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CORSO DI DOTTORATO IN GENETICA E
MEDICINA MOLECOLARE
XXII CICLO

microRNAs in the control of lymphocytes response

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Chapter 1: Introduction

The discovery of very short non-coding RNAs as regulators of gene activity began almost 15 years ago when Victor Ambros and colleagues studying larval development in the nematode worm *C. elegans* discovered a gene, *lin-4*, that encoded short RNA transcripts able to inhibit translation of the messenger RNA of a different gene, *lin-14*, by binding to its 3' untranslated region (UTR) (figure 1) [1]. In hindsight, *lin-4* was the first of the microRNA (miR o miRNA) class of genes to be characterized. Since this initial observation, more than 700 miRNAs (miRNA registry at http://www.mirbase.org/) have been identified in mammalian cells and they have been shown to be involved in a wide of physiological responses, including development, differentiation and homeostasis [2,3].

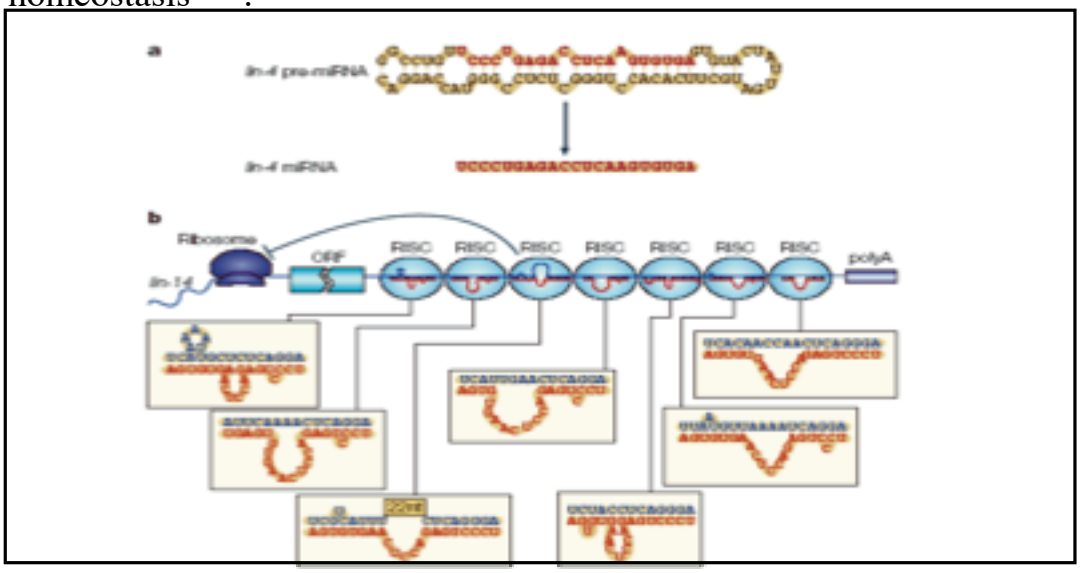


Figure 1 - The molecular hallmarks of *lin-4*, the founding member of the microRNA family. (a) The precursor structure and mature miRNA sequence of *lin-4*. (b) Sequence complementarity between *lin-4* (red) and the 3′-untranslated region (UTR) of *lin-14* mRNA (blue). *lin-4* is partially complementary to 7 sites in the *lin-14* 3′ UTR; its binding to these sites of complementarity brings about repression of LIN-14 protein synthesis. From Lin He et al. (2004). MicroRNAs: small RNAs with a big role in gene regulation. Nature Reviews Genetics, 5:522-531.

1.1 Biogenesis of microRNAs

miRNAs are short single-stranded RNA molecules of 19–23 nucleotides in length. Approximately half of all human miRNA genes are contained within the introns of protein-coding or non-protein coding genes, while others reside apart from known genes, or in the exons of untranslated genes[4]. The transcription of most miRNA genes is mediated by RNA polymerase II (Pol II), although a minor group of miRNAs that are associated with Alu repeats can be transcribed by Pol III (for miRNA biogenesis scheme see figure 2)[5-7]. A range of Pol II-associated transcription factors control miRNA gene transcription[8]. Thus, Pol II-dependent transcription allows miRNA genes to be elaborately regulated in specific conditions and cell types. Furthermore, some miRNA primary transcripts encode only a single mature miRNA, while other loci contain clusters of microRNAs that appear to be produced from a single primary transcript[4]. The primary transcript, pri-miRNA, is an imperfect hairpin characterized by a stem-loop structure and two single strand RNA (ssRNA) basal segments. A nuclear protein complex called the "Microprocessor", consisting of the Drosha type III RNase, the double-stranded RNA-binding protein DGCR8 (DiGeorge syndrome critical region gene 8, or Pasha), and others proteins, interact with pri-miRNAs through the single strand RNA basal segments and the stem of ~33 bp, and assists Drosha to cleave the substrate ~11 base pair away from the ssRNA-dsRNA junction[9]. Drosha liberates an 60-to-80-nucleotide long hairpin-shaped precursor, pre-miRNA, with 2-nt 3′ overhang, which is recognized and exported to the cytoplasm by exportin-5 via a Ran-GTP-dependent mechanism[10].

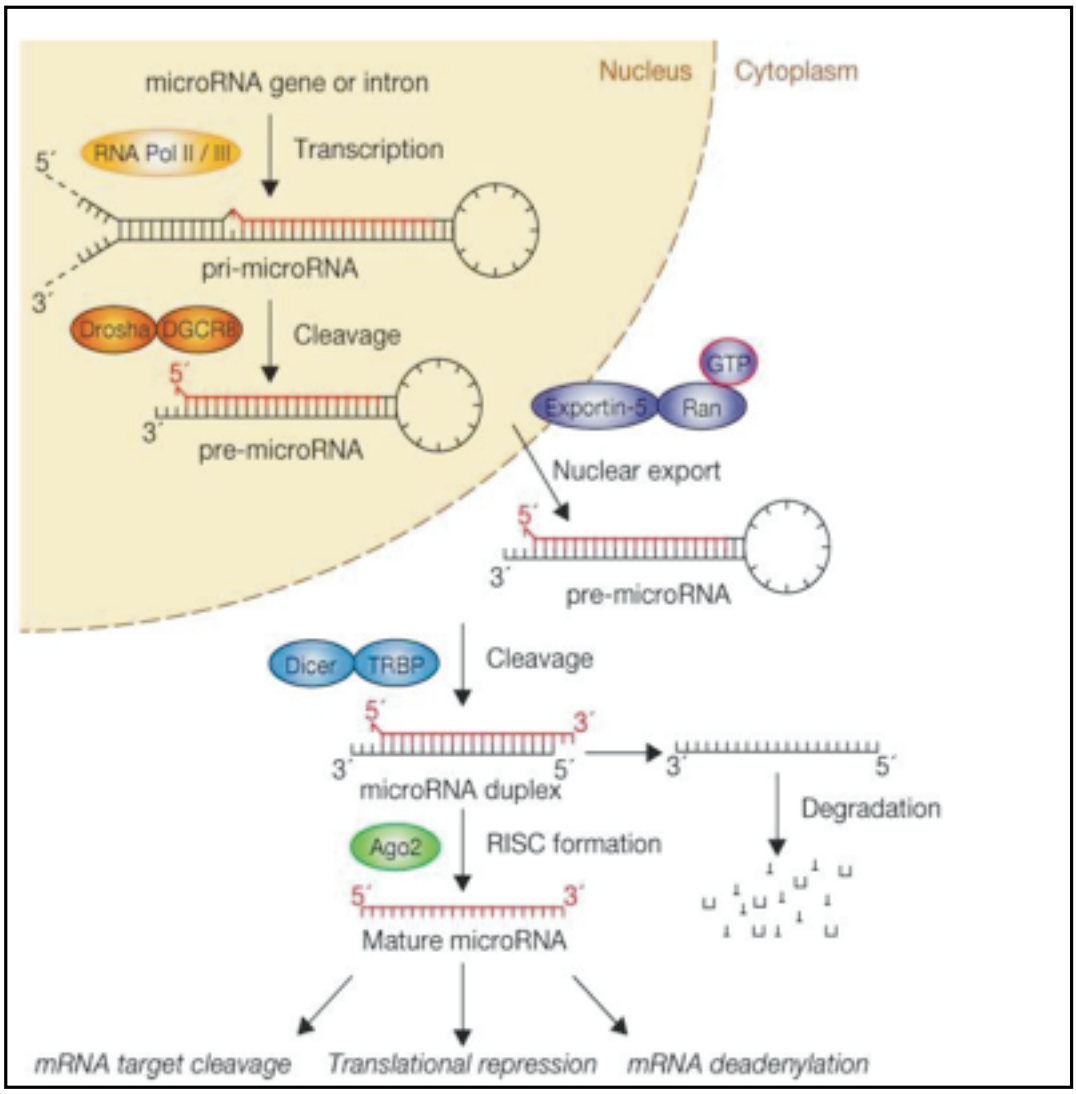


Figure 2 - The miRNA processing. In the nucleus, RNA polymerase II or III generates the primary miRNA transcript (pri-miRNA) that is then cleaved by the microprocessor complex Drosha–DGCR8 (Pasha). The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5–Ran-GTP. In the cytoplasm, Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs. From Winter J. et al. (2009). Many roads to maturity: microRNA biogenesis pathways and their regulation. Nature Reviews Molecular Cell Biology, 11: 228 – 234.

Recent studies show that pri-miRNA processing might be a co-transcriptional process[11-13]. The initial model was based on the finding that Drosha processing of intronic miRNA precedes the splicing of a host intron (figure 3a)[11]. Interestingly, the cleavage of the intron by

Drosha does not impair splicing[11]. This is consistent with a previous 'exon-tethering' model[14], which suggested that the exons of Pol II transcripts are cotranscriptionally assembled into the spliceosome. Thus, Drosha processing might take place after the transcript is tied to the splicing commitment complex (the early spliceosome complex), but before the intron is excised. Thus, cropping and splicing might be highly coordinated co-transcriptional processes. Supporting this, pri-miRNA and Drosha localize to the transcription sites and pri-miRNAs are enriched in the chromatin-associated nuclear fraction[12-13]. Apart from canonical intronic miRNAs, small groups of miRNA-like RNAs have been discovered in introns in flies and mammals[15-17]. These small RNAs are embedded in short introns, and their biogenesis does not require Drosha processing (figure 3b)[18]. Following the complex of splicing, the branch point of the lariat-shaped intron is resolved and the disbranched intron forms a hairpin structure that resembles pre-miRNA. Some precursors (mirtrons) contain extended tails at either the 5′ or 3′ end, which therefore require exonucleolytic trimming in order to become a substrate for nuclear export (figure 3b). Therefore, it is becoming clear that multiple non-canonical pathways can feed pre-miRNAs into the miRNA pathway through Drosha-independent processes.

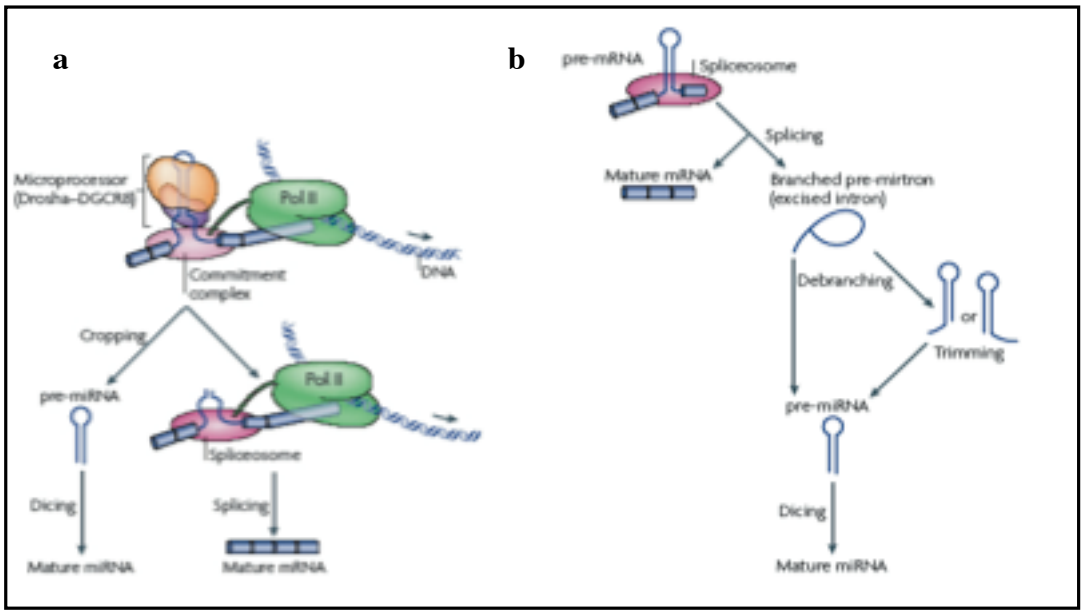


Figure 3 – Intronic miRNAs biogenesis pathway. (a) Canonical intronic miRNAs are processed co-transcriptionally before splicing. The miRNA-containing introns are spliced more slowly than the adjacent introns for unknown reasons. The splicing commitment complex is thought to tether the intron while Drosha cleaves the miRNA hairpin. The pre-miRNA enters the miRNA pathway, whereas the rest of the transcript undergoes pre-mRNA splicing and produces mature mRNA for protein synthesis. (b) Non-canonical intronic small RNAs are produced from spliced introns and debranching. Because such small RNAs (called mirtrons) can derive from small introns that resemble pre-miRNAs, they bypass the Drosha-processing step. Some introns have tails at either the 5′ end or 3′ end, so they need to be trimmed before pre-miRNA export. m7G, 7-methylguanosine. Modified from V. Narry Kim et al. (2009). Biogenesis of small RNAs in animals. Nature Reviews Molecular Cell Biology 10:126-139.

In the cytoplasm, the pre-miRNAs are further processed by a protein complex containing the Dicer type III RNase that cleaves the miRNA to its mature size[19-24]. In general, only one strand of the original precursor is kept as a mature microRNA. The mature miRNA becomes associated with a final protein complex called the miRISC (microRNA-induced silencing complex), which typically contains an Argonaute family protein, and which carries out the silencing of the genes targeted by the miRNA (figure 2). MiRNAs are believed to either block mRNA translation or reduce mRNA stability after imperfect binding of the

guide strand to miRNA-recognition elements (MREs) within target mRNA 3' UTR. Originally, it was believed that the specificity of this response was mediated by the "seed" region, which is localized at residues 2–8 at the 5' end of the miRNA guide strand. However, it now seems that this was an over-simplification and that miRNA targeting is influenced by additional factors such as the presence and cooperation between multiple MREs, the spacing between MREs, proximity to the stop codon, position within the 3' UTR, AU composition, and target mRNA secondary structure[25-28].

1.2 Mechanisms of microRNA action

The mechanism of miRNA-mediated gene regulation, and how it affects active translation (figure 4a), is a matter of controversy. The available data support two possible views: first, the translation of mRNAs is inhibited at the level of initiation, and the silenced mRNAs are occupied by few or no ribosomes[29]; second, the inhibition takes place at a step that is subsequent to initiation, and the silenced mRNAs sediment in the polyribosome fractions in a sucrose gradient. The first view is supported from several evidences:

i. AGO2, an Argonaute protein, binds the m7G (7-methylguanosine) cap of mRNAs through a domain that shares structural features with the translation initiation factor eIF4E, suggesting that when AGO2 is recruited to the 3′ UTR of a target mRNA by miRNAs, it hinders the m7G cap recognition by the translation apparatus[30] (figure 4b).

ii. miRNA translational inhibition is reversed, in an *in vitro* system, by increased levels of the eIF4F complex which includes the m7G cap-binding eIF4E translation factor[31].

iii. miR-2, *in vitro*, inhibits the assembly of the 48S complex, the translational complex that precedes the addition of the large ribosomal subunit to form the competent ribosome[32].

iv. eIF6, the ribosome inhibitory protein was co-purified with RISC[33].

Nonetheless, cellular subfractionation studies indicate that many miRNAs are found in the polyribosome fraction, which would be most consistent with a post-initiation inhibition by miRNAs; this could result from rapid degradation of the protein product encoded by the targeted mRNA, or from a high rate of ribosome drop-off during elongation, resulting in incomplete protein products that would be rapidly degraded (figure 4c)[34,35].

However, others observed variable levels of target mRNA degradation, and colocalization of the RISC component with mRNA degradation factors in the P bodies (figure 4d)[36]. Sequestration of mRNAs in the P bodies and degradation could be a step that follows blocking of translation, or a causative event in miRNA repression. GW182, a P-body component, has recently been shown to interact directly with AGO protein and to be recruited to the target mRNA in a let-7-dependent manner[37,38].

The detailed mechanisms of miRNA activity, including a full list of the protein components of the active miRISC complex for various miRNAs, await further elucidation. A recent paper underscores this point with the surprising observation that under some conditions, such

as cell cycle arrest, microRNAs can upregulate translation of a target protein[39].

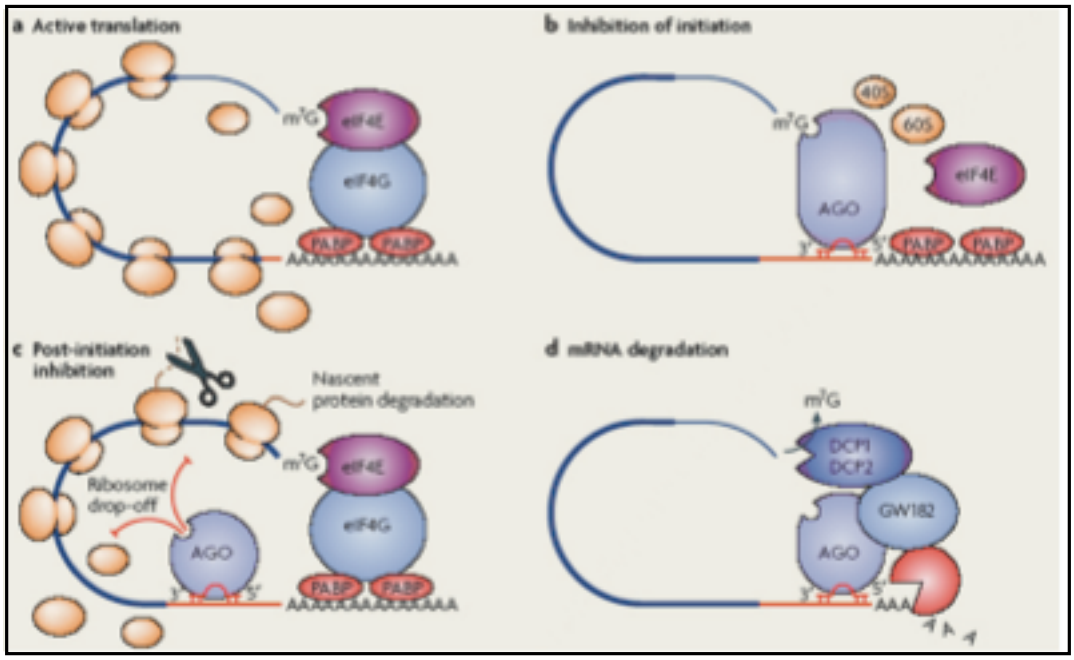


Figure 4 - Mechanism of miRNA-mediated gene regulation. (a) Active translation mechanism. (b) Translation of mRNAs is inhibited at the level of initiation. (c) The inhibition of mRNA target takes place at a step subsequently to initiation. (d) Sequestration of mRNA target in the P bodies determines RNA degradation. From Stefani et al. (2008). Small non-coding RNAs in animal development. Nature Reviews Molecular Cell Biology 9, 219-230.

