The Role of Cellular Micro-RNAs in Epstein-Barr Virus Induced Cellular Transformation and Oncogenesis

by

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Abstract

Micro-RNAs (miRNAs) are a class of non-coding RNA which post-transcriptionally regulate gene expression. Epstein-Barr Virus (EBV) can transform resting B-cells *in vitro* to establish continuously proliferating lymphoblastoid cell lines (LCLs) and is aetiologically linked to certain lymphomas. Little is known about the contribution of miRNAs to the transformation of B cells. We initially examined the regulation of the oncogenic miR-155, which is highly expressed in Hodgkin's lymphomas but was reportedly absent in Burkitt's lymphoma. We found that miR-155 was up-regulated by EBV-LMP1 expression, and that a reported defect of miR-155 processing in Burkitt's lymphoma was a misinterpretation of data. Next, to identify cellular miRNAs and genes modulated during EBV-induced transformation, we compared the expression profiles of resting B cells and B cells either infected with EBV or stimulated to proliferate with CD40L and IL4. This revealed that a large proportion of miRNAs and genes differentially regulated by EBV and not by CD40L/IL4 were modulated by EBV interaction with its CD21 receptor complex, but these changes were maintained or amplified in LCLs; and included a set of tumours suppressor genes selectively down-regulated by EBV. In addition, bioinformatics analysis indicated that EBV modulates the expression of multiple miRNAs predicted to target the same cellular genes.

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Abbreviations

ac-pre-miRNA	Ago2-cleaved pre-miRNA
ADAR	adenosine deaminase associated RNA
Ago	argonaute protein
AID	activation-induced deaminase
ALV	Avian Leukosis Virus
BARTs	*Bam*HI A rightward transcripts
B2M	beta-2-micoglobulin
BIC	B cell integration cluster
BCR	B cell receptor
BL	Burkitt lymphoma
BLIMP1	B-lymphocyte induced maturation protein 1
cDNA	complementary DNA
CDKI	cyclin dependent kinase inhibitor
C.elegans	Caenorhabditisis elegans
cHL	classic Hodgkin lymphoma
CLL	chronic lymphocytic leukaemia
CTL	cytotoxic T lymphocyte
CTR	control
DGCR8	DiGeorge critical region 8
DLBCL	diffuse large B cell lymphoma
DMSO	dimethylpyrocarbonate
DNA	deoxynucleic acid
dNTPs	deoxynucleotide triphosphates
DUSP	dual specificity phosphatase
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence

FACS	fluorescence-activated cell sorter
FCS	foetal calf serum
FoxO	forkhead transcription factor class O
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	germinal centre
GFP	green fluorescent protein
gp	glycoprotein
HCV	hepatitis C virus
HCMV	human cytomegalovirus
HIV	human immunodeficiency virus
HHV	human herpesvirus
HL	Hodgkin's lymphoma
HRS	Hodgkin-Reed Sternberg cells
HSV-1	herpes simplex virus-1
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IM	infectious mononucleosis
IRES	internal ribosome entry site
JAK	janus kinase
kbp	kilobase pair
KSHV	Karposi's sarcoma-associated herpesvirus
LCL	lymphoblastoid cell line
LCV	lymphocryptovirus
LMP	latent membrane protein
mAB	molecular antibody
MAPK	mitogen activated protein kinase

MICB	histocompatibility complex class I related chain B
miRNA	micro-RNA
mRNP	messenger ribonucleoprotein
NFκB	nuclear factor kappa B
NK cell	natural killer cell
NLS	nuclear localisation signal
NPC	nasopharyngeal carcinoma
ORF	open reading frame
PACT	protein activator of PKR
P-bodies	processing bodies
PBS	phosphate buffer saline
PCR	polymerase chain reaction
pi	post-infection
PFV1	primate foamy virus-1
PTEN	phosphatase and tensin homologue
PTLD	post-transplant lymphoproliferative disorder
PUMA	p53 up-regulated modulator of apoptosis
QPCR	quantitative polymerase chain reaction
Rb	retinoblastoma protein
RBP-Jκ	recombination binding protein J kappa
RISC	RNA-induced silencing complex
RLC	RISC loading complex
RLNs	reactive lymph nodes
RNA	ribonucleic acid
RNAi	RNA interference
RPM	revolutions per minute
RT	reverse transcription

SAM	significance analysis of microarrays
SCID	severe combined immunodeficient
SDS	sodium dodecyl sulphate
snoRNA	small nucleolar RNA
SV40	Simian virus 40
TCR	T cell receptor
TLR	toll like receptor
TM	transmembrane
TNF	tumour necrosis factor
TPA	12-O-tetradeconoylphorbal-13-acetate
TR	terminal repeat
TRBP	Tar RNA binding protein
tTA	tetracycline transactivator
USP	ubiquitin specific peptidase
UTR	un-translated region
VA RNAs	virus associated RNAs
VCA	viral capsid antigen
wt	wild-type

1. Introduction

1.1 Epstein-Barr virus (EBV) history

Epstein-Barr virus was identified in 1958 following the observation by surgeon Dennis Burkitt that a common childhood cancer in equatorial Africa was endemic to areas with specific climatic conditions and geographical locations [1]. This led him to hypothesise that the cancer may be caused by an insect-borne vector [2]. Using electron microscopy it was subsequently shown by Epstein, Anchong and Barr, that herpesvirus-like particles existed in a cell line established from a Burkitt lymphoma (BL) biopsy [3]; and formal identification of a new herpesvirus followed in 1965 [4]. Whilst this discovery was consistent with the hypothesis of an insect-borne infectious agent, it later transpired that it was not EBV itself that was carried by the insect but another co-factor in the development of BL, the malaria *plasmodium falciparum* parasite [5, 6]. Serological assays against the viral capsid antigen (VCA) were developed and indicated that greater than 90% of the adult population had antibodies against EBV and that the virus was associated with infectious mononucleosis (IM) [7, 8], which is now known to be a clinical manifestation of primary EBV infection. The growth transforming properties of EBV were identified after the virus was shown to induce proliferation in peripheral blood leukocytes *in vitro* and induce tumours in non-human primates [9, 10]. Thus, EBV was the first virus associated with the pathogenesis of a human cancer, establishing it as a ubiquitous oncogenic herpesvirus.

1.2 Classification of EBV

EBV (also known as human herpesvirus 4 (HHV4)) is a member of the Herpesviradae family [11]. This viral family is sub-divided into alpha, beta and gamma members based on genome homology. EBV is a member of the gamma sub-family which is further divided into two genera: gamma 1, also known as lymphocryptoviruses (LCV), and gamma 2, also known as rhadinoviruses. EBV is the prototype LCV and the only human virus of this genus. LCVs are exclusively found in primate species and are predominantly B lymphotropic.

1.3 EBV structure

EBV has a glycoprotein (gp)-rich envelope which surrounds a nucleocapsid composed of 162 capsomers and a protein tegument. The nucleocapsid encases a 184 kilobase pair (kbp) linear, double-stranded DNA genome wrapped around a toroid-shaped protein core [4, 12]. The genome is divided into short and long, largely unique sequence domains (US and UL) by reiterated 3kbp internal direct repeats (IR1) [13, 14]. The ends of the linear genome are flanked by 4-12 copies of tandem, reiterated, 500bp direct repeats called terminal repeats (TRs), which enable circularisation of the viral DNA into episomes [15-17]. The number of TRs can be an indicator of clonality as the precise number of TRs is determined during viral replication; therefore all EBV latently infected progeny cells will contain the same number of TRs as the originally infected parental cell [18].

EBV was the first herpesvirus to have its genome cloned and sequenced [19-21]. This was achieved using *Bam*HI (and *Eco*R1) restriction fragments from the B95.8 EBV strain, which consequently defined the nomenclature used to describe the viral genome [19]. *Bam*HI fragments were assigned a letter from A-Z in descending order of size. Open reading frames

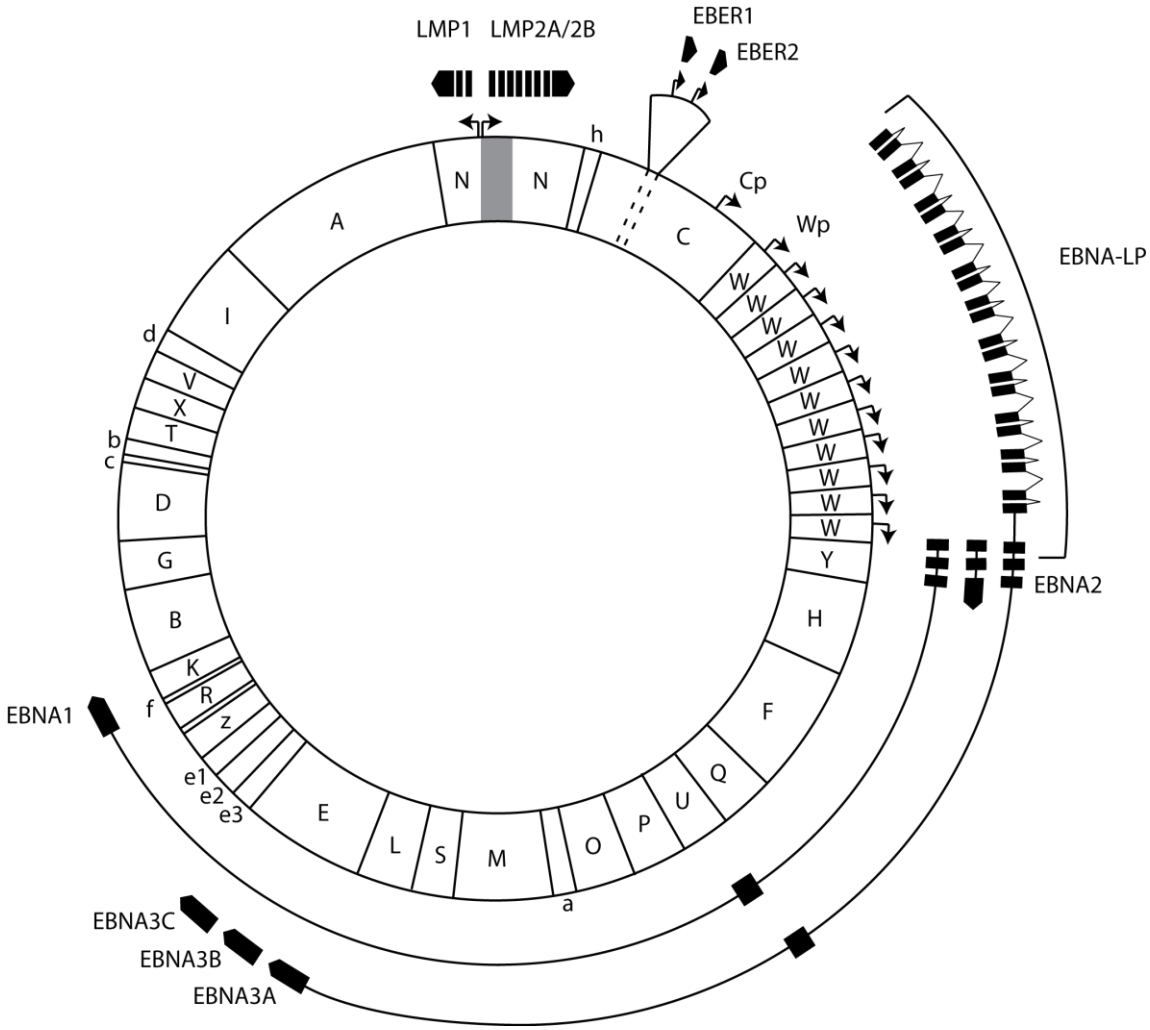


Figure 1.1: Schematic map of the EBV genome divided into *Bam*HI digest fragments. Arrows represent the latent promoters, filled boxes represent the genes encoding the latent proteins and EBERS. Grey boxes represent the terminal repeats (TRs)

(ORFs) were stated relative to their *Bam*HI fragment, direction of transcription and position relative to other ORFs located on the same fragment. For example: BHRF1 is located on the *Bam*HI H fragment, in the first rightward open reading frame.

All EBV strains have essentially the same genome organisation and most of the sequences are highly conserved. However, the two main subtypes of EBV, type 1 and type 2, differ primarily in the genes encoding the nuclear antigens (EBNAs) –LP, 2, 3A, 3B and 3C [22, 23]. The origins of the sequence variations between type 1 and type 2 EBV in EBNA2 and the EBNA3A, B and C antigens remains a mystery, with predicted differences in amino acid sequences of 47%, 16%, 20% and 28% respectively [24]. The two EBV subtypes can be further subdivided into different EBV strains according to local changes at polymorphic sites, such as the number of repeat sequences in several of the latent genes [25]. Many of the local polymorphisms distinguish between EBV strains of different geographical origin, consistent with a slow evolutionary drift of the virus in physically separated human populations. Type 1 EBV is epidemiologically dominant, except in areas endemic for BL (equatorial Africa and New Guinea) where infection with type 2 EBV is almost as prevalent as type 1 [26-31].

1.4 EBV tissue tropism

EBV is a B lymphotropic virus and its ability to effectively target B cells is determined by the expression of the C3d complement component receptor, CD21; this is the major viral receptor and it is widely expressed on B cells but its expression is lost upon terminal differentiation [32-35]. Thus, EBV is able to bind to and infect B cells at most stages of development with the exception of plasma cells. Infection of B cells is generally ‘non-productive’ for lytic virus replication, or ‘latent’, although lytic infection can occur in B cells [36]. There is also

evidence of rare lytic replication in mucosal epithelial cells of non-immuno-compromised patients [37]. The ability of EBV to infect CD21-negative epithelial cells is evident from the association of EBV with nasopharyngeal carcinoma (NPC) [38] and is supported by *in vitro* experiments which have demonstrated that EBV bound to the surface of B cells can efficiently infect epithelial cells by a process termed transfer infection [39, 40]. In addition, it is apparent from the broad range of malignancies associated with EBV that the virus is capable of infecting other cell types, including T cells [41] and natural killer (NK) cells [42, 43]. The degree to which CD21 negative epithelial, NK and T cells are normally infected *in vivo*, in the healthy immunocompetent host, and what role, if any, they play in the course of EBV infection and persistence is not clear.

1.5 Alternate patterns of latent infection

There are four well characterised patterns of EBV latent gene expression, latency III, II, I and 0. Figure 1.2 is adapted from Fields Virology [44] and shows the distinct patterns of viral gene expression observed during latency. The four latency states of EBV are not prescriptive as EBV latent gene expression in tumours can be variable and may not strictly adhere to any of the four categories of viral latency [45, 46] .

1.5.1 Latency III

Infection of resting B cells *in vitro* results in a pattern of viral latent gene expression termed latency III. This pattern of viral gene expression involves the expression of 9 or more latent genes encoding: each of six nuclear antigens, EBNAs 1, 2, -LP, 3A, 3B and 3C from the viral promoters Cp or Wp; the three latent membrane proteins (LMPs) 1, 2A and 2B are expressed from their own promoters. In addition, the RNA polymerase III-driven non-coding

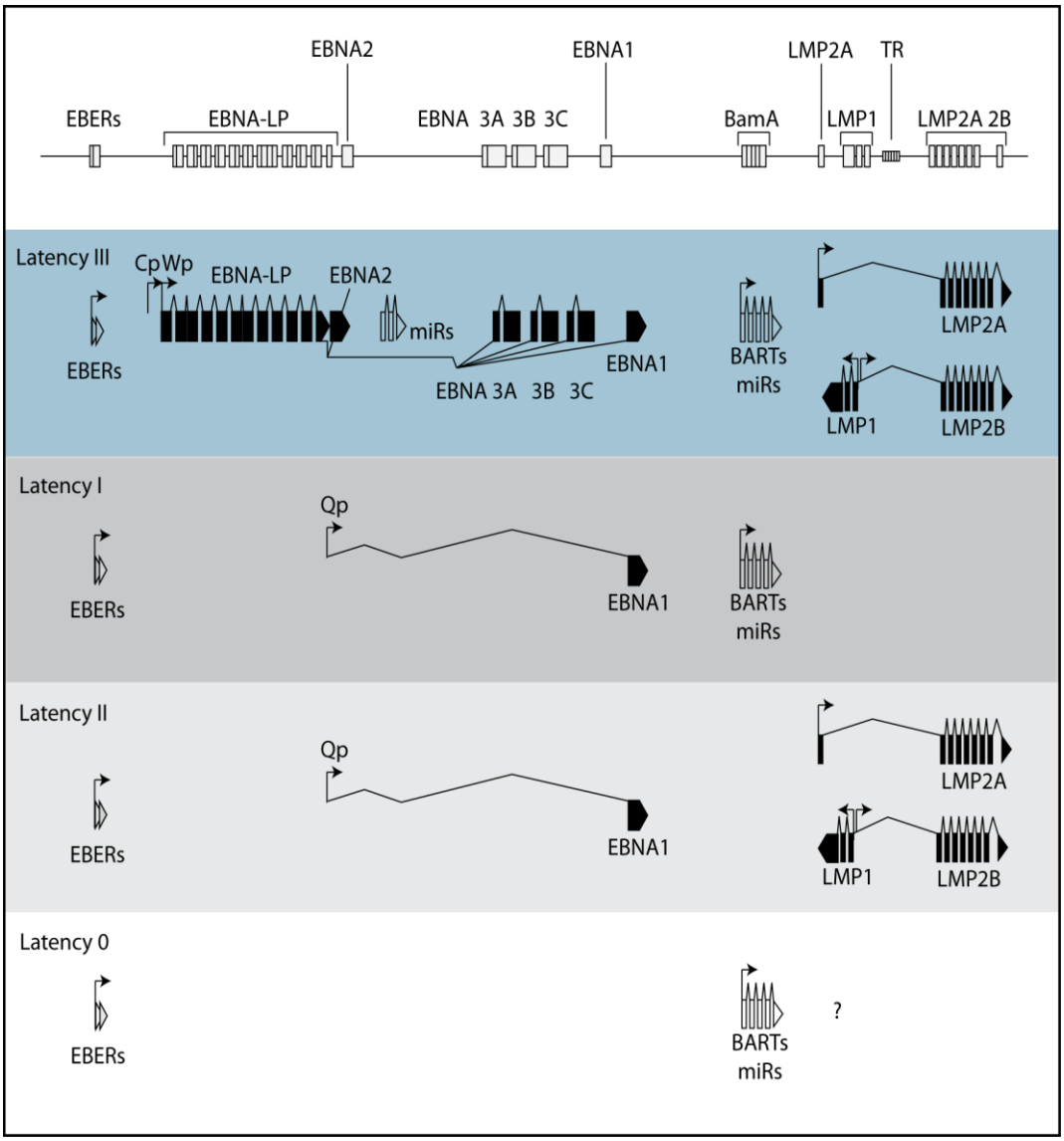


Figure 1.2: Schematic diagram of EBV latent transcription in different forms of latency. Adapted from Fields et al 2001 [44]. Boxes represent EBV latent ORFs; shaded boxes represent ORFs which are translated into viral protein; open boxes represent non-coding viral RNAs. A linear EBV genome is shown above with the relative positions of the latent genes and TRs marked.

EBER RNAs are expressed [47] along with the EBV encoded miRNAs: the BamHI A rightward transcripts (BARTs) and the BHRF1 miRNAs [48]. The latency III pattern of gene expression is also known as the growth-transforming programme (more details in section 1.9.2) as it transforms resting B cells into proliferating blasts ('lymphoblastoid') with a phenotype similar to that of B cells proliferating in response to antigen. The latency III transcripts are also detected in the tonsils of patients with IM, suggesting that this pattern of viral latent gene expression also occurs during primary infection *in vivo* [49].

1.5.2 Latency II

Latency II is a restricted pattern of viral latent gene expression in which the following latent genes are expressed: EBNA1 along with the EBERS, as well as the BARTs and BART miRNAs. Variable expression of the LMPs 1, 2A and 2B are also seen from EBNA2 independent promoters in the BamHI N region of the genome [50-53]. The viral promoters Cp and Wp are silent in latency II infected cells and selective expression of EBNA1 initiates from an alternative viral promoter Qp, located downstream of the *Bam*HI Q fragment [54]. Latency II was originally identified in the epithelial tumour NPC [55, 56] and was subsequently also found to be expressed in the Reed-Sternberg cells (HRS) of EBV positive Hodgkin's Lymphoma (HL) [57] . A latency II pattern of EBV gene expression has also been detected in the tonsillar GC B cells of IM patients [58], suggesting it may play a role in primary infection.

1.5.3 Latency I

Latency I is a restricted form of viral latency first identified in BL [59], in which only EBNA1, EBERs and the BART miRNAs are known to be expressed [60]. The EBNA promoters Cp and Wp are silent, as in latency II, and the LMP promoters are also silent and gene expression is initiated from the Q promoter (Qp) [54]. Latency I has also been detected

in replicating peripheral blood memory B cells from IM patients, suggesting that this latency state may be important for viral persistence *in vivo* [61].

1.5.4 Latency 0

It is believed that EBV persists in the memory B cell compartment in normal carriers in a form of viral latency termed latency 0. In this form of latency, latent gene expression is restricted to only the EBERs and the BARTs [44, 62].

1.6 EBV latent gene products

1.6.1 EBNA1

EBNA1 is encoded by the BKRF1 ORF. All EBNA1 mRNAs have been shown to contain an IRES (Internal Ribosome Entry Site) in the U exon. This IRES allows efficient translation of EBNA1 irrespective of which viral promoter is used to initiate EBNA1 transcription. Consequently, the varying 5'UTRs of EBNA1 generated from alternate promoter usage (Y_3-U-K from Cp and Wp, and Q-U-K from Qp) do not affect the efficiency of EBNA1 protein expression [63].

EBNA1 is the only latent protein to be expressed in all EBV-associated malignancies. EBNA1 is essential for EBV episome maintenance which it mediates through diffuse association with mitotic chromosomes [64]. EBNA1 is also expressed during the lytic cycle; lytic EBNA1 mRNAs initiate directly from the lytic F promoter, located just up-stream of Qp.

EBNA1 is believed to exist as a dimer which binds to the latent origin of replication (oriP) to initiate DNA replication [65]. The oriP contains two distinct binding sites for EBNA1: the family of repeats (FR), which consist of 20 copies of a 30bp EBNA1 binding repeat; and a dyad symmetry (DS) region, which contains 4 overlapping EBNA1 binding sites [66, 67]. On

binding EBNA1 dimers, DNA replication initiates from within DS and terminates within FR [68]. Binding of EBNA1 to FR may also enhance transcription from Cp and Wp, and have a long range effect on the transcription of the LMPs [69-71]. Conversely, EBNA1 binding to two low affinity sites down-stream of the Qp promoter results in decreased transcription, suggesting that EBNA1 negatively regulates its own transcription during latency I and II [72, 73].

In the prototype B95.8 strain the EBNA1 protein is 641aa in length and contains a glycine-alanine (gly-ala) rich region within the amino terminal which varies in length among different EBV isolates. This gly-ala repeat inhibits proteasomal degradation, thereby preventing presentation by the major histocompatibility complex (MHC) class I and evading the host immune response [74, 75]. EBNA1 is non-essential for growth-transformation although transformation efficiency is significantly reduced (>1000 fold) in the absence of EBNA1, presumably due to the essential role EBNA1 plays in maintenance of the EBV episome [76]. The contribution of EBNA1 to the pathogenesis of EBV associated tumours is also ill defined; there are conflicting reports regarding the ability of EBNA1 to increase the incidence of lymphoma in transgenic mice with constitutive EBNA1 expression in B cells [77-79]. EBNA1 has also been identified as a possible enhancer of cell survival [80], possibly due to its ability to prevent p53 stabilisation though binding to the p53 binding partner ubiquitin specific peptidase (USP) 7 [81].

1.6.2 EBNA2

EBNA2 is encoded within the BYRF1 ORF. It was the EBNA2 deleted non-transforming virus, P3HR1 which first demonstrated the essential role of EBNA2 in EBV-mediated growth transformation [82]. EBNA2 contains a number of important structural features: a negatively

charged amino terminus which may play a role in homodimerisation; a polyproline repeat region of variable size; a highly type divergent central region; a RBP-Jκ interaction domain; a highly conserved, imperfect Arg-Gly repeat which can serve as nuclear localisation signal (NLS); a negatively charged transactivation domain; and a C-termianl Lys-Arg-Pro-Arg NLS. Only the negatively charged N terminal domain and the imperfect Arg-Gly repeat are conserved between type I and type II viruses. It is proposed that the sequence differences in EBNA2 are the cause of the reduced transformation efficiency of the type 2 viruses relative to the type 1 viruses [82, 83].

The essential role of EBNA2 in B cell transformation is due to its ability to act as a potent transcriptional activator of viral and cellular genes. Three of the EBNA2 structural elements, the RBP-Jκ binding domain, the acidic activation domain and the homotypic association domains have been identified as essential for the transformation and transcriptional properties of EBNA2. The RBP-Jκ binding domain mediates promoter modulation in a similar manner to the critical development protein, Notch 2 and thus EBNA2 can be described a functional orthologue of an activated Notch receptor. RBP-Jκ, either alone or with the ski-interacting protein (SKIP), tethers EBNA2 to its response elements up-stream of viral and cellular promoters. EBNA2 has been shown to activate the viral promoter Cp and the promoters of the LMPs. In resting cells, RBP-Jκ is thought to repress promoters through an interaction with a histone deacetylase complex (HDAC) co-receptor complex. The binding of EBNA2 to RBP-Jκ replaces the repressive interactions and through its transactivation domain, recruits activators of transcription. Cellular genes which EBNA2 has been able to transactivate include CD21, CD23, c-fgr, cMyc, RUNX3 and EBII/BLR2 [84-88].

The acidic activation domain contains a homologous sequence to the herpes simplex virus (HSV)-encoded transcriptional activator protein, VP16. Both EBNA2 and VP16 bind a range

of transcriptional activators including p300/CBP, TFIIE and p100 [89, 90]. The scaffolding protein p100 is complexed with the acidic domain of EBNA2 in LCLs and mediates EBNA2's transactivation of genes through the interaction of p100 with c-myb and PIM-1 [91, 92]. EBNA2 dependent activation is likely to involve chromatin remodelling and histone acetylation, as evidenced by the interaction of EBNA2 with p300/CBP and SWI/SNF [93-95].

Finally, either of the regions bordering the polyproline repeat can mediate homotypic association which is pivotal to EBNA2's ability to form complexes that can recruit transcription factors [96]. Deletion of both of these regions results in a null transforming phenotype [97].

1.6.3 EBNA-LP

The EBNA-LP ORFs are located within the 5'UTR of all Wp and Cp initiated transcripts and are only translated when W_0 or C_2 exons splice to an alternate splice acceptor, W1', creating an AUG translation initiation codon [98]. EBNA-LP is transcribed from Cp or Wp initiated C_1, C_2 or W_0 exons, followed by a variable number of W_2W_1 repeats and unique Y_1 and Y_2 exons. The size of EBNA-LP varies between different EBV isolates which show differing numbers of repeat domains [99]. EBNA-LP is one of the first latent proteins to be expressed during primary B cell infection, along with EBNA2 [47]. The expression of EBNA-LP is not believed to be essential for transformation, as recombinants expressing a truncated protein lacking the unique C-terminus retain some transforming capabilities [100, 101]. Experiments performed with recombinants with mutated EBNA-LP ORFs are required to confirm the role of EBNA-LP in the transformation of B cells by EBV. The major function of EBNA-LP appears to be as a co-activator of EBNA2 mediated transcription of viral and cellular genes

[102]. EBNA-LP enhances EBNA2 mediated transactivation of the LMPs and Cp [103] and co-transfection of EBNA2 and EBNA-LP can induce G_0 to G_1 transition in primary B cells [104]. EBNA-LP is a phosphorylated protein with serine 35 in the W_2 exon serving as the major phosphorylation site for this protein. Substitution of serine 35 reduced EBNA-LP's ability to induce LMP1 following co-transfection with EBNA2 into Akata cells, indicating that the phosphorylation of EBNA-LP is critical for its function as co-activator of EBNA2 [105]. The phosphorylation of EBNA-LP may be required to control its activity depending on the requirements of the cell as its phosphorylation is cell-cycle specific, with phosphorylation increasing in G2 and being maximal in the G2/M phase [106]. *In vitro* assays have indicated that EBNA-LP phosphorylation can be mediated by casein II kinase, p34cdc2 and DNA-PK [105, 107].

EBNA-LP is highly associated with the nuclear matrix fraction of the cell and has been detected in both ND10 bodies (also known as nuclear bodies) and spread throughout the nucleus [99]. Immunofluorescence studies have shown that EBNA-LP co-localises with promyelocytic leukaemia protein (PML), retinoblastoma protein (Rb) and heat shock proteins (Hsp72/73) in the nucleus [108-110]. However, a cytoplasmic localisation for a proportion of EBNA-LP molecules has also been reported [111]. Studies in cell-free systems have also indicated an association between EBNA-LP and the tumour suppressor proteins, Rb and p53 [112]. However, this association has not been shown in LCLs and it has also been reported that the transfection of EBNA-LP does not affect Rb or p53 function, therefore the biological significance of these interactions is questionable [113].

1.6.4 EBNA3 family

The ORFs for the EBNA3 family of proteins are tandemly arranged in the *Bam*HI E fragment of the viral genome and all three proteins share a number of structural features: hydrophilic N-terminus; a type divergent region; a region of short sequences rich in negatively and positively charged aa residues; and a proline rich C-terminus containing repeated polypeptide domains [44]. The genes encoding the three proteins are postulated to have arisen as a result of gene duplication during virus evolution [114, 115]. The type 1 and type 2 EBNA3A, B and C proteins share 84%, 80% and 72% aa sequence homology respectively, however, this does not appear to have any correlation with the transformation efficiency of the virus [116, 117]. The EBNA3 proteins are transcribed from either the Cp or Wp viral promoters. The ORFs for each of the EBNA3 genes consists of a unique, short 5' exon and a unique, long 3' exon (BERF1, BERF2b and BERF4) [117, 118]. Despite only a few copies of the EBNA3 mRNAs per cell, the stability of these proteins results in their intranuclear accumulation [115, 119, 120]. The EBNA3 proteins are highly immunogenic and provide a major target for CD8 positive cytotoxic T-lymphocytes (CTLs) [121, 122].

The EBNA3 family of proteins are believed to act predominantly as a set of transcriptional activators and repressors and similar to EBNA2, the EBNA3s can regulate the transcription of cellular and viral genes [123-125]. An important function for the EBNA3s is likely to be the modulation of EBNA2 and Notch activity through competition for the transcriptional repressor RBP-Jκ [126]. Consistent with this, over-expression of EBNA3A in an LCL results in dissociation of EBNA2 from RBP-Jκ and consequent down-regulation of CD21, CD23, cMyc and G0/G1 cell-cycle arrest [127]. EBNA3C expression in LCLs reduces DNA binding by RBP-Jκ and represses the viral Cp and LMP1 promoters in an RBP-Jκ dependent manner [123, 128]. EBNA3C and EBNA3A can also repress cellular gene promoters independently of

RBP-Jκ when tethered to DNA via the DNA binding domain of the yeast GAL4 protein [129, 130]. Additionally, the EBNA3 proteins have also been shown to modulate the expression of a number of cellular genes. EBNA3B expression in EBV negative DG75-BL caused an increase in the expression of vimetin, CD40 and Bcl-2, but a down-regulation in CD77 [131]. EBNA3C expression in EBV-negative BJAB B cell line induced an up-regulation of the EBV receptor, CD21 [132].

Only EBNA3A and EBNA3C are essential for transformation, and this may be attributed to the pro-survival properties of these two genes [133, 134]. However, a recent report suggests that EBNA3A deficient LCLs can be produced from EBNA3A deleted EBV but these cells are more sensitive to apoptosis and show reduced proliferation rates, suggesting that EBNA3A may be important but not critical for transformation [135]. The EBNA3s have been shown to have an anti-apoptotic function *in vitro*, with EBNA3A and EBNA3C capable of down-regulating the expression of the pro-apoptotic protein Bim [136]. Using a recombinant virus it has been shown that the proliferation and survival of LCLs is dependent on EBNA3A expression [137]. Accordingly, cell lines generated from Wp-restricted BL, which express the EBNA3s in the absence of EBNA2 or LMPs, are significantly more resistant to apoptosis than latency I BL cell lines [138], although the relative contributions of the EBNA3 proteins and the vBCL2 protein, BHRF1, which is also highly expressed in Wp BLs, remains unclear [139]. The pro-survival properties of the EBNA3s suggest that they may contribute to the oncogenicity of EBV infected cells. Indeed, EBNA3C has been reported to cooperate with oncogenic Ras in the transformation of rat embryo fibroblasts [140], and deregulates many cell cycle control pathways in EBV infected cells [141, 142].

