to ATLL cells. We in fact observed an increase in cell death of C91PL and MT-2 cells when miR-34a was subtracted using a "sponge" construct (Figure 17). This contrasts with observations made in epithelial cell lines, where induction of miR-34a through p53 promotes growth arrest, senescence and apoptosis (Raver-Shapira et al., 2007; Tarasov et al., 2007; Welch et al., 2007; Yamakuchi et al., 2008; Yamakuchi and Lowenstein, 2009). Many T-cell lines, including Jurkat, contain mutations in the p53 gene (Cheng and Haas, 1990), while most ATLL cells and HTLV-1-transformed cell lines are characterized by high levels of functionally inactive p53 without mutations in the p53 gene (Yamada and Kamihira, 2005). This property has been attributed to effects of Tax on several pathways that influence p53 stability and function, although other mechanisms might be important in the context of ATLL cells, which are characterized by very low Tax expression (Tabakin-Fix et al., 2006). The observation that miR-34a is upregulated in ATLL and HTLV-1-infected cell lines suggest that expression of this miRNA may also be regulated by p53-independent pathways in the context of T-cells. As described in the Introduction, a role for Tax in upregulation of miR-146a and miR-130b through the NF-κB pathway has already been established. Given the ability of Tax to influence many other transcription factors (Hall and Fujii, 2005; Tabakin-Fix et al., 2006), it is expected that further investigations will yield additional Tax-responsive miRNAs. The complex effects of p30, p12 and HBZ on transcription factors, including CREB, Myc, NFAT, STAT5, p300, AP-1 (Matsuoka and Green, 2009; Nicot et al., 2005), indicate that they also likely to influence the profile of miRNAs. Furthermore, the fact that some of the viral proteins have opposite effects on a given transcription factor suggests that the expression of some miRNAs will depend on the balance in viral protein activities. One example is the CREB pathway, which is activated by Tax and repressed by p30 and HBZ. In addition to transcriptional effects, p30 also influences expression of some cellular mRNAs at the level of nuclear export (Taylor et al., 2009), thus expanding its potential impact on miRNA expression. We investigated the effect of transient ectopic expression of Tax and HBZ on miR-34a expression. Our results suggest that these 2 viral proteins are not responsible for the high levels of miR-34a found in

HTLV-1 transformed cells. The role of p12, p30 and combinatorial effects of multiple viral proteins on miR-34a expression control remain to be investigated.

In addition to adding to our understanding of HTLV-1 pathogenesis, unravelling the complex interactions between HTLV-1 and the miRNA regulatory network may yield useful markers for monitoring the course of infection from healthy carrier status to ATLL and novel targets for therapeutic intervention. miR-34a represents an interesting candidate biomarker as suggested by the recent identification of increasing levels of miR-34a in serum of patients with progressive liver disease (Cermelli et al., 2011).

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