1.3 Regulation of miRNA biogenesis

Expression profiling studies indicate that most miRNAs are under the control of developmental and/or tissue specific signalling[40]. Precise control of miRNA levels is crucial to maintain normal cellular functions, and dysregulation of miRNA is often associated with human diseases, such as cancer[41].

Transcriptional control. Transcription is a major point of regulation in miRNA biogenesis. Numerous Pol II-associated transcription factors are involved in transcriptional control of miRNA genes. For instance,

MyoD1 (myogenin and myoblast determination), SRF (serum response factor), Mef2 (myocyte enhancer 2) bind upstream of miR-1 and miR-133 loci and induce the transcription of these miRNAs during myogenesis[42,43]. Some miRNAs are under the control of tumour-suppressive or oncogenic transcription factors. The tumour suppressor p53 activates the miR-34 family[44], whereas the oncogenic protein MyC transactivates or represses a number of miRNAs that are involved in the cell cycle and apoptosis[45,46]. Epigenetic control also contributes to miRNA gene regulation; the miR-203 locus frequently undergoes DNA methylation in T-cell lymphoma but not in normal T lymphocytes [47].

Post-transcriptional regulation. Drosha and Dicer processing confers another important point of regulation. As it is mentioned above, miR-1 and miR-133 share common *cis* and *trans* regulation mechanism, but the relative abundance of miR-1 or miR-133 differs dynamically in the heart and skeletal muscle at distinct stage of development, which reflect a higher order of processing regulation[42,43]. miR-21 is induced in response to bone morphogenetic protein (BMP)/transforming growth factor-β (TGF β) signalling by regulating the Microprocessor activity, without transcriptional activation[48]. It was proposed that SMAD proteins activated by BMP/TGFβ interact with Drosha and DDX5 (also known as p68) to stimulate Drosha processing. Although the detailed mechanism for this remains unclear, the Drosha mediated processing of miR-21 is strongly enhanced and the abundance of miR-21 increases (figure 5 b). Drosha-mediated cleavage can also be regulated for individual miRNAs: heterogeneous ribonucleoprotein particle A1 (hnRNPA1) binds specifically pri-miR-18a and facilitates its

processing. Interestingly loss of (hnRNPA1) has impact only on miR-18a, but not on other miRNAs that are located in the same miR-17 genomic cluster (figure 5c) [48-50]. How many of such regulatory factors exist is unclear, but it is plausible that nuclear RNA binding proteins influence miRNA processing through specific interactions with a subset of pri-miRNAs.

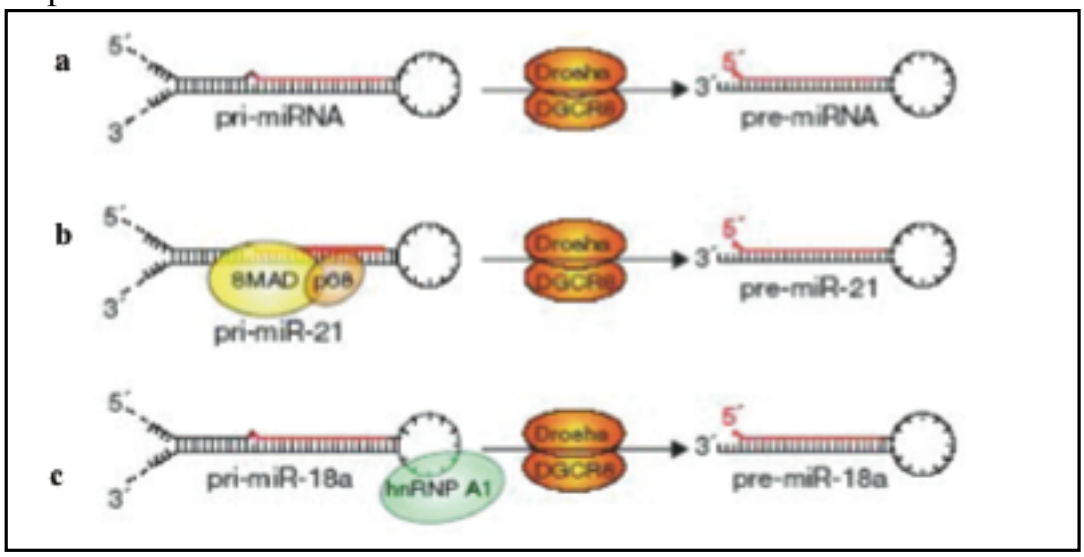


Figure 5 – miRNAs posttrascriptional regulation. (a) The microprocessor complex Drosha–DGCR8 cleaves the pri-miRNA, releasing the pre-miRNA. (b) TGF-beta signalling induces SMAD binding to the miR-21 precursor and enhances its efficient processing by Drosha. (c) Interaction of pri-miR-18a with hnRNP A1 facilitates cleavage of this specific miRNA by Drosha. Modified from Winter J. et al. (2009). Many roads to maturity: microRNA biogenesis pathways and their regulation. Nature Reviews Molecular Cell Biology, 11: 228 – 234.

The let-7 miRNAs show interesting expression regulation [51] The primary transcript of let-7 (pri-let-7) is expressed in both undifferentiated and differentiated ES (embryonic stem) cells, whereas mature let-7 is detected only in differentiated cells, indicating that let-7a might be post-transcriptionally controlled[52-54]. Similar post-transcriptional inhibition of let-7 also takes place in tumour cells[54]. Recent studies show that an RNA binding protein, LIN-28, is

responsible for the suppression of let-7 biogenesis[55-58] (figure 6). Several different mechanisms of LIN-28 action have been proposed: blockage of Drosha processing [55,56], interference with Dicer processing [58,59] and terminal uridylation of pre-let-7[58]. Given the cytoplasmic localization of LIN-28 and its strong interaction with pre-let-7 (but not with pri-let-7)[58], LIN-28 is likely to function mainly in the cytoplasm by interfering with pre-let-7 processing and/or by inducing terminal uridylation of pre-let-7. The "u tail" (~14 nt) that is added to the 3′ end of pre-let-7 blocks Dicer processing and facilitates the decay of pre-let-7 [59]. It is unknown how widespread this type of regulation is and which enzyme is responsible for pre-miRNA uridylation.

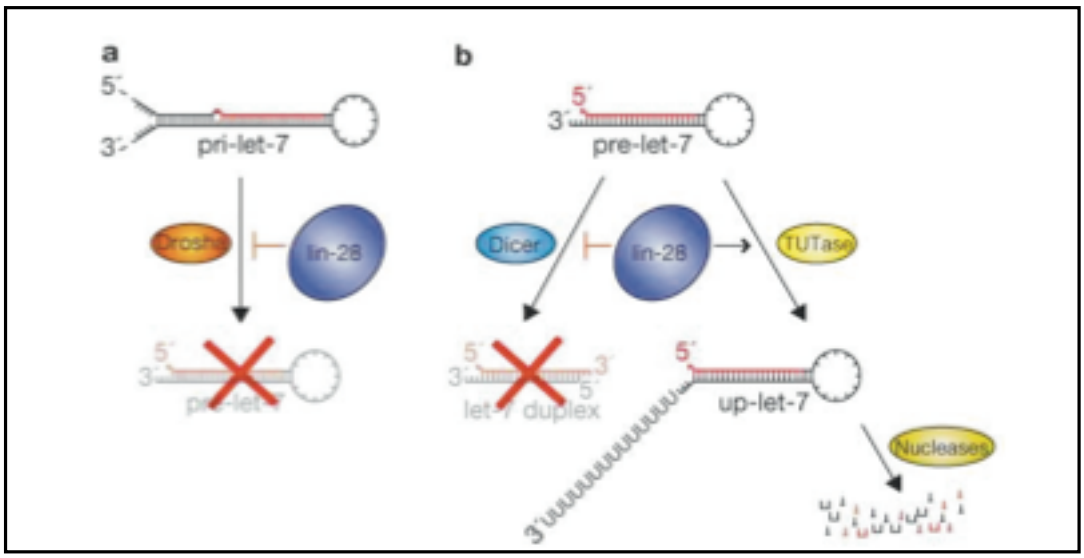


Figure 6 - Different mechanisms suppress the maturation of let-7 by the RNA-binding protein Lin-28. (a) Lin-28 inhibits Drosha-mediated processing of pri-let-7. (b) Lin-28 inhibits Dicer-mediated cleavage of pre-let-7 and recruits a terminal uridylyl transferase (TUTase) to pre-let-7. The uridylated up-let-7 is not processed but is degraded by nucleases. From Winter J. et al. (2009). Many roads to maturity: microRNA biogenesis pathways and their regulation. Nature Reviews Molecular Cell Biology, 11: 228 – 234.

Turnover of miRNA is a largely unexplored area. RNA decay enzymes might target not only mature miRNAs but also the precursors (pri-miRNAs and pre-miRNAs). Once bound to Ago proteins, mature miRNAs seem to be more stable than average mRNAs; the half-life of most miRNAs is greater than 14 hours [60]. However, certain miRNAs (for example, miR-29b) might be degraded much more rapidly than other miRNAs, which suggests a specific recognition of miRNA sequences by nucleases. The 3′→ 5′ exonuclease ERI1 (also known as THEX1) was previously shown to be responsible for the degradation of siRNAs in *C. elegans* [61]. A group of exoribonucleases named small RNA degrading nuclease (SDN) proteins were recently reported to affect the stability of miRNAs in plants[62]. However, it remains unclear which nucleases are responsible for miRNA degradation in animals. RNA editing is another possible way of regulating miRNA biogenesis. The alteration of adenines to inosines, a process that is mediated by adenine deaminases (ADARs), has been observed in miR-142 [63] and miR-151[64]. Because the modified pri-miRNAs or pre-miRNAs become poor substrates of RNase III proteins, editing of the precursor can interfere with miRNA processing. Editing can also change the target specificity of the miRNA if it occurs in miRNA sequences [65].

Feedback circuits in miRNA networks. miRNA biogenesis is controlled by multiple layers of feedback loops that involve the biogenesis factors, the miRNAs themselves and their targets [66]. Two types of feedback circuits are frequently observed: single-negative feedback and double-negative feedback. Single-negative feedback usually results in stable or oscillatory expression of both components,

whereas double-negative feedback serves as a bistable switch that results in mutually exclusive expression. Levels of Drosha and Dicer are controlled by single negative feedback to maintain the homeostasis of miRNA production [67,68]. Drosha constitutes a regulatory circuit together with DGCR8; Drosha downregulates DGCR8 by cleaving DGCR8 mRNA, whereas DGCR8 upregulates Drosha through protein stabilization (figure 8a,b). This loop seems to be highly effective because even when the DGCR8 gene copy number is reduced by one-half in *Dgcr8* heterozygous cells, an almost normal level of DGCR8 protein is produced and miRNA levels are also unaffected.

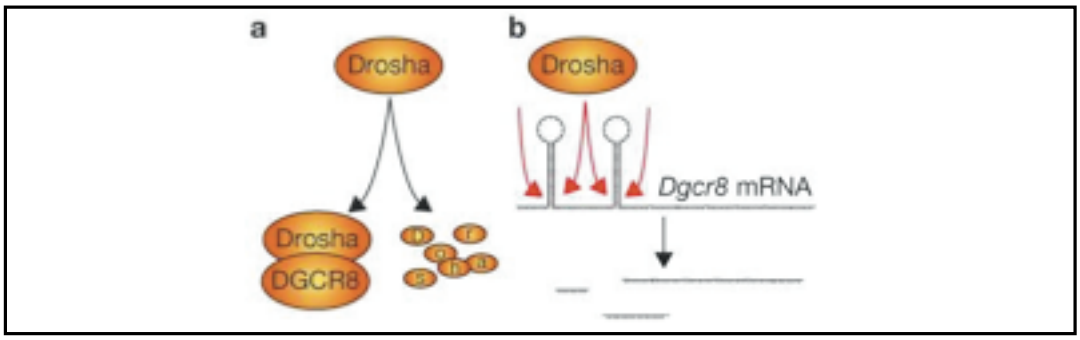


Figure 7 – miRNAs feedback regulation. (a) DGCR8 enhances the protein stability of its partner Drosha. (b) Drosha cleaves two hairpin structures in the *Dgcr8* mRNA, which is subsequently degraded. From Winter J. et al. (2009). Many roads to maturity: microRNA biogenesis pathways and their regulation. Nature Reviews Molecular Cell Biology, 11: 228 – 234.

Human Dicer is controlled by its own product, let-7, which binds to the 3′ UTR and coding region of the Dicer mRNA [69,70]. This might explain why Dicer knockdown is often more transient and moderate than knockdown of other genes. Furthermore, numerous examples of single-negative feedback have been described in worms, flies and mammals, in which a transcription factor that transactivates miRNA is itself repressed by that same miRNA[66]. Double-negative feedback

control is also often used as an effective genetic switch of specific miRNAs during differentiation. One interesting example is the conserved loop that involves the miR-200 family and the transcriptional repressors ZEB1 and ZEB2 that functions in epithelial–mesenchymal transition [71]. miR-200 family suppresses the ZEB1 and ZEB2 protein synthesis, whereas blocks miRs maturation.

1.4 miRNA functions

Targeted deletion of Dicer, the essential miRNA-processing enzyme, in mice causes embryonic lethality before embryonic day (E) 7.5, suggesting an essential role for miRNAs in development[72]. ***Neurogenesis.*** miRNAs regulate key events during neurogenesis in multiple species. For example, in *C. elegans*, two bilaterally symmetric gustatory neurons, ASE left (ASEL) and ASE right (ASER) exhibit left and right asymmetric molecular features. Auto-regulatory feedback loops involving distinct miRNAs control the cell-fate decision between the two asymmetric states. The transcription factors die-1 or cog-1 specify ASEL or ASER fates, respectively, by activating genes that distinguish ASEL from ASER, including the miRNAs lsy-6 and miR-273, which repress the ASER or ASEL fate, respectively. These cascades reinforce transcriptional programs leading to left–right asymmetry[73].

Fragile X syndrome is one of the commonly inherited mental retardation syndromes. The gene responsible for fragile X syndrome, FMR1 (fragile X retardation 1), is located on human chromosome 10. This syndrome is caused by loss of an RNA-binding protein called

familial mental retardation protein, which has been reported to be regulated by miRNAs [74].

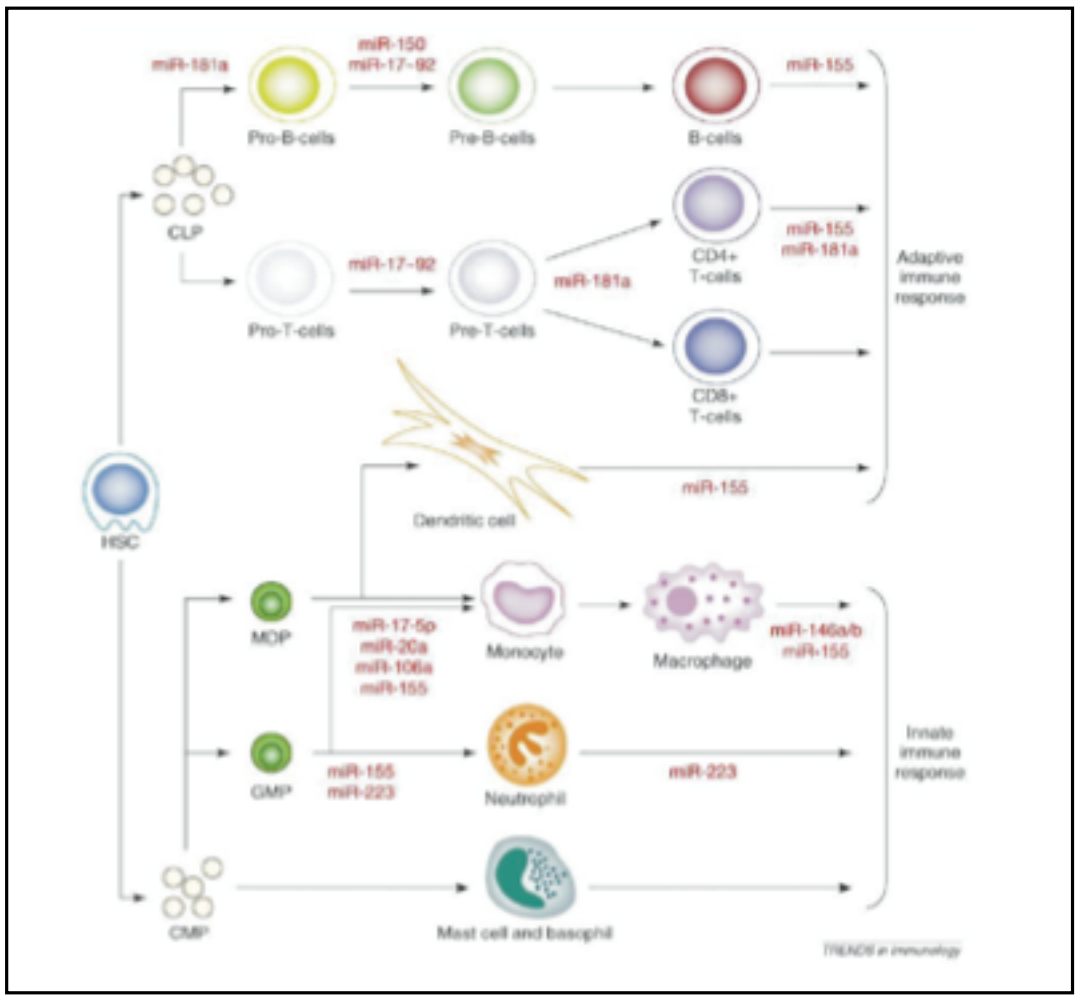
Tourette's syndrome is another neuropsychiatric disorder among humans in which the role of miRNA has been reported [75]. The 3'-UTR of the SLITRK1 gene contains the binding site of miR-189, which is mutated in some Tourette's syndrome patients [75]. In situ hybridization of SLITRK1 mRNA and miR-189 revealed coexpression in the neuroanatomical circuits most commonly implicated in Tourette's syndrome. This demonstrates how an miRNA can be involved in the establishment of a disease phenotype [76].

Irrespective of diseases, miRNAs are also involved in many other physiological functions. The expression of miR-375 takes place in murine pancreatic islets cells and plays an important role in regulation of the myotrophin gene and thereby glucose-stimulated insulin exocytosis [77]. Higher expression levels of miR-375 have been reported in pituitary glands of zebra fish embryoswhich indicates its possible involvement in neuroendocrine activities [78,79].