1.6.5 LMP1

LMP1 is an integral membrane protein encoded by the BNLF1 ORF. LMP1 is localised to the cell membrane, often in lipid rafts. EBNA2 can transactivate the LMP1 promoter and this is dependent on the interaction of EBNA2 with RBP-Jκ and PU.1 [143]. LMP1 consists of three domains: a short N-terminal, cytoplasmic, hydrophilic domain, 17aa residues in length; a hydrophobic transmembrane (TM) domain constituting 6 α helical, hydrophobic TM loops. The TM domain enables self aggregation and is essential for the signalling of LMP1 [144]; a C-terminal cytoplasmic tail which mediates signalling. The C-terminus contains two transformation effector sites: TES1, which is essential for transformation, and TES2 which significantly enhances transformation [145-147]. The C-terminus can also be divided into two C-terminal activating regions, CTAR1 and CTAR2 which overlap with TES1 and TES2 respectively [148]. CTAR1 and CTAR2 associate with proteins such as tumour necrosis factor (TNF) associated factors (TRAFs) and TNF receptor associated death domain (TRADDs) to induce signalling through a number of pathways, particularly NfκB and AP-1 [146, 149].

LMP1 mimics a constitutively active CD40 receptor [150, 151]. This has been demonstrated using a mouse model where expression of LMP1 in CD40 null mice partially restored the wild type (wt) phenotype [152]. Additionally, a CD40-LMP1 fusion protein, where the cytoplasmic tail of LMP1 was fused to CD40, prevented constitutive activation of LMP1 and produced a LMP1 protein dependent on CD40L stimulated for activation. The LMP1 signalling induced *in vivo* by CD40L completely substituted for CD40 [153]. The functional redundancy between LMP1 and CD40 can also be demonstrated *in vitro*, using LCLs generated with a recombinant EBV with inducible LMP1 expression. Upon removal of LMP1 expression, the LCLs stop proliferating and this inhibition of proliferation can be reversed by

treating the LCLs with CD40L [154]. Furthermore, LMP1 and CD40 up-regulate a similar repertoire of activation associated antigens and adhesion molecules in B cells such as CD21, CD23, CD40 and ICAM1 [155].

LMP1 is essential for B cell transformation and proliferation; furthermore, it can transform rat fibroblasts *in vitro*, and its expression in mice predisposes them to lymphoma [145, 154, 156-160]. Thus, LMP1 can be classified as a viral oncogene. Conversely, high expression of LMP1 can induce growth inhibition and apoptosis [161, 162]. Therefore, regulation of LMP1 expression is critical to cell survival and proliferation, which may explain why three EBV encoded BART miRNAs can down-regulate the expression of this crucial gene [163].

The cellular signalling pathways modulated by LMP1 are essential for its transforming and oncogenic properties. The $p16^{INK4a}$-RB pathway plays a critical role in preventing inappropriate cell proliferation and is often targeted by viral oncoproteins. Unlike other DNA tumour virus oncoproteins, such as papillomavirus E7 or adenovirus E1 [164-166], LMP1 does not bind directly to pRb but instead inhibits the transcription of $p16^{INK4a}$ by preventing the nuclear import of its transcription factor, Ets2 [167, 168]. Activation of NFκB is predominantly mediated by CTAR2 and is important for the survival of EBV transformed cells as inhibition of NFκB causes apoptosis. CTAR2 is also important in LMP1 mediated activation of the c-Jun N-terminal kinase (JNK) pathway, leading to activation of AP-1. Signalling through CTAR1 and CTAR2 can also induce p38 mitogen-activated protein kinase (MAPK) activation and this activation is essential for LMP1's ability to transform Rat-1 cells [169].

1.6.6 LMP2A/B

The LMP2 gene encodes two isoforms termed LMP2A and LMP2B. These two proteins are transcribed from spatially distinct promoters, 3kb apart in the EBV genome [170-173]. The transcripts each have a unique first exon but then share 8 common exons which encode the 12 TM domains and the C-terminus. Exon 1 of LMP2A contains an N-terminal cytoplasmic domain which mediates the signalling functions of this protein. LMP2B lacks this exon and therefore the signalling capabilities attributed to it. The first exon of LMP2B is non-coding and translation initiates at a methionine codon within exon 2 [170].

LMP2A and LMP2B are not essential for transformation of primary B cells [174-178], however, there is conflicting evidence which suggests LMP2A expression may enhance transformation [179, 180]. LMP2A can provide B cells with pro-survival and anti-differentiation signals and can cooperate with LMP1 to enhance the B cell survival signalling mediated by NFκB [181, 182]. LMP2A has also been shown to protect BL and gastric carcinoma cell lines from TGF-β and BCR cross-linking induced apoptosis [183, 184]. Additionally, LMP2A expression enabled surface Ig negative B cells to escape from the bone marrow and colonise peripheral lymphoid organs [185, 186].

The N-terminal domain of LMP2A is able to interact with the B lymphocyte Src family tyrosine kinases, particularly Lyn [187, 188]. Binding of Lyn results in the phosphorylation of LMP2A, the recruitment of Syk and activation of PI3K, Btk, BLNK and Akt [189, 190]. The signalling through Lyn and Syk has been shown to repress B cell entry into the lytic cycle after cross-linking CD19, MHC class II or the BCR [191, 192]. This indicates that one of the functions of LMP2A in latent infection is to prevent lytic activation of the virus.

Relatively little is known regarding the function of LMP2B, partly due to the technical problems involved in studying this isoform, such as the absence of a specific antibody. It is presumed that LMP2B must function in a different manner from LMP2A due to the lack of the signalling N-terminal cytoplasmic domain. Evidence suggests that the main function of LMP2B may be to antagonise LMP2A. Over-expression of LMP2B resulted in the co-localisation of LMP2A and LMP2B and prevented LMP2A mediated inhibition of lytic reactivation after BCR cross-linking [193, 194]. Co-localisation of LMP2A and LMP2B at the plasma membrane has been shown to disrupt the self association of LMP2A [195], suggesting a direct role for LMP2B in the modulation of LMP2A function.

1.6.7 EBERS

The EBV encoded RNAs, EBER1 and EBER2, are small non-polyadenylated RNAs 166 and 172 nucleotides in length respectively. The genes encoding the EBERs are situated adjacent to one another in the BamHI C region of the genome. EBER1 is transcribed by RNA polymerases II and III, whereas EBER2 is transcribed exclusively by RNA polymerase III [196, 197]. The EBERs are the most abundant EBV RNAs in the cell but they are not expressed equally in LCLs, with EBER1 having an approximately 10 fold greater level of expression than EBER2 [196, 197]. The EBERs are located in the nucleus where they are found in ribonucleoprotein (RNP) particles complexed to cellular proteins [198]. Despite the fact that the EBERs only share 54% sequence homology, they are predicted to have similar secondary structures and therefore may function in a similar manner. The EBERs have similar primary and secondary structures to the Adenovirus non-coding viral associated (VA) RNAs and the cellular U6 RNAs, hence these RNAs may provide clues to the function of the EBERs [199, 200]. VA RNAs have been shown to enhance adenoviral lytic replication due to their ability to promote translation of viral mRNAs [201]. The EBERs can functionally substitute

for VA RNAs in late stage Adenovirus 5 lytic cycle [202, 203]. Like VA RNAs, the EBERs can bind to and inhibit the interferon induced protein kinase PKR *in vitro*, providing one possible mechanism thorough which EBERs may function [204].

The expression of EBERs in all forms of latency suggests an important role for these RNAs in transformation; nevertheless, recombinant viruses have clearly demonstrated that the EBERs are not essential for transformation. The data regarding the contribution of the EBERs to transformation is conflicting. Originally it was demonstrated using an EBER knockout EBV that loss of the EBERs had no effect on transformation efficiency or LCL phenotype [205]. However, subsequent reports revealed impaired growth rates and transformation efficiency with EBER knockout or EBER2-only knockout EBV [206, 207].

There is increasing evidence to suggest that the EBERs may contribute to the oncogenicity of EBV. EBER expression in EBV negative B cell lines has been reported to increase the malignant phenotype of cells by decreasing their susceptibility to apoptosis, up-regulating Bcl-2, and enabling the cell lines to cause tumours in severe combined immunodeficiency (SCID) mice [208-212]. Additionally, it has been proposed that the EBERs may offer a growth advantage to BL cells through their ability to up-regulated IL10, which may act as an autocrine growth factor and suppress cytotoxic T cells [213, 214].

1.6.8 EBV miRNAs

EBV has been reported to encode at least 42 viral miRNAs, although new miRNAs may still be discovered as prediction and detection methods improve. The first EBV miRNAs to be discovered were the three BHRF1 miRNAs (BHRF1-1, -2 and -3) which flank the BHRF1 ORF and these miRNAs are found exclusively in B cells with latency III or Wp-restricted types of latent infection and, in the case of miR-BHRF1-2 and miR-BHRF1-3 only, during

lytic cycle [215, 216]. The remaining EBV miRNAs are located within the introns of the BART region and are consequently called the BART miRNAs. The BART miRNAs are located in two clusters (cluster 1 and 2). The B95.8 strain of EBV has a deletion in the BART region, leaving only 5 of the BART miRNAs in the genome. This deletion has no apparent effect on the transformation efficiency of this strain, suggesting that most of the BART miRNAs are non-essential for transformation. Conversely, the high degree of evolutionary conservation between the miRNAs of EBV and those of the rhesus LCV indicate that they have an important function at some stage of the EBV life cycle.

There have only been a limited number of EBV miRNAs targets identified, hence the role of these miRNAs in transformation and the pathogenesis of EBV remains to be elucidated. The EBV miRNA targets which have been identified include cellular as well as viral targets, indicating a potential for these miRNAs to control a wide variety of processes. The viral targets of the EBV miRNAs include LMP1, LMP2A and the EBV lytic cycle polymerase BALF5 [163, 217, 218]. Interestingly, BALF5 is targeted by miR-BART-2 which is located in an anti-sense position relative to the 3'UTR of BALF5 and represents the first example of a viral antisense miRNA [219]. An antisense miRNA will have 100% complimentarity to its target gene and will therefore cause mRNA cleavage in a siRNA-like mechanism, which is believed to a very efficient method of gene repression.

Two cellular gene targets of EBV miRNAs have so far been identified: p53 up-regulated modulator of apoptosis (PUMA) and CXCL11, an interferon (IFN)-inducible T cell attracting chemokine, targeted by miR-BART-5 and miR-BHRF1-3 respectively [220, 221]. Although the data is still limited, a role for EBV miRNAs in protecting EBV infected cells from apoptosis and evading immune surveillance is beginning to emerge. This strongly suggests that EBV miRNAs may contribute to the pathogenesis of the virus. This is supported by the

high expression of a number of the EBV miRNAs found in primary tumour biopsies including NPC, BL, PEL, gastric carcinoma and AIDS-DLBCL [220, 222-224].

1.6.9 Other EBV latent transcripts

1.6.9.1 BARTs

The BARTs are a family of differentially spliced complimentary-strand transcripts which encode mRNAs for putative RK-BARF0, A73 and RPMS1 proteins. The introns from this region also encode a number of miRNAs, as discussed in section 1.6.8, and these miRNAs are currently the main known function of the BARTs. Recombinant virus experiments have shown that the BARTs are not essential for transformation and the BART coding region in the B95.8 strain of EBV has a deletion of the BART coding region without any apparent function consequence on transformation. The BART transcripts are expressed in all forms of latent and lytic infection and have been detected in tumour samples but their function remains elusive [51, 225-230]. Although the BARTs encode mRNAs, the proteins produced from these mRNAs have not been detected in EBV infected cells *in vivo* or *in vitro* [231]. Despite this, experimentally generated proteins from these mRNAs have been implicated in the modulation of numerous cellular pathways including notch signalling [230]. The significance of these interactions cannot be evaluated until the expression of these proteins in EBV infected cells has been determined.

1.6.9.2 BHRF1 and BALF1

BHRF1 and BALF1 are both homologues of the anti-apoptotic protein Bcl-2 [232-234]. They are also both lytic cycle antigens, however, the expression of either BHRF1 or BALF1 has also been reported in NPC, BL cell lines and LCLs [139, 233]. Using recombinant viruses it

has been demonstrated that BHRF1 is not essential for transformation [235, 236], yet inactivation of both BHRF1 and BALF1 eliminates the transforming capacities of EBV [237]. This suggests that there is a degree of functional redundancy between these two Bcl-2 homologues and highlights the potential importance of anti-apoptotic stimuli during the transformation of primary B cells.

1.7 EBV lytic cycle

The EBV lytic life cycle can be sub-divided into immediate early (IE), early and late lytic cycle by the expression of distinct lytic genes. BZLF1 and BRLF1 are two IE lytic proteins which function as transcription factors, activating their own, and one another's promoters (Zp and Rp) to amplify lytic induction stimuli [238-242]. The BZLF1 protein is a viral homolog of c-Jun and c-Fos, and activates expression of early lytic genes through binding to AP-1 or Z-response elements (ZRE) [243, 244]. The lytic cycle is believed to be induced *in vivo* when plasma cells encounter antigen and differentiate [245]. *In vitro,* the lytic cycle can be induced in an EBV positive cell line by a range of stimuli, including surface Ig cross-linking, phorbol ester simulation, sodium butyrate treatment or in some instances, ectopic BZLF1 expression [246-250].

Early lytic cycle genes are defined as those which are transcribed prior to viral DNA replication and include the EBV DNA polymerase, BALF5, and the major DNA binding protein, BALF2 [251, 252]. The expression of early lytic cycle proteins results in the replication of EBV genomes by rolling circle replication [253]. During early lytic cycle, EBV also expresses the Bcl-2 structural homologs, BHRF1 and BALF [234, 254, 255]. The expression of these genes during early lytic cycle is thought to protect cells from apoptosis during the lytic cycle *in vivo* [237, 255].

Late lytic cycle genes are expressed following viral DNA replication and mainly include viral structural proteins, such as capsid proteins, glycoproteins, and tegument proteins [251, 252]. The few non-structural genes expressed during late lytic cycle include BCRF1 which encodes for viral IL10, providing growth and survival signals to the cell [256-260]. In addition, IL10 has been shown to aid immune evasion by negatively regulating macrophage, T cell and NK cell function [261-264]; this is important for EBV lytic replication as many viral lytic genes are highly immunogenic [265-267].

1.8 EBV infection and persistence *in vivo*

Primary EBV infection generally occurs at an early age and is usually asymptomatic. However, if EBV infection occurs in adolescence or later, it can result in IM [268-270]. Primary EBV infection and lytic replication occurs within the B cells of the oropharynx [271, 272]. These infected tonsillar B cells in IM patients are heterogeneous in both morphology and viral antigen expression [273, 274]. Most cells express a latency III pattern of EBV gene expression, however, 2 smaller subsets of infected cells have been identified: one, which expresses only EBNA2 in the absence of LMP1 and a second group which displays a HL-morphology and expresses LMP1 in absence of EBNA2 [275]. Virus replication in the oropharynx and virus-driven proliferation and expansion of the B cell pool elicits a strong CTL response. Large numbers of $CD8^+$ CTLs are produced to bring the infection under control, leading to hyperactivation of the immune response and the symptoms of IM [276, 277]. Despite the strong CTL response, EBV is not completely cleared and EBV remains detectable as infectious virus in throat washings and as a latent infection in the B cell pool [278, 279].

EBV persists *in vivo* within memory B cells in a very restricted form of viral latency termed latency 0 (section 1.5.4), where only the EBERs and BARTs may be expressed [280]. The mechanism by which EBV gains entry into the memory B cell pool is still controversial but the most widely accepted model is the GC model (figure 1.3). The GC model predicts that

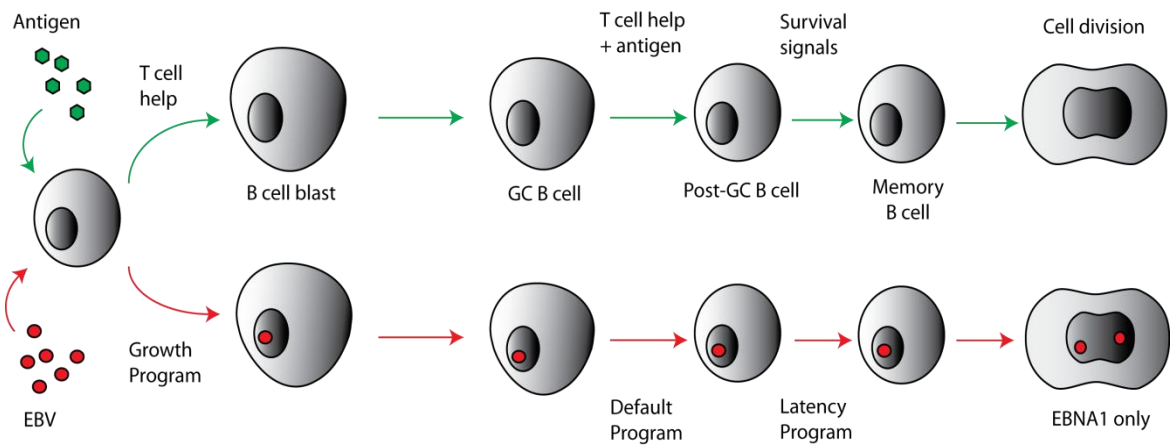


Figure 1.3: Diagram representing the germinal centre model of EBV persistence *in vivo*. This figure is adapted from Thorley-Lawson 2004 [281].

EBV infection of naïve B cells will drive the B cells to differentiate, mirroring the activation of naïve B cells by antigen [281]. Initial infection will result in expression of EBV's growth transforming programme (latency III) which will drive the naïve B cells to differentiate into GC B cells. As the B cell differentiates, EBV latent gene expression is down-modulated, with GC B cells expressing a latency II pattern of gene expression (called the default program). The LMPs then mimic the signals a B cell would normally receive in a GC, allowing the infected GC B cell to differentiate into a memory B cell, where EBV gene expression is further down-modulated to latency 0, also called the 'latency program' [281]. Thereafter, only when the memory B cells divide will EBNA1 be expressed to allow maintenance of the viral

episome. This model is based upon the observations of EBV gene expression found *in vivo* [58, 61, 62, 280, 282-286], and the similarity between antigen-activated B cell blasts and EBV infected B cell blasts [155, 287]. However, the mechanism by which EBV may drive naïve B cell differentiation is not known.

The alternative model for EBV infection and persistence *in vivo* is the direct infection model, where memory B cells are predicted to be directly infected by EBV, with no participation in the GC reaction [274, 275]. The direct infection model is supported by the persistence of EBV in X-linked lymphoproliferative (XLP) patients (these patients cannot form functional GCs), indicating that EBV can colonise a host without a need to transit a GC reaction [288]. Both models predict that once latently infected, the memory B cells have the potential to differentiate in response to antigen to produce plasma cells. These plasma cells are the site for vial lytic replication and they tend to localise near mucosal surfaces such as the oropharynx [36, 289]. Only a minority of plasma cells complete the lytic cycle and this occasional lytic replication and subsequent infection of new B cells serves to replenish the reservoir of infected B cells and maintain a lifelong EBV infection [245, 282].

1.9 EBV associated B cell malignancies

1.9.1 PTLD

Immunosuppression of transplant recipients to prevent graft versus host disease can cause the development of post-transplant lymphproliferative disorder (PTLD). PTLD represents a group of heterogeneous, abnormal B cell proliferations which are classified into three histological types: i) early lesions, which are usually of polyclonal or oligoclonal origin and are consistently EBV positive; ii) polymorphic lesions which may be polyclonal or monoclonal in

origin and are almost always EBV positive; iii) monomorphic lesions, these are monoclonal in origin and the most common subtype is diffuse large B cell lymphoma. (reviewed in [290]). In PTLD tumour biopsy material, EBV positive cells often only constitute a modest proportion of the tumour mass, with an extensive lymphoid infiltrate [291]. There is evidence to suggest that this infiltrate is necessary for the establishment of these tumours, possibly by supplying essential growth factors [292].

PTLD incidence varies with different types of organ transplant, with intestinal and heart/lung being the most common and bone marrow transplants being the least common [293]. Greater than 50% of PTLDs are associated with primary infection, therefore EBV seronegativity of the recipient is a risk factor for the development of PTLD; this may explain why paediatric recipients are at a greater risk of developing PTLD than adults [294, 295]. The onset of PTLD in an EBV negative recipient is usually within the first year post-transplant and these lesions respond well to withdrawal of immunosuppression [296, 297]. The onset of PTLD in EBV positive recipients is usually >1 year post-transplant and can be many years later [298].

Most early onset PTLD lesions display a latency III pattern of EBV gene expression, consistent with the outgrowth of growth transformed B cells in the absence of a cytotoxic T lymphocyte (CTL) response. Interestingly, antibody staining of PTLD sections has revealed a degree of heterogeneity in the EBV latent gene expression, similar to the varied EBV expression seen in the B cells of IM-tonsils [282].

Late onset PTLDs present with a much greater heterogeneity in EBV gene expression, with latency I and II and III patterns of gene expression all reported. Furthermore, the development of monomorphic lesions is likely to involve additional genetic changes and this is supported by the detection of mutations in p53, c-myc, N-ras and bcl-6 in tumour biopsies [299-303].

These cellular gene expression changes may explain the greater heterogeneity of EBV gene expression seen in late onset and monomorphic tumours, as they may no longer require the EBV latent antigens to drive proliferation and protect from apoptosis.

The treatment of PTLD usually involves the reduction of immunosuppression in combination with the monoclonal CD20 antibody drug rituximab or antiviral drugs which prevent lytic replication such as acyclovir. However, the efficacy of acyclovir is limited as EBV is usually present in a latent rather than a lytic state. If the reduction of immunosuppression fails then irradiation, chemotherapy and surgical resection can be used [304]. Additionally, T cell immunotherapy directed against EBV antigens is a very effective treatment for PTLD, is non-toxic to the patient and carries a lower risk of damage or loss of the transplanted organ [305, 306]. However, T cell immunotherapy is not a widely used treatment for PTLD, primarily due to the cost of providing such a tailored treatment.

1.9.2 Hodgkin's Lymphoma

There are two subtypes of HL, classical HL (cHL) and nodular lymphocyte-predominant HL (NLPHL), the latter of which only accounts for approximately 5% of HL. NLPHL is rarely associated with EBV, whereas cHL is associated with EBV in approximately 40% of cases in the west, although this can be up to 90% in Central and South America [307, 308]. HL is one of the most frequent cancers is the western world with an incidence of approximately 3 per 100,000 per year [308]. The tumours of cHL are characterised by the mononucleated Hodgkin cells and the unusual, multi-nucleated Reed-Sternberg cells (HRS cells). The HRS cells are only a small percentage of the tumour mass (~1%) with the remaining mass being comprised of reactive infiltrate. There is evidence to suggest that this reactive infiltrate is not only attracted by the HRS cells, but also necessary for their survival [309-312].

HRS cells are believed to be of B cell origin and are characterised by a dramatic down-regulation of B cell markers which originally hindered the identification of the cellular origin of HRS cells. Sequencing revealed that the HRS cells contained rearranged and somatically mutated immunoglobulin variable (IgV) genes, with some cells showing clearly destructive mutations which ought to have caused apoptosis in the germinal centre [313]. This led to the hypothesis that HRS cells originate from crippled germinal centre (GC) cells which somehow escape apoptosis [314]. Indeed, one of the postulated roles for EBV in the development of HL is to rescue germinal centre cells with crippling IgG mutations from apoptosis. EBV expresses a latency II pattern of gene expression in HRS cells and it is likely to be to the LMPs which are responsible for the rescue of GC B cells from apoptosis [53, 273, 315, 316]. Signalling through the CD40 and the BCR are two major physiological survival and selection signals for GC B cells [317]. As discussed in sections 1.6.5 and 1.6.6, LMP1 can mimic many of the signals normally supplied by an activated CD40 receptor and LMP2A can deliver BCR-like survival signals [152, 185]. This hypothesis is supported by two observations: firstly, comparison of clonal IgV region sequences in HL cases with the EBV status of the tumour revealed that almost all tumours with crippling mutations were EBV positive [318]; secondly, EBV can rescue GC B cells with crippling mutations from apoptosis *in vitro*.[319-321].

1.9.3 Burkitt Lymphoma

The form of BL originally described by Denis Burkitt in 1958 is now called endemic BL (eBL) and it occurs with high incidence in areas of equatorial Africa and Papua New Guinea where malaria is holoendemic [1, 2]. eBL has almost a 100% association with EBV and each tumour cell carries monoclonal EBV episomes [3]. In contrast, sporadic BL, which is a histologically identical tumour, occurs at lower incidence in other parts of the world; this is also a paediatric tumour although it presents more frequently in the abdomen rather than the

jaw. The association of sporadic BL with EBV varies with location and ranges from 15-85% [322]. AIDS-associated BL was discovered in the 1980's and has identical histology and similar sites of presentation as the sporadic form, however, it only presents in HIV positive adults. The association of AIDS-associated BL with EBV is approximately 30-40% and the lymphoma usually presents early in disease progression when patients are still relatively immunocompetent [323].

BL is characterised by a c-myc translocation to the Ig locus were it is constitutively expressed. The most common translocation which occurs is to the heavy chain Ig locus (8:14) and this is found in approximately 80% of patients. The alternative translocation is termed the 'variant' translocation and places c-myc under the control of the Ig_L, κ or γ loci [322, 324]. c-myc is a proto-oncogene and a transcriptional regulator involved in proliferation, differentiation and apoptosis [325]. It is c-myc which drives the proliferation of BL cells, however, it also makes the cells sensitive to apoptosis [326-329]. It is this sensitivity to apoptosis which led to the hypothesis that EBV is required to counteract the sensitivity to apoptosis induced by constitutive c-myc expression. EBV gene expression in BL is typically latency I [51, 59], therefore the essential EBV encoded growth-transforming proteins are absent. It is unlikely that EBV, in such a restricted form of latency, is contributing substantially to the proliferation of the BL cells, thus, an anti-apoptotic role is more likely. This is supported by the observation that EBV loss clones are more sensitive to apoptosis than EBV positive clones [330]. Additionally, EBV loss clones are more radily generated from late passage BL cell lines (>2 years old) than early passage lines, indicating that cellular gene expression changes are required to enable the cells to tolerate loss of the virus. As discussed in sections 1.6.1 and 1.6.7, EBER expression in BL has been associated with apoptosis resistance and EBNA1 has been implicated in the destabilisation of p53 [81, 213].

1.10 Growth transformation of B cell *in vitro*

Infection of resting B cells *in vitro* with EBV results in the growth transformation of the resting B cell into proliferating B cell blasts (LCLs) which phenotypically resemble B cells proliferating in response to antigen. Infection of B cells *in vitro* to produce LCLs has been a valuable model for studying the transforming capacities of the virus for many years.

1.10.1 Primary infection of B cells *in vitro*

Infection of B cells involves the binding of EBV, via its most abundant glycoprotein, gp350, to the C3d complement receptor, CD21, at the B cell suface [35, 331]. Thereafter, a second EBV glycoprotein gp42 forms part of a trimeric complex with gp85/gp25 and binds HLA class II, initiating viral fusion with the cell membrane [332-334]. EBV binding to CD21 can activate resting B cells and this may be important for subsequent viral gene expression. For example, CD21 signalling in B cells has been shown to induce NFκB activation and IL6 production in a protein kinase C dependent manner [335-339].

EBV episomes appear within the nuclei of infected B cells approximately 16 hours post infection [47]. The Wp promoter initiates transcription and through differential splicing produces EBNA2 and EBNA-LP, which are detectable 12-24 hours post-infection [47, 340, 341]. EBNA-LP enhances the transcriptional changes initiated by EBNA2 which causes an up-regulation of the B cell activation marker CD23 and a G0-G1 cell-cycle transition, as marked by the induction of cyclin D2. In addition, EBNA2 activates Cp which consequently becomes the dominant promoter for EBNA transcription 24-48 hours post-infection, whilst Wp-initiated transcription declines [47, 341-343]. The Cp generated mRNA transcripts are highly spliced; via alternative RNA splicing these Cp transcripts can produce expression of all the EBNAs: 1, 2, -LP, 3A, 3B and 3C [63, 344] (figure 1.4, adapted from Qu and Rowe 1992

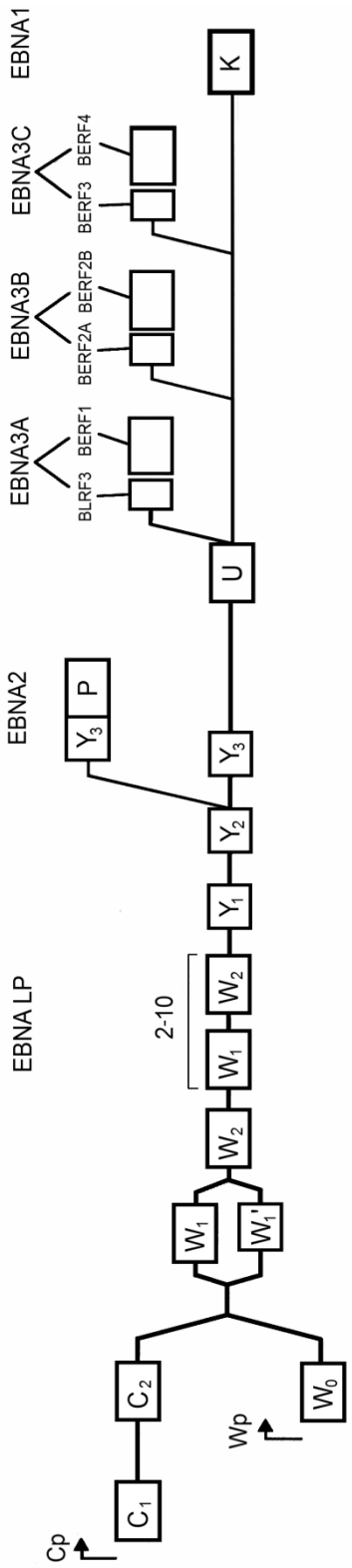


Figure 1.4: Diagram showing the differential splicing of EBV latent and viral transcripts in an EBV transformed B lymphocyte. This figure is adapted from Qu and Rowe 1992. Open boxes represent ORFs and arrows represent the viral promoters Cp, Wp.

[345]). It has been reported that EBNA1 binding to oriP also enhances Cp mediated transcription of the EBNAs [69]. Conversely, the EBNA3s may inhibit EBNA2 mediated transactivation [126]. EBNA2 and EBNA-LP also activate the LMP promoters, with LMP1 expression rising approximately 5 days post-infection [341, 346].

1.10.2 Growth transformation

The growth transforming programme of EBV (latency III) involves the expression of 9 latent genes, only 4 of which are essential for transformation: LMP1, EBNA3C, EBNA3A and EBNA2 [82, 133, 159, 160]. An additional two latent genes are important for growth transformation: EBNA1 and EBNA-LP. EBNA1 is non-essential for growth transformation but is essential for maintenance of the EBV genome in the proliferating blasts [76]. EBNA-LP is also non-essential for transformation, although the transformation efficiency is significantly reduced in B cells infected with an EBNA-LP mutant EBV relative to wild type virus [101]. The latent proteins of EBV produce changes in cellular gene expression which enable progression through the cell-cycle and increase cell survival (see section 1.6). Cellular gene expression changes are essential for transformation as EBV hijacks the cells normal signalling pathways to mimic antigen stimulation, thus producing a latently infected proliferating B cell blast (reviewed [347]). These cellular gene expression changes include the modulation of well characterised tumour suppressor genes and oncogenes, such as MDM2/p53, Bcl2 and Bim [81, 136]. The modulation of cellular genes during transformation which are known to contribute to the development of cancer may predispose the B cells to further genetic alterations, allowing the progression from transformed B blast to tumour cell.

The process of transformation is distinct from the process of immortalisation. Early stage passage LCLs are unable to form colonies in soft agar or produce tumours in nude mice.

However, late passage LCLs (typically passage >180) which have survived a so called 'proliferative crisis' are able to form colonies in soft agar, produce tumours in nude mice, show high expression of telomerase and have genetic aberrations [348]. Therefore, the process of transformation by EBV is not sufficient to produce a cell line with a tumourogenic phenotype, further cellular gene expression changes are required. This point is supported by the fact that some LCLs do not survive the proliferative crisis, presumably because they have not attained the cellular gene expression changes necessary for immortalisation.

Transformation *in vivo* is likely to occur during primary infection as patients with IM show detectable latency III lymphoblastoid like cells in lymphoid tissues [275]. Patients with early stage PTLD also have detectable latency III lymphoblastoid B cells in the lymphoid tissue [349]. Late stage PTLD, which often presents with a much more pronounced tumour phenotype, is associated with tumours which contain genetic aberrations. It is therefore likely that early passage LCLs are a good model for B cells during primary infection (IM) and early stage PTLD, whilst late passage LCLs more closely resemble late stage PTLD.