Pregnancy. Recently, Chim et al. have reported the existence of placental miRNA in maternal plasma, which opens up new possibilities of using the miRNAs as molecular markers for pregnancy monitoring. Four placental miRNAs (miR-141, miR-149, miR-299-5p and miR-135b) were found to be present at higher levels in maternal plasma during the predelivery period than after delivery. The measurement of miRNA in maternal plasma for prenatal monitoring and diagnosis would be an interesting future research direction [80].

1.5 miRNA in Immune System

Regulation of the immune system is vital to preventing many pathogenic disorders including autoimmune disease and cancers. Mammals have developed a complex system of checks and balances for immune regulation in order to maintain self tolerance while allowing immune responses to foreign pathogens, most of which are not fully understood. Recently, it has become evident that miRNAs play an important role in regulating immune response, as well as immune cell development. Amazingly, a relatively small number of specific miRNAs are coming to light as important regulators of the immune system (figure 8) [81-84].



1.5.1 miRNA during the Innate Immune Response

miRNAs have an important role in modulating innate immune responses, the first line of defense to bacterial, viral, and other pathogens. During inflammatory response, several hundred genes are involved and a process to achieve pathogen clearance and at the same time avoid consequences of dysregulated gene expression must be tightly regulated. Recent studies have shown several miRNAs, such as miR-155, miR-146, and miR-223, regulate the acute inflammatory response after the recognition of pathogens by the Toll-like receptors (TLRs) [85-88].

miRNA and Macrophage/Monocytes. To examine the potential involvement of miRNAs in regulation of the innate immune response, Taganov et al. analyzed expression of 200 miRNAs after exposure of human monocytic THP-1 cell line to LPS [85]. They showed three miRNAs were up-regulated, namely miR-146, miR-132, and miR-155. Further, induction of miR-146 by the TLR system displayed dual occurrence: TLRs that recognize bacterial constituents and reside on the cell surface (like TLR2, TLR4, and TLR5) trigger miR-146 induction; those TLRs that mainly sense viral nucleic acids and localize intracellulary (TLR3, TLR7, and TLR9) have little effect on miR-146 expression.

Figure 8 - Overview of the role of miRNAs during the differentiation and maturation of immune cells. miRNAs have been shown to regulate multiple steps in the development of immune cells including lymphocytes (B and T cells) and myeloid cell (monocytes and neutrophils). CD, cluster designation; CLP, common lymphocyte progenitor; CMP, common myeloid progenitor; GMP, granulocyte monocytic progenitor; HSC, hematopoietic stem cell; MDP, myeloid dendritic progenitor. From Mark A. Lindsay (2008). microRNAs and the immune response. Trends in Immunology Vol.29 No.7:343-351.

Promoter analysis of the miR-146a revealed that it is regulated by NF-κB and may function as a negative regulator of IRAK1 (IL-1 receptor-associated kinase 1) andTRAF6 (TNF receptor-associated factor 6) expression [85]. As it is well known that TRAF6 and IRAK1 are two key adapter molecules in the downstream of TLRs, which can trigger the activation of IκB kinase (IKK) and the Jun kinase (JNK), then in turn, activate NF-κB and activating protein (AP)-1 transcription factors, and finally result in up-regulation of immune-responsive genes [89]. In addition to TLRs stimulations, Perry's study verified miR-146a expression was related to IL-1b-induced responses [88]. They demonstrated an important feedback mechanism during severe inflammation: IL-1b-induced increases in miRNA-146a expression negatively regulate IL-8 and RANTES release. Recent study found this miRNA to be associated with psoriasis, a chronic inflammatory skin disease, indicating that alterations in the fine-tuning of innate immune responses by miRNAs may contribute to inflammatory disorders[90]. Taken together, miR-146a now was thought to be a negative regulator during the innate immune responses. Moschos et al. measured the differential mature miRNA expression profile during the innate immune response to aerosilized LPS in the mouse lung [91]. They found 12 miRNAs (miR-21, -25, -27b, -100, 140, -142–3p, -181c, 187, -194, -214, -223, and -224), which were involved in the innate immune response, were significantly up-regulated in a time dependent fashion. Unlike the LPS-induced response in THP-1 cells and mouse macrophages expression of miRNA-146 or -155 were not found to upregulate[85,86]. They speculated the reasons behind these differences could be a result of multiple factors including the presence of multiple

cell types within the lung or be related to the dynamics of the two models.

miRNA and Granulocyte. miRNA-223 was found to have crucial roles in regulating granulocyte proliferation and activation. Its expression was bone marrow-specific and was confined to myeloid cell lineages[92]. However, overexpression of miR-223 and knock-down of miR-223, studied by two independent groups, showed the opposite effects [93,94]. Fazi et al. firstly reported that miR-223 was a positive regulator of granulocyte differentiation by both overexpression and knock-down experiments. In their study, two transcription factors named NFI-A (nuclear factor I-A) and C/EBPα (the CCAAT enhancer proteins), had been implicated to regulate miR-223 expression. These two factors compete for binding to the miR-223 promoter. NFI-A is required to maintain miR-223 at low levels, then during differentiation NFI-A is replaced by the transcription factor C/EBPα, which induces high expression of miR-223 [93]. This, in turn, represses the expression of NFI-A post-transcriptionally. The role of C/EBPα in regulating miR-223 expression was confirmed by Fukao et al., who demonstrated C/EBPα can combine with PU.1 (another myeloid specific transcription factor) to enhance the promoter activity[95]. More recently, observations in miR-223 knockout mice revealed the negative role of miR-223 in regulating progenitor proliferation and granulocyte differentiation and activation[94]. They also found a critical target of miR-223 in early myeloid progenitors, Mef (myeloid ELF-1-like factor) 2c, a transcription factor that promotes myeloid progenitor proliferation, or the insulin-like growth factor receptor (IGFR). Although paradoxical

results exit, the role of miR-223 in regulating granulocyte production and the inflammatory responses was indispensable.

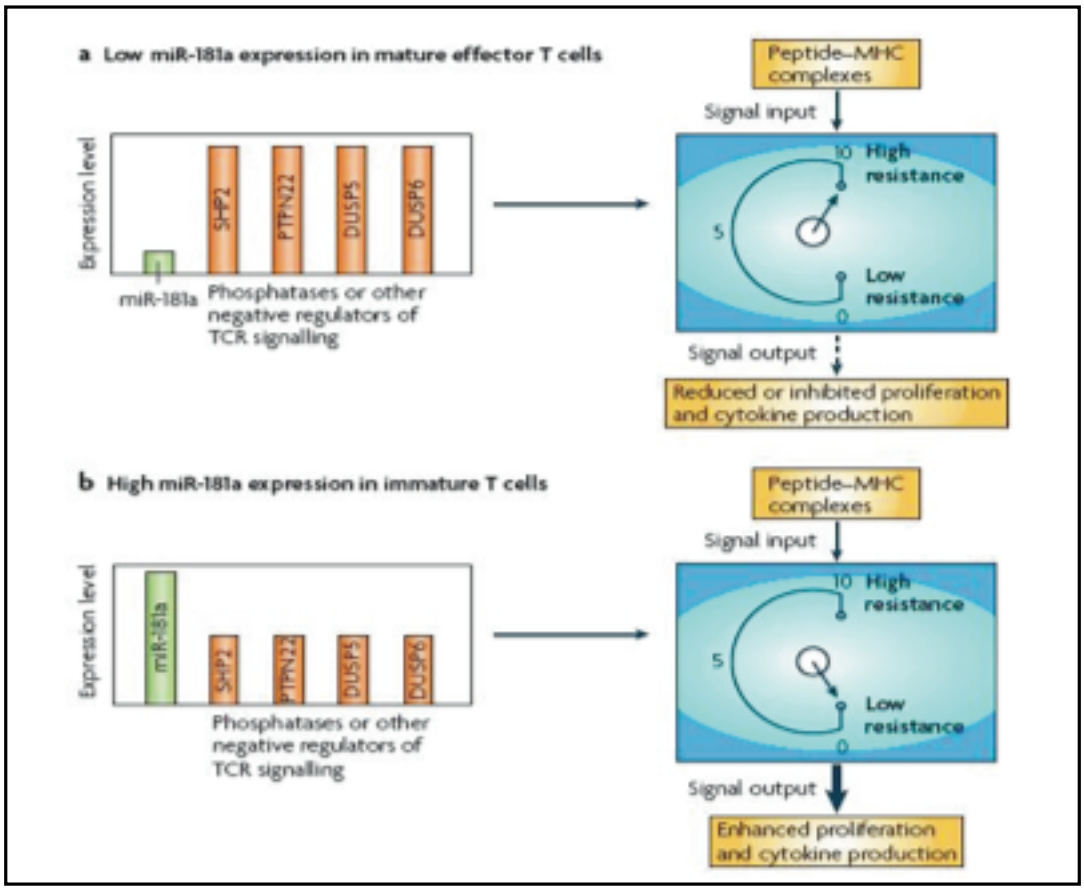
1.5.2 miRNAs in the Adaptive Immune Responses

In addition to regulating innate immune responses, miRNAs also have an important role in modulating adaptive immune responses, a central feature of which was activation and subsequent clonal expansion of antigen specific lymphocytes.

miRNA and T Cell Differentiation and Activation. In mice with a deficiency in Dicer in early T-cell progenitors (under the lck-driven cre transgene) the percentages of different double-negative, double-positive and the CD4–CD8 lineage (i.e. whether the transitioning cells became CD4 or CD8 single-positives) decisions also appears to be intact, albeit with a 10-fold reduction in total thymocyte numbers past the double-negative stage[96]. Instead, later deletion of Dicer with a CD4-drive cre transgene results in smaller reductions in the number of total thymocytes, at the single- positive stage[97]. Although the precise molecular mechanism for the discrepancy between these studies is still not well characterized, the differential response in the numerical impact on T-cell differentiation depending on the timing of Dicer excision suggests that miRNA do not have a non-redundant role in any specific developmental event, but rather create a delayed numerical reduction as the result of diminished proliferation and increased susceptibility to cell death[96,97]. Dynamic regulation of miRNA expression in several distinct stages of T cell was observed by Neilson et al. [98]. They found the degree of miRNA variation, across the T-lymphocyte developmental progression is striking, suggesting global miRNA levels are correlated

to the level of T cell differentiation. Recently, the miRNA expression profile in antigen-specific naïve, effector and memory CD8 T cells was analyzed using three different methods[99]. Among miRNAs, expressed in all the T cell subsets, the frequency of seven miRNAs (miR-16, miR-21, miR-142-3p, miR-142-5p, miR-150, miR-15b, and let-7f) alone accounted for 60% of all miRNAs. Compared with naïve cells, global downregulation of miRNAs (including six out of the seven dominantly expressed miRNAs) was observed in effector T cells, and in memory T cells the miRNA expression levels tended to come back up though they were still lower than in naïve cells. It seemed that the level of most miRNAs expression appear to inversely correlate with the activation status of the cells, exception of miR-21. Since regulation of miRNA expression was found to characterize the stage-specific development of thymocytes, miRNAs may modulate the differentiation status of antigen-stimulated T cells[99]. As stated above, different development stages and cell types of T cells have distinct miRNA expression profiles. However, studies on the relationship between the expression and functions of these miRNAs are limited. In 2007, Li et al. demonstrated that TCR sensitivity and signalling strength can be modulate posttrascriptionally by miR-181a (figure 9)[100]. The expression of miR-181a is higher in immature T cell populations such as double positive DP thymocyres, but lower in the more differentiated T cell population such as Th1 and Th2 effectors cells. The inhibition of this miRNA significantly impairs DP cell sensitivity and efficiently obstructs positive and negative selection, whereas increasingly miR-181 expression in mature T cells augments their sensitivity to peptide antigens[100]. Moreover, quantitative regulation of T cell sensitivity by

miR-181a enables mature T cells to recognize antagonists-the inhibitory peptide antigens-as agonists. Mechanism study demonstrated that this did not result from changes in the expression of surface receptors but instead involved the coordinated down regulation of multiple phosphatases. Multi-target regulation by miR-181a is required for fine-tuning T cell sensitivity, thus, miR-181a was thought an intrinsic modulator of T cell sensitivity and selection [100]. In addition, Neilson et al. also proved miR-181a was involved in positive selection of T cell through repressing the expression of BCL-2, CD69, and the T cell receptor [98]. These findings suggest that further characterization of the molecular networks controlled by miR-181a would probably yield additional insights about other possible functions for miR-181a in adaptive immune responses.



miRNA and B Cell. The essential role of miRNA in B-cell differentiation was first revealed in mice with a haematopoietic defect in Ago2, encoding an Ago protein indispensable for miRNA biogenesis and function[101]. Deficiency of Ago2 did not affect the generation of early pro-B cells, but significantly impaired further pre-B-cell differentiation and the succeeding peripheral B-cell generation. In agreement with this, a subsequent study where the whole miRNA network was ablated by employing the B-cell-specific deletion of a conditional allele of Dicer has demonstrated that B-cell differentiation is almost completely blocked at the pro- to pre-B-cell transition, at least partially as a result of the deregulation of a pro-apoptotic molecule, Bim[102]. Moreover, Dicer deficiency in B cells also resulted in sustained terminal deoxynucleotidyl transferase expression throughout B-cell maturation, altering the generation of the antibody repertoire[102].

Figure 9 - Modulation of the antigen sensitivity of T cells by the miR-181a dimmer switch (or rheostat). The expression of miR-181a is regulated during T-cell development and maturation, and the level of miR-181a expression correlates with T-cell sensitivity to antigens, suggesting that miR-181a might act as an intrinsic rheostat or dimmer switch to tune T-cell sensitivity to antigens during T-cell development and maturation. (a) In mature effector T cells, microRNA-181a (miR-181a) is expressed at low levels and therefore the signalling rheostat is tuned to high resistance. That is, by decreasing miR-181a expression and de-repressing the negative signals that are controlled by the negative regulators of T-cell receptor (TCR) signalling (such as SH2 (SRC homology 2)-domain-containing protein tyrosine phosphatase 2 (SHP2), protein tyrosine phosphatase, non-receptor type 22 (PTPN22), dual-specificity protein phosphatase 5 (DUSP5 and DUSP6) the signal output, such as T-cell proliferation and cytokine secretion induced by peptide–MHC complexes, is dramatically reduced or completely turned off. (b) By contrast, in immature T cells, such as $CD4^+CD8^+$ thymocytes, miR-181a is expressed at high levels and thus the rheostat is tuned to low resistance. That is, by increasing miRNA expression and repressing the negative signals that are controlled by the negative regulators of TCR signalling, the signal output from the identical stimulation is dramatically enhanced. From Lodish et al. (2008). Micromanagement of the immune system by microRNA. Nature Reviews Immunology, 8: 120-130.

Whereas these findings provided important insights as to how the miRNA network could impact B-cell differentiation and function, recent studies have begun to explore the role for individual miRNA in controlling different aspects of B-cell biology.

Similar to miR-181a, miR-150 also shows a temporo-spatial expression pattern during the development of B cell [103-106]. High-level expression of miR-150 in the spleen and the thymus suggested that it might participate in B and/or T lymphopoiesis. Indeed, over expression of miR-150 in haematopoietic stem cells blocked B lymphopoiesis by inhibiting the transition from the pro-B to the pre-B cell stage and greatly impaired the formation of mature B cells, but had little effect on the formation of either mature CD8- and CD4-positive T cells or granulocytes or macrophages [106]. This observation was consistent with another study, which also proved the importance of miR-150 in B cell formation using gain- and loss of function mouse models [104]. Further, Xiao et al. demonstrated a transcript factor-Myb, which is highly expressed in lymphocyte progenitors, might be a crucial target of miR-150. Transgenic mice lacking c-Myb looked very similar to the transgenic mice with ectopic expression of miR-150 in the progenitors-abnormal B cell development and whole sale loss of B1 cells [107]. Although Myb has a key role in both B cell and T cell development, over expression or deletion of miR-150 in mice affects only the development of B cells, but not T cells.

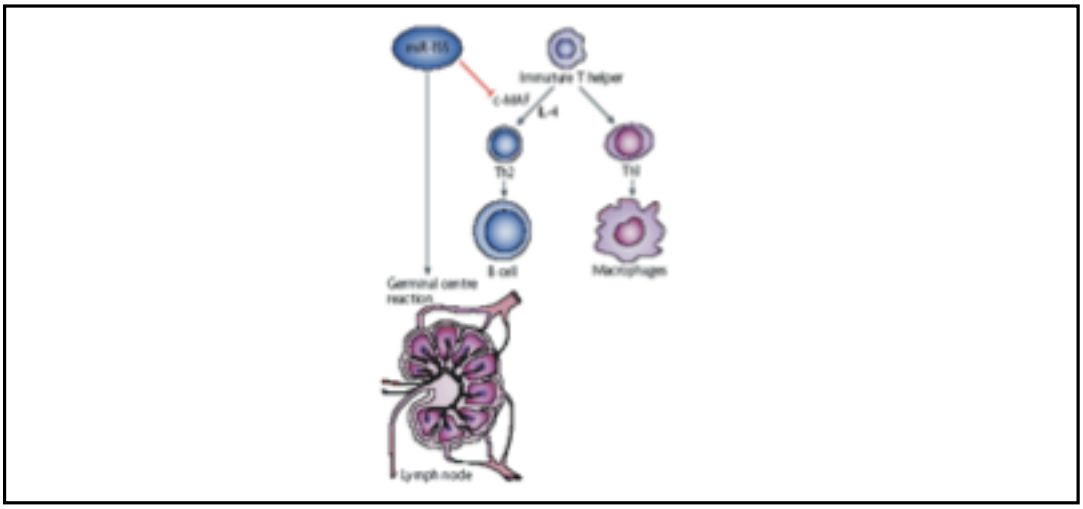
1.5.3 miRNA-155: a regulator of B - and T-cell maturation and the innate immune response

MiR-155 represent a typical multifunctional miRNA. To date, increased evidence points out that miR-155 is involved in numerous biological process including haematopoiesis, inflammation and immunity. Deregulation of miR-155 has been found to be associated with different kinds of cancer, cardiovascular disease and viral infections.

MiR-155 is processed from an exon of non-coding RNA transcribed from BIC, the *B*-Cell *I*ntegration *C*luster located on chomosome 21. A central role for miR-155 in the regulation of T- and B cells responses during the acquired immune response has emerged from studies in knockout mice; Rodriguez et al. found these mice are immunodeficient and fail to develop protective response to virulent *Salmonella typhimurium* infection after immunization with a non-virulent strain of these bacteria. As for B-lymphocytes, immunized knockout mice produced significantly reduced amounts of IgM and switched antigen-specific antibodies, indicative of impaired B cell responses [108,109]. Interestingly, miR-155 knockout mice also undergo age-related lung airway remodelling, which is characterised by increased collagen deposition, smooth muscle mass and inflammatory cell infiltrate within the broncholaveolar lavage [108]. These phenotypic observations seem to be in part mediated through involvement of miR-155 in B-cell production of isotype-switched, high affinity IgG1 antibodies and during the development of B cell memory[109,110]. The observed reduction in the size of the germinal centers suggested that reduced IgG1antibody production and B-cell memory was the result of a failure

to select high affinity plasma B cells [109,110]. Examination of the mechanism showed that miR-155 attenuated the expression of the transcription factor PU.1, which was shown to down regulate IgG1 levels [110]. In addition, miR-155 targets the cytidine deaminase AID, a critical enzyme that mediates class-switch recombination and somatic hypermutation [111,112]. These AID studies have used mutant mice in which the miR-155 binding site in the 3′ UTR of AID is abolished.

As with B cells, miR-155 seems to be involved in T cell differentiation [108,109]. Thus, naïve T cells derived from miR-155 knockout mice were shown to have an increased propensity to differentiate into Th2 rather than Th1 cells, with the concomitant production of Th2 cytokines such as IL-4, IL-5 and IL-10 [108,109]. It has been speculated that this results from miR-155 targeting of c-Maf (musculoaponeurotic fibrosarcoma), a transcription factor that is known to be a potent transactivator of the IL-4 promoter, a key cytokine in the development of Th2 cells [108]. With regard to the acute immune response, the T lymphocytes had an impaired response and showed attenuated IL-2 and interferon γ (IFNγ) release in response to antigens (figure 10)[108,109].



In monocytes, macrophages and myeloid dendritic cells, miR-155 increases substantially after exposure to a variety of inflammatory stimuli [86,113,114]. Direct recognition of microbial products by Toll-like receptors, particularly pathogen-associated molecular motifs derived from bacteria or viruses, leads to miR-155 upregulation. Furthermore, a plethora of immunoregulatory cytokines, such as tumor necrosis factor and interferons, also induce miR-155 upregulation in macrophages. A link between miR-155 and the innate immune response was suggested from studies showing increased expression after LPS (via TLR-4) and lipoprotein (via TLR- 2) stimulation in monocytes or macrophages and in the splenocytes of mice that had been inoculated with *Salmonella enteritidis*–derived LPS [86,87]. Increased miR-155 expression was also seen after activation of the innate response by viral- and bacterial-derived nucleotides including poly (I:C) (via TLR-3) and CpG (viaTLR-9), although increased expression in response to IFNβ and IFNγ seemed to be secondary to the autocrine release of TNF-α [86].

Tili et al. showed the relationship between miR-155 and TNF-α that is the main cytokine produced by macrophages in response to LPS.

They found the level of miR-155 in macrophage oscillated rapidly following TNF-α stimulation, which is similar to the oscillatory activity of NF-κB.

Figure 10 – Role of miR-155 in lymphocyte development. miR-155-null mice are characterized by complex defects in homeostasis of the immune system and globally impaired immune responses. Among the defects that were characterized in detail, the loss of miR-155- mediated inhibition of the transcription factor cMAF led to increased production of interleukin4 (IL-4) and T helper-2 (Th2) cells. The germinal centre reaction was disrupted, resulting in impaired T cell-dependent antibody responses. Modified from Stefani et al. (2008). Small non-coding RNAs in animal development. Nature Reviews Molecular Cell Biology 9, 219-230.

This implied miR-155 might exert both positive and negative effects by acting post-transcriptionally to regulate expression of different target proteins, such as, FADD (Fas-associated death domain) and Ripk1 (receptor interacting serine-threonine kinase 1). Further, miR-155 was found to enhance the production of TNF-α, which suggested the positive role of miR-155 to regulate the release of inflammatory mediators during the innate immune response. This hypothesis is supported by the results of an array analysis of gene expression in the *Eμ-miR-155* transgenic mice that over-expressed miR-155 in B cells, which demonstrate an elevated level of serum TNF-α [87].

Interestingly, a recent report has shown that LPS induced strong but transient miR-155 expression in mouse bone marrow cells and indicated that this is likely to drive granulocyte/monocyte expansion [115]. The possible involvement of miR-155 in the development of acute myeloid leukaemia (AML) was revealed from studies of the effect of long-term miR-155 over expression. In these studies, viral mediated transfection of miR-155 into haematopoietic stem cells (HSCs) and engraftment into lethally irradiated mice produced some of the pathological features characteristic of myeloid neoplasia [115]. This led these authors to speculate that the well-established link between inflammation and cancer might involve chronic up-regulation of miR-155, which could predispose these individuals to the development of myeloproliferative disorders. The pro-inflammatory transcription factors, AP-1 and NF-κB, have both been reported to regulate miR-155 expression [109,86,117].Thus, in macrophages, the action of TLR-3 and TNFα is mediated via AP-1[86], whereas the response to LPS is via NF-κB [116]. Similarly, BCR cross-linking in a human B-cell line was shown

to induce miR-155 expression via activation of the extracellular-regulated kinase (ERK) and c-jun N-terminal kinase (JNK) pathway and the subsequent recruitment of FosB and JunB to AP-1 binding site [117].

1.6 Aim

Recently, it has become evident the importance of miRNAs in regulating immune response, as well as immune cell development.

During my phD fellow I focused my attention on the relationship B-lymphocytes-microRNA; in particular I examined two the different topics: the microRNA activation in response to oncogenic virus infection and cloning of new microRNA within *IBTK* gene located in the genomic sequence 6q14.1, which is a hot spot of chromosomal rearrangements in lymphoproliferative disorders.

Epstein–Barr virus (EBV), which infects over 90% of the adults, appears to have evolved to exploit the normal biology of B-cell development in order to persist as a life-long asymptomatic infection. However, EBV can contribute to oncogenesis. Indeed, it is frequently found in Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma and lymphoproliferative diseases in immunesuppressed individuals [118,119]. EBV-related oncogenesis is principally associated with latency during which only a limited subset of the full repertoire of viral genes are transcribed. Of the genes expressed during viral latency in EBV-associated diseases, LMP1 is the one most implicated in tumor formation[120,121]. LMP1 is invariably expressed in Burkitt's lymphoma, it is required for EBVmediated transformation of lymphocytes in vitro and it transforms rodent fibroblasts. LMP1 is a six-transmembrane constitutively active signaling molecule that functionally mimics members of the cellular tumor necrosis factor receptor (TNFR) family. While the transmembrane domains are required for aggregation and constitutive activation of LMP1[122-125], two cytoplasmic domains, i.e.

the C-terminal activator regions 1 and 2 (CTAR-1 and -2), are critical for the transforming properties of LMP1[126-129].

Together, these signaling domains act through cellular TNFR-associated factors (TRAFs) and other cell-signaling molecules to activate, three transcription factors, namely, nuclear factor-κB (NF-κB), activator transcription factor-2 (ATF)-2 and AP-1, via c-Jun N-terminal kinase (JNK)[125-129]. As mentioned above miR-155 plays a critical role in lymphocyte activation in vivo[108,109] and is induced by a variety of immune cell stimuli, i.e. toll-like receptor (TLR) ligands, TNF-α, interferon-beta (IFN-β) and antigens [B-cell receptor (BCR) engagement][130]. The mechanism by which miR-155 is regulated after TLR and IFN signaling in macrophages is still unknown. Here we show that LMP1 upregulates the expression of miR-155 mainly by activating the NF-κB pathway, which suggests that miR-155 can cooperate in the EBV-mediated transformation of B cells.

Quinto and Scala lab have recently characterized the Inhibitor of Bruton's tyrosine kinase (IBtk) gene (accession number AL050333) that encodes three adaptor proteins in cell signalling [131]. The IBTK gene is 77,58 Kb and includes 29 exons with two promoters and transcriptional start sites that result in the expression of three IBTK transcripts, named IBTKα , IBTKβ and IBTKγ [131]. IBtkγ has been characterized as a ligand inhibitor of Tec kinases, such as Btk, Itk, and Akt, which regulate signal transduction upon specific stimuli [131,132]. As inhibitor of proliferation, IBTK is a candidate tumor suppressor gene. Consistently, the human IBTK gene is located in the genomic sequence 6q14.1, which is a hot spot of chromosomal rearrangements in lymphoproliferative disorders[133]. In this study, we have addressed

whether the IBTK gene might play a role in transcription regulation as source of miRNAs. By bioinformatics analysis, we identified four putative precursors of miRNA (pre-miR) encoded by three distinct introns and the 3' un-translated region (3'UTR) of the IBTK gene. Of them, only the pre-miR-IBTK3 encoded by intron 26 occurred in vivo, and was the effective substrate of RNase III Dicer. The presence of homologous in other primates suggests an evolutionary conserved role of pre-miR-IBTK3. Based on this evidence, IBTK miRNA is a novel member of the wide miRNAs genomic network.

Chapter 2: MATERIALS AND METHODS

2.1 Cell culture

EBV-negative human B cells (DeFew) and EBV immortalized human B cells MC3 (kindly provided by Prof. Giuseppe Scala, University 'Magna Grecia' Catanzaro) Devozione, Cap (kindly provided by Dr Giuseppina Ruggiero, University 'Federico II', Naples), were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen) and 2mM glutamine. Murine embryonic fibroblasts (MEFs) and HEK 293 referred to as LinX, stably transfected with the helper vector, [LinX cell line, Open biosystem, (http://www.openbiosystems.com/RNAi/LinX/)] were cultured in DMEM medium (GIBCO-Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 2mM glutamine.

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Paque gradient (GE Healthcare Europe, Munich, Germany) from buffy coats. Healthy subjects blood was diluted 1:1 in PBS and stratified on Ficoll solution with a 3:1 v/v ratio. After 30 min centrifugation at 1200 x g, PBMCs were recovered and resuspended in RPMI-1640 medium supplemented with 10% FCS.

2.2 RNA preparation and northern blot

Total RNA was extracted using Trizol reagent (Invitrogen) according to supplier's protocol. Northern blot analysis was carried out on 15 μg of total RNAs. Briefly, all RNA samples were dissolved in loading buffer [0.05% bromophenol blue, 0.05% cyanol, 5% Ficoll (type 400), 80% formamide and 7M urea], boiled for 5 min at 95°C and loaded onto

15% polyacrylamide gel under denaturizing conditions [15% acrylamide-bisacrylamide 19/1, 45mM Tris, 45mM boric acid, 1Mm EDTA pH 8, 7M urea, 0.01% N,N,N',N'-tetramethylethylenediamine (TEMED) and 0.1% ammonium persulfate (APS)]. Samples were resolved by electrophoresis for 90 min at 150V and transferred onto nylon membranes (Hybond N^+, Amersham/GE Healthcare, Little Chalfont, UK) by capillary blot. The nylon membranes were then equilibrated in 1M NaCl and pre-hybridized in 6X SSC, 5X Denhart's solution (1X Denhart's solution= 0.1% Ficoll, 0.1% polyvinyl pyrrolydone and 0.1% bovine serum albumin), 5 mg/ml of sheared salmon sperm ds-DNA (Ambion, Austin TX, USA) at 42 °C for 2 h. After pre-hybridization, $1x10^6$ CPM/ml of [γ^{32}P-ATP] radiolabeled oligonucleotide probe was added and the hybridization carried out overnight at 42 °C. The membranes were washed twice in 2X SSC, 1% SDS at 42°C for 30 min and exposed either by autoradiography or by phosphorimage screen (Amersham/GE Healthcare). The signals were quantified with image-scanning or by Image-J software analysis.

For Northen Blot in figure 24, the nucleotide sequence of probes was as follows:

IBTK1-25, for the nucleotides 1-25 of predicted pre-miRIBTK3 5'-TGGTAGTAAAATGATGAGCAAAGAC-3';

IBTK26-50, for the nucleotides 26-50 of predicted pre-miR-IBTK3, 5'-ACATGCCAGAAGCTAATATGAACAC-3';

IBTK51-75 for the nucleotides 51-75 of predicted pre-miRIBTK3 5'-TCACCAGACTCACTGAGCACCCAAT-3';

IBTK76-100, for the nucleotides 76-100 of predicted pre-miR-IBTK3 5'-AATTTCAATTAATTTACTCATTCAT-3'.

2.3 Western blot analysis

The cell pellets were resuspended in 1ml of lysis buffer [10mM HEPES, pH 7.4, 150mM KCl, 1mM EDTA pH 8,1% Triton X-100, 1mM DTT, 1mM orthovadanate, 1mM NaF and protease inhibitor cocktail (Roche, Basel, Switzerland)] at 4°C for 20 min. Then, 40 μg of proteins, of each sample, were resuspended in Laemmli buffer[134] and resolved by SDS–PAGE (running gel: 0.4M Tris, pH 8.8, 12% acrylamide/bis-acrylamide 37/1, 0.1% SDS, 0.01% TEMED, 0.1% APS; staking gel: 0.07M Tris pH 6.8, 5% acrylamide/bis-acrylamide 37/1, 0.1% SDS, 0.01% TEMED, 0.1% APS). The proteins were transferred onto nitrocellulose membranes (Hybond ECL, Amesham/ GE Healthcare) by electroblotting. Membranes were blocked in 5% of 'non-fat milk' (BioRad, Hercules CA, USA) 0.5% BSA in phosphate buffer solution for 2 h at room temperature and immunoblotted with monoclonal antibody against LMP1 (kindly provided by Dr Dong Yun Lee, McArdle Laboratory for Cancer Research, University of Wisconsin-Madison), mouse polyclonal antibody against γ-tubulin (Sigma Chemical Company, St Louis MO, USA) mouse monoclonal antibody against p65 (SantaCruz, CA, USA), rabbit polyclonal antibody against IkBα (SantaCruz), rabbit polyclonal against H3 (SantaCruz) or rabbit polyclonal antibody against PU.1 (Santa Cruz). Specific secondary antibodies (Sigma Chemical Company), horse-radish peroxidase conjugated, were used for protein detection by enhanced chemiluminescence (ECL, Amesham/GE Healthcare) followed by autoradiography (Hyperfilm ECL, Amesham/GE Healthcare). We used the Image-J software for densitometric analysis.

2.4 Cloning and mutagenesis of BIC/miR-155 promoter

The human BIC/miR-155 promoter region extending from 1783 to 1, from 1380 to 1, from 1065 to 1 relative to the start site was isolated from MC3 genomic DNA by PCR using the following primers:

Fw AGCTGAGCTCGAAAGGTCACCCTAGAATTG
(-1783),
Fw AGCTGAGCTCGATCTGGCACATGGTAAATG
(-1380),
Fw AGCTGAGCTCCAGTCACATGTTGATGAGGC
(-1065),
Rev CATGAAGCTTATCCGCTCCCTTCCCGAG (+1).

The isolated fragments were digested with SacI and HindIII and cloned into SacI and HindIII cut pGL3basic (Promega, Madison, WI, USA). The entire promoter region was sequenced and no discrepancies were identified relative to the Genebank genomic sequence (http://www.ncbi.nlm.nih.gov/GenBank). Mutagenesis of the pLuc1380 and pLuc1783 reporter plasmid was carried out with the 'Expand Long Template PCR system' (Roche) and the oligonucleotides listed belove.

NF-κB1:
Fw 5'-GTAAATTAAGTACTATGCTCGAGCCAGCTCTGACATG-3';
Rev 5'-CATAGTACTTAATTTACAGATGGCTCAGGTTGGTTAAG-3'.

NF-κB2:
Fw 5'CAACCTAGAATGAGAAATGCTCGAGTCAGAAAGGC
ATTGTAGG-3'
Rev 5'-CATTTCTCATTCTAGGTTGAACTATACCTCCCTT

CTCCCAG TG-3'

AP-1:

Fw 5'-GGCGCCTGGTCGGTTATCTCGAGCAAGTGAGTT ATAAAA-3';

Rev 5'-ATAACCGACCAGGCGCCT TTTCTGCAACCC-3';

In each case, the core transcription factor binding site was replaced by a XhoI restriction site. Mutations were initially screened by digesting with XhoI and then verified by sequence analysis.

2.5 Reporter analysis

For each reporter plasmid, $2x10^6$ MEFs were distributed in 6 cm dish plates containing 5ml RPMI1640 (Invitrogen) 10% FBS (Cambrex, East Rutherford, NJ, USA), 2mM glutamine. FuGene (Roche) of 6 μl were added to 300 μl of DMEM and incubated for 5 min at room temperature. Each reporter vector of 0.5 μg plus 0.2 μg of β-galactosidase vector were then added to the mixture, the tubes were shacked and incubated at room temperature for 15 min. For each transfection, the mixtures were then added to each plate and plates were incubated at 37°C, 5% CO_2 for 48 h. Cells were harvested 48 h later and assayed for luciferase activity. Results are presented as average of three independent experiments. For each reporter plasmid, $2x10^6$ DeFew were distributed in 6 cm dish plates containing 5ml RPMI1640 (Invitrogen) 10% FBS (Cambrex), 4mM glutamine. FuGene (Roche) of 8 μl were added to 300 μl of DMEM and incubated for 5 min at room temperature. Each reporter vector of 1μg plus 0.5 μg of β-galactosidase vector were then added to the mixture, the tubes were shacked and incubated at room temperature for 15 min. For each transfection, the

mixtures were then added to each plate and plates were incubated at 37°C, 5% CO_2. After 6 h each sample was split in two, and one of them was treated with 10 ng/ml of phorbol myristoylated acetate (PMA). Cells were harvested 48 h later and assayed for luciferase activity. Results are presented as average of three independent experiments.

2.6 Chromatin immunoprecipitation assay

MC3 or DeFew cells ($5x10^6$ cells) were exposed to 1% formaldehyde for 10 min at 37°C to obtain protein–DNA cross-linking. The nuclear fraction was sonicated to yield chromatin fragments of 200–1000 bp; 5% of the total volume was removed from each sample and used as the input fraction. Chromatin was pre-cleared by pre-incubation with a DNA salmon sperm/protein A-agarose 50% slurry (Upstate, Temecula, CA, USA) for 1 h at 4°C. The agarose was centrifuged, and the pre-cleared chromatin supernatant was incubated with the antibodies (3 μg) against RNA pol II (mAb anti RNA polymerase II, Active Motif, Rixensart, Belgium), p50 or p65 (pAb anti p50 or pAb anti p65, kindly provided by Dr N. Rice, Frederick Cancer Research and Development Center, Frederick, MD, USA), against histone 3 (pAb anti H3, SantaCruz), overnight at 4°C. The protein–DNA-antibody complexes were collected by the addition of the salmon sperm DNA– protein A-agarose (2 h at 4°C) and washed, and protein– DNA cross-linking was reversed (4 h at 65°C). DNA was purified by phenol/chloroform extraction and ethanol precipitationand aliquots (25%) of the purified materials underwent PCR (5 min at 94°C, 1 min at 94°C, 1 min at 52°C, 1 min at 72°C for 40 cycles, 5 min at 72°C).