It is clear that EBV latent gene expression is not sufficient to produce LCLs with full tumourogenic potential, despite the fact that LMP1 alone can transform rat fibroblasts. However, the cellular gene expression changes which are required for transformation are not fully characterised and the link between transformation and immortalisation of LCLs is not understood. The profiling of cellular gene expression changes which occur during transformation will provide insight into the mechanism of EBV induced growth transformation. These gene expression changes may also be important for EBV associated tumourogenesis, pre-disposing the B cells to further genetic aberrations which enable immortalisation to occur. miRNAs are post-transcriptional regulators of gene expression and the extent to which EBV modulates the expression of the host cellular miRNA system was not

known at the outset of this thesis. The relationship between EBV and cellular miRNA expression during EBV-mediated growth transformation of resting B cells may prove to be a novel method which EBV utilises to alter cellular gene expression during transformation and tumourogenesis.

1.11 The discovery of microRNAs

miRNAs are members of the RNA interference pathway (RNAi) which mediate post-transcriptional gene regulation of target genes, and were first identified in Caenorhabditisis elegans (C. elegans) by Rosalin Lee et al and Bruce Wightman et al in 1993 [350, 351]. The lin-4 gene had previously been shown to control the protein expression of LIN-14 post-transcriptionally and the 3'UTR of lin-14 was also shown to be necessary for this negative regulation [352]. Cloning of the C.elegans lin-4 gene revealed that it was non-protein coding, although it did contain two RNAs (one 61nt and the other 21nt in length), which both contained a sequence complimentary to a repeat sequence in the lin-14 3'UTR [350]. A reporter gene containing the section of the lin-14 3'UTR with complimentary binding sites for the lin-4 RNAs revealed that the lin-4 RNAs were able to repress protein expression from these sites [351]. These experiments were the first examples of miRNA expression and gave the first insights into miRNA mode of action. However, it was not until the year 2000 when Reinhart et al [353] discovered let-7a, whose expression was conserved from worms to mammals [354], that the importance of miRNAs was realised.

1.12 The biogenesis of miRNAs

miRNAs are predominantly transcribed in the nucleus by RNA polymerase II as a large capped and polyadenylated primary transcript termed a pri-miRNA [355, 356]; however, rare

instances of miRNAs transcribed by RNA polymerase III have been reported [357]. The majority of miRNAs are localised in intergenic regions, although approximately 25% are localised in known protein coding genes either within introns or exons [358]. Pri-miRNAs can be transcribed from their own promoters [355, 356] and are often located in close proximity where they are transcribed as single polycistronic primary transcripts, also termed 'pri-miRNA clusters' [358, 359].

pri-miRNAs are processed in the nucleus by the RNase III enzyme, Drosha, to form an approximately 80nt long stem-loop containing transcript termed a pre-miRNA [360]. Drosha forms a complex, often termed the 'microprocessor complex', with the DiGeorge syndrome critical region gene 8 (DGCR8) protein [361-363]. DGCR8 is believed to enhance Drosha substrate recognition [362]; the double-stranded stem and 3' overhang, but not the hairpin loop, are critical for pre-miRNA recognition [364, 365]. Drosha processing of intronic pri-miRNAs occurs co-transcriptionally and does not inhibit RNA splicing, as a continuous intron is not required for splicing [366, 367]. However, the regulation of exonic pri-miRNA Drosha processing remains to be determined. Interestingly, splicing can substitute for Drosha processing in a small number of instances if the miRNAs released by splicing are an appropriate size to form a hairpin resembling a pre-miRNA. These miRNAs which can circumvent Drosha processing are called 'mirtrons', and following nuclear export by Exportin 5, they are processed normally in the cytoplasm by Dicer-1 [368-370].

The pre-miRNA is exported from the nucleus to the cytoplasm by Exportin 5 in a complex with Ran-GTP [371]. A RNA stem of >16 bases and a short 3' overhang are the structural requirements for Exportin 5 mediated pre-miRNA recognition [372, 373]. The mechanism regulating the export of the pre-miRNAs from the nucleus remains to be elucidated, and

whether or not nuclear export is used by a cell as a means of controlling mature miRNA expression is still debated [374-376].

Once in the cytoplasm, pre-miRNAs are taken up by the RISC loading complex (RLC) composed of, the RNase III enzyme Dicer-1; the double stranded RNA domain binding proteins Tar RNA binding protein (TRBP) and protein activator of PKR (PACT); and the argonaute-2 (Ago-2) protein [377-380]. TRBP and PACT are non-essential for Dicer-1 mediated pre-miRNA cleavage but they facilitate miRNA processing by stabilising Dicer-1 and recruiting Ago-2 [379, 381].

Once the RLC has formed, the pre-miRNA is bound and processed by one of two possible mechanisms: (i) the pre-miRNA is cleaved by Dicer-1 to produce the mature miRNA duplex, which is subsequently loaded into the RISC complex (figure 1.5), or (ii) the pre-miRNA is cleaved by Ago-2 to produce an Ago2-cleaved-pre-miRNA (ac-pre-miRNA). ac-pre-miRNAs contain a nick in the 3'arm of the hairpin which is postulated to facilitate passenger strand dissociation of 5' miRNAs which do not contain mis-match regions in the centre of the miRNA duplex. The ac-pre-miRNAs are then processed by Dicer-1 to produce the mature miRNA duplex, which is subsequently loaded into the RSC complex, as in (i) [382].

Dicer-1 cleaves the hairpin loop of the pre-miRNA or ac-pre-miRNA to produce an approximately 22nt long mature miRNA duplex with a 2nt 3' overhang [383-386]. Unlike Drosha, Dicer-1 was believed to be essential for the biogenesis of all miRNAs [387, 388]; however, recent work by Bosse et al [389] suggests that a subset of miRNAs may be cleaved in a Ago-2-dependent, Dicer-1-independent manner.

Once the pre-miRNA has been cleaved by Dicer-1, the RLC dissociates and the mature miRNA duplex is unwound by a helicase and loaded into the miRNA RNA-induced silencing

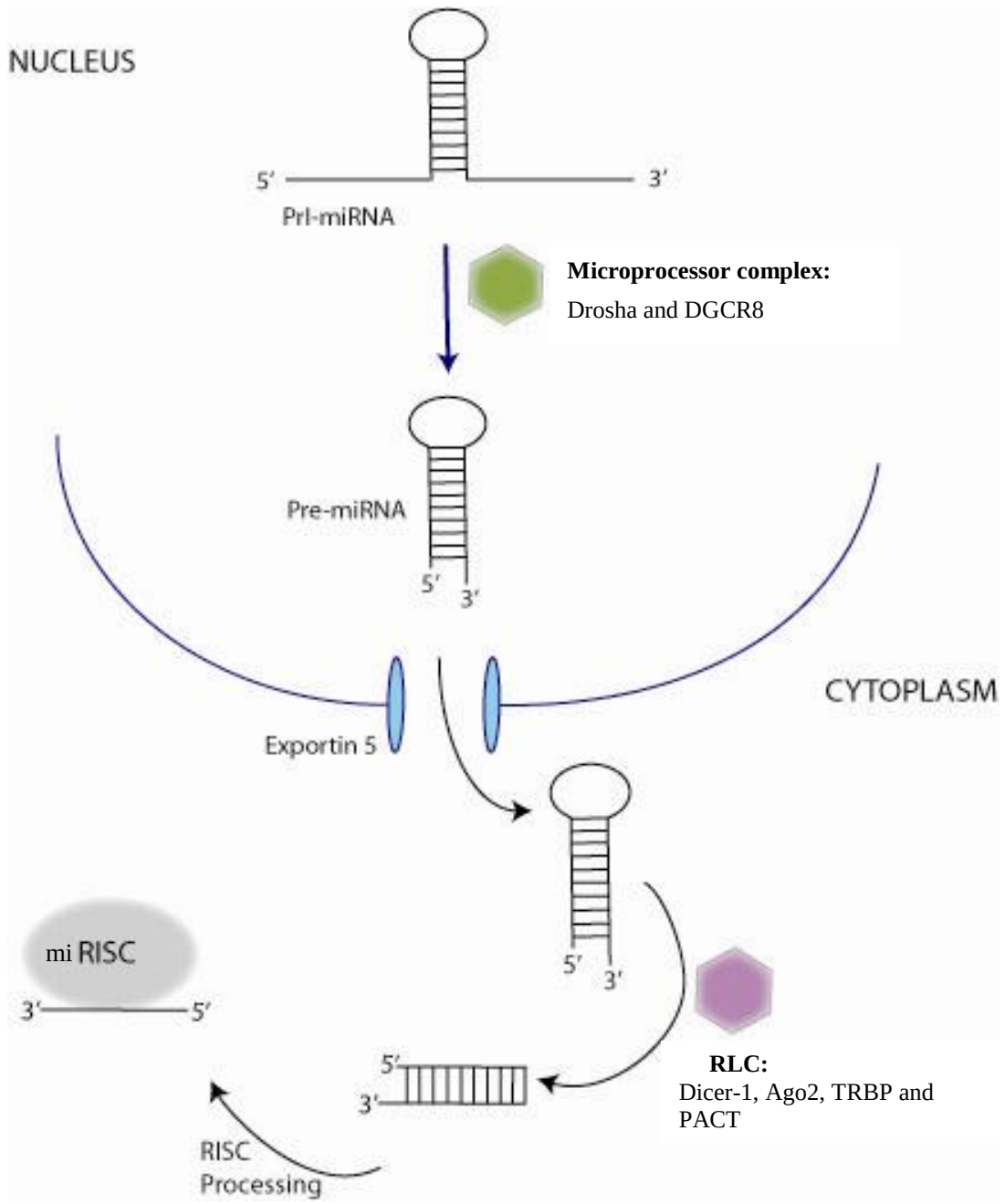


Figure 1.5: Simplififed diagram representing miRNA biogenesis

complex (miRSC). Multiple helicases have been associated with the miRNA biogenesis machinery. However, whether one universal helicase or multiple helicases are responsible for miRNA unwinding is still not clear [390-393]. Which strand of the miRNA duplex is selected for miRISC loading (the guide strand), and which strand is degraded (the passenger strand), is thought to be determined by the asymmetry of the miRNA duplex. The miRNA with the least stable base pair at its 5' end is usually the guide strand incorporated into the miRISC [394, 395]. The selection of the guide strand is not 100% efficient, and low levels of the passenger strand can be detected in the miRISC, the passenger strand miRNAs are termed the minor miRNA (miRNA*) species [396]. It is not yet clear whether miRNA* species contribute to gene regulation or simply represent an irrelevant by-product of miRNA processing [396, 397].

1.13 Regulation of miRNA biogenesis

The expression of pri-miRNA and mature miRNA transcripts have often been observed to show no correlation *in vitro* or *in vivo* [375, 398-401]. This led to the hypothesis that the processing of miRNAs could be regulated as a means of controlling mature miRNA expression and function [359, 399]. The control of miRNA processing has been a rapidly expanding field throughout the course of this research and has revealed that miRNA post-transcriptional regulation is an important determinant of miRNA function.

Numerous methods can be employed to modulate the miRNA biogenesis pathway, and the most significant three methods will be described briefly: (i) regulation of Drosha and Dicer-1 expression; (ii) cellular localisation and; (iii) ADAR editing.

(i) Drosha and Dicer-1 expression: The biogenesis of miRNAs can be affected by the expression of the two RNase III enzymes responsible for miRNA processing. Mouse models

have demonstrated that a loss or increase of either Drosha or Dicer-1 expression, results in a corresponding, non-specific increase or decrease in global miRNA expression respectively [402]. A global decrease in miRNA expression is associated with cancer and has been shown to enhance tumour development [398, 402]; moreover, the expression of Dicer-1 and Drosha has been reported to be altered in several cancers [403, 404]. In addition to expression regulation, the processing efficiency of Drosha has been shown to be enhanced by p53 binding. In many cancers, mutations are commonly found in the transcriptional domain of p53, which have been shown to reduce or prevent the ability of p53 to enhance the processing of pri-miRNAs. This is postulated to be another tumour suppressive function of p53 [405], and may represent another mechanism by which miRNA expression is regulated post-transcriptionally.

(ii) Sub-cellular localisation: miRNAs are processed initially by Drosha in the nucleus, followed by Dicer-1 in the cytoplasm; thus, for miRNA processing to occur, the pri-miRNA and pre-miRNA transcripts need to be localised in the nucleus and cytoplasm respectively. The sub-cellular localisation of miRNA precursors has not been extensively examined. Nevertheless, isolated reports have observed certain pri-miRNAs predominantly located in the cytoplasmic, rather than the nuclear cell fraction [406, 407], where they will not have access to Drosha for processing. Pre-miRNAs have also been shown to accumulate in the nucleus where they cannot be further processed by Dicer-1, although the cause of the pre-miRNA accumulation remains to be determined [398]. Collectively, these observations suggest that the sub-cellular localisation of miRNA precursors may be a method by which mature miRNA expression is modulated; this is significant because it may partially explain the tissue specific expression of selected miRNAs observed *in vivo* [408-410]. Furthermore, it is not only miRNA processing which may be modulated by sub-cellular localisation. Mature miR-29b

has been shown to contain a hexanucleotide motif at its 3' terminus, which acts as a NLS, causing an accumulation of miR-29b in the nucleus. This raises the possibility that the localisation of mature miRNAs may also be regulated as a means of controlling miRNA function [411].

(iii) ADAR editing: The enzyme, adenosine deaminase acting on RNA (ADAR), promotes the hydrolytic deamination of adenosine residues to inosine on dsRNA substrates [412, 413]. ADAR editing of pri-miRNAs and pre-miRNAs has been demonstrated *in vivo* [414, 415]. ADAR editing can alter the secondary structure of pri-miRNAs and pre-miRNAs [416], which is essential for Drosha and Dicer-1 recognition, and therefore subsequent miRNA processing. Indeed, ADAR editing has been shown to prevent Drosha or Dicer-1 recognition and processing of various miRNAs [416, 417]. Additionally, ADAR editing of pre-miR-367 was shown to be specifically localised to the proximal 5' 'seed' region of the miRNA. This resulted in altered miR-367 target recognition, demonstrating for the first time that ADAR editing can also affect the target specificity of miRNAs [418].

A picture is emerging of a highly complex system whereby the expression of miRNAs can be regulated by a host of cellular factors and conditions. A mechanism of miRNA transfer from one cell to another via exosomes has also recently been proposed, providing another possible mechanism whereby mature miRNA expression could be controlled [419-421]. Additional mechanisms by which miRNA biogenesis may be regulated include miRNA sequestration [422], miRNA biogenesis inhibitors [423] and even control by the cytoskeleton [424]. These mechanisms are currently less well characterised, but they demonstrate the complexity of the miRNA expression system and how limited our current knowledge still is.

1.14 Mode of action of miRNAs

Following transcription, mRNAs are assembled into messenger ribonucleoprotein (mRNP) particles and exported into the cytoplasm for translation. The fate of these mRNAs is often determined by cis-acting elements in the 3'UTR of the mRNA, such as miRNA binding sites and AU rich elements, which can respond to the requirements of the cell [425, 426]. Delayed translation results in mRNA accumulation in cytoplasmic processing bodies (P-bodies), which are also the site of mRNA degradation [427, 428]. Whether a mRNA is degraded or released for translation is believed to be governed, at least in part, by the cis-acting elements in the mRNAs 3'UTR [429, 430]. The role of miRNAs in the control of mRNA fate has been the focus of much of the current research in the field, and clues to the mechanism of miRNA mediated gene silencing are emerging.

miRNA inhibition of gene expression is mediated by the miRISC, which is primarily composed of Ago proteins and the mature miRNA [431-433]. Ago proteins are ubiquitously expressed proteins, defined by their piwi-argonaute-zwille (PAZ) and PIWI domains which bind miRNAs at the 3' and 5' terminus respectively [434, 435]. There are 8 Ago genes in humans, which encode 4 Ago proteins (Ago 1-4) and 4 PIWI proteins [436]. All four of the Ago proteins mediate translational repression, whereas only the Ago-2 protein contains intrinsic slicer activity [432, 437-439].

mRNA cleavage is believed to only be initiated when there is perfect complementarity between a miRNA and its mRNA target [440, 441]. This is exemplified by plants where, unlike mammals, miRNAs typically have 100% complementarity with target mRNAs and induce Ago-2 mediated mRNA cleavage and degradation [442-444]. However, perfect complementarity between a miRNA and its target mRNA is not always sufficient to induce

mRNA cleavage, suggesting that there may be additional requirements for a miRISC to catalyse cleavage [445]. miRNAs in mammals typically show imperfect complementarity to target mRNAs and they therefore function primarily through other mechanisms of translational repression.

miRNA mediated gene repression and the mechanisms which regulate it are poorly understood. Recent research has revealed some mechanistic detail; however, most models are still contested, with no preferred model yet being identified. It is clear that miRNAs can induce translational repression, mRNA destabilisation or both. It was originally thought that translational repression was the primary mode of action of miRNAs, but more recent work has suggested that miRNA induced mRNA destabilisation is also important for miRNA mediated gene repression in mammals [446].

(ii) Translational repression: The majority of literature regarding the mode of miRNA mediated translation repression has focused on determining whether inhibition occurs during translational initiation or post-translational initiation. Although there is currently greater evidence to suggest that inhibition occurs at the stage of translation initiation, a mechanism whereby miRNAs mediate inhibition post-initiation, instead of or as well as at the stage of initiation, cannot be discounted.

miRNAs cannot mediate translational inhibition of target mRNAs containing an IRES, suggesting that translational inhibition is inhibited in a 5’cap dependent manner [447]. Further studies have also demonstrated a dependence on the 5’cap and the poly(A) tail for translational inhibition [448-450].

In C.elegans, lin14 and lin28 mRNAs have been shown to associate with fewer polysomes following translational repression by the let7a and lin4 miRNAs; suggesting that translational

initiation was inhibited [451], although this observation is contrary to previous reports [452, 453]. Moreover, there is evidence to suggest that miRNAs may inhibit the association of target mRNAs with ribosomes, preventing the initiation of translation [454, 455]. Finally, a possible interaction between Ago-2 (as part of the miRISC) and the 5'cap of target mRNAs has been reported, preventing the binding of translation initiation factor eIf-4E, thereby inhibiting translation initiation [454].

Collectively, these data demonstrate that the mechanism by which miRNAs mediated translational repression is still largely obscure; however, current research suggests that miRNAs may inhibit the initiation of translation or they may employ multiple mechanisms to exert their effect.

(iii) mRNA destabilisation: It is accepted that miRNAs mediate gene silencing through both inhibition of translation and increasing mRNA destabilisation [448, 456-460]. By destabilising the mRNA, miRNAs indirectly enhance the mRNA degradation of target genes. It is worth noting that this increased mRNA destabilisation and subsequent mRNA degradation is distinct from the mRNA cleavage and degradation caused by RNAi which is only induced when a miRNA has perfect complementarity to a mRNA.

Degradation of target mRNAs is known to require deadenylation of mRNA targets prior to 5' decapping [461]. It has been shown that miRNAs mediate deadenylation of target genes through a mechanism that is still uncertain [460, 462-464]. Different degrees of mRNA destabilisation apply to different mRNAs, as demonstrated by miRNA inhibition and ectopic expression studies [458, 459, 465, 466]; although the majority of genes only show small decreases in mRNA and protein expression, consistent with a fine-tuning role for the majority of miRNAs.

The Ago proteins, the poly(A) binding protein (PABP) and the processing body (P-body) associated protein, GW182, are all required for miRNA mediated deadenylation [461, 463, 464]. Knockdown of GW182 has been shown to abrogate miRNA mediated inhibition of translation and mRNA deadenylation, suggesting a critical role for this protein in all aspects of miRNA mediated gene silencing [467]. All Ago proteins can interact directly with GW182 [468], indicating that GW182 may be recruited to target mRNAs by Ago proteins in the miRISC.

GW182 has been shown to directly interact with the deadenylation complex, CAF1-CCR4-NOT1 [469] and PABP. These interactions are postulated to facilitate the recruitment of the proteins required for deadenylation to the miRISC. The current model for miRNA mediated deadenylation therefore involves the miRISC functioning as scaffold proteins for deadenylation facilitating proteins.

Following deadenylation, mRNAs will be decapped and then degraded. Components of the mRNA degradation machinery are concentrated in P-bodies, and reports have linked P-bodies to miRNA mediated gene silencing as they have been shown to contain Ago proteins and mRNAs targeted by miRNAs for translational repression [470-472]. Interestingly, one report has even suggested that miRNA target genes can be temporarily stored in P-bodies and released following cellular stress [473]. Based upon these results, a model for miRNA mediated gene silencing involves the targeting of mRNAs to P-bodies, which increases the mRNAs association with the mRNA degradation machinery, causing mRNA degradation. However, the role of P-bodies in miRNA mediated gene silencing is still contested; numerous reports indicate that P-bodies may be a consequence, rather than a cause, of miRNA mediated mRNA degradation [467, 474, 475]. Further investigation into this area is required before the role of P bodies in miRNA gene silencing can be resolved.

The sequence of translational repression and deadenylation are also contested, and it is possible that a cyclic model of translational repression and deadenylation will explain the conflicting observations [476].

(iii) Alternative modes of miRNA action: miRNAs are believed to predominantly function through binding to target sequences in the 3'UTR of mRNAs with imperfect complementarity to induce translational repression; however, more recently, alternative modes of miRNA action have been observed. miRNA binding sites have been identified and validated in the 5'UTRs [477] and protein coding regions of genes [478]. These sites have been shown to induce efficient translational repression of target genes, despite their localisation. Whether these examples represent a common mode of action for miRNAs is not clear, as these results are contrary to those of Gu et al [479], who demonstrated that miRNA sequences were no longer effective at repressing translation following modification of the stop codon to extend the coding sequence through the miRNA binding sites.

Additionally, there have been many reports of miRNA mediated up-regulation of gene expression [480-483]. miR-10a has been reported to enhance the translation of a ribosomal protein through non-canonical binding to the 5'UTR, indicating that the mode of action of this miRNA may be influenced by the position of the binding site and/or the degree of complementarity with the mRNA [481]. There are several reports of miRNAs using non-canonical binding sites in mRNAs to mediate translational repression, and the extent to which miRNAs utilise such sites warrants further investigation [353, 484-486]. Interestingly, miRNAs have been reported to repress gene expression in actively proliferating cells but to up-regulate gene expression in cells arrested in G0/G1 [480, 487]. Vasudevan et al [480] suggested that the switch from an inhibitory to an activating miRISC may be caused by the association of Ago-2 with different proteins; Ago-2 was found to associate with Fragile X

mental retardation protein 1 (FXR1) in 'activating' but not 'repressing' miRISCs. Cellular proliferation has also been linked to a loss of miRNA mediated translation inhibition, by the observation that mitogen stimulated lymphocytes express shorter 3'UTRs than resting lymphocytes, and that these short 3'UTRs lack conserved miRNA binding sites [488].

Collectively these data suggest that miRNA mediated gene regulation is complex and may be influenced by the requirements of the cell. It has been postulated that miRNAs may merely act as guides for the miRISC, and that the protein constituents of the miRISC, influenced by the cellular environment, determine the consequence of the miRNA:mRNA interaction; be that translational repression, mRNA destabilisation or even the activation of translation.

1.15 miRNA target site specificity and prediction

One of the major challenges for miRNA research in mammals has been the bioinformatic prediction of miRNA target genes. In plants, miRNA target prediction has proved to be very effective, as plant miRNAs have near perfect complimentarity to the mRNA target site [489, 490]. In contrast, mammalian miRNAs bind with imperfect complimentarity to target mRNAs, making the prediction of miRNA target genes very complex. Investigations into the rules which dictate miRNA mediated translational repression have yielded a partial understanding of miRNA:mRNA interaction and have enabled the development of a host of miRNA prediction programs which are constantly being refined as new experimental evidence regarding miRNA:mRNA interactions becomes available [491-498].

(i) The principles of miRNA binding: Watson-Crick base pairing between the target mRNA and nucleotides 2-7 at the 5' termini of the miRNA is considered crucial for determining miRNA binding and translational repression, and this 6nt sequence is called the miRNA

‘seed’ sequence [499]. There is much data to support the requirement of contiguous base pairing of the miRNA seed sequence to its target, mostly from mutational studies [500-502]. The importance of the miRNA seed sequence for miRNA function was first identified computationaly following the discovery that only the miRNA seed sequences are conserved across different species [459, 503]. Studies showing that mutuation of the miRNA seed sequence abolished miRNA function provided conclusive evidence that miRNAs function predominantly through seed sequence recognition [478] Collectively, there is strong evidence that the miRNA seed sequence is a significant factor in determining miRNA mediated translational repression. However, as previously discussed, miRNA targets have been identified which do not demonstrate conventional miRNA:mRNA binding in the seed sequence [353, 481, 484-486], suggesting that additional factors which influence miRNA binding are yet to be identified.

The 3’ portion of the miRNA can also enhance the repression of miRNA targets which already contain a strong 5’ pairing [499, 501, 504, 505], although strong 3’ pairing cannot compensate for a weak 5’ miRNA:mRNA pairing interaction [501].

The presence of multiple miRNA binding sites, either for the same miRNA or multiple miRNAs, is generally required for efficient translational repression of mRNA targets [441, 499, 504, 506]. This is in contrast to miRNA induced cleavage of target mRNAs with 100% complementarity binding sites, where only one miRNA target site is usually sufficient [441, 506].

(ii) miRNA target prediction programs: Predicting miRNA target genes has proved challenging due to the variability of miRNA target sites. Numerous miRNA target prediction

programs have been developed and there are three key principles which are used to identify miRNA target genes:

(a) sequence matching, where most miRNA target prediction programs devise a scoring system based on the degree of miRNA:mRNA complementarity. Pairing in the seed region of the miRNA will be given a greater weight for matches, or penalty for mis-matches, than the rest of the miRNA as this region is considered the most important determinant of miRNA action. The programs usually encompass a set of rules defined by mutational studies. For example, Enright et al [491] state that there can be no mis-matches at positions 2 or 4, fewer than 5 mis-matches between positions 3 and 12, and there must be at least one mis-match between positions 5 and the 3' terminus of the miRNA. Each program may define a slightly different set of parameters and rules, resulting in a different predicted gene list.

(b) Thermodynamic calculations are employed by most miRNA prediction programs which requires the calculation of the free energy for each predicted miRNA target interaction to be estimated. The free energy is calculated using an algorithm to generate all suboptimal secondary RNA structures between the minimum free energy and an arbitrary upper limit [491, 507]. A limitation of this form of analysis is that the proteins which facilitate the miRNA:mRNA interaction are not yet defined, and the contribution of these proteins to the free energy therefore cannot be calculated. With the current literature suggesting that the protein components of the miRISC can alter under different cellular conditions [487], the contribution of these proteins to the miRNA:mRNA interactions may be significant, and may even explain the reported change in miRNA function observed under different cellular conditions [480].

(c) Evolutionary conservation of the predicted miRNA target site is the final common principle upon which miRNA target predictions are based. Some target prediction programs (such as Target Scan [492]) allow the user to view highly conserved and/or low and non-conserved miRNA target genes. Other programs, such as miRanda [491], account for the degree of evolutionary conservation in the overall target site score value. Placing a different emphasis on evolutionary conservation may produce significant differences in the number of gene targets predicted. Additionally, discounting a predicted target site because it is not evolutionary conserved may not be an effective method for identifying miRNA target sites in humans, as it is reasonable to assume that some miRNA:mRNA interactions will be species specific. Likewise, it possible that some miRNA target sites in mRNAs will be evolutionary selected against as the resulting interaction is detrimental for gene expression.

1.16 The function of miRNAs in mammals

The discovery of miRNAs in 1993 prompted the re-evaluation of how cellular processes are regulated. Subsequent investigations have revealed that miRNAs are important regulators of a host of cellular processes, including the regulation of the immune response [508, 509], apoptosis [510], proliferation [511] and cellular differentiation [512]. The majority of the 1048 currently identified mature miRNAs in the human genome [513] have no known function, therefore, the true extent of miRNA mediated gene regulation in humans remains uncertain. Computational analyses predict that approximately 30-90% of known protein coding genes in humans are regulated by miRNAs [514, 515], suggesting that miRNAs have the potential to influence the expression of a significant portion of the cellular proteome.

(i) Cellular differentiation and immune regulation: The apparent tissue specific expression of miRNAs suggested that miRNAs may have important roles in the regulation of cellular

differentiation. This hypothesis was supported by the observation that embryonic development was accompanied by a significant increase in cellular miRNA expression [516]. Additionally, mouse embryonic stem cells which are Dicer-1 deficient, and therefore cannot produce mature miRNAs, are defective in differentiation [387]. The disruption of miRNA expression has been shown to affect the differentiation of a wide variety of cell types, including neuronal cells [459, 517, 518], epithelial cells [519], myoblasts [520], adipocytes [521] and lymphocytes [512, 522]. The modulation of lymphocyte differentiation by miRNAs is one of the ways by which miRNAs modulate immune function.

miRNAs have been shown to regulate the lineage differentiation of B and T cells; Dicer-1 deficient mice show defects in T cell differentiation [388, 523]. More specifically, miR-181a expression can affect the differentiation of B and T lymphocytes [512, 524]. Mouse studies demonstrated that over-expression of miR-181a facilitated B cell differentiation and resulted in a 2 fold reduction in the number of circulating T lymphocytes, whilst also disrupting the distribution of $CD4^+$ and $CD8^+$ T cells [512, 525]. Furthermore, the two miRNAs processed from the miR-142 gene (miR-142-3p and miR-142-5p) were shown to substantially alter the lineage differentiation of haematopoietic progenitor bone marrow cells from mice [512]. The expression of miR-142 in these cells resulted in a 30-40% increase in the T cell lineage. The importance of miR-142-3p and miR-142-5p in T cell immune function was strengthened when miRNA profiling revealed that 60% of all the miRNAs present in T cells are constituted by just 7 miRNAs; two of which were miR-142-3p and 142-5p [526]. This observation suggests that although the majority of miRNAs have fine tuning role in cellular gene expression, a small subset in each tissue may be significant mediators of cellular signalling.

Recently, the contribution of miRNAs to B cell differentiation was investigated by Zhang et al [524] who demonstrated a specific pattern of miRNA expression at each stage of B cell

differentiation. This led to the identification of the miR-30 family and miR-9 as miRNAs whose expressions were elevated in GC B cells, and decreased in plasma cells. These miRNAs were shown to effectively target B-lymphocyte induced maturation protein 1 (BLIMP1), a gene important for B cell differentiation, thereby aiding the regulation of the GC to plasma cell stage of B cell differentiation [524]. Collectively, these data demonstrate that miRNAs are significant mediators of B and T cell differentiation, and consequently immune regulation.

In addition to the modulation of lymphocyte differentiation, miRNAs have been demonstrated to regulate T cell activation. For example, miR-181a was demonstrated to fine tune the sensitivity of T cell receptor signalling (TCR) during thymic development. This was achieved through miR-181a mediated regulation of multiple phosphatases which act as negative regulators of the TCR signalling pathway [284]. The regulation of TCR strength by miR-181a was shown to be capable of altering the T-cell threshold and sensitivity to antigens; thus, miR-181a is an example of a miRNA which is able to modulate the expression of multiple target genes to control a complex signalling pathway.

miRNAs may also have a role in the regulation of the immune response, demonstrated by miRNA expression profiling of monocytes following toll-like receptor (TLR) activation. miR-146a/b were shown to be induced by ligands of TLRs which recognise bacteria (TLR2, TLR4and TLR5), and these miRNAs were also shown to be capable of inhibiting the expression of two adaptor molecules in the TLR and interleukin-1β (IL1-β) pathways; TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1). Consequently, both miR-146a/b were proposed to play a role in dampening the TLR signalling response to bacterial products, preventing excess inflammation [508]. The importance of miR-146a/b in controlling the inflammatory response was supported by the

observation that the expression of these miRNAs is altered in the chronic inflammatory disease, psoriasis [527]. However, the effect of LPS stimulation on mouse lungs revealed 12 miRNAs whose expression was significantly altered following LPS stimulation *in vivo*, and miR-146a/b were not altered in this model [528]. It is clear that miRNAs are modulators of the immune response but which miRNAs are involved and what genes are specifically regulated by miRNAs remain obscure.

It has been shown that miRNAs are involved in the host-response to viral infection. Interestingly, miR-122 has been shown to actually enhance the translation of hepatitis C virus (HCV), possibly through aiding RNA folding as the seed sequence of miR-122 is not required for the enhancement of HCV replication [482]. Consequently, miR-122 is a therapeutic target for the treatment of HCV infection, and the development of chemical inhibitors of miR-122 are currently being investigated [529]. Interferon-β (IFN-β) signalling has been shown to up-regulate the expression of 8 miRNAs which have seed matches in the HCV genome, and therefore may contribute to the viral defence against this gene [530]. IFN-β signalling was also shown to down-regulate the expression of miR-122, suggesting that the cellular miRNA expression profile is altered in response to viral infection as a host-defence mechanism [530]. Additionally, miR-32 is able to repress the replication of the retrovirus primate foamy virus type 1 (PFV1) by negatively regulating the expression of five PFV1 mRNAs *in vitro* [531]. Furthermore, a suppressor protein encoded by PFV1 (Tas) can counteract the repressive effect of miR-32, although this effect may not be specific to miR-32, as Tas showed a broad negative effect on miRNA and siRNA function in a plant system [531]. These data demonstrate the wide ranging functions of miRNAs in the modulation of the immune response, including anti-viral, and in some instances pro-viral responses, providing an additional level of complexity to the host-innate immune response.