2.7 Preparation of nuclear extracts and electrophoresis mobility shift assay

MC3 cells (5- to 6x10^6 cells) were washed with cold phosphate buffered saline and harvested. The cell pellet was resuspended in extraction buffer containing 10mM HEPES, pH 7.9, 10mM KCl, 1.5mM $MgCl_2$, 0.1mM EGTA, 0.5mM DTT, 0.5mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, passed through a needle, kept on ice for 45 min, and centrifuged (15 min at 14 000 r.p.m. at 4°C). The nuclear pellet was then resuspended in high salt extraction buffer containing 10mM HEPES, pH 7.9, 0.4mM NaCl, 1.5mM $MgCl_2$, 0.1mM EGTA, 0.5mM DTT, 0.5mM phenylmethylsulfonyl fluoride, 10 μg/ aprotinin, 10 μg/ml leupeptin and incubated for 45 min at 4°C. The nuclear extract supernatant was obtained by centrifugation (30 min at 14 000 r.p.m. at 4°C), protein concentration was determined and 5 μg aliquots were stored at -80°C until used. Double-stranded synthetic oligonucleotides were radiolabeled using [γ^{32}P]ATP (3000 Ci $mmol^{-1}$; Amersham Biosciences) and T4 polynucleotide kinase (Fermentas, Burlington, ON, Canada). The binding reaction was carried out for 20 min at room temperature with 5 μg of nuclear proteins in 10% glycerol, 60mM KC1, 1mM EDTA, 1mM dithiothreitol and 0.25 μg/μl poly[dI–dC] containing 50.000 cpm of radiolabeled probe and a 80-fold molar excess of unlabeled competitor oligonucleotide when indicated. For supershift experiments, 4 μg of specific anti-p65 or anti-p50 were added to the binding reaction and incubated for 30 min, after which we added the radiolabeled probe. DNA–protein complexes were separated by 5% non-denaturing polyacrylamide gel and revealed by either autoradiography or phosphorimage screen (Amersham Biosciences).

2.8 Transient transfection

DeFew cells ($3x10^6$ cells) were centrifuged, washed in PBS and resuspended in 300 μl of electroporation medium (RPMI 1640 medium, 20% FBS and 2mM Glutamine). Cells were mixed with 20 μg of plasmid expressing LMP1 (pcDNA3-LMP1), or LMP2 (pcDNA3-LMP2) or both and subsequently transferred in electroporation cuvette. Cells were electroporated twice at 220 V, 960 μFa and incubated 5 min on ice. Cells were plated in 10 ml of RPMI 1640 medium, 10% FBS and 2mM glutamine for 2 days. After 2 days, they were harvested and assayed for miR-155 and LMP1 expression.

2.9 Viral generation, infection and time course

The retroviral vector LMP1pBABE-puro was kindly provided by Prof. Eiji Hara (University of Tokushima, Japan). LinX cell line, stably transfected with the helper vector, were grown in DMEM medium 10% FBS, 2mM glutamine and 100 μg/ml hygromicin. Transfections with LMP1pBABEpuro or pBABEpuro (empty vector) were as follows: 6 cm plate of semi-confluent (about $1x10^6$ cells) cells were transfected and 7.5 μg of DNA vector incubated in 20 μl of FuGene and incubated in 4 ml of medium without antibiotic for 24 h. After incubation, the medium was harvested, replaced with 4ml of fresh medium and the LinX cells incubated for 24 h for a second round of viral generation. DeFew cells ($4x10^6$ cells) were centrifuged, washed in PBS and resuspended in 4 ml of virus containing medium plus 4 μg/ml of Polybrene (Sigma). The infection was performed by spinoculation, namely by centrifugation at 3500 r.p.m. for 90 min at 20°C. After centrifugation, the cells were resuspended in 10 ml of RPMI and

incubated at 37°C, 5% CO_2. Twenty-four hours later, the cells underwent a second round of infection. After 48 h, cells were treated with 1 μg/ml of puromicine to start selection of positive clones. For the time course, DeFew cells ($4x10^6$ cells) were centrifuged, washed in PBS and resuspended in 12 ml of virus containing medium plus 12 μg/ml of Polybrene (Sigma). The infection was performed by spinoculation, namely by centrifugation at 3500 r.p.m. for 90 min at 20°C. After centrifugation, the cells were resuspended in 10 ml of RPMI and incubated at 37°C, 5% CO_2. Twentyfour hours later, 1 μg/ml of puromocine was added to cells to start selection. Cells were harvested at 6, 24, 48 and 72 h after infection and assayed for miR-155 and LMP1 expression.

2.10 Transient transfection of MC3

MC3 cells ($3x10^6$ cells) were centrifuged, washed in PBS and resuspended in 300 μl of electroporation medium (RPMI 1640 medium, 20% FBS and 2mM glutamine). Cells were mixed in a tube with 20 μg of pRc/CMV-HAIkBα-S32/36A (kindly provided by Prof. Ileana Quinto, University Magna Grecia, Catanzaro) and subsequently put in electroporation cuvette. Cells were electroporated twice at 220 V, 960 μFa and incubated 5 min on ice. Cells were plated in 10 ml of RPMI 1640 medium, 10% FBS and 4mM glutamine for 2 days. After 2 days, they were harvested and assayed for miR-155, p65 and IκBα expression.

2.11 Prediction of potential miRNAs

Search for putative miRNAs generated by the genetic locus IBTK was performed by Pro-MirII software (http://cbit.snu.ac.kr/~ProMiR2/Index.php). The pre-miR-IBTK nucleotide sequences were predicted according to the following cut-off values: Window size: 100 base pairs; Shift size: 10 nucleotides; ProMir values: 0,017; Conservation score ≥ 0; Free Energy (ΔG) ≤ -20; GC-ratio = 0.3-0.7; Entropy ≥ 1.8.

2.12 DNA plasmids

To generate T7 expression plasmids of pre-miR-IBTK1, pre-miR-IBTK2, pre-miRIBTK3 and pre-miR-IBTK4, the genomic DNA from MC3 cells was amplified by PCR with appropriate primers and cloned in the HindIII/XhoI-digested pcDNA3 vector (Invitrogen) under the T7 promoter. The regions of the IBTK gene encompassed the nucleotides 82971042-82970943 for pre-miR-IBTK1, the nucleotides 82963276-82963177 for pre-miR-IBTK2, the nucleotides 82945044-82944945 for pre-miR-IBTK3 and the nucleotides 82937481-82937382 for pre-miRIBTK4.

Forward (FW) and reverse (RW) primers were as follows:

pre-miR IBTK1 FW:

5'-CCCAAGCTTTTGTATTGAAATGTCATAAAACC-3';

pre-miR IBTK1 RW:

5'-GGCCTCGAGGGGAAAGTTAAATTTTTAAAATCA-3';

pre-miR IBTK2 FW:

5'-CCCAAGCTTGAATGAAGCTACCCCCTTGCTTGG -3';

pre-miR IBTK2 RW

5'-GGCCTCGAGGAGGGAGATAGTTCTTTGGGA-3';

pre-miR IBTK3 FW:

5'-CCCAAGCTTATATGTTCAATGAGAGCAAGG-3';

pre-miR IBTK3 RW:

5'-GGCCTCGAGGAAACAAATGTTACTTGAAAC- 3';

pre-miR IBTK4 FW:

5'-GGCCTCGAGGATTTTTAAAAAGCAACAAGAATC-3';

pre-miR IBTK4 RW:

5'-GGCCTCGAGGCCGCCTGATAAATACTGTGGCCC-3'.

2.13 Dicer RNase assay

IBTK transcripts corresponding to predicted pre-miR IBTK were transcribed with T7 RNA polymerase and [^{32}P]α-UTP (3000 Ci/mmol) using the Riboprobe System - SP6/T7 (Promega); ApaI-digested pre-miR-IBTK pcDNA3 plasmids were used as template. RNA samples (10^6 cpm) were incubated with or without the recombinant human Dicer (2 Units) (Genlantis) in a reaction buffer (10 μl) containing 20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM ATP, at 37°C for 2 h. The RNA products were separated by 15% PAGE in 8M Urea TBE, and analyzed by autoradiography, as described [135].

2.14 Comparative analysis of nucleotide sequence

The nucleotide sequence of *Homo sapiens* pre-mir-IBTK3 was aligned with the genome of *Pan troglodytes, Pongo pygmaeus, Macaca mulatta, Bos taurus* and *Canis familiaris* to identify homologous nucleotide sequences by BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The pre-miR-IBTK3

homologues of *Homo sapiens, Pan troglodytes, Macaca mulatta, Bos taurus* and *Canis familiaris* were analysed for the identity percentage by CLUSTALW software (http://www.ebi.ac.uk/Tools/clustalw2/index.html) [136].

Chapter 3: Epstein-Barr virus latent membrane protein 1 trans-activates miR-155 transcription through the NF-κB pathway

3.1 The expression of miR-155 is regulated by LMP1

We measured miR-155 expression in three EBV immortalized cell lines (MC3, Devozione and Cap) and in two EBV-negative B-cell lines (EW36 and DeFew), a T-cell lymphoma cell line (Jurkat) and NB4 from acute promyelocytic leukemia. As shown in figure 11, miR-155 was expressed at relevant levels only in the EBV-positive cells according to Mrazek et al. [137]. In the EBV-negative B cells, miR-155 expression was not affected by stable transfection of the EBV nuclear factor 2 (EBNA2) (figure 11), a viral transacting factor that activates the expression of such cellular and viral promoters as the HIV-1 long terminal repeat [138] and the interleukin-6 promoter [139].

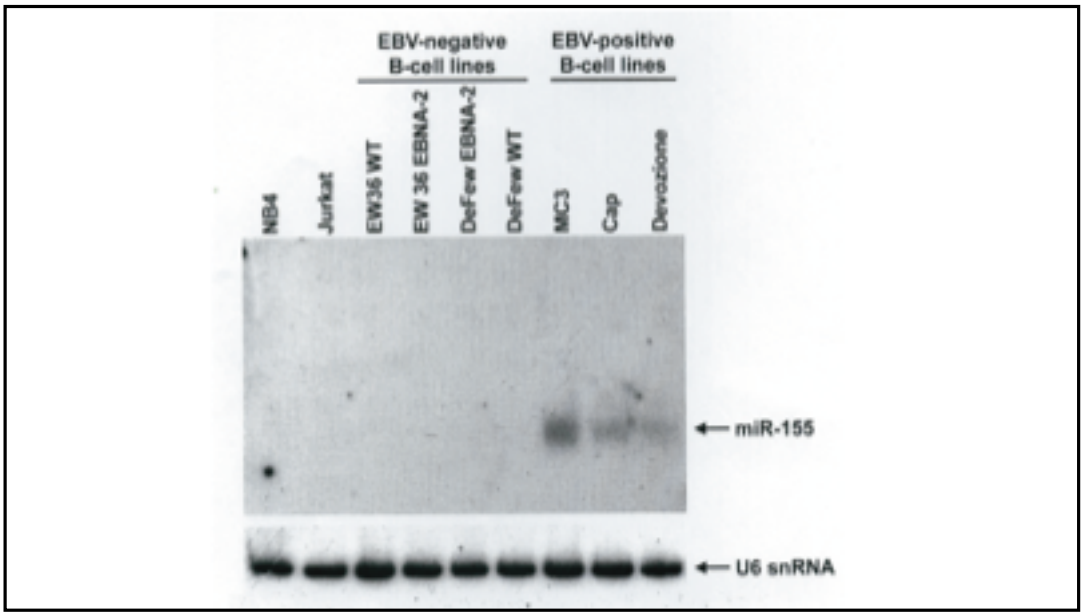
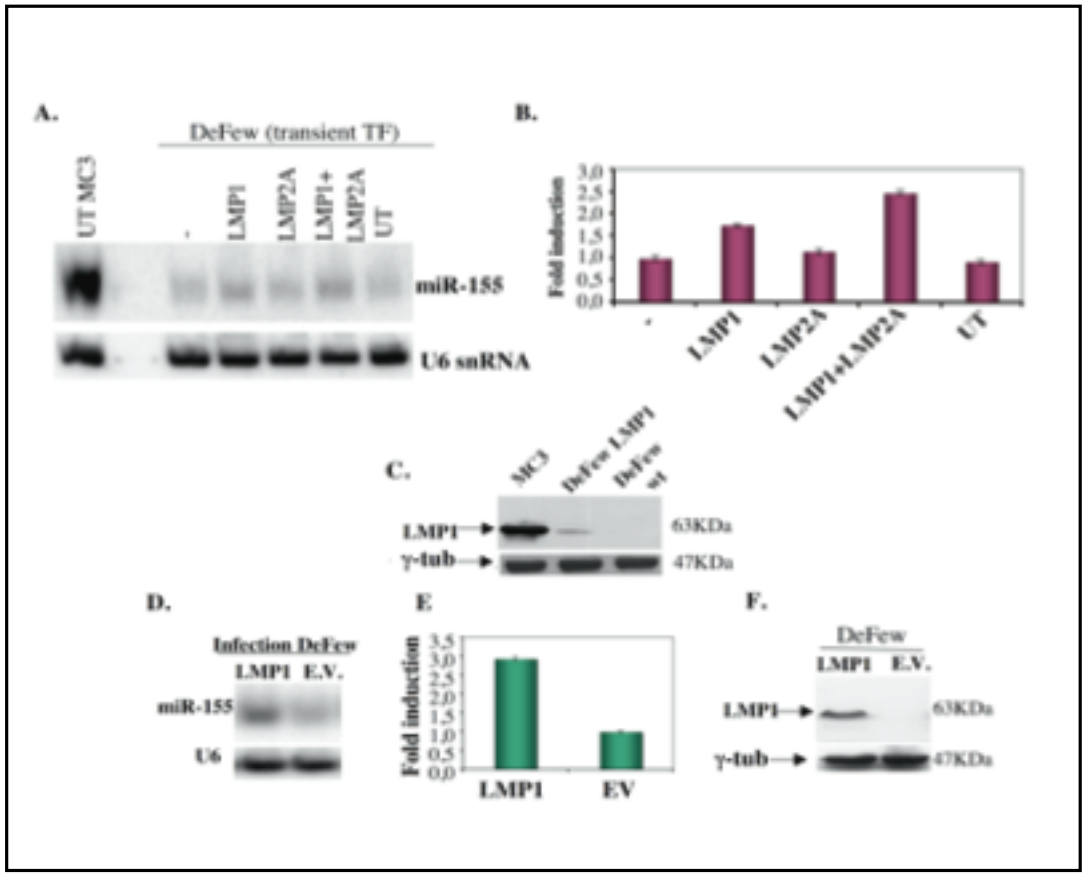


Figure 11- miR-155 is upregulated in EBV-immortalized cell lines. Northern blot analysis on total RNAs obtained from 9 cell lines. The blot was analyzed for the expression of mature miR-155 and normalized by the ubiquitous small nuclear RNA U6. Note that basal levels of miR-155 expression in DeFew cells are not visible in this blot due to the brief exposure.

To evaluate whether LMP1 induces miR activation, we transiently transfected a vector expressing LMP1, LMP2 or both in DeFew cells (EVB-negative B cells). There was a 1.6-fold induction of miR-155 expression in LMP1-transfected cells, no induction in LMP2-transfected cells and a 1.7-fold induction in cells transfected with LMP1 and LMP2 (figure 12A and B). The significance of this finding is underestimated because only a few cells were transfected by LMP1 expression vector. Indeed, LMP1 was much lower in transiently transfected DeFew cells than in MC3 cells (figure 12C). To overcome this problem, we infected the DeFew cells with a retroviral vector expressing LMP1 (pBABEpuroLMP1). As shown in figure 12D, we obtained a higher expression of LMP1, at 72 h post-infection, resulting in about 3.5-fold induction of miR-155 expression compared to DeFew cells infected with the empty virus (figure 12E and F).



3.2 miR-155 expression can be driven through activation of two NF-κB elements in the BIC/miR-155 promoter

The foregoing results suggested that the LMP1-mediated induction of miR-155 occurred via a transcriptional mechanism. A recent study showed that the miR-155 promoter contains an AP-1 active site, located at 40 nt upstream from the TATA box and a NF-κB site located at 1150 nt upstream from the transcription starting site [117]. The AP-1 active site plays a key role in activation of miR-155 expression induced by BCR [117]. To identify potential cis-elements active on miR-155, we isolated a promoter region of ~1.9 kb of the BIC gene, which encodes miR-155 [140], from MC3 cells and cloned it into a luciferase reporter plasmid. We looked for cis element homologies using TESS (http://www.cbil.upenn.edu/cgi-bin/tess) or TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html), and identified some potential transcription factor binding sites, among which, an additional NF-κB site located at 1697 nt upstream from the transcription starting site (figure 13).

Figure 12 - LMP1 enhances the expression of miR-155 in an EBV-negative B-cell line. (A) A representative northern blot analysis on total RNAs obtained from DeFew cells transfected (TF) as indicated or untransfected (UT). The blot was analyzed for the expression of mature miR-155 and normalized by the ubiquitous small nuclear RNA (U6). (B) Graphic representation of the northern blot results as average of three independent experiments. (C) Western blot analysis of LMP1 expression in the transfected cells compared to MC3 cell immortalized by EBV. Input proteins were equalized by detecting the endogeneous γ-tubulin. (D) Northern blot analysis of total RNAs obtained from DeFew cells at 72 h post-infection with retroviral vector expressing LMP1 or empty vector. The blot was analyzed for the expression of mature miR-155 and normalized by U6. (E) Graphic representation of the northern blot results as average of three independent experiments. (F) Western blot analysis of the expression of LMP1 upon infection with the retroviral vector. Input proteins were equalized by detecting the endogeneous γ-tubulin.

Figure 13 - The promoter of miR-155 encoding Bic gene contains two putative NF-κB binding sites. It is drown a partial nucleotide sequence of Bic promoter (from + 150 to – 222; from – 1064 to – 1912) containing the putative cis elements with higher score. The TATA box (-25 in pink), the AP1 site (-40 in red) the SP1 site (- 97 in blue), the two NF-κB binding sites (-1150; -1697 in green), and the PU.1 site (-1403 in purple).

Because NF-κB signaling plays a central role in B-cell physiology, we focused on the two NF-κB cis-elements. We next examined by northern blot the induction of miR-155 expression in DeFew cells treated with PMA, which induces NF-κB by activating protein kinase C (PKC) and related phosphorylation of inhibitor-κBa (IκBα), thereby leading to its degradation [141]. PMA (25 ng/ml) increased miR-155 expression by about 3-fold (figure 14A and B); miR-155 expression peaked 36 h after treatment and decreased thereafter (figure 14A and C). The latter

finding is in accordance with the pattern of PKC-activated NF-κB expression [141].