(ii) Regulation of cell proliferation: There is accumulating evidence to suggest that miRNAs have a central role in cell-cycle regulation. For example, miR-34 family members have been shown to be direct targets up-regulated by p53 [532], and the ectopic expression of miR-34 induces cell-cycle arrest [533]. A family of miRNAs sharing seed region homology with miR-16 were shown to negatively regulate cell-cycle progression from G0/G1 to S phase [534]. Interestingly, the transcription factor c-myc, which regulates cell proliferation and apoptosis, has been shown to up-regulate a cluster of six miRNAs called the miR-17-92 cluster. Two of these miRNAs (miR-17-5p and miR-20a) were found to negatively regulate E2F1, a target of c-myc whose down-regulation is required for cell-cycle progression [511, 535]. Furthermore, the miR-106b family were also shown to target the E2F1 transcription factor, as well as the cyclin dependent kinase inhibitor p21/CDKNIA, promoting cell-cycle progression [536, 537].

Multiple reports have linked the expression of miRNAs to the control of cell-cycle regulation and apoptosis, and not surprisingly, the majority of these reports have been in the context of cancer development and are therefore discussed in the following section.

1.17 miRNAs and cancer

The role of miRNAs in mammalian cells is exemplified by the apparent deregulation of miRNA expression observed in numerous diseases, particularly cancer. Approximately half of all known miRNAs are located at fragile sites and regions associated with cancer [538]. Furthermore, miRNA expression increases in a spatial and temporal manner during embryonic development [516, 539], suggesting that miRNAs are involved in regulating the process of cellular differentiation. This led to the hypothesis that the global down-regulation of miRNAs observed in most cancer reflects a loss of cellular differentiation [398, 540],

which is a common characteristic of cancerous cells. This hypothesis is strengthened by reports which demonstrate that Dicer-1, Drosha and DGCR8 knock-down tumour cell lines show a global decrease in miRNA expression, coupled with an enhanced tumourogenic phenotype [402]. Additionally, miRNA biogenesis associated genes such as Dicer-1, Drosha and Ago-2, show high frequency copy number abnormalities in cancer tissue relative to healthy tissue [398, 541].

It has been reported that expression of the oncogene c-myc causes a global down-regulation of miRNA expression, perhaps representing an additional mechanism by which c-myc enhances tumourogenesis [542]. The global miRNA down-regulation induced by c-myc had a notable exception, which was the miR-17-92 cluster of miRNAs, and the 6 miRNAs in this cluster are generally accepted to be oncogenic miRNAs, or 'oncomiRs' [543, 544].

(i) oncogenic miRNAs: As the number of functional studies of miRNAs has increased, a subset of miRNAs which display unambiguous oncogenic phenotypes have been indentified, most notably, miR-155, the miR-17-92 cluster and miR-21.

(a) miR-155: miR-155 is encoded by the non-coding gene, B cell integration cluster (BIC), which was found to be a common integration site of Avian Leukosis Virus (ALV) of chickens, commonly in conjunction with c-myc activation in tumours [545]. ALV causes the up-regulation of BIC expression through integration at the BIC locus where the provirus drives expression of BIC through promoter insertion. A chimeric BIC RNA can be detected which contains the viral LTR R-U5-leader-gag sequence of AVL spliced to exon 2 of BIC, driving miR-155 expression through ALV integration [545]. The identification of miR-155 as a miRNA elevated in conjunction with c-myc in tumours led to the hypothesis that miR-155 enhanced c-myc induced lymphomagenesis [545]. Indeed, transgenic mice with a miR-155

transgene expressed in B cells (Eμ-mmu-miR-155) were studied, and 7/7 of the mice showed preleukemic pre-B cell proliferation in spleen and bone marrow which later progressed to high grade B cell neoplasms [546]. Thus, miR-155 was the first miRNA classified as oncogenic [547], and its over-expression has since been linked to many cancers [548-550], particularly lymphomas [374, 551-556].

(b) miR-17-92: The miR-17-92 cluster is regarded as an oncogenic polycistron as its expression is up-regulated in both haematopoietic and solid tumours [160, 557-559]. The human miR-17-92 cluster is located on 13q31.3, a locus which is frequently amplified in lymphoma [557, 560, 561]. The miR-17-92 polycistron encodes 6 mature miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a; the sequences of these miRNAs are highly conserved across all vertebrates [560]. The miR-17-92 locus was shown to be a common integration site in mouse models of retroviral induced leukaemia, which was further indication that this miRNA cluster had an oncogenic role [559, 562].

Myc was demonstrated to be a potent transcriptional activator of the miR-17-92 cluster [535]. Additionally, mouse models have shown that the miR-17-92 cluster of miRNAs enhances c-myc induced tumourogenesis through inhibition of apoptosis, strongly suggesting an oncogenic function for these miRNAs [544, 563]. The anti-apoptotic phenotype induced by miR-17-92 over-expression in c-myc-induced tumours has been demonstrated to involve the targeted down-regulation of the tumour suppressor gene, phosphatase and tensin homologue (PTEN), by miR-19a/b [563, 564]. Inhibition of PTEN results in the activation of the pro-survival Akt-mTOR pathway, thereby antagonising the apoptotic phenotype induced by c-myc [563, 564]. An anti-apoptotic phenotype for the miR-17-92 cluster was also observed when expression of miR-17-92 was demonstrated to inhibit hypoxia-induced apoptosis. Moreover, the expression of miR-17-92 were down-regulated by wild type p53 under hypoxic

conditions [565]. Finally, miR-17 and miR-20 have been demonstrated to inhibit the expression of the E2F1 transcription factor, enhancing cell proliferation. Collectively these data demonstrate a clear oncogenic function for the miR-17-92 cluster.

(c) miR-21: miR-21 expression has been reported to be increased in breast cancer [566], pancreatic cancer [549], head and neck cancer cell lines [567], cervical cancer tissue [568], NK-cell lymphoma [556] and DLBCL [551], suggesting an oncogenic role for this miRNA. The inhibition of miR-21 has been shown to induce apoptosis in a number of cell lines [566, 569, 570], including multiple myeloma cells, where miR-21 was up-regulated by STAT3, and it was shown to have an anti-apoptotic effect on the cells [571]. miR-21 knockdown was also demonstrated to induce apoptosis of breast cancer tumour cells in a xenograft mouse mode, which was partly ascribed to indirect miR-21 mediated up-regulation of Bcl2 [566]; indicating an anti-apoptotic function for miR-21 in tumour development. miR-21 has also been demonstrated to enhance proliferation of tumour cell lines *in vitro* [566, 572, 573]. In addition to the up-regulation of the oncogene Bcl2, miR-21 has been shown to target and repress protein expression of the tumour suppressor genes PTEN [556, 573] and tropomyosin 1 [574]. Therefore, miR-21 may contribute to tumour development by enhancing cellular growth and protecting cells from apoptosis via the regulation of known oncogenes and tumour suppressor genes.

(ii) Tumour suppressor miRNAs: The global down-regulation of miRNAs in cancer could be interpreted to signify that the majority of miRNAs have a tumour suppressive function. Nevertheless, current investigations have only identified a small number of miRNAs whose repression is favourable for tumour development. It may be that the combined effect from multiple down-regulated miRNAs is required to enhance tumourogenesis. However, it has been observed that B cell malignancies do not typically exhibit a global down-regulation of

miRNAs, although they do have miRNA expression profiles which are distinct from healthy tissue [524]; suggesting that a global down-regulation of miRNAs is not always required for tumour development.

The miR-15a and 16 cluster is located at 13q14, a region frequently deleted in cancer [575], most notably in CLL [576], where loss of miR-15a and miR-16 expression was shown to cause an increase in the expression of the anti-apoptotic protein Bcl2 [510]. Consistent with this, re-expression of miR-15a and miR-16 in CLL cell lines induced apoptosis [510]. In a model of gastric cancer, miR-15a/16 down-regulation was shown to facilitate multi-drug resistance in tumours; this function could be partially ascribed to the loss of miR-15a/16 mediated suppression of Bcl2 [577]. Consequently, it has been proposed that miR-15a/16 mimics could be used to treat multi-drug resistance (MDR) in gastrine cancer, as re-expression of miR-15/16 has been shown to reduce the MDR of gastiric cancer cell lines, partially via the up-regulation of Bcl2 [577]. Additionally, miR-15a/16 were down-regulated by c-myc, and the re-expression of these miRNAs in c-myc induced tumours in mice was detrimental for tumour growth [542], possibly due to the pro-apoptotic function of these miRNAs. The tumour suppressive functions of miR-15a/16 may extend to an anti-proliferative effect, as expression of these miRNAs results in cell-cycle arrest [534] and re-expression of miR-15a/16 in lung cancer cell lines causes cell-cycle arrest, in an Rb-dependent manner [578].

Additional miRNAs which have been demonstrated to exert a tumour suppressive function include the let7 family of miRNAs, which have been shown to negatively regulate the oncogene Ras in lung tumours [579, 580]. Expression analysis has suggested that miR-143 and miR-145 may have tumour suppressive functions as expression of these miRNAs is significantly decreased in a number of solid tumours including colorectal cancer, breast

cancer and cervical cancer [568, 581], as well as B cell malignancies [582]. A possible role for miR-143 in the negative regulation of the oncogene KRAS has been reported in colorectal cancer cell lines, suggesting that the loss of miR-143 in cancer may facilitate un-regulated KRAS expression [583].

(iii) miRNAs and therapy: The deregulated expression of miRNAs has enabled miRNA expression to be used as biomarkers for disease diagnosis [584] and has the potential to be exploited for gene therapy. miRNAs hold significant promise for gene therapy due to their small size and consequent ease of delivery, and their cytoplasmic localisation negates the need for nuclear delivery mechanisms. However, the recently described exosome-mediated transfer of miRNAs [419, 420, 585] will need to be investigated further before the potential toxicity of miRNA gene therapy can be fully evaluated.

Gene therapy involving miRNAs would involve inhibition of oncomiRs, such as miR-21, using 'antagomiRs', and re-expression of tumour suppressive miRNAs, such as the miR-15a/16 cluster, using miRNA mimics [456]. The mode of action of miRNAs also poses a significant advantage for gene therapy as miRNAs target a large number of proteins in multiple pathways, therefore, modulation of only one or two miRNAs could significantly affect the expression of many oncogenic pathways; consistent with the current view of cancer as a disease which involves the deregulation of multiple pathways [586].

miRNA gene therapy is in its infancy, however, the liver specific miR-122 is already entering phase II clinical trials for the treatment of HCV infection (discussed in section 1.15 [529, 587]) and there are a number of pre-clinical and clinical trials pending for miRNA gene therapy in cancer treatment [588]. For example, Rosetta Genomics are currently designing a miRNA based therapy aimed at treating liver cancer, inspired by an *in vivo* mouse model

which demonstrated a halt in disease progression or total relapse of hepatocellular carcinoma in mice treated with miR-26a mimics [588]. Indeed, *in vitro* and *in vivo* models have demonstrated a sound scientific logic for miRNA based therapy [510, 542, 577, 579], although, until the results of the pending clinical trials are available, the true potential of miRNA therapy will remain uncertain.

1.18 The function of miRNAs in Viruses

It is currently believed that only DNA viruses encode miRNAs [48, 589], however, this is controversial for HIV [589-591]. The herpesvirus family has the most prevalent expression of miRNAs [48, 592-594], although primate polyomavirus and human adenoviruses have also been shown to encode miRNAs [48, 589, 595, 596]. It has been proposed that the presence of miRNAs in the genomes of herpesviruses may be due to the characteristic long-term latent infections of this virus family [597]; as evidence suggests that the non-antigenic miRNAs are able to modulate numerous host and viral pathways to facilitate latent infection, immune evasion and lytic replication [485, 598, 599].

Viral miRNAs have a similar genome structure, biogenesis and mode of action to cellular miRNAs [48]. Viral miRNAs have been shown to target cellular, as well as viral genes [163, 221], suggesting that the interplay between cellular and viral miRNAs is complex, with both host and viral miRNAs capable of regulating cellular and viral mRNAs. Despite much research over recent years, the number of experimentally validated viral miRNA targets is still only modest. Nevertheless, the few known viral miRNA targets suggest that viral miRNAs may play a significant role in the control of immune evasion (i) and apoptosis resistance (ii).

(i) immune evasion: human cytomegalovirus (HCMV) miR-ULL112 has been shown to target the NK cell ligand, major histocompatibility complex class I related chain B (MICB),

inhibiting NK cell recognition of HCMV infected cells [485]. Likewise, the EBV encoded miR-BHRF1-3 has been shown to down-regulate the CTL chemoattractant CXCL11, reducing the recognition of EBV infected cells by CTLs [220]. In addition, the antigenic T antigens expressed by SV40 are targeted by SV40 encoded miRNAs to reduce CTL recognition of infected cells, indicating that viral miRNAs target host and viral genes to evade the immune response [600].

(ii) apoptosis resistance: Herpes simplex virus-1 (HSV-1) encoded miRNAs, miR-LATs, can down-regulate components of the pro-apoptotic TGF-β pathway, including TGF-β and SMAD-3 [601]. KSHV encoded miRNAs have also been demonstrated to target genes which result in the down-regulation of the TGF-β signalling pathway via the down-regulation of the tumour suppressor gene, Thrombospondin-1 [602]. Additionally, EBV encoded miR-BART5 has also been shown to target the pro-apoptotic protein, p53 up-regulated modulator of apoptosis (PUMA) in NPC, as a means of protecting cells from apoptosis [221].

The discovery of viral miRNAs has added a layer of complexity to the viral-host interaction, and a greater understanding of how viral miRNAs modulate the cellular environment is required to fully understand viral infection and persistence.

1.19 Cellular miRNAs and EBV

When this project began in 2007, there was little information available regarding EBV-mediated regulation of cellular miRNA expression. Only one study to our knowledge had documented the expression of cellular miRNAs following EBV infection of resting B cells and this study had used subtractive hybridisation and northern blotting to identify miRNA species in LCLs [603]. However, the sensitivity of this assay was limited and only 7 cellular

miRNAs were identified as up-regulated by EBV infection of resting B cells (miR-155, miR-21, miR-29b, miR-146a, miR-23a, miR-27a and miR-34a), and no miRNAs were identified as down-regulated following EBV infection. Thus, a comprehensive investigation into EBV-mediated regulation of cellular miRNA expression had not yet been performed.

Aims and objectives

It is evident that growth-transformation of resting B cells by EBV requires the modulation of cellular gene expression, including genes associated with oncogenesis, such as tumour suppressor genes and oncogenes. It is the EBV-mediated modulation of cellular gene expression which is likely to lead to immortalisation of LCLs in culture, a process which is analogous to the multi-pathway deregulation required for tumourogenesis. miRNAs are capable of regulating a large number of genes in multiple pathways, therefore, the modulation of the cellular miRNA environment by EBV may enable the virus to modulate the multiple signalling pathways required to facilitate the maintenance of viral latency, viral persistence, and possibly contribute the development of EBV associated-malignancies. The objective of this thesis was to investigate the role of cellular miRNAs in EBV-mediated B cell transformation.

In results chapter I, we investigate the regulation of the oncogenic miRNA miR-155 by EBV, a miRNA which was known to be abundantly expressed in HL. We also sought to investigate the reported processing defect of miR-155 in BL and to determine whether the biogenesis of this miRNA was regulated by EBV.

In results chapter II, we sought to identify the extent to which expression of particular miRNAs were regulated by EBV during B cell transformation. We used sensitive QPCR-arrays to measure the cellular miRNA expression profile of EBV infected B cells and CD40L

+ IL4 stimulated B cell blasts. A comparison between proliferating B cell blasts and EBV transformed B cells was designed to enable the identification of miRNAs whose modulation by EBV was specific to transformation rather than transient activation and proliferation of a resting B cell. The expression and function of miRNAs whose expression changes were transformation specific were subsequently investigated.

In results chapter III we profiled the cellular gene expression of EBV infected and CD40L + IL4 stimulated blasts. The aims of results chapter III were twofold: firstly, to identify transformation associated genes whose expression may have been modulated by transformation associated miRNAs from results chapter II; secondly, to identify candidate tumour suppressor genes whose down-regulation by EBV may aid transformation and contribute to the development of EBV associated malignancies. The expression and function of candidate tumour suppressor genes was further investigated using a lentiviral based expression system.

2. Materials and Methods

2.1 Cell Culture

2.1.1 Maintenance of cell lines

B cells, B cell lines and virus producing 2089 cells were grown in cell culture medium which contained medium (RPMI1640 medium, Sigma) supplemented with 10% v/v foetal calf serum (FCS), 2mM glutamine (Gibco), and 5000U penicillin/streptomycin (Gibco). Mutu-BL were grown in normal BL medium with 20nM Bathocuproine disulfonic acid (BCS) (Sigma), 50µM α-thioglycerol (Sigma) and 10mM HEPES buffer (Sigma).

293-FT cell culture media contained DMEM medium (Invitrogen) supplemented with 10% v/v bovine calf serum (BCS), which was previously heat inactivated at 56°C for 1 hour, 5000U penicillin/streptomycin (Gibco) and 1.2mg of G418. The lentivirus production media consisted of DMEM medium (Invitrogen) supplemented with 10% v/v BCS, which was previously heat inactivated at 56°C for 1 hour, 5000U penicillin/streptomycin (Gibco). All cells were grown at 37°C, 5% CO_2 in 25cm^2 flasks (suspension cells) or 100mm plates (adherent cells).

2.1.2 Cryopreservation of cells

Approximately 1x10^7 cells were pelleted by centrifugation at 1,400rpm for 5 minutes and re-suspended in 1ml of medium (RPMI1640 medium, Sigma) supplemented with 20% v/v foetal calf serum (FCS), 10% v/v dimethylsulphoxide (DMSO), 2mM glutamine (Gibco) and 5000U penicillin/streptomycin (Gibco). Cells were placed in a Mr Frosty container surrounded by a sponge soaked in propan-2-ol and stored for at least 4 hours at -80°C before being transferred to the vapour stage of a liquid nitrogen freezer at -180°C for long term storage.

2.1.3 Recovery of cells from liquid nitrogen

10ml of cell culture medium was pre-warmed to 37°C. Cells were then quickly defrosted at 37°C to minimise exposure to DMSO and then added drop-wise to the pre-warmed media. Cells were then pelleted by centrifugation at 1,400 RPMI for 5 minutes and re-suspended in 10ml of tissue culture medium in a 25cm^2 flask and cultured at 37°C and 5% CO_2.

2.2 Isolation of B cells

2.2.1 Separation of PBMCs from whole blood

Blood from buffy coat samples (Blood Transfusion Service, Birmingham) was diluted with sterile PBS to a total volume of 125ml and layered underneath lymphoprep separation medium (Lymphoprep, Robbins Scientific) in a 250ml centrifuge tube (Corning). This was then centrifuged at 1800rpm for 30 minutes and slowed without a brake to maintain a gradient. Peripheral blood mononucleocytes (PBMCs), which form a distinct buffy coloured layer at the interface between the serum and the red blood cells were extracted using a 10ml pipette. The PBMCs were washed twice in sterile PBS by centrifugation at 1600 rpm for 10 minutes and then 1200 rpm for 10 minutes. PBMCs were then re-suspended in 10ml of RPMI 1% v/v FCS and counted. The number of B cells was estimated based on the assumption that 5% of the PBMCs were B cells. PBMCs were either stored overnight at 4°C or processed immediately for CD19$^+$ B cell isolation.

2.2.2 CD19 selection of B cells from PBMCs

CD19 is a B cell specific maker and can be used to isolate resting B cells from peripheral blood. Magnetic polystyrene beads coated in monoclonal antibody to CD19 (Pan B CD19 Dynabeads (Dynal)) were used to positively select B cells from PBMCs by exposure to a magnet. The number of CD19 Dynabeads needed was calculated (4 Dynabeads per B cell)

and added to the PBMCs at a concentration of 10^7 Dynabeads per ml in a 14ml round bottomed tube and incubated at 4°C for 30 minutes on a roller. The B cells bound to the Dynabeads were then removed from the PBMCs by exposure to a magnet for 2 minutes. Non-B cells were removed with a Pasteur pipette and the B cells bound to the Dynabeads were washed 5 times with 10ml of RPMI 1% v/v FCS. The B cells bound to Dynabeads were then re-suspended in 1ml RPMI 1% FCS and 50ul of Detatchabead CD19 (Dynal) was added to the cells in a 14ml round bottomed tube and incubated at room temperature for 45 minutes on a roller. 9ml of RPMI 1% v/v FCS was then added to the suspension and the Dynabeads removed from the B cells by exposure to a magnet. The Dynabeads were washed once with 10ml of RPMI 1% v/v FCS and the total number of $CD19^+$ B cells counted.

2.2.3 Analysis of cell purity by fluorescence activated cell sorting (FACS)

The purity of the B cell preparation was assessed by staining cells with the alternative B cell marker, CD20. $2x10^6$ $CD19^+$ B cells were each washed 3 times in PBS in a FACS tube. Both the CD21 antibody (Mouse CD21/RPE-Cy5, Dako) and the isotype control (Mouse IgG1/RPE-Cy5, Dako) were diluted 1/20 with 5% v/v heat inactivated goats serum (HINGS). Cells were mixed with the antibody and incubated on ice for 30 minutes in the dark. Cells were then washed 3 times with PBS and re-suspended in either 1ml of PBS if they were going to be analysed immediately or in 1ml of 2% paraformaldehyde in PBS and stored at 4°C in the dark until used.

2.3 EBV infection experiments

2.3.1 Preparation of EBV stocks

293 cell clones stably transfected with the recombinant B95.8 EBV genome were plated into 6 well plates 24-48 hours prior to transfection. When the cells were 70% confluent they were transfected with 0.5µg of BZLF1 and 0.5µg of BALF4 (gp110) plasmids [604, 605] per well using Lipofectamine (Invitrogen) reagent in Optimem minimal medium (Gibco) according to manufacturer's instructions and incubated for 3 hours at 37°C, 5% CO_2. 1ml of cell culture medium was then added to each well and the supernatant was harvested 72hours later, centrifuged at 2000rpm for 5 minutes and filtered using a 0.45µm filter to remove cellular contaminants. This supernatant was either used directly or stored as aliquots at -80°C. 50µl of virus supernatant was kept for virus quantification.

2.3.2 Recombinant EBV

Recombinant EBV was provided as 293 producer clones deleted for either LMP1 [606] or gp42 [604] and these viruses were prepared, titred and used to infect B cells via the same methods as the wtEBV (sections 2.3.1, 2.3.3 and 2.3.4)

2.3.3 Quantitation of EBV titre by Q-PCR

An equal volume of lysis buffer (100mg/ml proteinase K (Roche), 10mM Tris-HCL pH 8.8, 1.5ml $MgCl_2$, 50mM KC:, 0.1% v/v Triton X-100) was added to the virus supernatant and incubated at 55°C for 1 hour followed by 99°C for 10 minutes to heat inactivate the proteinase K. quantitative PCR was then carried out for the EBV polymerase gene (BALF4) (see section 2.8.3) 5µl of processed viral supernatant was added per well of a 96 well to a quantitative PCR reaction to determine the number of EBV genomes per ml of virus supernatant.

2.3.4 B cell infection with EBV

CD19-positive B cells were spun down at 1200rpm for 5 minutes and re-suspended in an appropriate volume of virus supernatant to give the desired m.o.i and incubated at 4°C for 2 hours. B cells were then spun out of the virus supernatant at 1200rpm and re-suspended in cell culture medium ($2x10^6$ B cells/ml) and cultured at 37°C and 5% CO_2. At each time point between $2x10^6$ and $7x10^6$ cells were harvested for RNA and between $8x10^6$ and $20x10^6$ cells were harvested for protein.

2.4 Generation of B cell blasts

$5x10^6$ $CD19^+$ resting B cells per well of a 6 well plate were cultured in cell culture medium with 50ng/ml of soluble trimeric human recombinant megaCD40L (Alexis Biochemicals) and 50ng/ml interleukin 4 (IL4) (ProSpec-Tany). For comparison, $5x10^6$ B cell blasts were also generated using mouse fibroblast cells stably transfected with CD40L (L-CD40L cells), as previously described [607] and 50ng/ml of IL4 (ProSpec-Tany) in culture medium. Medium was changed twice a week.

2.5 Stimulating B cells with anti-CD21

$1x10^7$ $CD19^+$ resting B cells were spun down and re-suspended in 500µl of 10% heat inactivated goat serum (Hings) with 12µl of CD21 mouse monoclonal antibody (Abcam) and incubated at room temperature for 1hour. Cell culture medium was then added to the cells and they were cultured at 37°C, 5% CO_2.

2.6 Stimulating ERK phosphorylation in LCLs

LCLs were washed in culture media, counted and re-suspended in culture media at a concentration of 0.5 x 10^6 cells per ml. ERK phosphorylation was stimulated in LCLs following the addition of 20µg/ml of 12-O-tetradeconoylphorbal-13-acetate (TPA), dissolved in DMSO, to the culture media. Cells were cultured for 24 hours at 37°C, 5% CO_2. Cells were harvested for protein (section 2.17.1) and lysed in mirVana™PARIS™ cell disruption buffer, plus 100µl/ml of phosSTOP (Roche), a cocktail of phosphatase inhibitors designed to prevent the loss of phosphorylation in protein samples. Sample preparation for western blot was otherwise the same as described in section 2.17.1.

2.7 RNA extraction

All procedures were performed on ice with RNase free eppendorff tubes and filter tips. Total RNA was extracted using the mirVana™ PARIS™ kit (Ambion, Europe) according to the manufacturer's instructions. Briefly, between 5 x 10^5 and 1 x 10^7 cells were harvested, washed once with PBS and re-suspended in 300-600ul of ice cold Cell Disruption Buffer and an equal volume of Denaturing Solution and then extracted with an equal volume of Acid-Phenol:Chloroform. The aqueous phase was extracted and 1.25 volumes of 100% ethanol was added to the lysate which was then passed through a filter cartridge. The bound RNA was washed three times in the Wash Buffers and then eluted in 50-100ul of 95°C Nuclease Free Water (Ambion, Europe). The RNA concentration was determined using a Nanodrop (Thermo Scientific) according to manufacturer's instructions and the RNA samples were aliquotted and stored at -80°C.

2.8 Reverse Transcription

2.8.1 Principle of Taqman miRNA Reverse Transcription

The reverse transcription of miRNAs requires primers specifically designed to bind to the 3' portion of the mature miRNA sequence. These primers have a stem-loop structure which shows increased specificity and sensitivity over linear primers (figure 2.1). This is predicted to be a result of the spatial constraint and base stacking interactions of the stem-loop structure. The spatial constrain of the primers is believed to be the reason why these primers cannot bind genomic DNA or miRNA precursors. The base stacking interactions may improve the thermal stability of RNA primers/miRNA interaction over those of conventional linear primers which may be of particular importance when using relatively short primers for miRNA reverse transcription [608].

2.8.2 Reverse transcription of miRNAs

cDNA synthesis of miRNAs was performed using the Taqman® Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. 1-10ng of total RNA was added to a reaction mix containing 115nmoles dNTPs, 0.05U of MultiScribe™ Reverse Transcriptase, 3.8U of RNase Inhibitor, 1.5µl of 10X Reverse Transcription Buffer, 4.16µl of nuclease free water and 3µl of a specific miRNA RT primer (table 1) to give a total volume of 15µl per reaction in a thin walled, 0.5ml PCR tube. The Reaction mix was incubated at 16°C for 30 minutes, 42°C for 30 minutes and then 85°C for 5 minutes in a thermal cycler and the cDNA was then stored at -20°C.

2.8.3 Reverse transcription of EBV genes

200ng of RNA was heated to 90°C for 3 minutes to denature the RNA which was then incubated on ice. The RNA was added to a reverse transcription reaction mix containing 5U

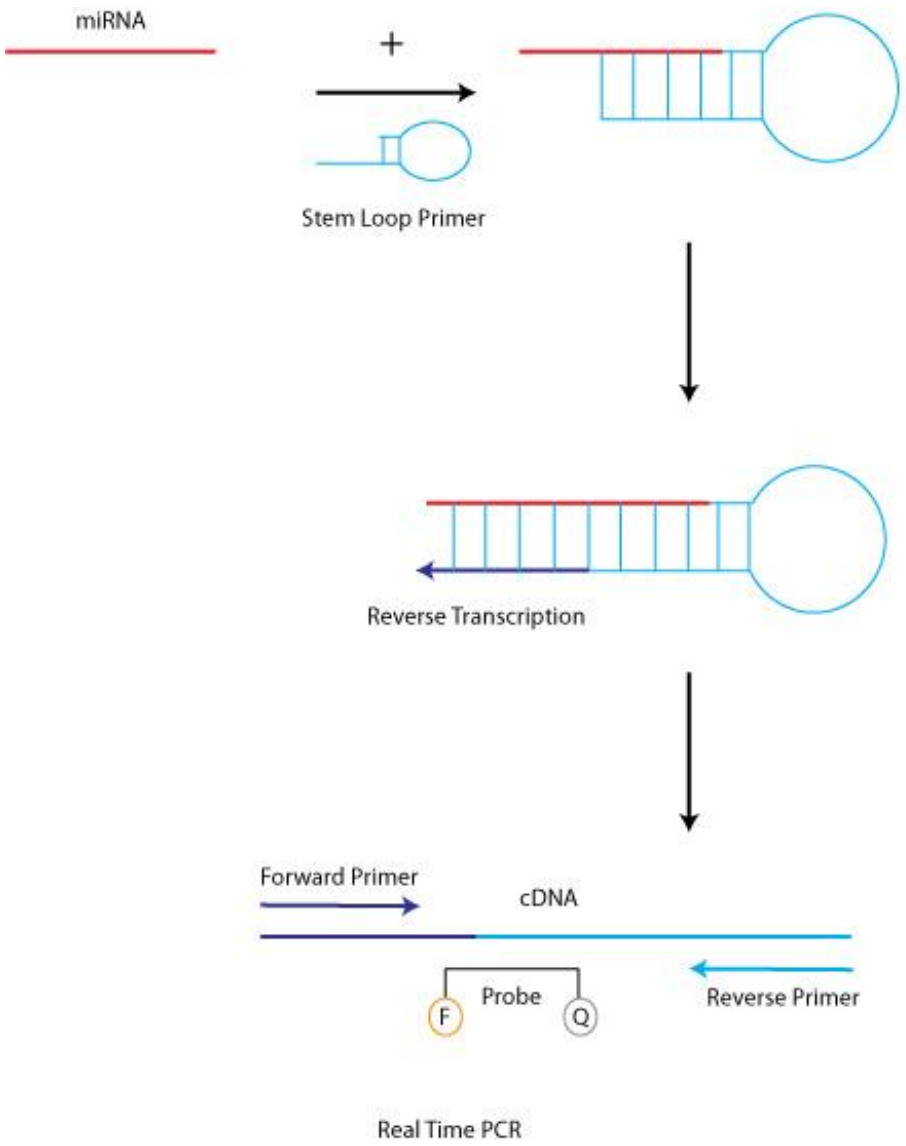


Figure 2.1 Diagram representing the Taqman miRNA real time PCR system. This figure is adapted from Chen et al 2005 [608]. The reverse transcription primers are stem-loop primers specific for each miRNA to be assayed. These stem-loop primers are specific for the mature miRNA and allow extension of the cDNA providing enough length for Taqman real time primers and probe to bind in the real time amplification step. In traditional Taqman quantitative real time PCR design, the probe consists of a quencher (Q) dye and a Fam labelled flourescer dye (F).

miRNA	Applied Biosystems ID	Included in Taqman miRNA Array?	Primer Target Sequence
has-miR-155	000479	Yes	UUAAUGCUAAUCGUGAUAGGGG
has-miR-155	002623	No†	UUAAUGCUAAUCGUGAUAGGGGU
has-miR-155*	002287	No	CUCCUACAUAUUAGCAUUAACA
has-miR-148a	000470	Yes	UCAGUGCACUACAGAACU UUGU
hsa-miR-125b	000449	Yes	UCCCUGAGACCCUAACUUGUGA
has-miR-132	000457	Yes	UAACAGUCUACAGCCAUG GUCG
has-miR-31	001100	Yes	GGCAAGAUGCUGGCAUA GCUG
hsa-miR-95	000433	Yes	UUCAACGGGUAUUUAUUG AGCA
has-miR-28-5P	000411	Yes	AAGGAGCUCACAGUCUAU UGAG
has-miR-15a	000389	Yes	UAGCAGCACAUAAUGGUU UGUG

Table 1: Taqman QPCR miRNA assay sequences. miRNA assays from Applied Biosystems with miRNA target sequence (for reverse transcription)

† miRNA sequences which were changed on miRBase in 2008 , after the Taqman miRNA Array had been performed so subsequent experiments with the individual assays used primers with a slightly different sequence to those on the array

miRNA	Applied Biosystems ID	Included in Taqman miRNA Array?	Target Sequence
has-miR-199a-3p	002304	No†	ACAGUAGUCUGCACAUUG GUUA
has-miR-193a-3p	002250	No†	AACUGGCCUACAAAGUCC CAGU
has-miR-200b	002251	No†	UAAUACUGCCUGGUAAUG AUGA
has-miR-34a	000426	Yes	UGGCAGUGUCUUAGCUGGUUGU

Table 1 continued: Taqman QPCR miRNA assay sequences. miRNA assays from Applied Biosystems with miRNA target sequence (for reverse transcription)

† miRNA sequences which were changed on miRBase in 2008 , after the Taqman miRNA Array had been performed so subsequent experiments with the individual assays used primers with a slightly different sequence to those on the array

Avian Myeloblastosis Virus Reverse Transcriptasr (AMV-RT), reaction buffer (50mM Tris-HCL pH 8.5, 30mM potassium chloride (KCL), 8mM magnesium chloride ($MgCl_2$), 1mM dithiothreithol (DTT) (Roche), 200uM dNTPs, 1µM of the 3' specific primer (Alta Bioscience) (table 2) in a total volume of 20µl. The reaction was incubated at 42°C for 1 hour and then 90° for 5 minutes in a thermal cycler and the cDNA was stored at -20°C.

2.8.4 Reverse transcription of cellular genes

20-400ng of total RNA was added to a reaction mix containing 15nmoles dNTPs, 0.05U of MultiScribe™ Reverse Transcriptase, 3.8U of RNase Inhibitor, 1.5µl of 10X Reverse Transcription Buffer, 6.16µl of nuclease free water and 50pmoles random hexamers in a total volume of 15µl. The Reaction mix was incubated at 16°C for 30 minutes, 42°C for 30 minutes and then 85°C for 5 minutes in a thermal cycler and the cDNA was then stored at -20°C.

2.9 Taqman Quantitative PCR (QPCR)

2.9.1 Principle of real time PCR using Taqman probes

Real time PCR is a method which allows the accurate quantitation of a specific gene as the PCR amplification occurs. The data is collected during the exponential (log) phase of RNA/DNA amplification when the quantity of PCR product is directly proportional to the amount of nucleic acid template.

2.9.2 Quantitative PCR of miRNAs

miRNAs were reverse transcribed individually (singleplex) as described in 2.7.2 and this cDNA was used for real-time amplification to quantitate the miRNA. A 20µl reaction was set up in one well of a 96 well Optica Reaction Plate (Applied Biosystems) containing 10µl of

Taqman 2X universal PCR master mix, No AmpErase, no UNG (Applied Biosystems), 1µl of miRNA specific primer and probe mix (Table 1) (Applied Biosystems), 7.67µl nuclease free water (Ambion) and 1.33µl of miRNA specific cDNA. The plate was then sealed with Optical Adhesive Film (Applied Biosystems) and thermocycling and fluorescence detection were carried out in a 7500 Real-Time PCR System (Applied Biosystems).The samples were heated to 95°C for 10 minutes to activate the ApliTaq® Gold polymerase enzyme, followed by 40 cycles of denaturation at 95°C for 15 seconds and primer annealing and extension at 60°C for 60 seconds.

The data was analysed using the provided 7500 system software using the ddCt method of quantification[609]. Small nucleaolar RNAs are used as suitable endogenous controls for the miRNA reverse transcription and QPCR steps of the assay. The nucleolar RNAs RNU48 or Z30 (Applied Biosystems) were assayed separately from the miRNAs (singleplex) as both probes were FAM labelled. Relative expression of the miRNAs was then calculated relative to a calibrator sample. The dCt values were used to calculate the significance of certain results using the student T test.

2.9.3 Quantitative PCR of EBV genes

The titre of EBV viruses produced was determined by quantitative DNA-PCR using a probe specific for the EBV polymerase (pol) BALF5 gene. This probe was labelled with FAM at the 5’ end and with TAMRA at the 3’ end (Eurogentiec) [610]. The Q-PCR reaction was set up in a 25µl reaction volume containing primer and probe combinations for BALF5 (see table 2) Viral number was quantified using serial dilutions of DNA from the reference BL cell line Namalwa-BL which is known to contain two integrated copies of EBV per cell.

QRT-PCR assays for the EBV latent transcripts (with the exception of the EBER1 and EBER2 transcripts) and for the lytic cycle transcripts are described in [227]. Briefly, the

Target	Primer	Sequence	Final conc.	B98.8 coords
EBV pol	5' primer	5'-AGTCCTTCTTGGCTAGTCTGTTGAC-3'	200nM	154828-154804
	3' primer	5'-CTTTGGCGCGGATCCTC-3'	200nM	154738-154754
	probe	5'(FAM)CATCAAGAAGCTGCTGGCGGCCT(TAMRA)-3'	200nM	154779-154757

Table 2: Primer and probe sequences for QPCR titre of EBV

endogenous control used in these assays was GAPDH and the probe for this gene was labelled at the 5' end with VIC and labelled at the 3' end with BHQ (Applied Biosystems). All EBV latent gene probes were labelled at the 5' end with FAM and at the 3' end with TAMRA, thus allowing the expression of the gene of interest and the endogenous control to be measured at the same time, in the same well of the QPCR plate (multiplexed). The plate was then sealed with Optical Adhesive Film (Applied Biosystems) and thermocycling and fluorescence detection were carried out in a 7500 Real-Time PCR System (Applied Biosystems).The samples were heated to 95°C for 10 minutes to activate the ApliTaq® Gold polymerase enzyme, followed by 40 cycles of denaturation at 95°C for 15 seconds and primer annealing and extension at 60°C for 60 seconds.

A full list of EBV transcripts investigated, along with their primer and probe combinations, are shown in table 3. EBV coordinates are based on the revised B95.8 sequence [611].

2.9.4 Quantitative PCR of cellular genes

Quantitative PCR of cellular genes was performed on cDNA generated using random hexamers as described in section 2.8.4. Primers and probes for the gene BIC have been described previously [612] and were synthesised by Alta Biosciences and Eurogentec respectively (Table 4). Commercially available QPCR assays were purchased from Applied Biosystems for the following genes; Drosha, Dicer1, SPINT2, NR4A3, DUSP6, and Beta-2-

Transcript (cell line)	cDNA primer (B95.8 coordinates)	Primer/probe combinations	Final conc	Oligonucleotide sequence (B95.8 coordinates)
Cp-initiated (Oku LCL)	5'-CCTAGGCCCTGAAGG-3' (17631-17626/14832-14824)	5' primer (C_1C_2)	1µM	5'-AATCATCTAAACCGACTGAAGAAACAG-3' (11467-11479/11626-11639)
		3' primer (W_1W_2)	1µM	5'-GAGGGGACCCTCTGGCCC-3' (14709-14701/14619-14612)
		Probe (W_1)	200nM	5'-(FAM)ACCGCCGTGAAGGCCCTGGACCAAC(TAMRA)-3'
Wp-initiated (X50-7)		5' primer (W_0)	100nM	5'-CGCCAGGAGTCCACACAAAT-3' (14391-14410)
		3' primer (W_1W_2)	100nM	5'GAGGGGACCCTCTGGCC-3' (14709-14701/14619-14612)
		Probe (W_1)	200nM	5'-(FAM)ACCGCCGTGAAGGCCCTGGACCAAC(tamra)-3' (14564-14588)
LMP1 (X50-7)	5'TAGATAGAGAGCAATAATG AGAGCAG-3' (168841-168864)	5' primer (ex2)	300nM	5'GCACGGACAGGCATTGTTC-3' (168893-168912)
		3' primer (ex3)	300nM	5'-AAGGCCAAAAGCTGCCAGAT-3' (168893-168912)
		Probe (ex 2-3)	200nM	5'(FAM)TCCAGATACCTAAGACAAGTAAGCACCCGAAGA T(TAMRA)-3' (168951-168965/169042-169060)
GAPDH	5'-GATCTCGCTCCTGGAA-3'	Commercial PCR assay (Applied Biosystems)	-	5'-GAAGGTGAAGGTCGGAGTA-3'
			-	5'-GAAGATGGTGATGGGATTTC-3'
			-	5'-(FAM)CAAGCTTCCCTTCTCAGCC(TAMRA)-3'

Table 3 Primer and probe sequences used for QPCR analysis of EBV latent gene transcripts

micoglobulin. All commercially available QPCR assays were FAM labelled and used at a concentration of 1/20.

Target	Primer	Sequence	Final conc.
BIC NR_001458.5	5' primer	5'-TCAAGAACAACCTACCAGAGACCTT-3'	200nM
	3' primer	5'-TCCTGGTTTGTGCCACCAT-3'	200nM
	probe	5'-6-(FAM)ACCTTGGCTCTCCCCACCCAATGGA(TAMRA)-3'	900nM

Table 4: BIC QPCR primer and probe sequences, previously described by van den Berg et al [612]

2.10 Taqman micro fluidic cards

2.10.1 Principle of Taqman microfluidic cards

Taqman microfluidic cards allow QPCR of many different genes on a single 364 well microfluidic card. A cDNA specific for the type of microfluidic card being run is made and then added to 8 ports of the card. Once centrifuged, the cDNA is distributed evenly to each of the 364 wells on the card to give a 1µl volume per well. Each well has a primer for a specific gene target dried into the bottom so the cDNA re-suspends this primer to give the final ready to run cDNA/primer mix.

2.10.2 Multiplex reverse transcription for Taqman® Array Human miRNA panel v1

A pre-designed Taqman human cellular miRNA array early release, version 1 (Applied Biosystems) was used to measure the expression of cellular miRNAs. The array contained primers for 365 human miRNAs. All reactions contained primers for two endogenous controls, RNU46 and RNU48.

cDNA synthesis of miRNAs was performed using the Taqman® Reverse Transcription Kit according to the manufacturer's instructions. Briefly, eight independent multiplex reverse

transcription reactions were prepared for each sample with eight different pre-defined multiplex primer pools. For each reverse transcription reaction, 100ng of RNA was added to a reaction mix containing 20nmoles dNTPs, 0.1U MultiScribe™ Reverse Transcriptase, 2.5U RNase inhibitor, 1µl of 10X Reverse Transcription Buffer, 1µl of miRNA multiplex RT primers and 3.675µl of nuclease free water, to give a total volume of 10µl per reaction in a thin walled, 0.5ml PCR tube. The Reaction mix was incubated at 16°C for 30 minutes, 42°C for 30 minutes and then 85°C for 5 minutes in a thermal cycler and the cDNA was used immediately.

2.10.3 Quantitative PCR for Taqman® Array Human miRNA panel v1

The Taqman miRNA array cards were loaded and run according to the manufacturer's instructions. Briefly, cDNA was diluted 62.5 fold by the addition of 40µl of nuclease free water. This was combined with 50µl of Taqman universal PCR master mix (Applied Biosystems) to give a 100µl total cDNA/master mix. 100µl of this RT reaction-specific cDNA/master mix was added to the corresponding port on the microfluidic card (figure 1.2).

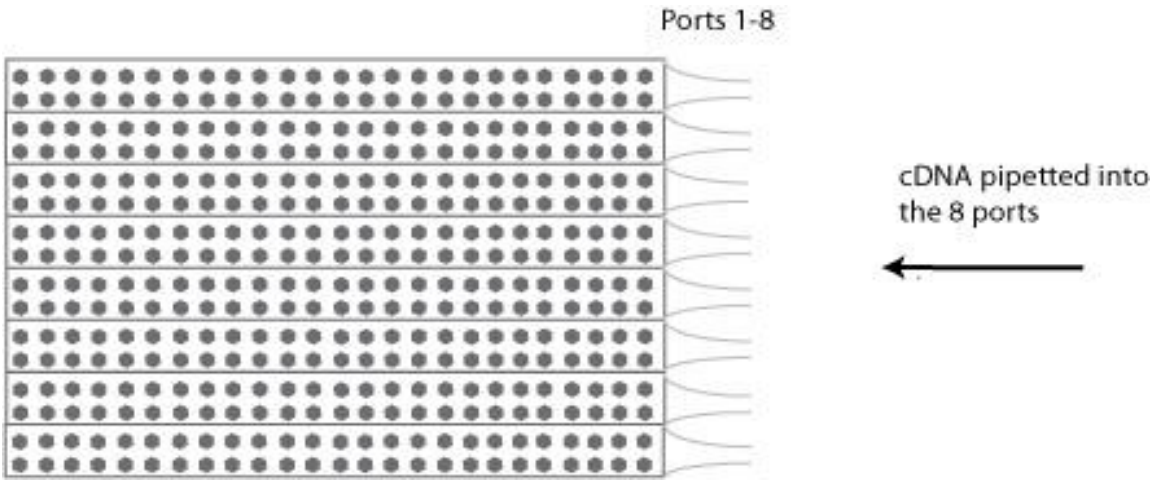


Figure 2.2: Diagram representing the Taqman microfluidic card system. Taqman microfluidic cards contain 394 wells fed by 8 ports with each port feeding approximately 50 wells. Each well has quantitative PCR primer and probe mix for a single gene or miRNA dried into the bottom which is re-suspended when the cDNA and mastermix are added. This allows 394 individual, singleplex QPCR reactions to occur on one card.

The card was then centrifuged to evenly distribute the cDNA/master mix between the wells and the card was then sealed and loaded into a 7900HT PCR machine with a Low Density Array upgrade (Applied Biosystems) and run at 50°C for 2 minutes, 95.4°C for 10 minutes and then for 40 cycles of 97°C for 30 seconds followed by 59.7°C for 1 minute. Data was analysed using the supplied SDS2.2.2 software (Applied Biosystems) according to manufacturer's instructions using the ΔΔCt method [609] and exported into Microsoft Excel for further analysis.

2.10.4 Taqman® Custom Array

To validate by QPCR the expression of genes of interest from the Affymetrix Exon array (section 2.11) we used a Taqman® Custom Arrays (Applied Biosystems). These custom arrays are 384 well micro fluidic cards with primers of your choice dried into the bottom of each well. We used format 48 cards which allow the quantitation of 47 unique assays (Table 5) plus one mandatory control (GAPDH) for 8 unique samples per card.

2.10.4.1 Reverse transcription for the Taqman® custom array

For each sample, 50ng of total RNA in a total volume of 5µl was added to 10µl of master mix containing 115nmoles dNTPs (Applied Biosystems), 0.05U of MultiScribe™ Reverse Transcriptase (Applied Biosystems), 3.8U of RNase Inhibitor (Applied Biosystems), 1.5µl of 10X Reverse Transcription Buffer (Applied Biosystems), 6.16µl of nuclease free water (Ambion) and 50pmoles random hexamers (Applied Biosystems).

Gene Name	Applied Biosystems Assay Number
7A5	Hs00766186_m1
ELOVL6	Hs00225412_m1
CD300A	Hs00381974_m1
C3orf37	Hs00220172_m1
CARD6	Hs00261581_m1
NETO2	Hs00983152_m1
CCL25	Hs00171144_m1
OASL	Hs00388714_m1
FCGR2B	Hs00414000_m1
TXNDC5	Hs00229373_m1
MAP2K6	Hs00992389_m1
IFI44	Hs00197427_m1
MX2	Hs00159418_m1
OAS3	Hs00196324_m1
CABLES1	Hs00292828_m1
IFIT1	Hs00356631_g1
IL12RB2	Hs00155486_m1
RSAD2	Hs00369813_m1
TRIM25	Hs00231947_m1
PIK3R3	Hs01103591_m1
CHI3L2	Hs00187790_m1
LGALS3BP	Hs00174774_m1
MX1	Hs00895598_m1
USP18	Hs00276441_m1

Gene Name	Applied Biosystems Assay Number
LY6E	Hs00158942_m1
IFI6	Hs00242571_m1
PTP4A3	Hs00845785_m1
RNASE6	Hs00377819_m1
CECR1	Hs00602615_m1
IFIT3	Hs00382744_m1
OAS2	Hs00159719_m1
GPR155	Hs00400624_m1
IFITM1	Hs01652522_m1
STK3	Hs00169491_m1
LGMN	Hs00559848_m1
SCL44A1	Hs00223114_m1
NR4A3	Hs00235001_m1
NR4A1	Hs00172437_m1
DUSP6	Hs00169257_m1
SPINT2	Hs00173936_m1
PLSCR1	Hs00275541_m1
PLAC8	Hs00930936_m1
PRDM1	Hs01068508_m1
BCL2LII	Hs00197892_m1
BACH2	Hs00222364_m1
B2M (endo)	Hs00187824_m1
PPIA (endo)	Hs99999904_m1

Table 5: List of genes and Taqman assay numbers used for the custom designed Taqman cards. These genes were identified on an Affymetrix exon array. Genes highlighted in yellow are potential tumour suppressor genes, highlighted in green are genes of other interest in the group and highlighted in grey are extra endogenous controls to GAPDH. The rest of the genes were up-regulated by EBV and not CD40L simulation of resting B cells and were included for the work of Heather Long (Cancer Sciences, The University of Birmingham) as a collaborative study.

2.10.4.2 Quantitative PCR for Taqman® custom array

The Taqman miRNA array cards were loaded and run according to the manufacturer's instructions. Briefly, for each sample, total cDNA (15µl) was diluted with 35µl of nuclease free water to make a 50µl total cDNA volume. This was combined with 50µl of Taqman universal PCR master mix (Applied Biosystems) to give a 100µl total cDNA/master mix. 100µl of this RT reaction-specific cDNA/master mix was added to one of the eight ports on the microfluidic card. The card was then centrifuged to evenly distribute the cDNA/master mix between the wells and the card was then sealed and loaded into a 7900HT PCR machine with a Low Density Array upgrade (Applied Biosystems) and run at 50°C for 2 minutes, 95.4°C for 10 minutes and then for 40 cycles of 97°C for 30 seconds followed by 59.7°C for 1 minute. Data was analysed using the supplied SDS2.2.2 software (Applied Biosystems) according to manufacturer's instructions using the ddCt method [609] and exported into Microsoft Excel for further analysis. All miRNA QPCR data in results I was normalised to the snoRNA endogenous control Z30 and in results chapters II and III, unless otherwise stated, data was normalised to the snoRNA endogenous control RNU48. All data expressed relative to an LCL in all Results I was LCL-PER and in results II and III, where not otherwise stated, was LCL A6

2.11 GeneChip Human Exon 1.0 ST Array

Gene expression profiling, using microarray technology, allows the measurement of thousands of genes and can be used to identify genes which are differentially expressed between multiple samples. To search for genes which were differentially expressed between EBV and CD40L blasts, the gene expression of resting B cells, EBV day 7 and CD40L day7 blasts was profiled using the GeneChip Human Exon 1.0 ST Arrays (Affymertix).

Each exon array can be analysed to give two different levels of gene expression information: the first is 'gene level analysis', which produces the conventional gene expression changes from microarrays. Each of the ~30,000 human genes is represented at least once and in many cases several times on the array; the second is an 'exon level analysis' which produces a list of gene expression changes which distinguish between different gene isoforms. The exon array contains an average of 4 probes per exon, which corresponds to approximately 40 probes per gene, allowing the expression of each exon, and therefore each gene isoforms, to be profiled.

RNA samples were sent to an in-house microarray service where the samples were prepared and the microarray was run. Briefly, 100ng of RNA was used to transcribe single stranded cDNA using oligo dT primers. A second strand was then synthesised and the double stranded cDNA purified. This double stranded cDNA was used as a template to generate fluorescently labelled cRNA by *in vitro* transcription, which was purified and fragmented before being hybridised to the microarray chip.

2.11.1 Sample preparation

RNA was extracted from resting B cells isolated from peripheral blood, B cells stimulated with CD40L and IL4 for 7 days or infected with EBV at an m.o.i of 100 and harvested 7 days post-infection, using the mirVana™PARIS™ kit (Applied Biosystems, section 2.7).

2.11.2 Microarray analysis

Data was analysed by bioinfomatician Wenbin Wei, using the Significance Analysis of Microarrays (SAM) analysis [613], to produce a gene level analysis of the microarray data. SAM analysis assigns a score to each gene based on the change in gene expression relative to the standard deviation of repeated measurements. The analysis uses gene specific T-tests to account for gene specific variation, as fluctuations in the signal to noise ratio are gene

specific. To maximise the significance of the statistical analysis, multiple permutations of the repeated measurements are compared; this data is used to estimate the percentage of genes identified by change, the false discovery rate (FDR). The parameters of SAM analysis can be defined by the user and SAM analysis of our data set a FDR of 0.05 and a minimum fold change of 1.5.

2.12 Transfection of miR-148a inhibitors and mimics into suspension cells

2.12.1 miR-148a inhibitor

miR-148a inhibitors (Applied Biosystems) are single stranded nucleic acids which bind to and inhibit miRNA function. Each miRNA inhibitor is designed to be complementary in sequence to its target miRNA, resulting in efficient binding following transfection. The inhibitors are chemically modified to increase the stability of the inhibitor and to prevent disassociation of the miRNA:inhibitor complex in the miRISC, enabling efficient inhibition of miRNA function.

To control for the transfection of the miR-148a inhibitors, a miRNA negative control was used, called anti-miR-negative control-1 (Applied Biosystems). This control inhibitor was a random sequenced molecule, tested extensively in human cell lines and tissues and validated not to produce any identifiable affect.

2.12.2 miR-148a mimic

To ectopically express miR-148a, Pre-miR™-precursor molecules (Applied Biosystems), referred to as ‘miRNA mimics’, were transfected into B cell lines. miRNA mimics are double stranded miRNA molecules which have been designed to ensure that they will only be taken

up by the miRISC complex in the correct orientation, resulting in production of only the mature miRNA of interest.

To control for the transfection of the miR-148a mimic, a miRNA mimic negative control was used, called Pre-miR™-negative control-1 (Applied Biosystems). This control mimic was a random sequenced molecule, tested extensively in human cell lines and tissues and validated not to produce any identifiable affect.

2.12.3 Transfection

To estimate the transfection efficiency of miRNA mimics and inhibitors, a siRNA fluorescently labelled with DY-547 (siGLO, Dharmacon) was transfected into an appropriate B cell line, and the percentage of cells transfected was measured by flow cytometry 24-48 hours post-transfection.

B cell cultures were passaged 24 hours prior to transfection to ensure that cells were in the optimal growth phase. Cells were washed 2 x ice cold PBS, counted, and re-suspended in Optimem medium (Gibco) to a concentration of 2 x 10^7 cells/ml. 500µl of cells were transferred to a sterile, 4mm electroporation cuvette (Geneflow) containing the stated concentration of miRNA mimic, inhibitor, or control RNA. When the co-transfection of the pMIR-REPORT luciferase system and miR-148a inhibitors were performed, 5µg of β-gal and either the luciferase or luciferase-148a plasmids were added to the electroporation curvette, along with the stated concentration of miR-148a inhibitor or control inhibitor. Electroportation was carried out using a Bio-Rad Gene Pulser II, with a capaticitance of 975µF and a voltage of 270. Transfected cells were transferred immediately to 9ml of pre-warmed cell culture medium and incubated at 37°C, 5% CO_2 for 24-48 hours. If the cells were to be prepared for western blotting, dead cells and debris were removed by carefully layering of the cells over 3ml of lymphoprep (Axis Shield) and centrifugation at 1,600RPM for 30

minutes without the brake. Live cells were then collected from the interphase between the BL medium and lymphoprep, and washed twice in fresh culture medium.

2.13 DNA extraction

DNA was extracted from approximately $5x10^6$ cells. Cells were washed with PBS to remove serum and DNA was extracted using the Qiagen DNeasy kit according to manufacturer's instructions. DNA was then eluted in 100µl of nuclease free water (Applied Biosystems) and the concentration determined using the Nanodrop (Thermo Scientific) according to the manufacturer's instructions. DNA samples were typically diluted to a concentration of 100ng/µl and stored in aliquots at -20°C

2.14 PCR amplification of DNA

2.14.1 PCR amplification of cDNA

A 50µl reaction mix was set up in a 0.5ml microfuge tube containing 100ng of cDNA, 2.5U Taq high fidelity enzyme bland (Roche), Expand 1x PCR buffer with 1.5nM $MgCl_2$ (Roche), 200µM dNTP, 300nM of each primer. An Eppendorf Thermocycler was used to hot start the PCR at 94°C for 5 minutes and then to incubate the samples through 35 cycles of 95°C to denature the DNA strands, 45-60°C to allow the primers to anneal to the DNA and 72°C to allow extension of the product. The annealing temperature and incubation time at each stage varied according to the specific primers and product size of each PCR. The PCR products were separated on agarose gel (section 2.15).

2.14.2 Sequencing PCR

200-500ng of DNA was put into a 10µl reaction volume sequencing reaction, containing 3.2pmol of sequencing primer (table 6) and nuclease free water. The samples were then

subjected to a full, automated DNA sequencing analysis using a 3700 DNA Analyser (Applied Biosystems) as part of a service provided by the functional genomics laboratory, School of Biosciences, The University of Birmingham. Olignucleotide primers were synthesised by Alta Biosystems, The University of Birmingham.

Plasmid	Position	Primer	Sequence
Pmir-REPORT-luciferase-148a	5' primer	pmirREPORT Forward	5'–AGGCGATTAAGTTGGGTA–3'
FT(DUSP6)UTG	5' primer	FT-UTG Forward	5'-AAAGGATCCACCATGATAGATACGCTCAGACCCGTGC-3'
	3' primer	FT-UTG Reverse	5'TTTTGTACATTAATTAATCACGTAGATTGCAGAGAGTCCACC-3'

Table 6: Primer sequences used to sequence the final lentivirus and luciferase vectors. The pmir-REPORT luciferase-148a plasmid and the FT(DUSP6)UTG lentivirus plasmid were sequenced following cloning.

2.15 Agarose gel electrophoresis

The DNA solution was mixed with 6 x gel loading buffer (0.25%w/v xylene cyanol, 0.25% w/v bromophenol blue, 30% v/v glycerol) and the DNA fragments separated by gel electrophoresis at 120 volts through a 1 x TBE (0.09M Tris-borate, 0.002M EDTA), 1% agarose gel (molecular biology grade) (Eurogentec). A1kb DNA ladder (GibcoBRL) was also loaded into the gel so the size of the PCR products could be determined. After electrophoresis the gel was stained in a bath of 1 x TBE containing 0.5µg/ml ethidium bromide and the DNA visualised with UV light on a transilluminator. A Polaroid photograph of the gel was often taken for record.

2.16 DNA extraction and purification form agarose gels

PCR products were visualised using a UV light box and the desired band cut out the gel using a sterile scalpel and placed into a sterile 1.5ml eppendorf. The gel fragment was then weighed

and the DNA extracted using the DNA gel extraction kit (Quiagen) according to the manufacturer's instructions. DNA was eluted in nuclease free water (Ambion).

2.17 Western blotting

2.17.1 Preparation of gel samples

Protein preparations were made from between $4x10^6$ and $1.5x10^7$ cells. Cells were washed twice in PBS to remove FCS and then pelleted by centrifugation at 1,200rpm for 5 minutes and lysed in 100-200µl of lysis buffer (Ambion). The viscosity of samples was reduced by disruption of genomic DNA by sonication using an ultrasonic cell disruptor (Misonix). The protein concentration was determined using a Bio-Rad DC protein assay kit according to the manufacturer's instructions. The desired concentration of protein (usually 2mg/ml) along with an equal volume of gel sample buffer (0.4M sodium 2-mercaptoethanesulfonate (MESNA), 125mM Tris-HCL pH 6.8, 20% v/v glycerol, 4% v/v SDS and 0.004% v/v bromophenol blue) were added together and heated to 100°C for 5 minutes. Gel samples were either used immediately or stored at -20°C.

2.17.2 SDS-PAGE

Pre-cast NuPAGE® Novex® Bis-Tris 10 well gels (Invitrogen) were loaded with approximately 20-40µg of protein alongside 10µl of Seeblue plus 2 pre-stained protein standards (Invitrogen). Electrophoresis was carried out in MOPS SDS running buffer (Invitrogen) at 78V for 3 hours in the XCell *Sure*Lock™ Mini-Cell (Invitrogen).

2.17.3 Blotting

Invitrolon™ polyvinylidine difluoride (PVDF) membranes (Invitrogen) were prepared by soaking in 100% methanol for 5 seconds and then soaking in blotting buffer (25mM TRIS-BASE, 192mM glycine and 20% v/v methanol) for 5 minutes. Once the dye front reached the

end of the gel, samples were blotted onto the pre-soaked PVDF membrane in an XCell II™ Blot Module (Invitrogen) blotting cassette. Onto the cathode side of the blotting cassette was placed 2 sponges and a sheet of filter paper (Invitrogen) soaked in blotting buffer. The gel was then placed onto the filter paper followed by the PVDF membrane and another pre-soaked filter paper. Finally 3 more pre-soaked sponges were added to the blotting cassette to ensure air bubbles were between the gel and the PVDF membrane. The blotting cassette was then filled with blotting buffer and the tank outside of the cassette filled with distilled water to allow cooling of the gel during transfer. The gel was blotted at 30V, 250mA for 100 minutes in a XCell *Sure*Lock™ Mini-Cell (Invitrogen)..

After blotting, the blotting cassette was disassembled and the PVDF membrane incubated in I-Block/Tween buffer (IBT) (0.4% w/v casein powder (I-Block™, Applied Biosystems), 0.05% v/v Tween, 0.02% sodium azide (NaN_3)) on a shaker at room temperature for 1 hour to prevent non-specific antibody binding. PVDF membranes were then incubated with primary antibody (table 7) diluted with blocking buffer either over night at 4°C or at room temperature for 2 hours depending on the antibody used. PVDF membranes were then washed 3 times for 2 minutes in PBS Tween. The appropriate secondary antibody conjugated to either peroxidise or alkaline phosphatase (table 7) and diluted in either blocking buffer or 5% w/v milk respectively, and then incubated with the PVDF membrane at room temperature with shaking for 1 hour. The membrane was then washed 5 times for 5 minutes in PBS Tween.

2.17.4 Chemiluminescence detection of antibody staining

Enhanced chemiluminescence (ECL) was used to visualise antibody bound proteins probed with a peroxidise-conjugated secondary antibody. 0.5ml of each of the two ECL reagents A and B (Amersham) were mixed together and the 1ml of combined reagents added to the PVDF membrane and incubated for 1 minute in Saranwrap. The excess reagents were then

removed from the membrane which was then sealed in the Saranwrap and placed in an autoradiograph cassette. Kodak X-Omat AR film (Kodak) was exposed to the membrane for between 30 seconds to 1 hour depending the strength of the luminescence signal.

Alkaline Phosphatase (AP) chemiluminescence was also used to visualise antibody bound proteins probed with an AP-conjugated secondary antibody (see table 7). PVDF membranes were incubated with 2 x 7ml AP buffer (0.1M diethanolamine pH 9.5, 1mM MgCl2,) with shaking for 2 minutes and then transferred to Saranwrap where 0.8ml of CDP-star™ developer (Invitrogen) was added to the membrane and incubated for 15 minutes. Excess reagent was then removed from the membrane and the Saranwrap sealed and then placed in an autoradiograph cassette for detection.

Target	Primary Antibody	Species	Working Dilution	Incubation	Secondary Antibody	Working dilution
LMP1	CS1-4 [614]	mouse	1/50	4°C over night	Anti-mouse IgG-peroxidase (Sigma)	1/1000
EBNA2	PE2 [615]	mouse	1/25	4°C, over night	Anti-mouse IgG-peroxidase (Sigma)	1/1000
EBNA3A	EBNA3A	sheep	1µg/ml	22°C, 2hours	AP-anti-sheep IgG (Biorad)	1/10,000
EBNA3C	EBNA3C	mouse	1/20	22°C, 2hours	AP-anti-mouse IgG (Biorad)	1/10,000
EBNA1	EBNA1	human	1/200	22°C, 2hours	AP-anti-human IgG (Biorad)	1/10,000
LMP2A	LMP2A	rat	1/10,000	4°C, 3hours	AP-anti-rat IgG (Biorad)	1/10,000
DUSP6	MK3 (Sigma)	mouse	1µg/ml	4°C over night	Anti-mouse IgG-peroxidase (Sigma)	1/1000
Phospho-ERK	p-ERK (Cell Signalling)	Mouse	1/2000	4°C over night	Anti-mouse IgG-peroxidase (Sigma)	1/1000
ERK1/2	ERK1/2 (Cell Signalling)	Rabbit	1/1000	4°C over night	Anti-rabbit IgG-peroxidase (Sigma)	1/1000
Calregulin	Calregulin (Santa-Cruz)	Goat	1ug/ml	22°C, 2hours	Anti-mouse IgG-peroxidase (Sigma)	1/100,000

Table 7: Antibodies used for western blotting

2.17.5 Stripping PVDF membranes

The secondary antibody used during western blotting can be removed (stripped) to allow re-probing of a blot with another primary and secondary antibody from a different species. The blots were stripped by incubation with Ponceau S stain (1% Ponseau S w/v (Sigma) in 3% v/v trichloroacetc acid (TCA)) at room temperature for 2 minutes with shaking. The Ponseau S stain was then washed off the membrane with tap water and the membrane was subsequently washed 3 times for 5 minutes in PBS Tween and re-blocked in blocking buffer for 1 hour. Blots were either re-probed immediately or stored at 4°C in Saranwrap to prevent drying out.