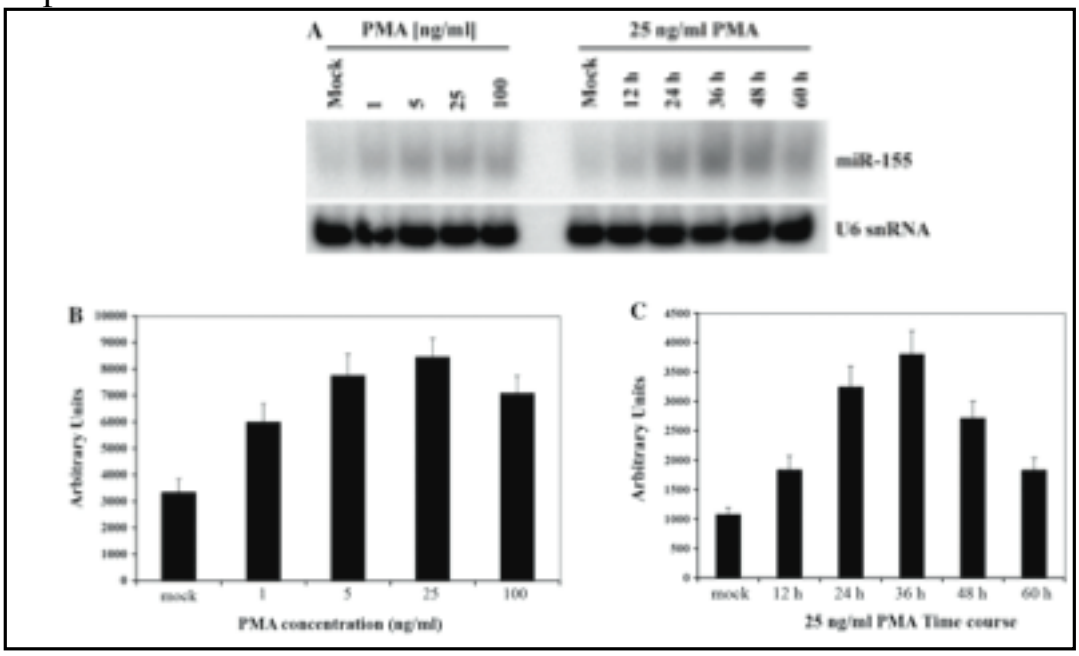


Figure 14 - PMA induces miR-155 expression in a dose- and time-dependent manner. (A) Left: northern blot analysis of RNAs from DeFew cells treated with different concentrations of PMA; Right: northen blot analysis of RNAs from DeFew cells at different times upon PMA treatment. The blots were analyzed for the expression of mature miR-155 and normalized by U6. (B and C) Graphic representation of the average of three independent northern blot experiments.

3.3 The NF-κB elements in the BIC/miR-155 promoter bind NF-κB factors *in vitro* and *in vivo* in MC3 cells

The NF-κB elements in the BIC/miR-155 promoter bind NF-κB factors *in vitro* and *in vivo* in MC3 cells. To determine the activity of the two NF-κB elements identified in the miR-155 promoter, we performed EMSA using nuclear extracts from MC3 cells. Briefly, the nuclear extracts were incubated with dsDNA oligonucleotides identical to the sequence of the NF-κB site 1 (-1150) or 2 (-1697). Both sites underwent an electrophoretic mobility shift, which disappeared when we used a 80-fold molar cold competitor, but not when we used the

same amount of a mutant competitor that is not bound by NF-κB complexes (Figure 15A). There was a supershift when the nuclear proteins were pre-incubated with antibodies against p65/RelA or p50, two members of the NF-κB family (Figure 15A).

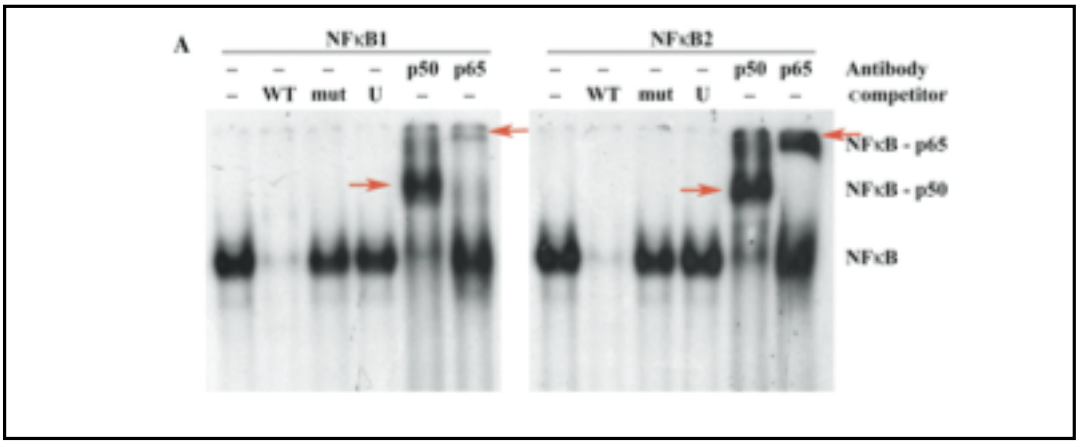


Figure 15 - The two putative NF-κB binding sites are bound by NF-κB complexes *in vitro*. (A) Electrophoretic mobility shift assay using nuclear extracts from MC3 cells (EBV-positive cells) on the two NF-κB binding sites. Left panel: NF-κB site 1 (–1150). Right panel: NF-κB site 2 (–1697). WT = 80-fold molar cold wild-type competitor; mut = 80-fold molar cold mutant competitor; U = 80-fold molar cold unspecific competitor; p50 = supershift with anti NF-κB p50 antibody; p65 = supershift with anti NF-κB p65 antibody. Supershift bands are indicated with red arrows.

Because LMP1 could activate the p52/p65 NF-κB complex (non canonical pathway), we repeated the experiment using an anti p52 antibody, and found no supershift under these conditions. In line with the hypothesis that the canonical NF-κB pathway is involved in the LMP1-mediated activation of miR-155, we found that miR-155 expression was inhibited in MC3 cell expressing an exogenous, undegradable form of IκBα [142], which is an inhibitor of the canonical NF-κB pathway (figure 16).

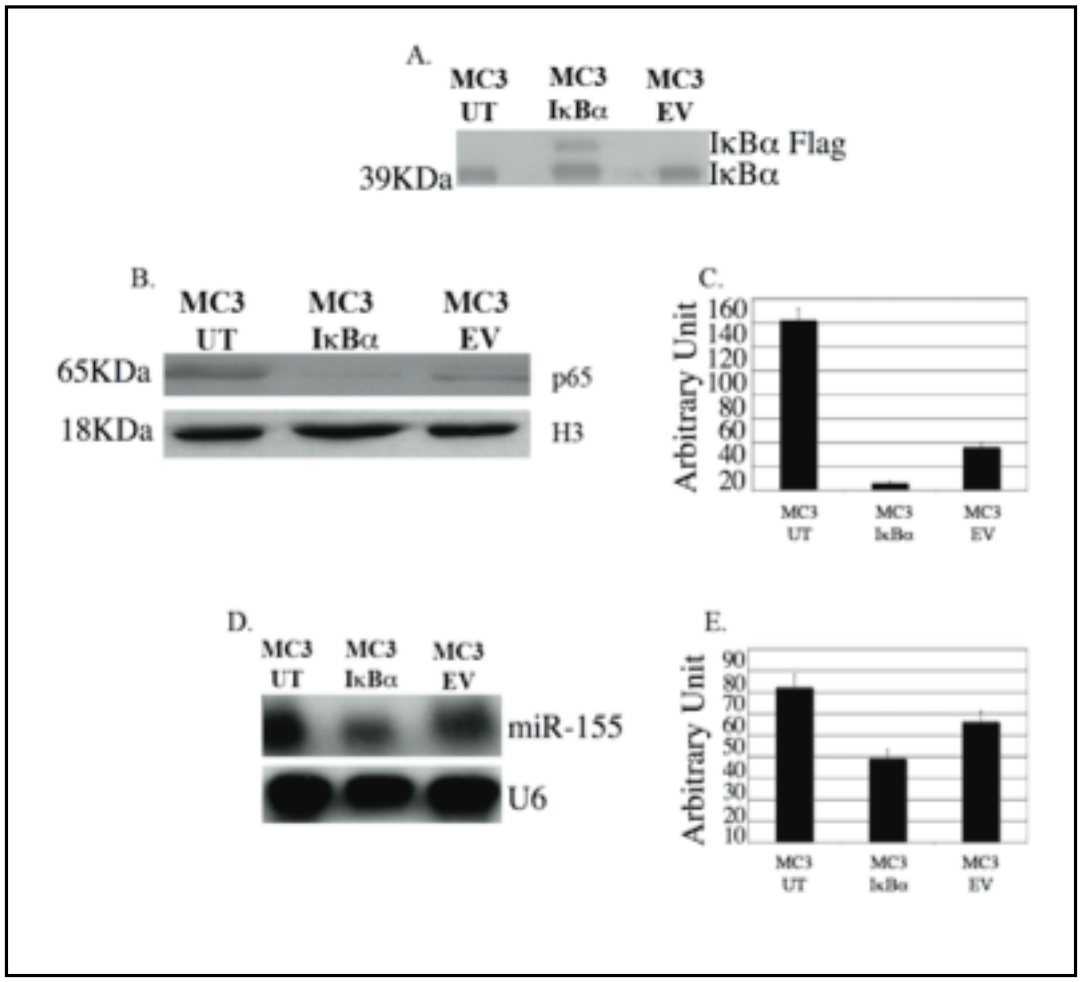


Figure 16 - Inhibition of NF-κB canonical pathway leads to a reduction of miR-155 levels in EBV immortalized MC3 cells. (A) Western Blot analysis of total protein extracts obtained from MC3 untransfected (MC3 UT), MC3 transfected with pRc/CMV-HA-IκBα-S32/36A (MC3 IκBα) and MC3 transfected with Empty Vector (MC3 EV). The blot was analyzed for IκBα expression. The exogenous protein runs higher than the endgenous because has HA-FLAG. (B) Western Blot analysis of nuclear extracts obtained from MC3 untransfected (MC3 UT), MC3 transfected with pRc/CMV-HA-IκBα-S32/36A (MC3 IκBα) and MC3 transfected with Empty Vector (MC3 EV). The blot was analyzed for p65 expression and normalized by H3. (C) Graphic representation of the Western blot showed in B results as average of three independent experiments. The nuclear p65 decreases when the undegradable form of IκBα is expressed. (D) Northern Blot analysis of total RNAs obtained from MC3 untransfected (MC3 UT), MC3 transfected with pRc/CMV-HA-IκBα-S32/36A (MC3 IκBα) and MC3 transfected with Empty Vector (MC3 EV). The blot was analyzed for miR-155 expression and normalized by U6. (E) Graphic representation of the Northern blot results as average of three independent experiments.

To determine whether the NF-κB p50/p65 complex is recruited *in vivo* on the BIC/miR-155 promoter, we examined the two NF-κB sites by chromatin immunoprecipitation assay (ChIP). We show that p65 but not p50 is engaged on the two NF-κB sites (figure 17A). However, we cannot rule out that p50 contributes to the active complex because it could simply be covered by DNA and p65, and thus inaccessible to the antibody. RNA pol II drives miR-155 transcription [5]. Therefore, we checked, in the same experiment if the BIC promoter was also engaged by the RNA pol II. As shown in figure 17B we found the RNA polymerase on the TATA box of BIC promoter together with p65 (figure 17A).

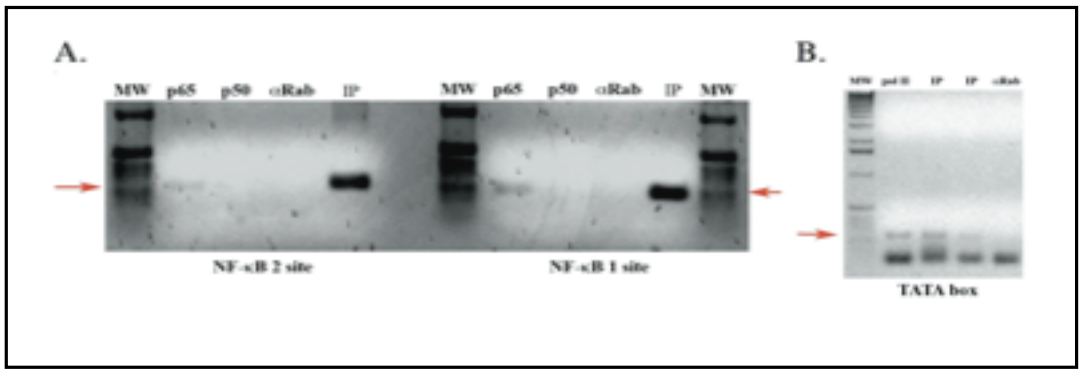


Figure 17 - The two putative NF-κB binding sites are bound by NF-κB complexes *in vivo*. (A) ChIP assay of chromatin extracted from MC3 cells using polyclonal antibodies directed against the NF-κB p65 or NF-κB p50. After immunoprecipitation, the DNA samples were purified and subjected to PCR with specific primers amplifying the NF-κB site 1 region (right panel) or NF-κB site 2 region (left panel). MW = molecular weight marker; p65 = pAB anti NF-κB p65; p50 = pAb anti NF-κB p50; αRab = unspecific polyclonal antibody anti rabbit immunoglobulin; IP input. The red arrows indicate the specific amplified band. (B) ChIP assay of chromatin extracted from MC3 cells using monoclonal antibody directed against the RNA pol II. After immunoprecipitation, the DNA samples were purified and subjected to PCR with specific primers amplifying the TATA box region. MW = molecular weight marker; pol II = mAB anti RNA pol II; IP = input. The two lanes represent two different amounts of input sample used for the PCR; αRab = unspecific polyclonal antibody anti rabbit immunoglobulin. The red arrow indicates the specific amplified band.

3.4 LMP1 requires intact NF-κB *cis*-elements to enhance miR-155 expression

The foregoing experiments provide compelling evidence that LMP1 drives miR-155 upregulation and that the miR promoter responds to NF-κB transacting factors. However, LMP1 can activate two different pathways: the NF-κB pathway and the Jun/Fos (AP-1) pathway [123,125–127] and the miR-155 promoter has a *cis*-element responsive to AP-1 40 nt upstream from the start site [117]. Using truncated forms of BIC promoter cloned upstream the luciferase gene reporter, we observed that only the first 1380 nt drive the maximum activation of the promoter by PMA or LMP1 (figure 18). This could indicate that the NF-κB site 2 is irrelevant in the LMP1-mediated activation of miR-155. To verify the effectiveness of the two NF-κB *cis*-elements in LMP1-enhanced miR-155 promoter, we used both the promoters cloned upstream luciferase gene reporter [pLuc1783 wild-type (wt) and pLuc1380 wt]. Subsequently, we mutated NF-κB site 1 (pLuc1783ΔNF-κB1 and pLuc1380ΔNF-κB), the AP-1 site (pLuc1783ΔAP-1 and pLuc1380ΔAP-1) and the NF-κB site 2 (pLuc1783ΔNF-κB2) using site-directed mutagenesis. All vectors were used for transfection and reporter assay in EBV-negative MEF. As shown in figure 18B, pLuc1380 wt was upregulated by about 5-fold when cotransfected with a vector expressing LMP1. In the presence of LMP1, pLuc1380ΔNF-κB expression was about 80-fold less, and the expression of pLuc1380ΔAP-1 about 6-fold less than pLuc1380 wt expression (figure 18B). These data would suggest that NF-κB site 1 plays a pivotal role in LMP1 activation and that substantial cooperation by the AP-1 site is required to attain maximum activation. Consistent

with these results, NF-κB seems to be central also for basal levels of miR-155 expression. Indeed, without LMP1, pLuc1380ΔNF-κB expression was about 17-fold lower than pLuc1380 wt expression, whereas pLuc1380ΔAP1 was downregulated by about 3-fold (figure 18B). When the reporter assays were repeated using the full-length vector pLuc1783 wt, induction was reduced (about 3-fold), in the presence of LMP1 compared to pLuc1380 wt (compare figure 18B and C), which is in line with the results reported in figure 18A. Surprisingly, all mutated forms of this vector (pLuc1783ΔNF-κB2, pLuc1783ΔNF-κB1 and pLuc1783ΔAP-1) were no longer activated by LMP1 expression (figure 18C). The graph in figure 18C is presented in logarithmic scale in order to show that the mutations in both NF-κB sites dramatically reduced the basal level of miR-155 promoter-driven luciferase expression, whereas the AP-1 mutation reduced basal levels to a much lesser extent. The latter observation is consistent with our data obtained with the truncated promoter. The expression of LMP1 was checked in each sample (figure 18D) for a further normalization of the luciferase assays. These data indicate that both NF-κB *cis*-elements play a central role in LMP1-mediated activation of the promoter and may suggest the possible presence of a negative regulatory region between the two *cis*-elements.

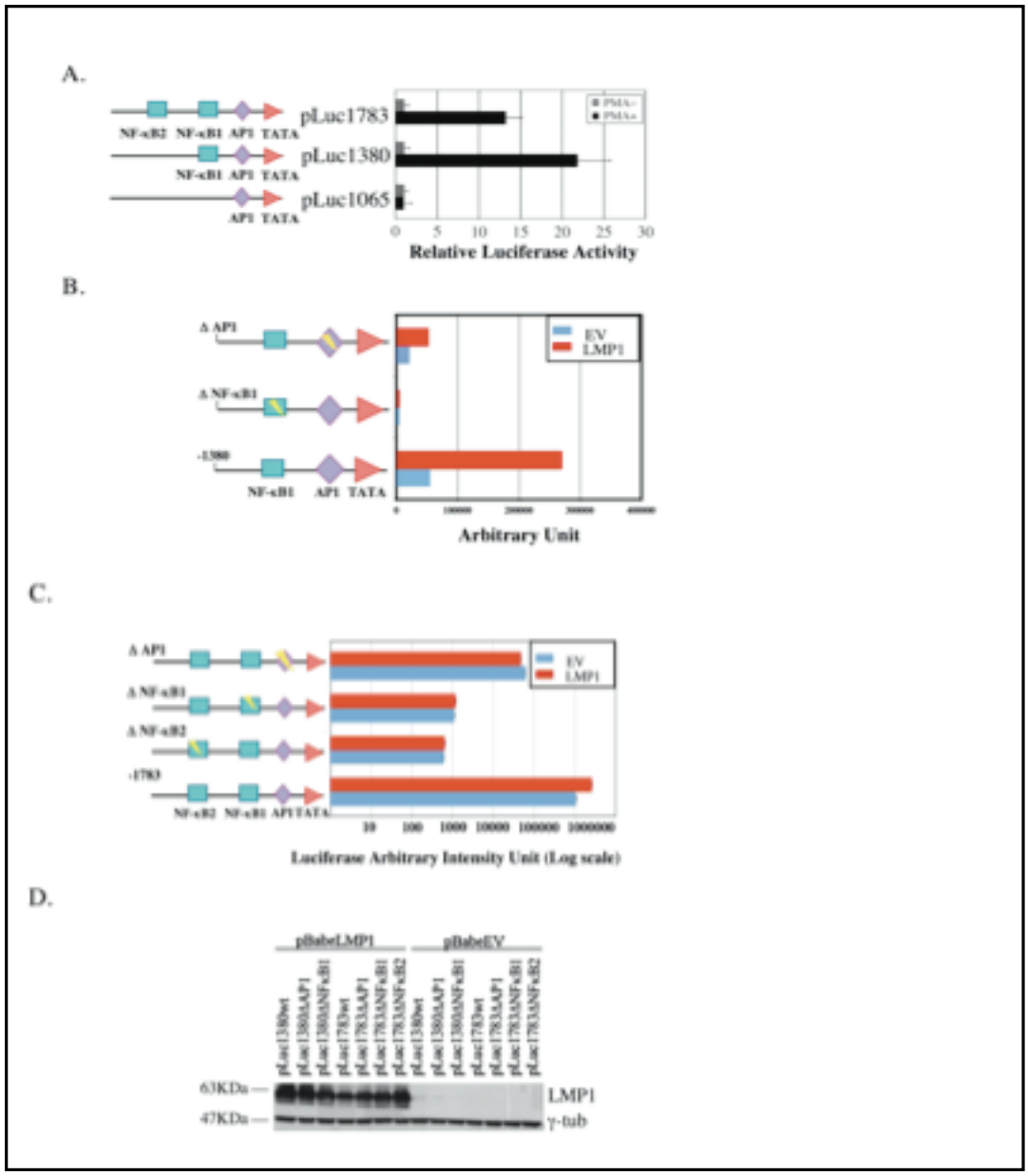
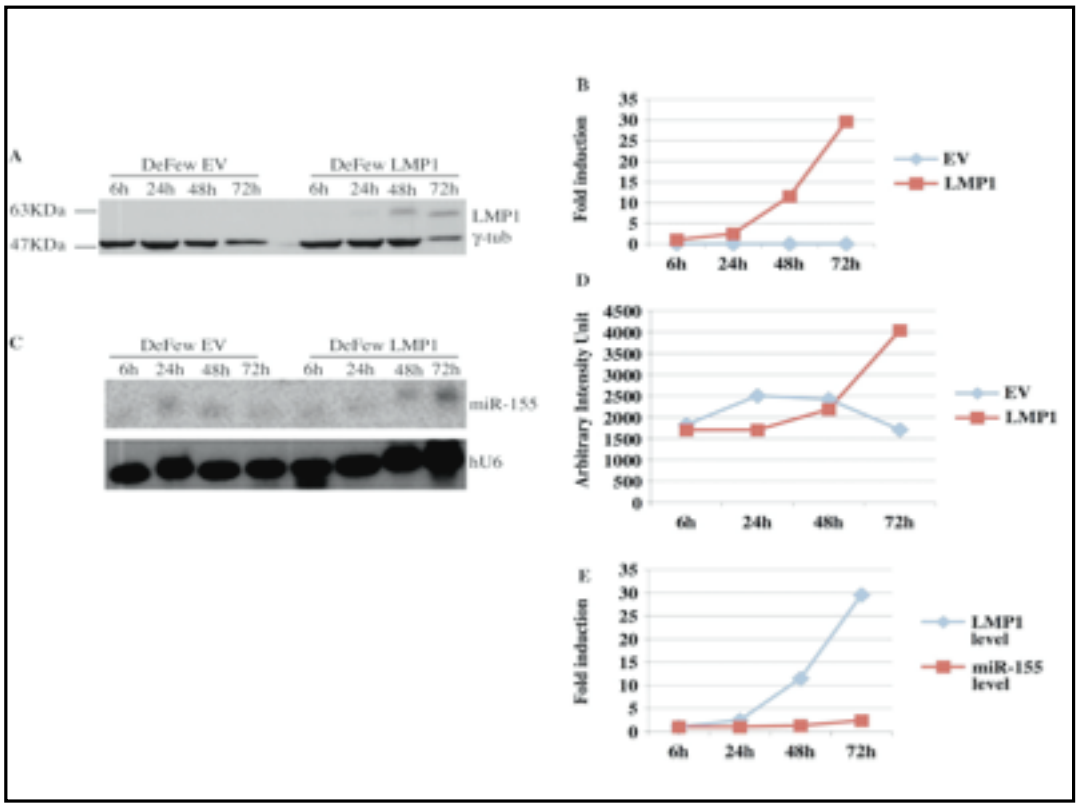


Figure 18 - NF-κB *cis*-elements are essential for basal and LMP1-mediated activation of miR-155 pomoter. (A) Luciferase assay performed in De Few cells using the full-length miR-155 promoter and two truncated forms. The transfected cells were treated or mock treated with 25 ng/ml of PMA. In presence of PMA the truncated form of the BIC promoter (pLuc1380) reaches the maximum of luciferase activity. The graphic representation of the luciferase assay is the average of three independent experiments. (B and C) The indicated miR-155 promoter luciferase vectors were co-transfected with pBABEpuroLMP1 or empty vector and incubated for 48 h in MEF cells. Luciferase expression was normalized by β-gal/μg of total protein and represented as average of three independent experiments. (D) Western blot analysis of LMP1 expression in the co-transfected MEF cells, normalized by γ-tubulin.

3.5 The binding of p65/RelA to both NF-κB *cis*-elements of miR-155 promoter is temporally correlated to LMP1 expression

To determine whether the exogenous expression of LMP1, in DeFew cells, is correlated temporally with the upregulation of the endogenous miR-155, we performed a time-course experiment in DeFew cells infected with pBABEpuroLMP1 or with the empty vector as control. Each time point was split into three for western blot, northern blot and ChIP analyses. LMP1 expression was observed 24 h post-infection and peaked at 72 h (figure 19A and B), whereas the upregulation of miR-155 occurred at 48 h post-infection and increased at 72 h (figure 19C and D). These results indicate that the upregulation of miR-155 is temporally dependent on the expression of LMP1 (figure 19E).



To further clarify the function of the two NF-κB *cis*-elements in the endogenous miR-155 promoter, we performed ChIP analysis followed by semi-quantitative PCR. There was constitutive binding of p65/RelA on both NF-κB sites (figure 20A, left panel and figure 20B). This binding did not increase in the cells infected with the empty vector (figure 20A, left panel). In contrast, in the cells expressing LMP1, the binding of p65/RelA on NF-κB 1 site increased 1.5-fold at 24 h and 3.5-fold at 72 h (figure 20A, left panel and figure 20B), whereas the binding of p65/RelA on the NF-κB 2 site increased 1.3-fold at 24 h and 2.5-fold at 72 h (figure 20A, left panel and figure 20B). At the same time points, the binding of RNA pol II on the TATA box of BIC promoter increased in the LMP1-infected cells but not in the control cells control and the assay was normalized against histone H3 (figure 20A and C, right panel). Taken together, these data and the results reported in figure 20 strongly suggest that LMP1 activates p65 binding on miR-155 promoter and this lead to upregulation of miR expression.

Figure 19 - Time-course of LMP1-mediated activation of miR-155. (A) Western blot analysis of protein extracts obtained at indicated times from DeFew cells infected with pBABEpuroLMP1 or empty vector. The blot was analyzed for the expression of LMP1 and normalized by γ-tub. (B) Graphic representation of the western blot results. (C) Northern blot analysis of RNAs obtained at indicated times from DeFew cells infected with retroviral vector expressing LMP1 or empty vector. The blot was analyzed for the expression of miR-155 and normalized by U6. (D) Graphic representation of the northern blot results. (E) Graphic representation of the correlation of miR-155 and LMP1 expression at indicated times.

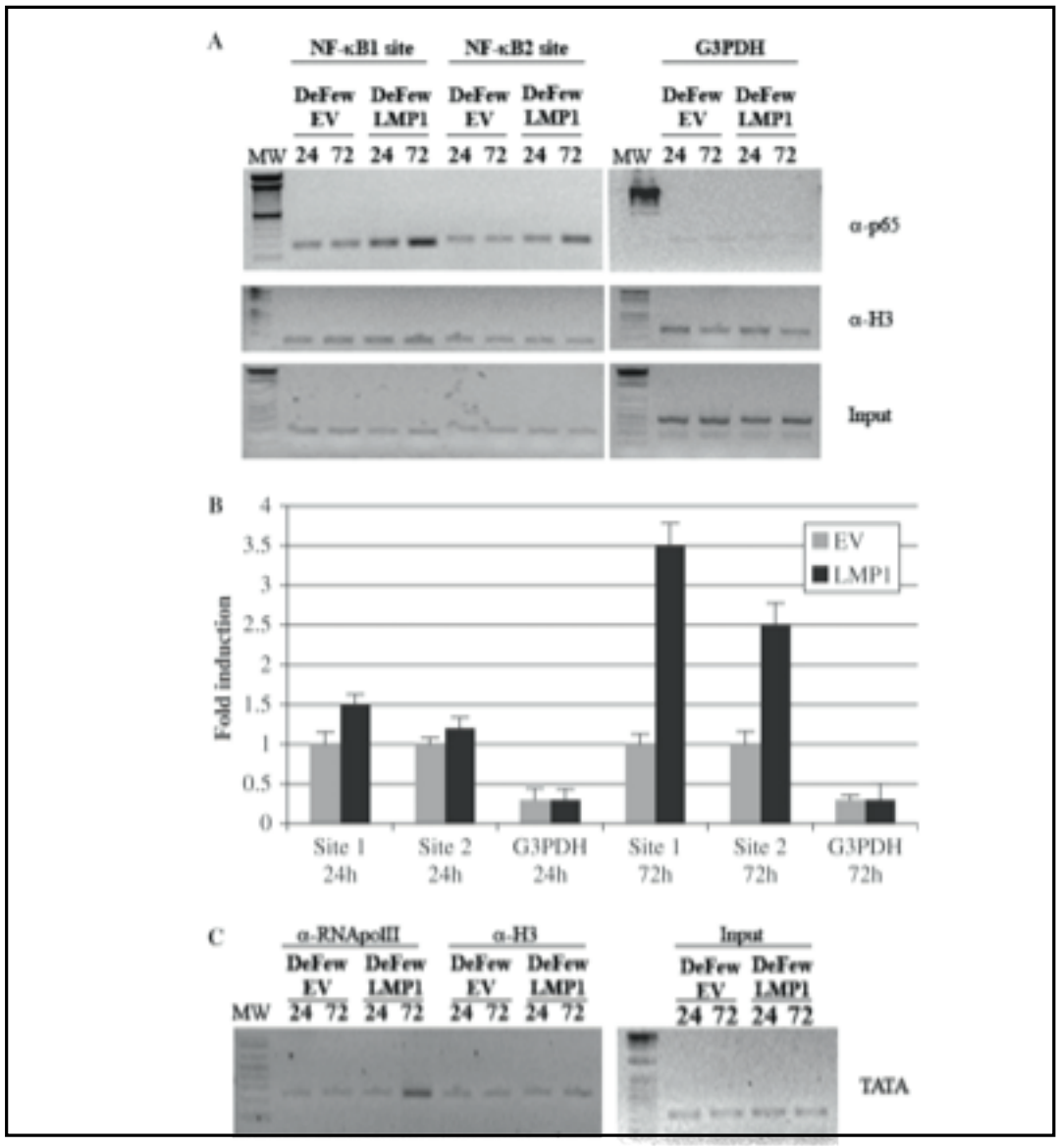
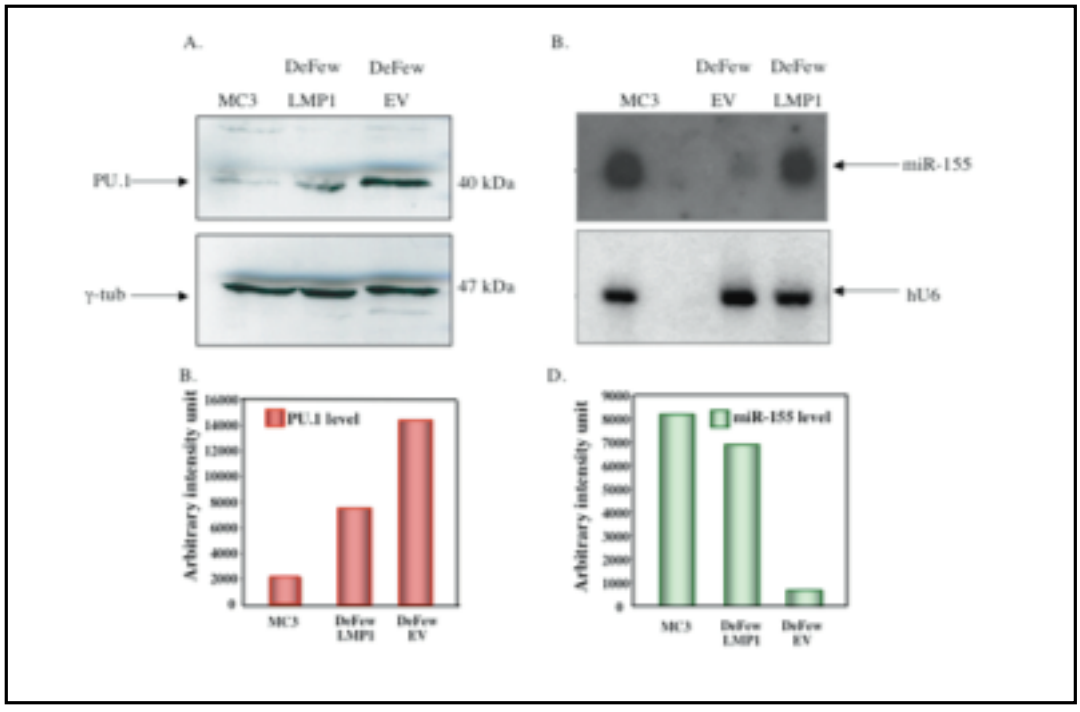


Figure 20 - Semi-quantitative ChIP on the BIC promoter at indicated times in DeFew cells after retroviral infection with a vector expressing LMP1 or empty vector. ChIP assay of chromatin extracted at the indicated times from DeFew cells infected with pBABEpuroLMP1 or empty vector. (A) left panel: After immunoprecipitation with the indicated antibodies, the DNA samples were purified and subjected to PCR with specific primers amplifying the NF-κB site 1 region or NF-κB site 2 region. MW = molecular weight marker; p65 = pAB anti NF-κB p65; ti NF-κB; H3 = pAb anti Histone H3. Right panel: After immunoprecipitation, the DNA samples were purified and subjected to PCR with specific primers amplifying the G3PDH promoter region. (B) Graphic of the results showed in panel (A; C) After immunoprecipitation with mAb directed against the RNA pol II, the DNA samples were purified and subjected to PCR with specific primers amplifying the TATA box region. MW = molecular weight marker; pol II = mAB anti RNA pol II; H3 = pAb anti Histone H3.

3.6 The protein levels of transcription factor PU.1 correlates with LMP1-induced miR-155 expression

It was recently reported that miR-155 targets PU.1 mRNA thereby affecting its translation [110]. Hence, we checked whether the levels of protein PU.1 could be lowered by LMP1 activation of miR-155. To this aim, we infected DeFew cells with the retroviral vector expressing LMP1 or with the empty vector as control. The results show a 2-fold decrease of PU.1 protein levels in the DeFew cells infected with LMP1-expressing vector versus empty vector (figure 21A and B). In the same experiment, we ran a northern blot analysis to evaluate miR-155 expression. As shown in figure 21C and D, miR-155 expression in LMP1-expressing DeFew cells was 10-fold higher than in the empty vector. Interestingly, PU-1 levels were very low in EBV-immortalized MC3 cells overexpressing miR-155.



3.7 Discussion

microRNAs, like transcription factors, regulate the expression of several genes post-transcriptionally, and are thus important players in such relevant cellular processes as cell growth, differentiation, cell signaling and apoptosis. miRNAs themselves are subjected to fine expression regulation, their transcription being driven by RNA pol II responsive promoters [5]. It is generally recognized that miRNAs play a central role in the molecular etiology and maintenance of cancer.

In this study, we describe the activation of miR-155, which is a potential oncomiR [143], by LMP1, a trans-membrane EBV protein considered to be the major oncoprotein of EBV. LMP1 is expressed during viral latency and it activates the signaling pathways NF-κB and AP-1 [129]. Using miRNA array, it has been reported that LMP1-induced the expression of numerous miRNAs, such as miR-146, whose expression is upregulated by the LMP1-induced NF-κB pathway [145,146]. Here we show that EBV-infected cells constitutively express high levels of miR-155, and that the exogenous expression of LMP1 in EBV-negative B cells enhances miR-155 expression in a time- and dose-dependent manner (figure 20).

Figure 21 - PU.1 protein levels correlate with LMP1-mediated activation of miR-155 expression. (A) western blot analysis of PU.1 expression in EBV-immortalized B cells (MC3) EBV-negative B cells (DeFew) infected with pBABEpuroLMP1 (DeFew LMP1) or pBABEpuro, and selected with 1 mg/ml of puromycin for 7 days (DeFew Empty Vector); (B) Graph of densitometric analysis of A normalized by γ-tubulin; (C) northern blot analysis of mir-155 expression in EBV-immortalized B cells (MC3) EBV-negative B cells (DeFew) infected with pBABEpuroLMP1 (DeFew LMP1) or pBABEpuro (DeFew Empty Vector) and selected with 1 mg/ml of puromycin for 7 days; (D) Graph of densitometric analysis of C normalized by U6.

Yin et al. [117] reported that the promoter of the BIC gene encoding miR-155 contains a conserved AP-1 element 40 bases upstream from the transcription start site that is essential for the induction of miR-155 mediated by activation of the BCR [117]. They also identified a putative NF-κB site at –1150 nt on the BIC promoter whose mutation did not affect miRNA enhancement by activation of the BCR [117]. We found an additional NF-κB putative binding site at –1697 nt on the BIC promoter. We show that the endogenous promoter is responsive to PMA (figure 14), a well recognized activator of the canonical inhibitor kinase kinase-dependent (IKK) [141] NF-κB pathway. Moreover, both putative NF-κB sites were able to bind *in vitro* the NF-κB proteins p50 and p65 in nuclear extract from MC3 cells (figure 15A).

Because LMP1 induces both the canonical IKK-dependent [147] and non-canonical NF-κB-induced kinase-dependent (NIK) [148] NF-κB pathways, we tested whether p52 (an NF-κB protein activated by a non-canonical pathway) was part of the binding complex, but we did not detect it. Accordingly, inhibition of the canonical NF-κB pathway by exogenous expression of IκBα led to reduced expression of miR-155 in MC3 (figure 16). This could indicate that the non-canonical pathway is activated only at the beginning of EBV infection when the virus needs to maximize this signaling pathway.

Furthermore, both the NF-κB sites bound to p65 *in vivo* as did the RNA pol II on the TATA box of the BIC promoter, in MC3, which indicates ongoing miR-155 transcription (figure 17A and B) suggesting that the two NF-κB sites are active in an EBV-positive background. However, PMA- and LMP1-driven induction was higher with a truncated form of the BIC promoter lacking the distal site (NF-κB site 2) than with the

full-length promoter (figures 18A). To shield light on the effectiveness of the two NF-κB *cis*-elements, we performed reporter assays using the full length (pLuc1783) or a truncated form (pLuc1380) of miR-155 promoters either wild-type or mutated in the NF-κB site 1, NF-κB site 2 or AP-1. Our results indicate that both NF-κB sites are pivotal for basal and LMP1-induced expression of miR-155 (figure 18B and C). Conversely, mutation of the AP-1 site in the pLuc1380 reduced the constitutive expression of the promoter, which in fact was still inducible by LMP1 albeit to a lesser extent (figure 18B). However, mutation of the AP-1 site in the pLuc 1783 abolished the LMP1-induced expression. Yin et al. [149] suggested that miR-155 promoter contains one or more CpG islands. The presence of putative CpG as well as *cis*-elements bound by negative regulatory protein(s) or promoter secondary structures could explain why the truncated form of the promoter, lacking this putative NRE, is inducible at higher extent by both PMA and LMP1.