2.18 Fluorescence activated cell sorting

Flourescence activated cell sorting (FACS) was used to isolate either green fluorescent protein (GFP) positive cells transduced with a GFP expressing lentivirus or to sort primary tonsillar B cells into sub-populations based on CD10 and CD77 expression. FACS was performed using the cell sorting service at the Institute of Biomedical Research (IBR) using a MoFlo cell sorter (Beckman Coulter).

To isolate LCLs successfully transduced with either a GFP positive DUSP6 or control (empty vector) expressing lentivirus, cells were sorted a minimum of 48 hours post transduction to allow maximal expression of GFP and FACS was then used to sort individually GFP-positive LCLs.

To sort centrocytes and centroblasts isolated from a tonsil, 200 x 10^6 tonsillar cells were stained with CD10 (CD10-PC5, (BD Pharmingen)) and CD77 (CD-77-FITC (BD Pharmingen)). The $CD10^+/CD77^+$ (centroblasts), $CD10^+/CD77^-$ (centrocytes) and $CD10^-/CD77^-$ (non-GCBCs) were isolated by FACS.

2.19 Bacteriology

Plasmid vectors were grown in DH5α bacterial cells. Small scale DNA preparation was used to screen colonies containing recombinant plasmids while large scale preparations were used to make large amounts of high purity DNA suitable for transfection.

2.19.1 Bacterial growth medium

Liquid bacterial cultures were grown in Lennox Broth (L-Broth) medium. 2.5% w/v L-broth (Fisher Scientific) was dissolved in 400ml distilled water and sterilised before use by autoclaving at 121°C, 15psi for 15 minutes.

Bacterial colonies were cultivated on L-Broth Agar (L-agar) plates. 1.5% w/v of agar (Fisher Scientific) was dissolved in 400ml of deionised water and sterilised by autoclaving at 121°C, 15psi for 15 minutes. The L-agar was re-melted by microwaving, cooled to approximately 50°C and ampicillin was added to make a final concentration of 100µg/ml. 20ml of L-agar was poured into Petri dishes and left to solidify at room temperature and was then ether used immediately or stored at 4°C for up to one week. Before use plates were dried at 37°C.

2.19.2 Generation of competent DH5α *E.coli*

DH5α E coli were spread onto an L-agar plate and grown at 37°C overnight. 10-12 colonies were then picked from the plate and used to inoculate 250ml of SOB medium. Cultures were grown for 24-36 hours at 18-20°C until their absorbance at 600nm reached 0.6 when they were then placed on ice. Bacteria were then pelleted by centrifugation at 3000rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet re-suspended in 80ml of sterilised, ice cold TB (10mM Pipes, 15mM calcium chloride, 250mM KCL, adjusted to pH 6.7 with potassium hydroxide (KOH) and then supplemented with 55mM manganese chloride) and incubated on ice for 10 minutes. The culture was then centrifuged again at

3000rpm for 10 minutes at 4°C and the pellet re-suspended in 20ml of ice cold TB. 1.5ml of DMSO was added and the mixture incubated in ice for a further 10 minutes. 200µl aliquots of competent bacteria were snap frozen in liquid nitrogen and stored at -80°C ready for transformation.

2.19.3 Transformation of DH5α E.*coli*

Aliquots of competent DH5α E.*coli* were thawed on ice and 100µl transferred to an autoclaved 1.5ml microcentrifuge tube containing 10ng of plasmid DNA or the product of a DNA ligation reaction. The mixture was incubated on ice for 30 minutes and then heat shocked for 90 seconds at 42°C. 900µl of L-Broth was added to the culture and incubated at 37°C in a shaker to allow the bacterial to grow. 30µl of the culture was then spread onto L-agar/ampicillin plates and incubated overnight at 37°C.

2.19.4 Preparation of plasmid DNA

A single bacterial colony was picked using a sterile loop and used to inoculate 3ml of L-Broth containing the appropriate antibiotic (usually ampicillin). Cultures were then incubated with shaking at 37°C for 6 hours. 0.5ml of the culture was then added to 50-200ml of L-Broth containing the appropriate antibiotic in a 250ml conical flask and incubated at 37°C in a shaker overnight. The next day the bacteria were pelleted by centrifugation at 15,000rpm for 10 minutes and plasmid DNA extracted using the midi or maxi prep system (Jetstar) according to the manufacturer's instructions. The DNA pellet was then air dried before re-suspension in 200µl of TE buffer. The concentration of the plasmid DNA was determined using a Nanodrop (Thermo Scientific) according to manufacturer's instruction. Plasmid DNA was typically diluted to 1µg/µl and stored at -20°C until required.

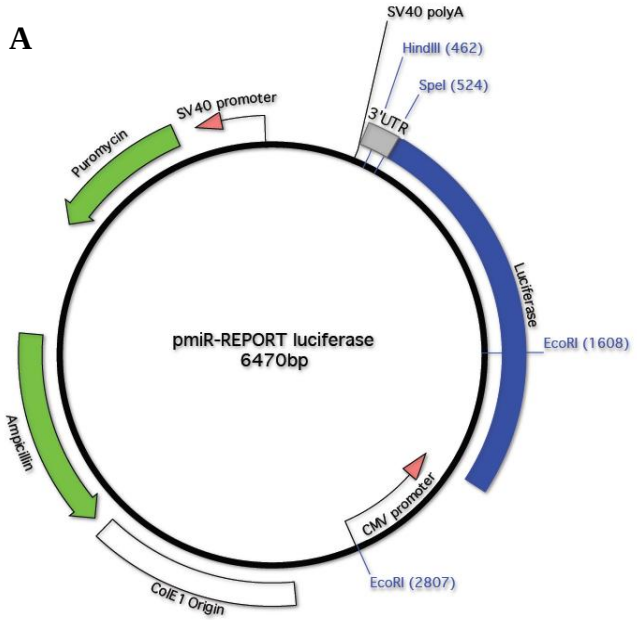
2.20 pMIR-REPORT™

The pMIR-REPORT expression reporter vector system (Ambion) consists of two mammalian expression vectors: pMIR-REPORT-luciferase and pMIR-REPORT-βgal. The pMIR-REPORT luciferase miRNA expression vector contains firefly luciferase under the control of a CMV promoter with a miRNA target cloning region down-stream of the luciferase sequence. This vector is designed for cloning putative miRNA target sites to evaluate miRNA regulation though the detection luciferase expression. pMIR-REPORT β-galactosidase reporter control vector is used for normalisation of transfection efficiency.

The pMIR-REPORT vectors were supplied as glycerol stocks and were cultured according to manufacturer's instructions.

2.20.1 Generation of pMIR-REPORT luciferase-hsa-miR-148a target

To evaluate the efficacy of the anti-hsa-miR-148a inhibitors following transfection into an LCL, we used the pMIR-REPORT system (Ambion) which included a luciferase vector for cloning miRNA target sites into the 3'UTR of the luciferease gene and a betagalactosidase (β-gal) plasmid used for co-transfection to normalise for transfection efficiency. To create a miR-148a responsive luciferase reporter we cloned a synthetic hsa-miR-148a binding site into the pMIR-REPORT luciferase vector (figure 1.3). This vector was designed to contain a binding site with 100% complimentarity to the hsa-miR-148a sequence in the 3'UTR of the luciferase gene for maximal binding efficiency and luciferase mRNA degradation/translational repression. Luciferase expression will then be inhibited by the high hsa-miR-148a expression in an LCL and the addition of anti-hsa-miR-148a inhibitors should therefore restore luciferse expression back to control vector (pMIR-REPORT luciferse with no insert) levels, allowing a read-out for the efficacy of the hsa-miR-148a inhibitors.



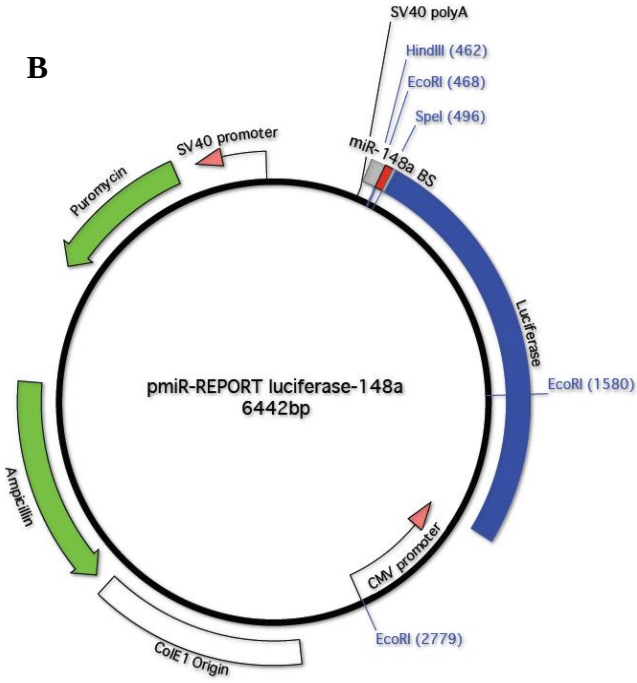


Figure 2.3: Schematic map of **A;** pmiR-REPORT-luciferase, and **B;** pmiR-REPORT-luciferase-148a. The miR-148a target site was cloned into the luciferase reporter in the multiple cloning site using *Spe*I and *Hind*III enzyme sites, enabling miR-148a to target the luciferase mRNA for degradation/translational repression. **C;** miR-148a binding site insert for pmiR-Report-luciferase. The *Eco*R1 restriction enzyme site is highlighted in green, *Spe*1 in red and *Hind*III in grey

C

5'-CTAGTACAAAGTTCTGTAGTGCACTGAGAATTCA-3' Coding Strand
3'-ATGTTTCAAGACTACACGTGACTCTTAAGTTCGA-5' Reverse Strand

The hsa-miR-148a target insert (figure 2.3) was designed to contain the reverse compliment of the hsa-miR-148a sequence (mature sequence accession number MIMAT0000243) taken from miRBase version 14 (http://www.mirbase.org/), and included an *Eco*R1 site to allow identification of clones containing the insert and Spe1 and Hind III sticky end sites for insertion into the vector.

2.21 FT(DUSP6)UTG lentivirus

2.21.1 Generation of FT(DUSP6)UTG lentivirus

To generate a lentivirus expressing DUSP6 under the control of a tetracycline repressor (TREX) which also constitutively expressed GFP for identification of DUSP6 lentivirus positive cells, DUSP6 was cloned into two lentivirral vectors designed by Marco Herold [616]. A DUSP6-HIS Topo vector was kindly donated to us by Toru Fuukawa [617] and was used as a PCR template for the lentivural cloning strategy and also served as a positive control for western blotting. The cloning strategy involved firstly inserting DUSP6 into an intermediate vector to pick up the tetracycline promoter and then the tetracycline promoter and DUSP6 were ligated into the final lentiviral vector which contained the tetracycline repressor protein and eGFP under the control of a ubiquitin promoter.

DUSP6 PCR primers were designed to contain a BamHI site at the 5’ end of the forward primer and at the 5’ end of the reverse primer they contained a BsrG1 site followed by a PACI site. The DUSP6 PCR product was first TA cloned into a Topo vector (Invitrogen) and then released by digestion with BsrG1 and BamHI restriction enzymes. DUSP6 was then ligated into an intermediate vector (FTGW, figure 2.4) where it replaced eGFP and was inserted downstream of a tetracyclin promoter (TREX-P) contained within the FTGW vector. DUSP6 was then cut of this intermediate vector along with the TREX-P and cloned into the final

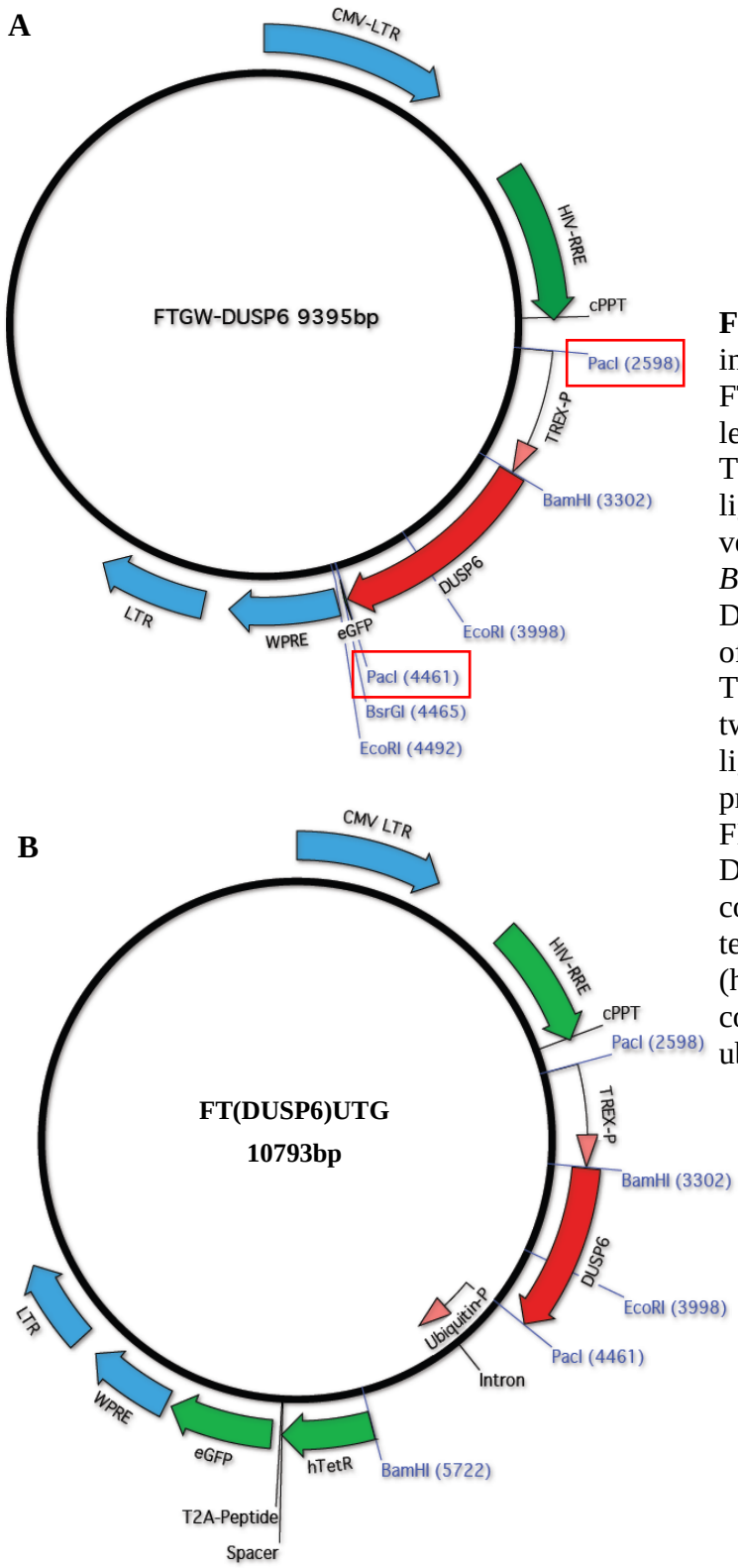


Figure 2.4: Schematic map of the intermediate cloning vector FTGW-DUSP6 and the final lentiviral vector FT(DUSP6)UTG. The DUSP6 PCR product was ligated into the intermediate vector FTGW using *Bsr*G1 and *Bam*HI sites to produce FTGW-DUSP6 (**A**) DUSP6 was cut out of FTGW-DUSP6 along with the TREX promoter (TREX-P) using two *Pac*1 sites (red boxes), and ligated into FIHt(INSR)UTG to produce the final lentivirus vector FIHt(DUSP6)UTG (**B**), where DUSP6 expression is under the control of the TREX-P and the tetracycline repressor protein (hTetR) and eGFP are constitutively expressed from the ubiquitin promoter (Ubiquitin-P)

vector (FHIT-IR5-UTG) to produce FT(DUSP6)UTG (figure 2.4). This final vector was packaged into a lentivirus and used to transduce selected LCLs. The majority of this cloning was kindly performed by Dr Jianmin Zuo.

2.21.2 Production of FT(DUSP6)UTG lentivirus

Lentivirus was produced using a second generation system and the lentivirus was pseudotyped with the VSV-G envelope glycoprotein (plasmid MD2G). 293-FT cells (Invitrogen) were plated out 24 hours prior to transfection to be 70-90% confluent on the day of transfection, in lentivirus production media. On the day of transfection, lentivirus production media was replaced 2 hours prior to transfection with 10ml of fresh, pre-warmed lentivirus production media. The following were mixed at room temperature for 5 minutes: (i) 1.5ml of Optimem (Gibco) was mixed with 36µl of Lipofectamine 2000 (Invitrogen) and (ii) 1.5ml of Optimem was mixed with 4µg of FT(DUSP6)UTG, 2µg of the envelope plasmid, MD2G and 6µg of the packaging plamid psPAX2. The contents of (i) and (ii) were then mixed and incubated at room temperature for 20 minutes. This transfection mix was then added to the 10ml of lentivirus production media on the 293-FT cells. 16 hours post-transfection the medium containing the transfection reagents was removed and replaced with 10ml fresh lentivirus production media plus 100µl of sodium pyruvate. 48 hours later, the media containing FT(DUSP6)UTG lentivirus was harvested by centrifugation at 1200RPM for 5 minutes to pellet any cells. Lentivirus supernatant was then filtered using a 0.45µM syringe filter. Lentivirus supernatant was either used immediately or stored at -80°C.

2.21.3 Transduction of LCLs with FT-DUSP6-UTG lentivirus

10ml of lentivirus supernatant was concentrated by ultracentrifugation prior to use. 10ml of lentivirus supernatant was added to a 14 ml thinwall polyallomer tube (Beckman). Tubes were weighed to ensure the centrifuge was balanced and then secured in the buckets of a SW40

swing rotor (Beckman) and centrifuged at 19000 RPM for 2 hours at 16°C in a Beckman ultracentrifuge. The supernatant was carefully removed and discarded until approximately 100-200µl of supernatant remained, which was used to re-suspend the lentivirus pellet. $5x10^5$ LCLs were pelleted by centrifugation at 1200 RPM for 5 minutes and the LCLs were re-suspended in the 100-200µl of concentrated lentivirus supernatant and incubated overnight at 37°C, 5% CO_2. LCLs were then cultured in culture media and transduction efficiency determined by flow cytometry for GFP expression.

2.22 LCL-DUSP6 growth assay

2.22.1 Trypan blue assay

To determine the number of viable stably transduced LCLs/ml either induced or un-induced to express DUSP6, a trypan blue assay was performed. 50µl of the culture was mixed with 50µl of trypan blue (Sigma) and the number of negatively stained cells/ml was determined using a haemocytometer. To ensure equal confluency in each sample at the beginning of the experiment, cells were pelleted by centrifugation at 1,200 RPM for 5 minutes and re-suspended at 0.2×10^6 viable cells/ml in culture media either containing 0.5µg/ml of tetracycline (DUSP6 induced), or with no tetracycline (DUSP6 un-induced) in one well of a 6 well plate. The number of viable cells were determined each subsequent day for 7 days.

2.22.2 Determination of cell growth by GFP

In this experiment, LCLs transduced with FT(DUSP6)UTG (LCL-DUSP6) were >99% positive by flow cytometry for GFP, following FACS. To monitor cell growth in the presence and absence of DSUP6 expression the LCL-DUSP6 GFP positive cell line was mixed at a 1:1 ratio with the parental, GFP negative, LCL to give a final cell concentration of 0.2×10^6

cells/ml. Cells were cultured in one well of a 6 well plate in the presence or absence of 0.5µg/ml of tetracycline, and GFP expression was measured after 7 days to determine whether the growth of the GFP positive DUSP6-LCL was altered following induced DUSP6 expression. If cell growth was inhibited then an outgrowth of the GFP negative, DUSP6 negative LCL would be expected, and vice versa.

3. Results Part I

Micro-RNA-155 regulation by EBV

3.1 Introduction

The observation which formed the basis of this aspect of the work came from a microarray experiment which showed the up-regulation of a gene called B cell integration cluster (BIC) following LMP1 expression in GC B cells [618]; BIC was the precursor (pri-miRNA) for the miRNA, miR-155. Although miRNA research was still in its infancy at this time, miR-155 was already regarded as a potentially oncogenic miRNA, or an 'oncomiR' [547], with many reports linking it to malignancies, particularly lymphomas [374, 545, 546, 554]. BIC and miR-155 were shown to be over expressed in HL [553, 612] and BIC was reported to have extremely low expression in BL [619]. A possible defect in the processing of BIC to miR-155 had also been demonstrated in BL cell lines [375]. There was some literature emerging regarding miR-155 and EBV, with miR-155 expression being reported to be high in EBV transformed LCLs [374, 603, 620]. Despite the observation that BIC was highly expressed in EBV transformed LCLs and one EBV associated lymphoma, no link between EBV and miR-155 in these malignancies had been investigated, providing a unique opportunity for further study. The broad aims of this part of the study were to (i) validate the regulation of BIC/miR-155 by LMP1, (ii) investigate the regulation of miR-155 by EBV in the context of the whole virus infection and (iii) further examine the BIC to miR-155 processing defect in BL reported by Kluiver et al [375].

3.2 Characterising miR-155 expression

Previously in the literature, miR-155 expression had been measured by northern blotting and in-situ-hybridisation in a variety of cell lines, tumour cell lines and primary tissue. These techniques lack the sensitivity and specificity for the miRNA. We chose to take advantage of a newly available Taqman RT-QPCR technique which claimed to show much greater sensitivity and specificity for the mature miRNA.

3.2.1 miR-155 expression in BL and HL cell lines

First, we sought to confirm the reported high expression of miR-155 in HL and low expression in BL, using a series of EBV positive and negative HL cell lines and a panel of latency I BL cell lines for QPCR measurement of miR-155 (Figure 3.1). With the exception of L540, the HL cell lines showed very high levels of miR-155 expression regardless of EBV status. The panel of BL cell lines show detectable but very low expression of miR-155 when compared with the LCL or HL cell lines but the expression level is variable in the BL cell lines, with the Ezema-BL cell line showing higher miR-155 expression than the resting B cells. These data are broadly consistent with published reports.

3.2.2 miR-155 expression in B cells

Previous studies into miR-155 expression in primary cells have not been able to detect miR-155 due to the lack of sensitivity of their techniques. The QPCR method of miRNA detection allowed us to detect and quantitate miR-155 in primary cells, which was previously not possible.

RNA in-situ-hybridisation for BIC (pri-miR-155), demonstrated that the few BIC positive cells detected in the tonsil and lymph node were located predominantly within germinal centres [612]. Since HL and BL both have GC phenotypes but very different expression levels

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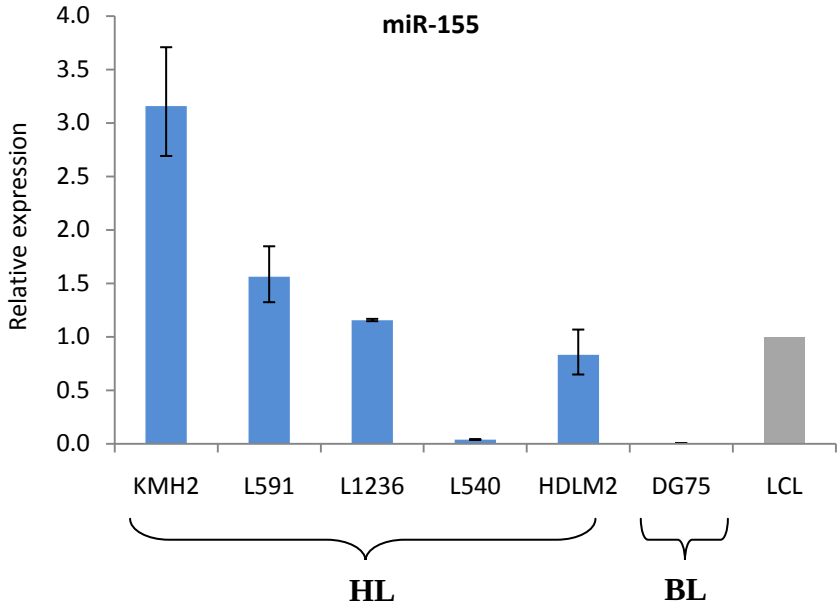

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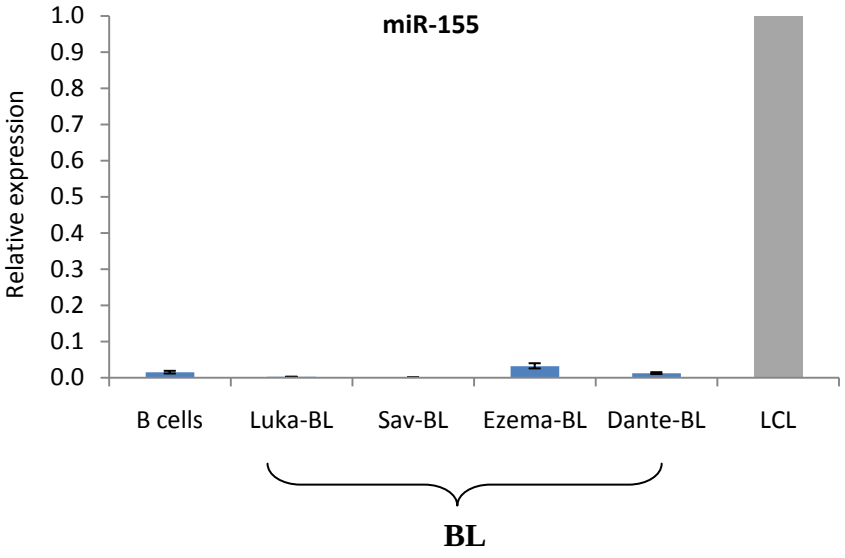

Figure 3.1: miR-155 expression by QPCR in HL and BL cell lines. **A**; Panel of HL cell lines, L591 is the only EBV positive HL cell line and DG75 is an EBV negative BL cell line. **B**; Panel of latency I BL cell lines and peripheral blood $CD19^{+}$ resting B cells. Error bars represent the range in fold change within one experiment calculated from the standard deviation of the ΔΔCt values.

of miR-155, we wanted to establish if either lymphoma represented the original cell type. To address this, we examined miR-155 expression in different populations of B cells isolated from healthy tonsils by co-staining for CD10 and CD77 (Figure 3.2). CD10 is a marker for GC B cells and CD77 staining subdivides the $CD10^+$ GC B cells into: $CD77^+$ centrocytes and $CD77^-$ centroblasts. miR-155 expression showed little variation between different B cell subsets, with no greater expression seen in the germinal centre B cells compared with the $CD19^+$ B cells. This suggests that the high expression of miR-155 in HL is a feature of the malignant cell rather than reflecting the normal cell origin.

3.3 Regulation of miR-155 by EBV

When we began this work in 2007 it was not known whether miR-155 expression was regulated by EBV, despite its high expression in LCLs and HL, a tumour which is often associated with EBV infection. The following work aimed to validate the observed regulation of BIC and miR-155 by LMP1 and further investigate their regulation by the virus.

3.3.1 miR-155 association with EBV latency in BL

Following on from the observation that miR-155 expression was low in latency I BL cell lines, we asked whether a latency III pattern of EBV gene expression in a BL cell line would result in up-regulation of miR-155, especially considering latency III cells express LMP1. We decided to examine a panel of Mutu-BL lines in addition to the Mutu-I cell line. These Mutu-BL clones included an EBV loss clone (EBV negative) and two clones which had drifted in culture from a latency I, to a latency III pattern of EBV gene expression. These Mutu-BL clones would allow the correlation of miR-155 expression and EBV latent gene expression in the same cell background. The EBV latent gene expression of the Mutu-BL clones was confirmed by western blot (Figure 3.3).

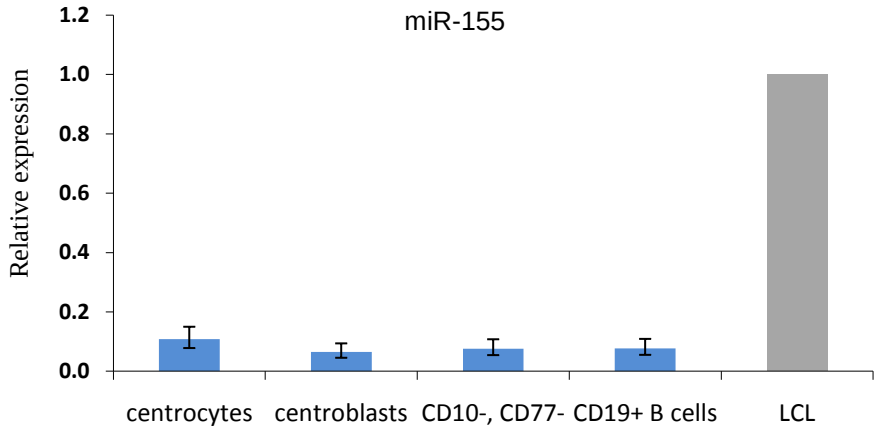

Figure 3.2. miR-155 expression by QPCR in different tonsillar B cell subsets. Populations of B cells were sorted by FACS staining from a non-IM tonsil. Germinal centre B cells were stained for CD10 and CD77. $CD77^+$ cells are centrocytes and $CD77^-$ cells are the centroblasts. $CD19^+$ cells are the total B cells and these were positively selected using $CD19^+$ beads. Data is expressed relative to an LCL. Error bars represent the range in fold change within one experiment calculated from the standard deviation of the ΔΔCt values.

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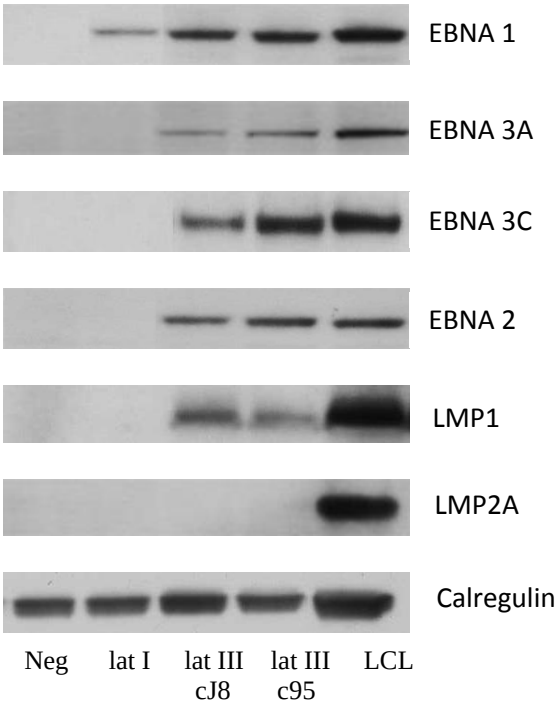

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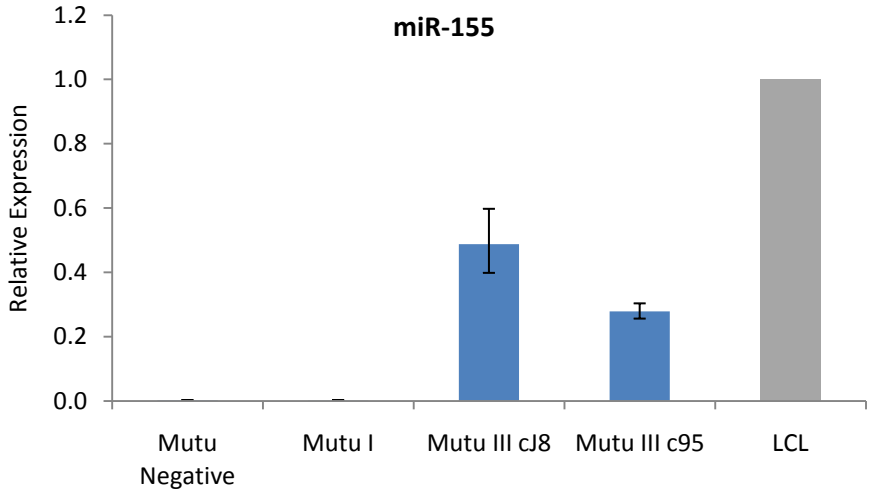

Figure 3.3. A; Western blot analysis of EBV latent gene expression and QPCR for miR-155 expression in a panel of Mutu-BL clones. Mutu I represents the biopsy phenotype of this tumour, while Mutu negative has lost the EBV genome and the two latency III clones (j8 and 95) have drifted in culture to express a latency III type of EBV gene expression similar to that seen in an LCL. **B**; The status of EBV was confirmed in the clones (neg; EBV negative, lat I; latency I; lat III; latency III and an LCL) using western blot to various EBV latent genes. Error bars represent the range in fold change within one experiment calculated from the standard deviation of the ΔΔCt values and data was normalised to the snoRNA, Z30.