Subsequently, we performed time-course experiments in EBV-negative cells infected with an exogenous LMP1. Our data show that the expression of LMP1 is temporally correlated with the activation of p65 binding on both NF-κB sites (figure 20), recruitment of RNA pol II and miR-155 induction (figures 19 and 20). All data reported give a clear indication that LMP1 enhances miR-155 expression, mainly, via activation of the canonical NF-κB pathway.

Our results, together with the previous data reported by Yin et al. [117], illustrate the exquisite regulation of this miR by various stimuli, and highlight its importance in B-cell physiology.

PU.1 is a downstream effector of miR-155 thanks to the presence of a miR-155 complementary seed sequence in the 3' UTR of PU.1 mRNA [110]. In the latter report, B cells lacking miR-155 generated reduced extrafollicular and germinal center responses and did not produce high-affinity IgG1 antibodies. PU.1 is highly expressed in miR-155-deficient B cells and its overexpression in wild-type B cells results in reduced numbers of IgG1-switched cells, which indicates that miR-155 plays a key role in antigen-driven B-cell maturation. PU.1 is a master gene of the Ets transcriptional factor family that promotes cell growth, differentiation and apoptosis thereby playing an important role in hematopoiesis [112]. It is both an oncogene [112] and a tumor suppressor gene that promotes apoptosis. Here we show that LMP1-mediated activation of miR-155 is correlated with diminished levels of the PU.1 protein (figure 21). Many mRNAs were predicted to be direct targets of miR-155. A recent work reported that miR-155 plays an important role in EBV-regulated gene expression of the infected cells [117]. It is thus possible that the phenotypic alterations and the oncogenic potentialities observed in miR-155-overexpressing mice[143] are the result of deregulation of other targets in addition to PU.1. *In silico* analysis indicated that miR-155 may target SOCS1[150] and WEE1[151] (Mallardo et al. unpublished data), which would better account for its oncogenic role. Probably, a miRNA can have two or more targets or even different targets depending on the cellular context. Studies to elucidate the molecular targets of miR-155 after LMP1 induction, and to determine their reciprocal role during EBV infection and EBV-mediated transformation are underway in our laboratory.

Chapter 4: Computational analysis and in vivo validation of a microRNA encoded by the IBTK gene, a regulator of B-lymphocytes differentiation and survival

4.1 *IBTK* gene encoded a new microRNA

To identify putative pre-miRs encoded by the IBTK gene, we performed the *in silico* analysis of the genomic nucleotide sequence of the IBTK genetic locus by using the Pro-MirII software that allows the probabilistic prediction of clustered, un-clustered, conserved and un-conserved [152]. We identified four potential miRNAs located in the introns 17, 21, 26 and in the 3' UTR of the IBTK gene, named pre-miR-IBTK1, pre-miR-IBTK2, pre-miR-IBTK3, pre-miR-IBTK4, respectively, according to chemical and physical properties, such as free energy, G-C ratio and entropy (figure 22).

A

Name	Genomic position (bp) From-To	IBTK gene region	Free Energy ΔG (Kcal/mol)	G-C Ratio	Entropy
pre-miR-IBTK1	82971042-82970943	Intron 17	-29.4	0.34	1.87
pre-miR-IBTK2	82963276-82963177	Intron 21	-37.3	0.4	1.96
pre-miR-IBTK3	82945044-82944945	Intron 26	-26.3	0.36	1.91
pre-miR-IBTK4	82937481-82937382	3'UTR	-27.8	0.32	1.86

B

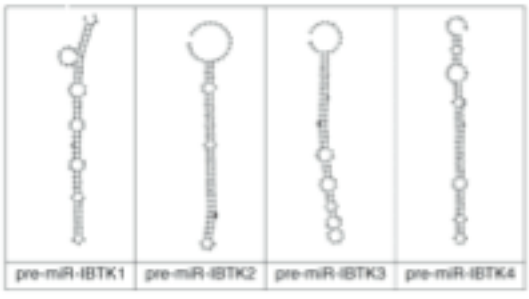


Figure 22 - Precursor bioinformatic analysis of the genetic locus IBTK. (A) Chemical-physical properties (free energy, G-C ratio, entropy) of the four predicted microRNA precursors encoded by the human genetic locus IBTK on the chromosome 6. (B) Bidimensional representation of the predicted microRNA precursors by using PromirII software.

To assess whether the predicted pre-miRs were substrates of RNase III Dicer, we generated expression vectors of pre-miR-IBTK sequences under the T7 promoter, which were used to produce the relative [^{32}P]α-UTP-labeled transcripts. By *in vitro* assay, pre-miR-IBTK3, and not the other pre-miRs, was cleaved by the human recombinant Dicer to generate 22-25 nucleotides RNA products (figure 23).

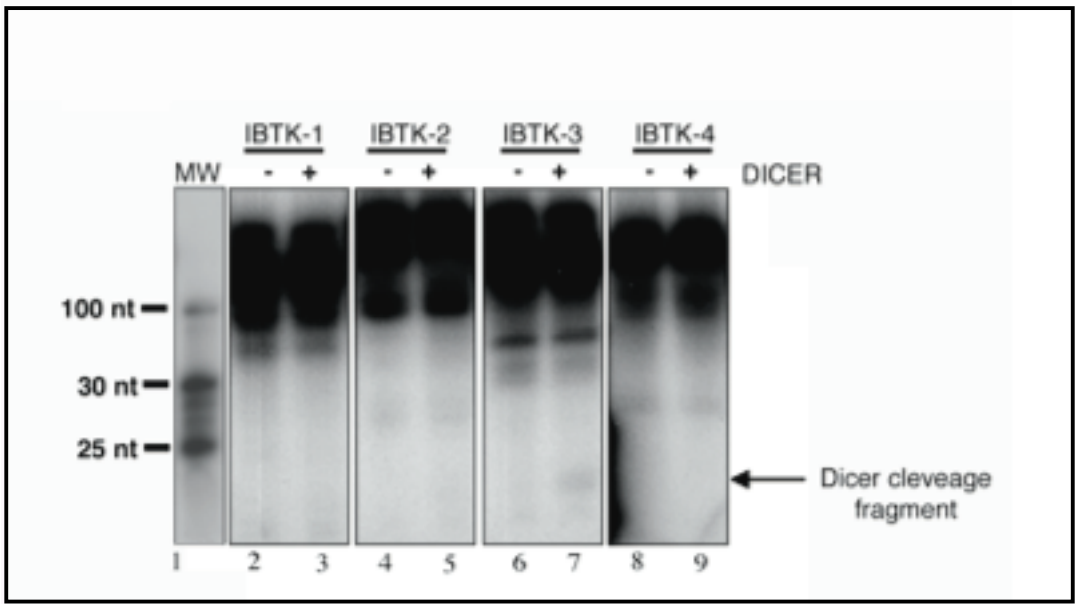


Figure 23 - *In vitro* Dicer RNase assay. *In vitro* transcribed and labeled pre-miR-IBTK1(lane 2,3), pre-miR-IBTK2 (lane 4,5), pre-miR-IBTK3 (lane 6,7), pre-miR-IBTK4 (lane 8,9),were incubated for two hours in the presence (3,5,7,9) or absences (2,4,6,8) of recombinant Dicer. Products were resolved on a 15% TBE-Urea gel and visualized by autoradiography. Arrow indicates the ~22 nt band generated by Dicer cleavage. MW (lane1) was exposed for 1 minutes, while the digests (lanes 3,5,7,9) were exposed for 15 minutes. Numbers to the left indicate the size of the associated RNA marker.

Then, we performed the Northern blotting analysis of total RNA extracted from human PBMCs to verify the *in vivo* occurrence of pre-miR-IBTK3 and relative products under physiological conditions. To this end, we used 25 nucleotides-probes annealing to four sequential regions of pre-miR-IBTK3 (figure 24A). All nucleotide probes

identified both the pre-miR-IBTK3 and IBTK miRNA (figure 24B), indicating that both arms of pre-miR-IBTK3 generated the RNA products *in vivo*.

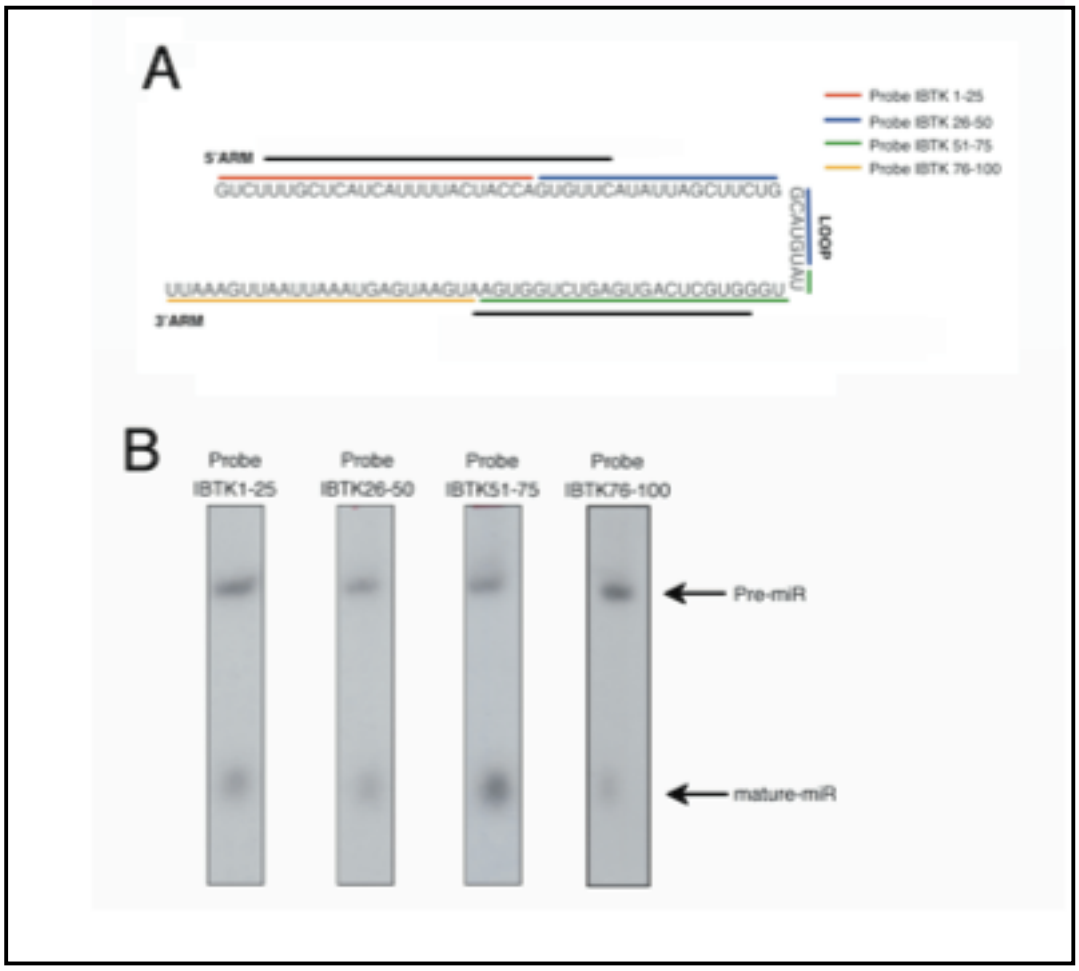


Figure 24 - Northern blot analysis of microRNAs encoded by genetic *locus* IBTK. (A) Schematic representation of the probes used in northern blotting analysis. (B) Northern blotting of total RNA extracted from PBMC.

To evaluate the grade of evolutionary conservation of pre-miR-IBTK3, we performed a CLUSTALW-based multiple sequence alignment of the nucleotide sequence of pre-miR-IBTK3 in different vertebrates. The pre-miR-IBTK3 of *Homo sapiens* showed a high identity degree with pre-miR-IBTK3 of *Pan troglodytes*, *Pongo pygmaeus* and *Macaca*

mulatta, and a lower identity degree with pre-miR-IBTK3 of *Bos Taurus* and *Canis Familiaris* (figure 25).

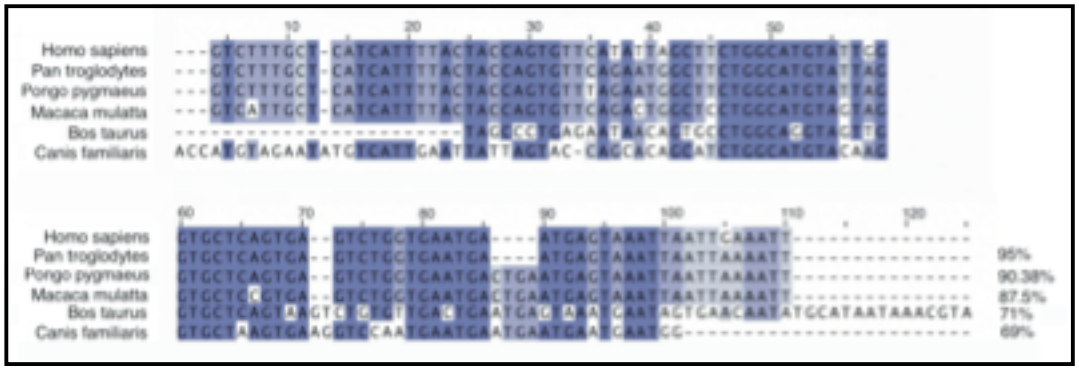


Figure 25 - Comparative analyses of pre-miR-IBTK3 among different species. Alignement of the nucleotide sequence of *Homo sapiens* pre-mir-IBTK3 with the homologs nucleotide sequences of *Pan troglodytes*, *Pongo pygmaeus*, *Macaca mulatta*, *Bos Taurus* and *Canis Familiaris*. The identity percentage among these sequences was calculated by using CLUSTALW software.

In fact, the identity percentage of *Homo sapiens* pre-miR-IBTK3 as compared to the pre-miR-IBTK3 of *Pan troglodytes*, *Pongo pygmaeus* and *Macaca mulatta* was 95%, 90.4% and 87.5%, respectively. Differently, the identity percentage compared with the pre-miR-IBTK3 of *Bos taurus* and *Canis familiaris* was 70% and 69%, respectively. Accordingly, the PromirII-based analysis of pre-miR IBTK3 nucleotide sequence of *Pan troglodytes*, *Pongo pygmaeus* and *Macaca mulatta* predicted a miRNA secondary structure (figure 26), which was not predicted for the homologue IBTK nucleotide sequence of *Bos taurus* and *Canis familiaris*.

A

Species	Free Energy ΔG (Kcal/mol)	G-C ratio	Entropy
Pan troglodytes	-25,4	0,36	1,91
Pongo pygmaeus	-24,5	0,35	1,91
Macaca Mulatta	-26	0,4	1,96

B

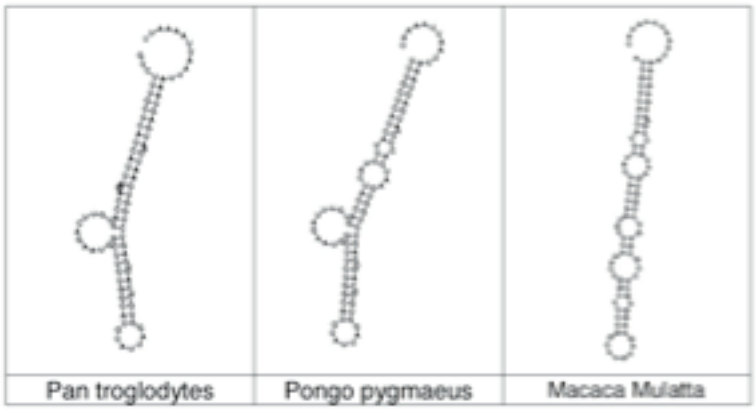


Figure 26 - Precursor bioinformatic analysis of the homologs pre-mir-IBTK3 sequences in *Pan troglodytes*, *Pongo pygmaeus*, *Macaca mulatta*. (A) The table shows the Chemical-physical properties (free energy, G-C ratio, entropy) of the predicted pre-mirIBTK3 in different species. (B) Bi-dimensional representation of the predicted pre-miRIBTK3 in primates *Pan troglodytes*, *Pongo pygmaeus* and *Macaca mulatta*.

4.2 Discussion

The IBTK genetic locus encodes the IBtk proteins α, β, γ [131]. We previously characterized IBtkγ as a repressor of Btk [131,132]. Btk is a Tec tyrosine kinase required for B-lymphocytes development and differentiation [153]. Mutations of the BTK gene inactivating the Btk activity caused X-linked agammaglobulinemia in humans [154] and X-linked immunodeficiency in mice [155]. In mature B-lymphocytes, Btk is required for calcium signalling and activation of the anti-apoptotic transacting factor NF-κB in response to the triggering of the B cell receptor for the antigen (Janda E. et. al., submitted); in this context, Btk promotes the antibody production, B cell differentiation and cell cycle progression. IBtkγ binds the pleckstrin homology (PH) domain of Btk and represses the Btk-mediated signalling leading to B-cell activation [131,132]. Based on this evidence, *IBTK* exerts a prevalent anti-proliferative action. Moreover, *IBTK* might play a more extended role in cell signalling since the IBtkα and IBtkγ proteins are expressed in distinct cell types and bind the PH domain o distinct Tec kinases, such as Btk, Itk and Akt [131]. Two promoters and several *cis* regulatory sequences were identified in the *IBTK* gene [131]; however, little is known on the *IBTK* transcription regulation. Recent studies have shown that miRNAs regulate cell proliferation and apoptosis and are involved in cancer development [156]. Indeed, more than 50% of miRNA genes are located either in cancer-associated genomic region, or in fragile sites, suggesting that miRNAs rearrangements may play a major role in the pathogenesis of human cancers [157, 158]. In particular, B and T cell neoplasia showed a high incidence of miRNAs rearrangements [159, 160],

indicating that specific miRNAs mutations may deregulate cell functions that play a role in B and T lymphocyte proliferation.

In this study, we have addressed whether the *IBTK* gene generates miRNAs in human PBMCs. We found that intron 26 of the human *IBTK* gene encodes the premiR IBTK3, which is cleaved by Dicer generating ~22-nucleotide-long products. This finding correlated with the occurrence of pre-miR IBTK3 and the relative miRNA products in human PBMCs. We also found that pre-miR IBTK3 is an evolutionary conserved function in primates since it is highly conserved in *Pan troglodytes*, *Pongo pygmaeus* and *Macaca mulatta*. The evidence of miRNA IBTK occurrence *in vivo* supports an expanded role of the IBTK gene as regulator of gene transcription through miRNA generation. As the human IBTK gene is located in the genomic sequence 6q14.1, which is a region of recurrent chromosomal aberration in lymphoproliferative disorders [1363, our findings suggest that aberrant production of IBTK miRNA might be involved in tumorigenesis. Identification of the primary structure of the IBtk miRNA *in vivo* will be required to identify its mRNA targets and specific function in gene regulation.

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