We examined the expression of miR-155 by QPCR and miR-155 was clearly up-regulated by a latency III pattern of gene expression but it was still less than half the miR-155 expression seen in an LCL (figure 3.3).

3.3.2 miR-155 expression following induced EBV latent gene expression in DG75-BL

To determine which latency III associated EBV genes were capable of up-regulating miR-155 in BL cell lines, we used previously established stable clones of the EBV negative BL cell line DG75 with inducible EBV latent gene expression under the control of a tetracycline activator [162]. These DG75 clones expressed either: an empty vector control (DG75-tTA), LMP1 (DG75-tTA/LMP1), LMP2A (DG75-tTA/LMP2A) or EBNA2 (DG75-tTA/EBNA2); and the gene expression was induced upon removal of tetracycline. We also examined inducible clones of the BL-like cell lines, BJAB-tTA and BJAB-tTA/LMP1. These inducible transfectants were assayed for miR-155 expression to determine the effects of LMP1 expression in different B cell backgrounds.

LMP1 expression in BJAB resulted in a modest increase in miR-155 expression to only approximately 10% of the expression seen in an LCL (figure 3.4). Whereas, LMP1 expression in DG75 caused a more significant increase in miR-155 expression, to approximately 20% of the expression seen in an LCL (figure 3.5). This result is more convincing when it is expressed relative to the un-induced DG75tTA/LMP1 control (figure 3.5, C), with an increase in miR-155 expression of 15 fold; thus verifying the up-regulation of miR-155 by LMP1.

Comparing the miR-155 expression in DG75 with induced LMP1 expression (figure 3.5) and latency III Mutu-BL (figure 3.3), it is clear that the latency III Mutu-BL express a higher level of miR-155. This is despite the fact that both Mutu III clones express less LMP1 than the induced DG75 by western blot. There could be many possible explanations for this but it does suggest that there may be other latency III associated genes which are involved in the up-

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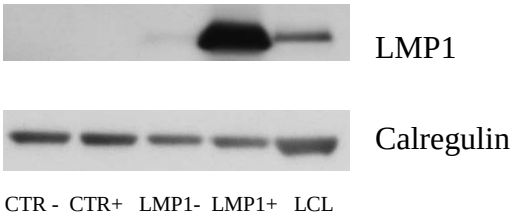

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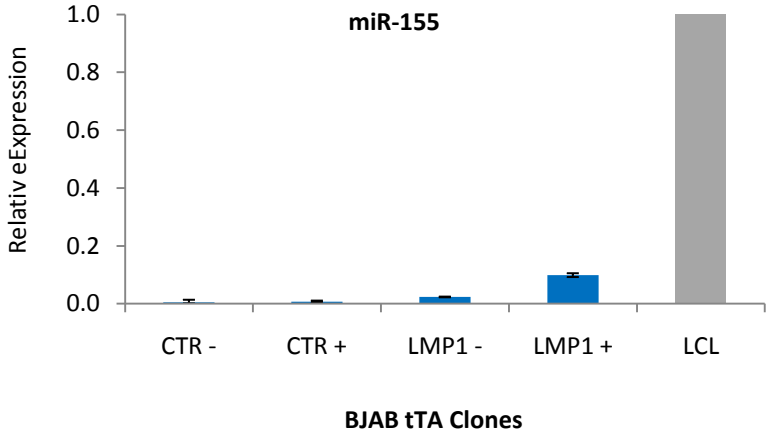

Figure 3.4: Western blots analysis of LMP1 expression and QPCR for miR-155 expression in BJABtTA/LMP1 clones. **A;** LMP1 induction in stable BJAB tTA and tTA/LMP1 transfectants either un-induced (-) or induced (+) by the addition or removal of tetracycline respectively. **B;** Representative QPCR data showing miR-155 expression relative to an LCL in BJAB tTA clones stably expressing either a control (CTR) or LMP1 expression vector under the control of a tetracycline responsive promoter. Samples are shown at 48 hours as either induced (+) or un-induced (-). Error bars represent the range in fold change within one experiment calculated from the standard deviation of the ΔΔCt values.

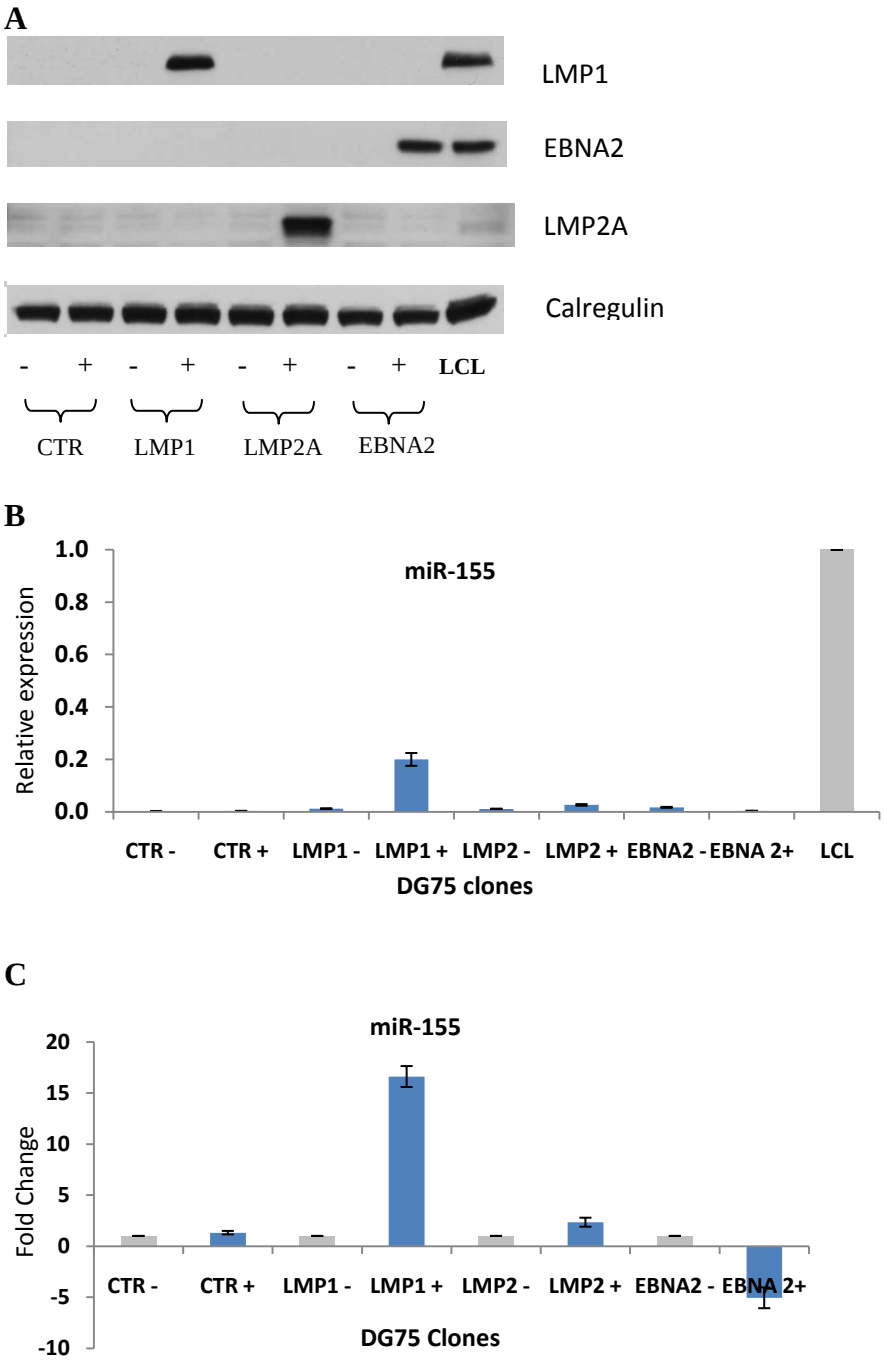

Figure 3.5: Western blot analysis of EBV latent gene expression and QPCR for miR-155 expression in DG75tTA clones. **A**; Western blots confirming the expression of LMP1, LMP2 and EBNA2 in the induced DG75 samples. **B**; Representative data showing miR-155 expression in DG75 clones stably expressing either a control (CTR), LMP1, LMP2A or EBNA2 expression vector under the control of tetracycline. Samples are shown at 48 hours as either induced (+) or un-induced (-). Data is expressed relative to an LCL. **C**;. The same data as for **(B)** but expressed relative to the un-induced controls. Error bars represent the range in fold change within one experiment calculated from the standard deviation of the ΔΔCt values.

regulation of miR-155 in BL cell lines, or that the effects of LMP1 are dependent on the cell context. LMP1 expression in BJAB resulted in a similar level of miR-155 expression to the induced DG75 (approximately 0.1 of an LCL), despite the fact that the induced BJAB showed greater LMP1 expression than DG75 relative to the same LCL (figures 3.4 B and 3.5 C). Collectively these data suggest that miR-155 expression does not directly correlate with the amount of LMP1 expression. However, induction of LMP2A or EBNA2 expression in DG75 did not result in the up-regulation of miR-155 in DG75, in fact, EBNA2 may have resulted in a 5 fold down-regulation (figure 3.5).

3.3.3 miR-155 expression during infection of primary B cells

We wanted to determine the time course of miR-155 up-regulation by EBV as LMP1 is not expressed until approximately 5 days post-infection (p.i) [341]. Therefore, the kinetics of miR-155 up-regulation p.i may support or oppose the hypothesis that miR-155 up-regulation by EBV is mediated exclusively by LMP1.

We began by measuring miR-155 expression in resting B cells infected with EBV or stimulated with CD40L and IL4 to produce activated, proliferating B cell blasts [621] which are phenotypically very similar to EBV-transformed LCLs. As LMP1 is in many respects a functional homolog of CD40 [622-624], we speculated that it may also be capable of up-regulating miR-155.

$CD19^+$ resting peripheral blood B cells from several donors were pooled and a sample of the B cells was kept as a day 0 control. The remaining cells were either infected with EBV at an m.o.i of 100 or stimulated with CD40L and IL4 to produce B cell blasts. The results confirmed the high expression of miR-155 in LCLs and also showed that miR-155 was up-regulated by EBV infection and CD40L stimulation within 24 hours (figure 3.6). This is

before LMP1 is expressed in resting B cells, suggesting other EBV genes, or simply resting B cell activation through virus binding, is sufficient to up-regulate this miRNA.

3.3.4 miR-155 regulation by LMP1-knockout EBV

The kinetics of miR-155 up-regulation suggested that miR-155 was up-regulated prior to LMP1 expression in B cells. Nevertheless, LMP1 expression in BL-cell lines was sufficient to cause a significant up-regulation of miR-155. This suggests that miR-155 may be up-regulated early in B cell transformation by virus binding or other EBV latent genes, but that LMP1 may be responsible for further up-regulation and maintenance of miR-155 expression. This hypothesis is further supported by the observation that CD40L stimulation of miR-155 decreased at later time points, whereas LMP1 stimulation increased over the time course (figure 3.6). To address this, we used an LMP1-knockout (LMP1KO) virus [606] and monitored miR-155 expression by QPCR. The LMP1KO virus is non-transforming and infected B cells are activated initially and after approximately 10 days the cells begin to die, therefore all time points were harvested within 10 days post-infection. B cells infected with an LMP1KO virus up-regulated miR-155 to a similar level as the wild type virus (wtEBV) at all time points harvested (figure 3.7, A). This suggests that LMP1 plays no role in the up-regulation of miR-155 in the early stages of transformation.

3.3.5 miR-155 up-regulation following virus binding

Previous data which examined miR-155 expression following individual latent gene expression in a variety of BL cell lines indicated that miR-155 up-regulation by another EBV latent gene was unlikely (section 3.3.2). Virus binding to B cells is mediated through the viral glycoprotein gp350 (BLLF) binding to the complement receptor CD21 on the B cell surface

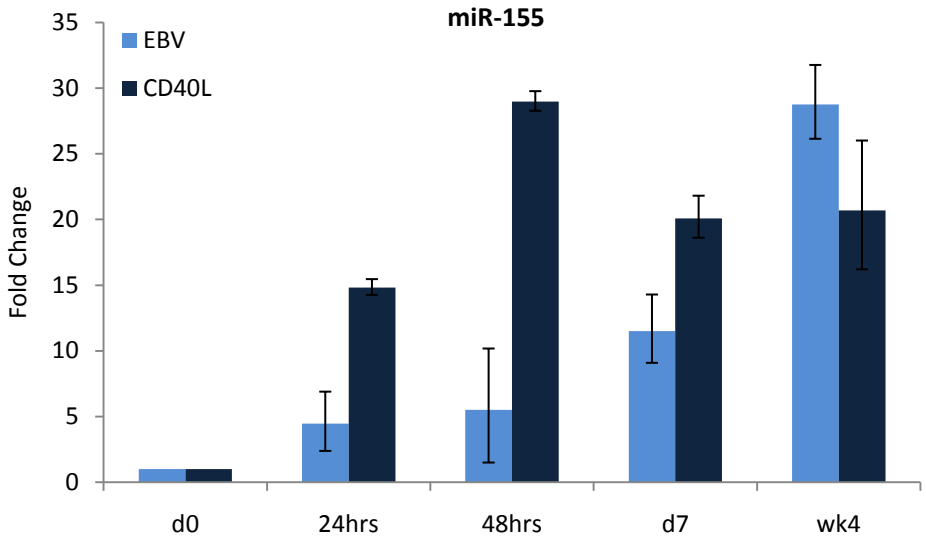

Figure 3.6: miR-155 expression by QPCR in primary B cells either infected with EBV or stimulated with CD40L and IL4. $CD19^{+}$ purified B cells were infected with EBV (m.o.i 100) or stimulated with CD40L + IL4 for 24 hours, 48 hours, 7 days (d7) or 4 weeks (wk 4). Data is expressed relative to the resting B cells and error bars represent the range in fold change within one experiment calculated from the standard deviation of the ΔΔCt values.

[331]. Penetration of the cell membrane requires a complex of three additional glycoproteins, including gp42 which binds to HLA II molecules to initiate virus-cell fusion [625]. A mutated EBV which has the gp42 protein deleted from the genome (Δgp42 virus) can bind to the B cell surface through gp350-CD21, but it cannot enter and subsequently infect the B cell [39]. B cells incubated with Δgp42 virus will therefore still bind virus as normal and the B cells may still be activated though the gp350-CD21 interaction, but no virus particles will enter the B cells. This allows the effect of just virus binding to the surface of the B cell to be examined, in the absence of any viral gene expression, and the effect on miR-155 expression to be measured.

Infection of B cells with wtEBV, LMP1KO and Δgp42 virus was performed to determine whether the up-regulation of miR-155 seen during early infection with wtEBV and LMP1KO virus was due to virus binding and activation of the resting B cells. Δgp42 virus infection of B cells consistently resulted in an up-regulated miR-155 expression. The up-regulation observed following Δgp42 infection was always lower than that seen following wtEBV or LMP1KO infections; however it was consistently a 5-10 fold up-regulation (figure 3.7, B). The up-regulation of miR-155 following B cell receptor (BCR) activation has been reported [612, 626, 627]. It appears, therefore, that B cell activation through virus binding may explain the early up-regulation of miR-155 by wtEBV, before LMP1 is expressed. The up-regulation of miR-155 by the Δgp42 virus complicates interpretation of the LMPKO virus data, as any up-regulation of miR-155 in the LMP1KO or wtEBV infections seen at 5-7 days pi will be masked by the effect of virus binding, and later time points are not possible due to the non-transforming nature of the LMP1KO virus. Therefore, influence of LMP1, or any other EBV gene, on the regulation of miR-155, cannot be determined using these recombinant viruses.

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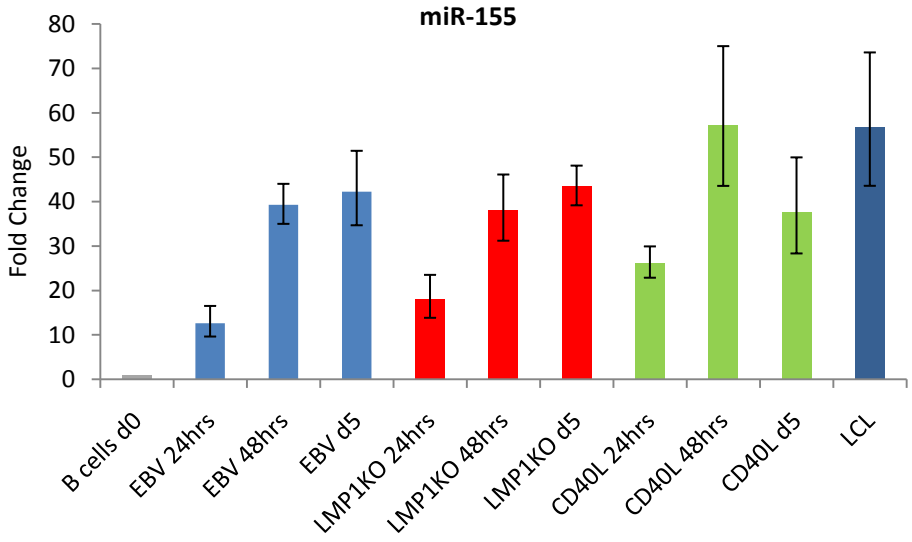

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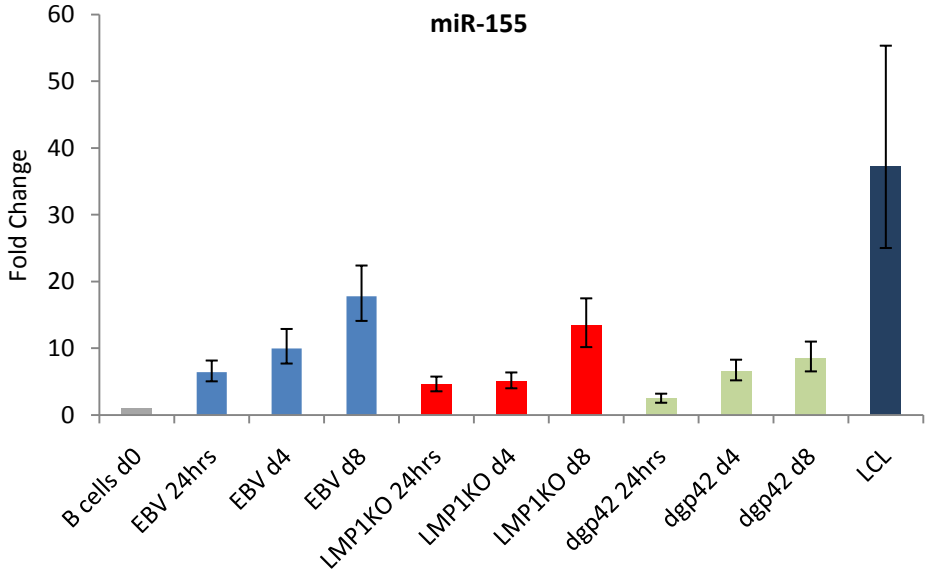

Figure 3.7 miR-155 expression by QPCR in primary B cells infected with LMP1KO, Δgp42 or wt EBV. **A;** resting B cells (d0) infected with either wt EBV (EBV), an LMP1 knock-out virus (LMP1KO) or stimulated with sCD40L and IL4 and harvested after 24 hours, 48 hours and 5 days. Data is expressed relative to the resting B cells. **B;** Resting B cells were infected with either wt EBV, LMP1KO virus or a GP42 knock-out virus (Gp42) and cells were harvested after 24hours, 4 days and 8 days. Data is expressed relative to the resting B cells. Error bars represent the range in fold change within one experiment calculated from the standard deviation of the ΔΔCt values.

3.4 Processing of BIC to miR-155 in BL

Kluiver et al reported in 2007 that stimulation of BIC expression (using anti-IgM) or even ectopic BIC expression in the EBV negative BL cell lines, Ramos and DG75, did not result in miR-155 expression and that this suggested a specific block in the processing of BIC to miR-155 in this tumour [375]. Two observations from our own data could be interpreted as evidence which supports a possible processing defect in BL: Firstly, latency III Mutu-BL expressed approximately 50% less miR-155 than an LCL which may represent inefficient processing of BIC to miR-155 in these cells. Secondly, LMP1 expression in BL cell lines caused an increase in miR-155 expression to approximately 0.1-0.2 of the miR-155 expression seen in an LCL, which could also represent inefficient processing of BIC in these cells. Additionally, the BJAB and DG75 cell lines showed variable increases in miR-155 expression which could represent variability in the processing defect in different cell backgrounds.

Collectively, the reported BIC processing defect and our own observations raised the following questions: is the miR-155 expression seen in latency III BL cell lines the result of increased BIC, and therefore miR-155 expression? Or, is BIC already expressed in BL and an EBV latent is gene required to stimulate the processing of BIC to miR-155? To address these questions we examined the expression of major components of the miRNA processing machinery and used a QPCR assay specific for spliced BIC mRNA, as previously described [612], to re-examine the BIC processing defect proposed by Kluiver et al [375].

3.4.1 Dicer-1 and Drosha expression in BL cell lines

The processing of BIC to miR-155 will involve a number of proteins which comprise the miRNA processing machinery. Two major components of the miRNA processing pathway are

the enzymes Drosha and Dicer-1. Drosha is a nuclear protein which cleaves the primary miRNA transcript and Dicer-1 cleaves the pre-miRNA transcript to produce the mature miRNA. Any defects in either of these proteins would result in defective miRNA processing. Increased expression of Drosha has been reported in cervical carcinoma [404], and loss of either gene was associated with increased tumourogenesis in a mouse model [402]. Thus, there is evidence to suggest that the expression of these enzymes may be altered in certain cancers, and the processing of miRNAs will consequently be altered.

To determine whether the reported BIC processing defect in BL was due to dysregualtion of the expression of either Drosha or Dicer-1, we used commercially available Dicer-1 and Drosha QPCR assays to measure the expression of these two genes in a variety of cell lines. Initially we measured Drosha and Dicer-1 expression in cells which could effectively process miR-155 to establish an estimate of functional Drosha and Dicer-1 expression; primary B cells, EBV and CD40L day 7 blasts plus an LCL were examined (figure 3.8) and these cells all expressed at least 50% of the Dicer-1 and Drosha expression of an LCL, therefore this level of expression is likely to be sufficient for BIC processing.

Next, we examined the DG75 tTA and DG75 tTA/LMP1 BL clones used in section 3.4.2. All DG75-BL clones showed similar levels of Dicer-1 and Drosha expression to the LCL, therefore these two miRNA processing enzymes are highly expressed at the transcript level in this BL cell line and are unlikely be the cause of a miRNA processing defect.

Kluiver et al also compared the processing of BIC in the epithelial cell line, 293 cells and DG75-BL, following ectopic BIC expression [375]. To assist in the interpretation of these data, we compared the expression of Drosha and Dicer-1 in 293 cells and DG75-BL. Neither cell expressed less Drosha or Dicer-1 than the LCL, indicating that the expression of these

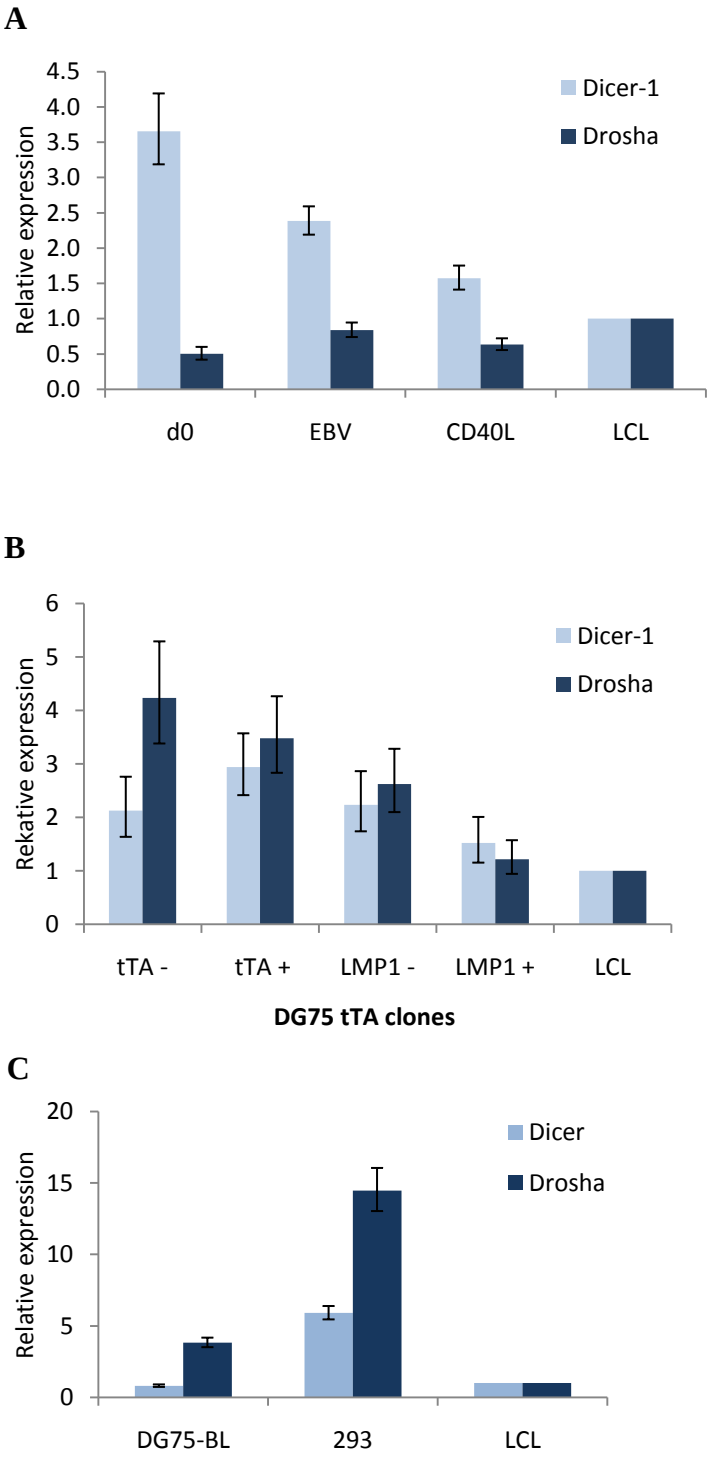

Figure 3.8 Dicer1 and Drosha expression by QPCR in primary B cells, DG75-BL tetracycline inducible clones and 293 cells. **A**; primary B cell infection, **B;** stable DG75 tTA cell lines expressing either an empty control or LMP1 expression vector with (+) or without (-) induction by removal of tetracycline, and **C**; 293 and DG75-BL expressed relative to an LCL. Error bars represent the range in fold change within one experiment calculated from the standard deviation of the ΔΔCt values.

two enzymes is unlikely to a contributing factor to the reported processing defect of BIC in DG75-BL.

3.4.2 BIC expression and EBV latent gene expression in Mutu-BL

BIC expression in the same panel of Mutu-BL cell lines used in section 3.3.1 mirrored the expression of miR-155 in these lines (figure 3.9). BIC expression is high in latency III BL cell lines and very low in the EBV negative and latency I, suggesting increased BIC transcription and subsequent processing are responsible for the elevated miR-155 expression seen in latency III BL. This result supports the observation by Kluiver et al [375] who also reported high BIC expression in the latency III BL cell line Raji.

3.4.3 BIC is processed in Ramos-BL after BCR stimulation

Van den Berg et al were the first to report that BIC was up-regulated in B cells after BcR stimulation with anti-IgM [612]. This observation was later repeated and expanded upon by Kluiver et al [375], who showed that BIC was up-regulated in Ramos-BL cells following treatment with anti-IgM but this did not result in miR-155 expression, further supporting the observation that BIC cannot be processed in BL. We re-examined this result using Ramos cell lines, including a transfectant expressing LMP1 under the control of a tetracycline activator, similar to the DG75 tTA cell lines used in section 3.3.2. This allowed us to compare BIC processing after anti-IgM treatment in the absence or presence of LMP1. The LMP1 expressing clone would serve as a positive control for BIC to miR-155 processing as we had previously shown that LMP1 was able to induce BIC to miR-155 processing in DG75-BL and BJAB cell lines. An empty vector Ramos tTA control cell line was used to control for the effect of tetracycline on BIC/miR-155 expression.

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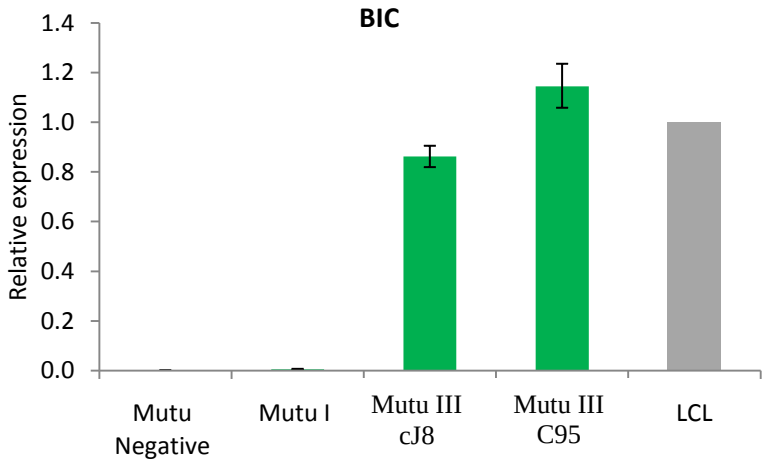

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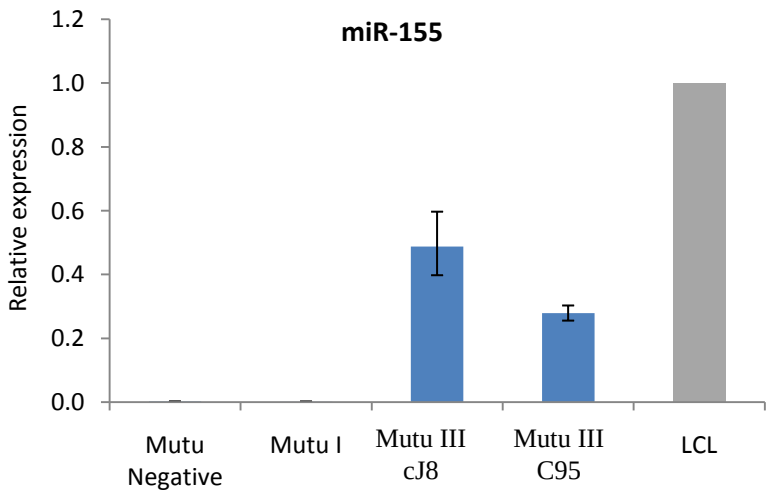

Figure 3.9: BIC and miR-155 expression by QPCR in a panel of Mutu-BL clones. **A**; BIC and **B**; miR-155 in four sub-clones of Mutu-BL by quantitative RT-PCR. Mutu I represents the biopsy phenotype of this tumour, while Mutu negative has lost the EBV genome and the two latency III clones (j8 and 95) have drifted in culture to express a latency III type of EBV gene expression similar to that seen in an LCL. EBV latent gene expression was confirmed (figure 3.3). Error bars represent the range in fold change within one experiment calculated from the standard deviation of the ΔΔCt values.

Both BIC and miR-155 were induced by anti-IgM treatment and by LMP1 expression in the Ramos tTA/LMP1 cell line (figure 3.10) which was in disagreement with the data reported by Kluiver et al [375], who used QPCR to measure BIC expression in their experiments; however, they used northern blotting to detect miR-155 expression, which may explain our differing results. QPCR is a very sensitive technique which is recognised to be more specific and sensitive then northern blotting [608]. miR-155 expression is very low in BL and our previous data on DG75 showed that a 10-20 fold up-regulation of miR-155 in a BL cell is still only 10-20% of the expression seen in an LCL. It is therefore possible that the northern blot assay in Kluiver's study was unable to detect the increase in miR-155 expression following anti-IgM treatment, whereas their QPCR assay was sensitive enough to detect the increase in BIC expression.

3.4.4 Ectopic expression of BIC in DG75-BL

In the Kluiver et al study [375], ectopic BIC expression in Ramos-BL did not result in miR-155 expression, despite very high levels of BIC mRNA after transfection. We were kindly donated some of the BIC PCDNA.3 expression vector used by Kluiver, which contained the full length BIC cDNA. We sought to confirm the results seen by Kluiver et al by re-examining the processing of BIC to miR-155 in DG75-BL and 293 cells, using the sensitive miRNA QPCR assay.

293 cells were transfected using lipofectamine with 1μg of PCDNA.3-BIC or PCDNA.3 and the cells were selected in 0.5mg/ml G418 for 3 weeks. DG75-BL cells were transfected via electropration with 5μg of PCDNA.3-BIC or PCDNA.3 and positive cells selected in 2mg/ml of G418 for 3 weeks. BIC and miR-155 expression in the stably transfected 293 and DG75-BL cell lines was measured by QPCR.

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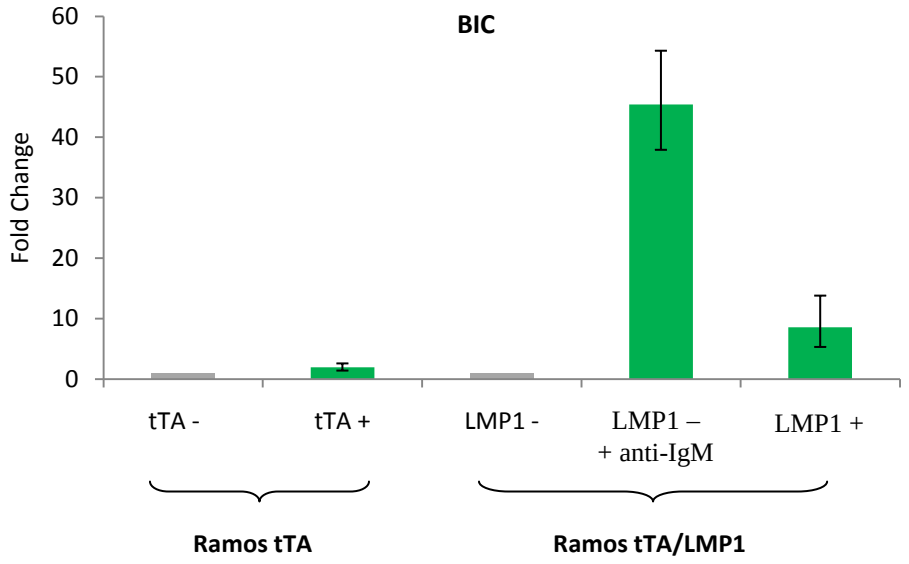

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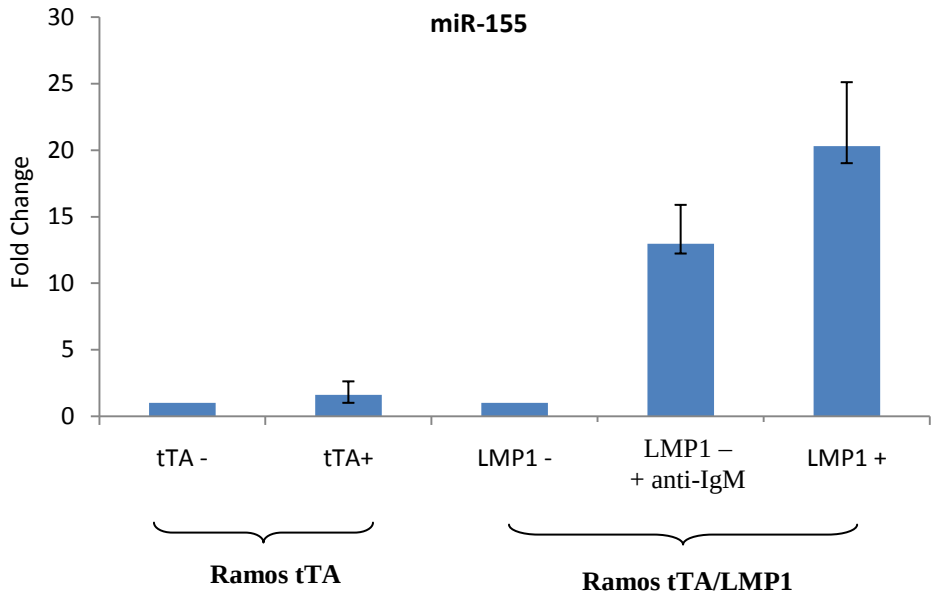

Figure 3.10: BIC and miR-155 expression in Ramos-BL cells following either LMP1 expression or BCR stimulation. **A;** BIC and **B;** miR-155 expression in induced (+) or uninduced (-) Ramos tTA and Ramos tTA/LMP1, and after BCR stimulation with anti-IgM. Data is expressed relative to un-induced controls (grey bars). Error bars represent the range in fold change within one experiment calculated from the standard deviation of the ΔΔCt values.

The data clearly show that BIC was successfully transfected and expressed in both the DG75-BL and the 293 cells. However, only the 293 transfectant appears to expressed miR-155, suggesting that the BIC expression vector was not being processed to miR-155 in the BL cell line (figure 3.11). This result is consistent with those of Kluiver et al who used northern blotting to detect miR-155 in their study. However, when the same data are expressed relative to an LCL (figure 3.12), it can be seen that the expression of BIC, in both the 293 and the DG75-BL cells, was substantially higher than an LCL (10-40 fold); whereas the miR-155 expression, in both 293 cells and DG75, was much lower than those seen in an LCL. This suggests that the BIC expression vector was not being processed efficiently in either cell line, indicating that there may be a problem with the vector itself which is causing artificially low processing efficiencies.

Another point to note about these experiments is that the comparative amounts of endogenous BIC and miR-155 in the cell lines is not a 1:1 ratio to begin with. For example, the expression of miR-155 by QPCR is approximately 9 Ct values higher in the DG75 cells compared with the LCL, which corresponds to an expression level 500 fold lower than the LCL. However, the BIC expression in the 293 cells is only approximately 4 Ct values higher than the LCL, which corresponds to an expression level only 16 fold less than an LCL. This discrepancy may be due to the different specificities and sensitivities of the two assays or may represent a real difference in the endogenous levels of BIC and miR-155 in the 293 cells. There is no reason to assume that the miR-155 expression levels should correlate exactly with BIC expression levels in a 1:1 ratio; the processing of pri-miRNAs is poorly understood and is further complicated in this instance by the fact that miR-155 is an exonic miRNA rather than an intronic miRNA.

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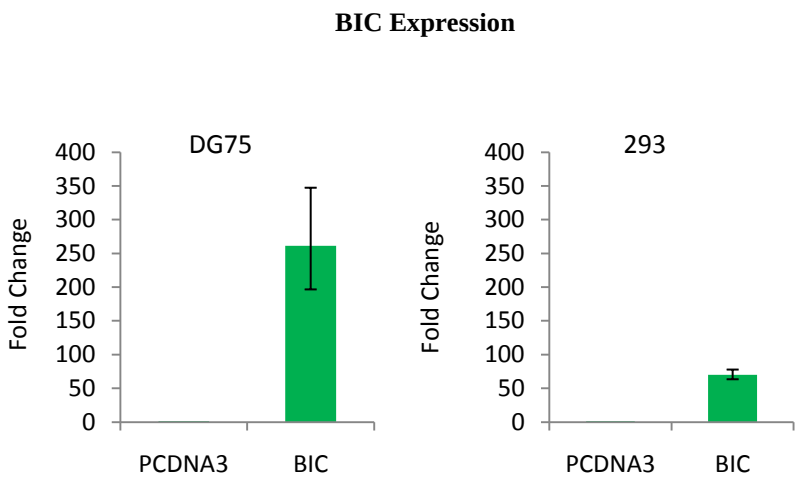

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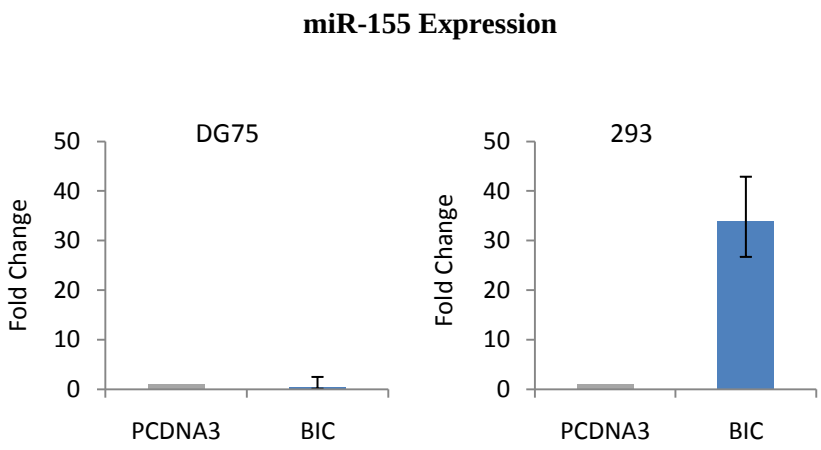

Figure 3.11: BIC and miR-155 expression by QPCR in DG75-BL and 293 cells following transfection of either PCDNA.3 or BIC-PCDNA.3. **A**; BIC and **B**; miR-155 expression in stably transfected DG75-BL and 293 cells expressing either PCDNA.3-BIC or PCDNA.3 expression vectors. Data is expressed relative to the PCDNA.3 control transfections. Error bars represent the range in fold change within one experiment calculated from the standard deviation of the ΔΔCt values.

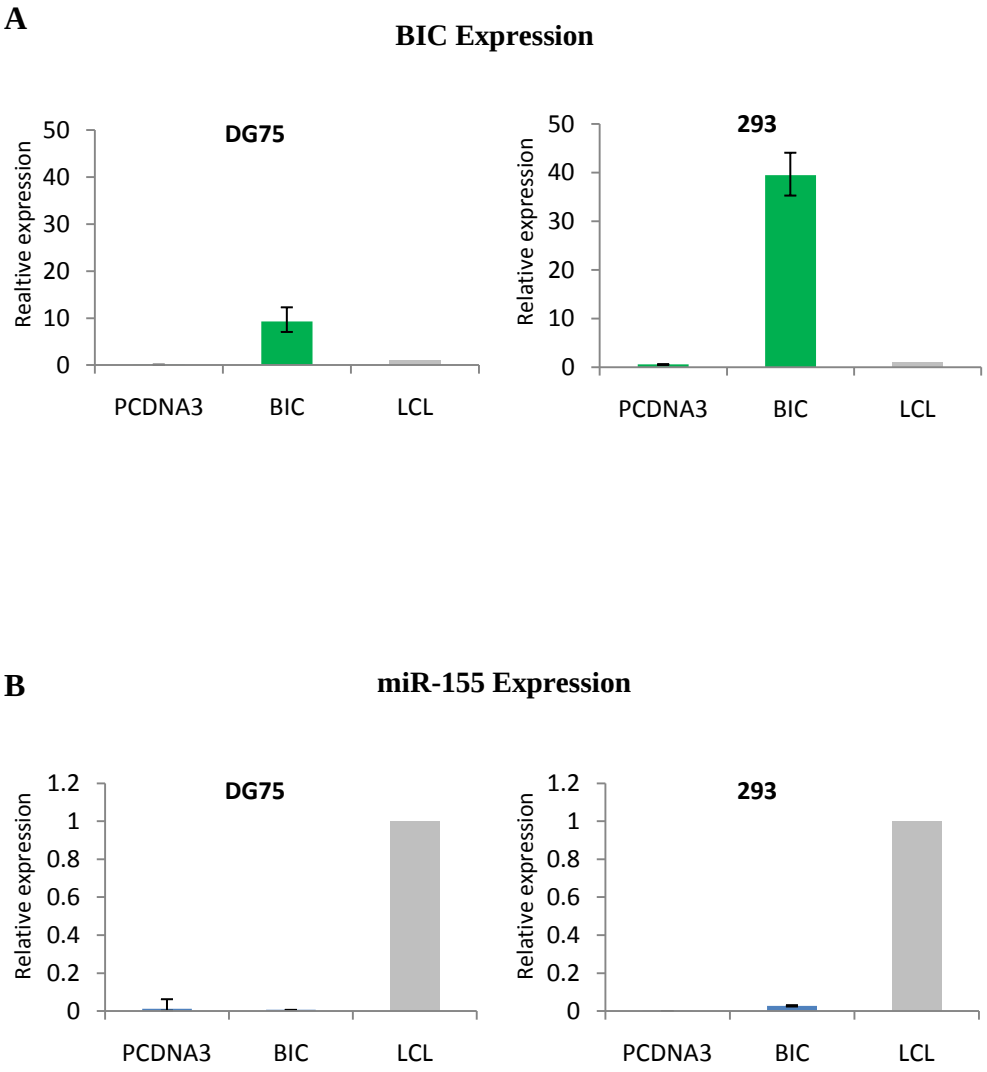

Figure 3.12: Data from figure 3.11, expressed relative to an LCL. **A**; BIC expression, **B**; miR-155 expression in stably transfected DG75-BL and 293 cells expressing either PCDNA.3-BIC or PCDNA.3 expression vectors. Error bars represent the range in fold change within one experiment calculated from the standard deviation of the ΔΔCt values.

3.4.5 Processing of BIC to miR-155*

During miRNA processing the pre-miRNA is cleaved by Dicer-1 to produce the mature miRNA duplex. The miRNA duplex is asymmetric and is believed to be preferentially 'loaded' into the RISC complex in a specific orientation which designates one strand (usually the 5' end) of the pre-miRNA as the mature miRNA strand due to its enhanced flexibility [394, 628]. It is also possible for the alternative arm (passenger strand) to be processed (the 3' arm) preferentially over the dominant arm. There is evidence to suggest that this alternative processing can be controlled in a tissue specific manner, with the 3' arm mature miRNA being expressed in one tissue and the 5' mature miRNA being expressed in a different tissue [396]. When there is evidence for tissue specific expression of both arms of the pre-miRNA, the two mature miRNAs are then called 3p and 5p to distinguish between them. There are miRNAs in which the alternative arm is processed all the time but at very low levels in comparison to the dominant arm, and in this case the alternative arm is designated the minor miRNA and is symbolised by a * (figure 3.13).

miR-155 has a minor miRNA (miR-155*) which has not been shown to have any significant level of expression in any tissue to date, hence its designation as a minor miRNA. We sought to measure the miR-155* expression in the BIC transfected 293 and DG75-BL cell lines to determine whether BIC was being processed to the alternative arm of miR-155, explaining the apparent processing defect of BIC in these experiments. There was a small increase in miR-155* expression in the DG75 BIC transfected cells but not enough to explain the lack of BIC processing in these cells as it was still very small in comparison to the expression of BIC (figure 3.13). miR-155* expression increases with miR-155 expression in the 293-BIC transfected cells as you would expect; the expression of the minor form of a miRNA is observed to increase and decrease with the expression of the major form as the ratio of

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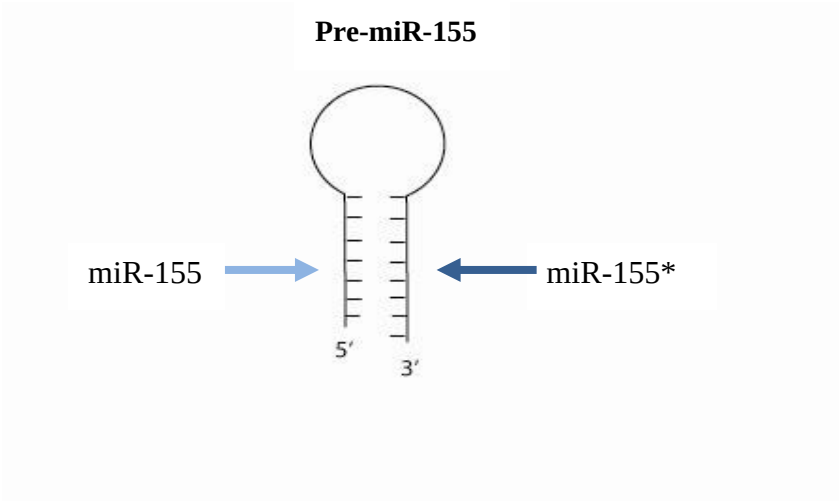

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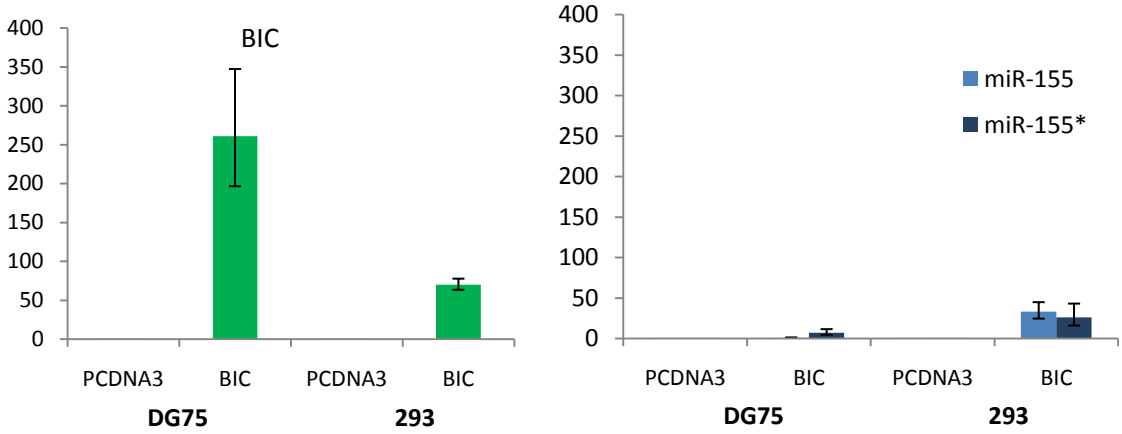


Sample	Ct miR-155	Ct miR-155*	Ct Z30
DG75 PCDNA.3	30.4	36.7	30.4
DG75 BIC	30.8	34.1	30.7
293 PCDNA.3	31.8	36.3	29.0
293 BIC	27.2	32	29.5

Table 8: miR-155 and miR-155* Ct values following BIC transfection. The mean Ct values from one experiment showing that miR-155* is expressed at a lower level than miR-155 in all samples. The snoRNA Z30 was used as the endogenous control.

Figure 3.13: Schematic of pre-miRNA. Graphs representing miR-155 and miR-155* expression in DG75-BL and 293 cells following BIC transfection. **A;** Diagram representing pre-miR-155 showing the 3' arm which can also be processed into the minor miR-155* **B;** QPCR data showing BIC expression, miR-155 and miR-155* expression in stably transfected DG75-BL and 293 cells expressing either PCDNA.3-BIC or PCDNA.3 expression vectors. Data is expressed relative to the PCDNA.3 controls. Error bars represent the range in fold change within one experiment calculated from the standard deviation of the ΔΔCt values.

major:minor miRNA remains the same. The mean Ct values from one experiment are shown in figure 3.15 to demonstrate that miR-155* is consistently expressed at a lower level than miR-155 and at no point does the expression of miR-155* become the dominant miRNA.

Discussion I

(a) Characterising miR-155 Expression

Using a panel of HL cell lines we were able to demonstrate high expression of miR-155 in HL relative to resting B cells, which was consistent with the results shown by van den Berg who used in-situ-hybridisation of paraffin embedded HL tissue to show very high expression of BIC [612]. We were also able to confirm the low expression of miR-155 observed by other groups [375, 626, 629] in BL cell lines after assaying a panel of BL cell lines expressing the original tumour biopsy pattern of EBV latent gene expression, latency I. The in-situ hybridisation data on HL tissue by van den Berg suggested that GC B cells may have a higher level of BIC expression than the other B and T cell tumour infiltrate. However, when we sorted CD10 positive GC B cells from a tonsil, neither the centrocytes nor the centroblasts showed higher miR-155 expression when compared with the CD19 positive tonsillar B cells. Our data indicate that GC B cells, which are argued to be the precursor cell for HL and BL [314, 630], express less miR-155 than HL cell lines and more miR-155 than the BL lines, suggesting that a change in miR-155 expression has occurred in both of these tumours.

It is worth noting, that during the course of my study there was a significant advancement of knowledge regarding the expression and function of miR-155, which led to a change in the interpretation of much of the pre-2007 data. The expression of miR-155 was considered to be very low/non-existent in resting B cells [631] with GC B cells expressing a low but detectable level of expression [553]. However, miR-155 is now regarded as having an important role in normal B cell function, germinal centre formation and immune responses [509, 632, 633], causing us to re-evaluate what a ‘low level’ of miR-155 expression is. Likewise, the apparent

lack of BIC/miR-155 expression reported in BL, with hindsight, appears to represent expression levels which may still be functionally significant as they are approximately 10% of those seen in B cells, where miR-155 plays an important functional role.

(b) miR-155 regulation by EBV

We were able to demonstrate a correlation between BIC and miR-155 expression and a latency III pattern of EBV gene expression in the BL cell line Mutu, consistent with the hypothesis that miR-155 expression is regulated by EBV gene expression. The initial observation that BIC expression could be up-regulated by LMP1, which came from a microarray experiment conducted at our institute [618], was then validated using a model of induced LMP1 expression in DG75-BL, which clearly resulted in increased BIC and miR-155 expression.

During the course of this work and after, several papers were published regarding the regulation of miR-155 by EBV and the function of this observed regulation. One paper published by Yin et al 2008 [634] examined the regulation of BIC and miR-155 expression by EBV. They demonstrated that an AP-1 promoter element upstream of the BIC transcription initiation site is involved in the transcription initiation of BIC in latency III BL cell lines. Since LMP1 and LMP2A have both been shown to signal through AP-1 [635], they examined the effect of LMP1 and LMP2A expression on miR-155 expression, using retroviruses expressing LMP1 or LMP2A. After transducing EBV negative BL cell lines, including Mutu-BL and Ramos-BL, they could not show any increase in miR-155 expression in the LMP2A expressing BL cell lines, in agreement with our results. They could only show a 2 fold increase in miR-155 expression with the LMP1 expressing retrovirus in EBV negative Mutu-BL and Akata-BL, but they saw no increase in Ramos-BL. This only partially agrees with the data presented in this chapter as we demonstrated a 10-15 fold increase in miR-155

expression relative to the un-induced controls following LMP1 expression. We also saw a significant up-regulation of miR-155 with LMP1 expression in Ramos-BL, and these differences may be the consequence of differing experimental design. Yin et al used retoviruses and drug selected the cells for 14 days prior to harvesting, whereas our DG75 and Ramos tTA/LMP1 transfectants were harvested 48 hours after induction. The time difference post-LMP1 expression may be important here as cells with a high BIC to miR-155 processing efficiency may have time to be negatively selected against, as suggested in another report published later that year [376].

Lu et al 2008 [636] also examined the regulation of miR-155 by LMP1 and LMP2, and they found that miR-155 was up-regulated approximately 7 fold after LMP1 transfection into DG75, and by less than 3 fold after transfection of LMP2A. Collectively, these results and my own strongly suggest that LMP1 can up-regulate miR-155 in BL cell lines but this alone is not sufficient to reproduce the miR-155 expression levels seen in latency III BL cell lines or LCLs. A more complicated mechanism of BIC/miR-155 expression regulation must be taking place in these cells which has yet to be elucidated.

We observed a strong up-regulation of miR-155 following B cell stimulation with CD40L and IL4 to produce proliferating B cell blasts. This result partially supports the previous data, which show an up-regulation of miR-155 by LMP1, as LMP1 is a functional homologue of an activated CD40 receptor. The strong up-regulation of miR-155 in CD40L blasts also suggests a possible role for miR-155 in B cell proliferation and activation as miR-155 is abundantly expressed in these proliferating blasts. EBV and CD40L blasts are phenotypically very similar; therefore, it is likely that miR-155 is regulating overlapping gene sets in these two blasts.

We have shown that miR-155 expression is up-regulated during EBV infection and transformation of primary B cells, a result also shown by numerous groups [603, 620, 636]. We also demonstrated a large increase in miR-155 expression 24 hours post EBV infection, which was not consistent with an up-regulation by LMP1. However, in BL cell lines, the only EBV gene that we, or anybody else, were able to identify as capable of up-regulating miR-155, was LMP1. Despite this, we could not demonstrate that LMP1 was essential for up-regulation of BIC/miR-155 during infection of primary B cells because an LMP1KO virus was as efficient at up-regulating miR-155 as the wtEBV. This result was further complicated by the observation that a Δgp42 virus, which was only capable of binding to the cell surface and not of entering the cell, was also able to up-regulate miR-155 expression in resting B cells. These results would suggest that miR-155 is up-regulated by EBV binding and subsequent activation of the B cell. Virus binding to the B cell may activate TLR signalling as EBV infection and CD40L stimulation of resting B cells has been shown to activate TLR signalling pathways [637], and miR-155 expression is induced by TLR signalling in macrophages [638].

(c) Processing of BIC to miR-155 in BL

The BIC to miR-155 processing defect in BL outlined by Kluiver et al [375] was the reasoning for the subsequent work in this chapter which aimed to re-examine this processing defect and potentially identify the mechanism behind it and how it may relate to EBV. Our data showed that miR-155 and BIC were up-regulated in the latency III BL cell line Mutu, a result later confirmed by other groups [639, 640]. This indicated that the reported processing defect of BIC in BL was apparently overcome through a latency III pattern of EBV gene expression; providing an interesting and novel opportunity to study the role of EBV in regulating the expression and processing of this oncogenic miRNA.

Initially, we measured the expression of Dicer-1 and Drosha, two essential components of the miRNA processing machinery, to rule out an obvious miRNA processing defect in the DG75 cell line. We measured the expression of Dicer-1 and Drosha during primary B cell infection and in the DG75 tTA cell lines, with and without LMP1 induction. There was no loss of expression of these genes in any sample. The expression of Dicer-1 and Drosha varied between samples with no apparent pattern. None of the cells showed very low levels of Dicer-1 or Drosha expression, with most showing higher level of expression than an LCL, which was capable of processing BIC to miR-155.

Next, we sought to characterise the proposed BIC processing defect in BL cell lines. We measured BIC expression in the panel of Mutu-BL cell lines and demonstrated increased BIC expression in the latency III Mutu-BL clones; consistent with an increased BIC transcription being the cause of the increased miR-155 expression.

In order to reproduce the BIC processing defect outlined by Kluiver et al we sought to repeat an experiment which they had published, where the Ramos-BL cell line was stimulated with anti-IgM to produce an increase in BIC transcription, with no subsequent miR-155 expression [375]. We were not able to reproduce this result as we observed increased BIC expression coupled with increased miR-155 expression in the Ramos-BL cells following anti-IgM treatment. Kluiver et al used northern blot to detect miR-155 expression, whereas we used QPCR, a much more sensitive technique, which may explain our differing results. miR-155 is expressed at low levels in BL, therefore an increase in miR-155 expression in these cell lines may still not be enough miR-155 expression for the northern blot to detect. Another group has since shown that BcR stimulation using anti-IgM results in both BIC and miR-155 expression in Ramos-BL, in agreement with our data [639].

Kluiver et al were aware of the limited sensitivity of their miR-155 northern blotting system so they decided to transfect the full length BIC cDNA in a PCDNA.3 vector, into Ramos-BL and 293 cells as a positive control. Their reasoning being that the supra-physiological levels of BIC expression caused by BIC transfection, should result in miR-155 expression high enough for a northern blot to detect if there was no BIC processing defect. They could not detect any miR-155 expression in the BIC transfected Ramos-BL whereas they could in the 293 cells, supporting their previous data which indicated a processing defect in the Ramos-BL cell line.

Kluiver et al kindly donated their PCDNA.3-BIC plasmid for us to use in an attempt to reproduce their data. After transfection of PCDNA.3-BIC into DG75-BL and 293 cells we observed the same apparent processing defect, with a large up-regulation of BIC expression in both cell lines but only the 293 cells showed an up-regulation of the mature miR-155. Thus far, none of the results in this chapter had shown any processing defect in BL cell lines, contradicting the data published by Kluiver et al and there was no loss of Dicer-1 or Drosha expression in DG75 which would explain a processing defect. A possible explanation for the lack of miR-155 expression in DG75-BL after BIC transfection was that BIC transcript was being processed into the alternative arm of the pre-miR-155, to produce the minor miR-155*. We used QPCR to measure the miR-155 expression along with miR-155* and the data showed no evidence of preferential processing of BIC to miR-155*, with the expression of miR-155 being at least 8 fold higher than miR-155* in all samples.

The fact that PCDNA.3-BIC could be processed to miR-155 in the 293 cells suggested that the PCDNA.3-BIC vector was producing a BIC transcript with the correct sequence and conformation to allow recognition by the processing machinery. We had shown that BIC was able to be processed in DG75 after LMP1 expression and we had also shown that Ramos-BL

was able to process BIC after anti-IgM treatment, indicating that the BL cell lines were able to process BIC to miR-155. We could rule out an alternative processing mechanism as we had demonstrated that BIC is not being preferentially processed into miR-155*. Additionally we demonstrated that Dicer-1 and Drosha were present in BL lines at similar levels to the 293 cells and an LCL, which collectively, would strongly suggest there was no BIC to miR-155 processing defect in BL cell lines. In support of this, when the PCDNA.3-BIC transfection data was expressed relative to an LCL, it can be seen that the BIC expression in the 293 cells is approximately 40 fold higher than the BIC expression in an LCL, however, the miR-155 expression is approximately 50 fold less than the expression seen in an LCL. This suggests that there is a possible flaw in the experiment because even this positive control cell line cannot process BIC to miR-155 efficiently. As there may be a problem with the experimental design or the BIC expression vector being used, it would be unwise to attempt to draw firm conclusions from these data.

There are several possible explanations for the lack of BIC processing after transfection with the PCDNA.3-BIC expression vector, but there are three which appear most likely: Firstly, miR-155 is unusual because it is an exonic miRNA and it is, therefore, not simply spiced out in the nucleus where it is accessible to Drosha for processing as intronic pri-miRNAs are. The mechanisms which govern miRNA processing and regulation are still not fully understood and whether or not they are coupled to the splicing of the pri-miRNA transcript is also unknown. This makes interpretation of pri-miRNA versus mature miRNA expression data very difficult to interpret, as in the absence of a proper understanding of the details of the miRNA processing pathway, there is actually no reason to assume that the expression of the two should correlate. If miRNA processing, particularly exonic miRNA processing, is in any

way coupled to splicing then it might be expected that the processing efficiency of a BIC cDNA expression plasmid would be low.

Secondly, the cellular localisation of BIC after transfection may affect processing efficiency as BIC would need to be present in the nucleus to have access to Drosha for the first processing step to take place. Lipid mediated transfection of epithelial cells and electropration of B cells may result in slightly different cellular localisation which could affect the ability of the BIC to be processed. An interesting report published by Eis et al highlighted the flaw in measuring BIC expression by QPCR [374]. BIC QPCR assays are usually designed across the spice junction of exons 2 and 3 (our QPCR assay included), therefore, the assay will only detecting the spliced BIC RNA. Eis et al [374] showed that un-spliced BIC RNA was predominantly nuclear whereas the spliced RNA was predominantly cytoplasmic, where it has no access to Drosha for the first processing step. It is likely that exonic miRNAs can be regulated transcriptionally and/or post-transcriptionally, with miR-155 potentially being processed from un-spliced BIC, spliced BIC pre-nuclear export, or spliced BIC RNA exported back into the cytoplasm. This again calls into question the methods and assumptions which are commonly used when measuring mature miRNAs and their precursors. It is very difficult to interpret pri-miRNA versus mature miRNA data until these questions have been answered.

The third possibility is the impact of drug selection on the processing of BIC in both cell lines. We and Kluiver et al used hygomycin drug selection to produce a stable cell line after BIC transfection, and it had since been reported by Zhang et al that transient transfection of BIC into Ramos-BL and 293 cells resulted in efficient BIC to miR-155 processing [376]. Zhang et al propose that drug selection in culture may allow for the clonal selection of cells which do not express miR-155 as it may be unfavourable for cell proliferation and/or survival. They imply that this clonal selection may be more pronounced in the BL cell lines than the

293 cells, hence the apparent processing defect observed by Kluiver et al selectively in the BL cell line. My own results would suggest that clonal selection may also be occurring in the 293 cells as the processing efficiency of BIC to miR-155 appeared to be very low in both 293 and DG75-BL cells when compared with an LCL, therefore, transient transfection of BIC may produce different results form those which we obtained following drug selection.

(d) miR-155 function

The function of miR-155 in normal B cells and EBV associated tumours is a rapidly expanding area of research with many significant breakthroughs occurring throughout the course of my work. The observation that miR-155 plays an essential role in germinal centre formation and immune responses [509, 632, 633] was an extremely important discovery which caused the miR-155 literature to date to be re-evaluated, as discussed earlier. Many targets of miR-155 identified through microarrays and reporter assays revealed that miR-155 targets many genes already known to be involved in B cell development and EBV signalling, such as PU.1 [633], IKKε [636] and multiple members of the bone morphogenic protein signalling pathway [641]. Another significant discovery was the identification of miR-155 orthologs in other oncogenic herpesviruses. KSHV encodes a miRNA (miR-K12-11) which possesses an identical seed sequence to hsa-miR-155 and has been shown to target some of same genes as miR-155, including BACH1 [642, 643]; miR-K12-11 is therefore classified as a functional ortholog of miR-155. It was later shown that an oncogenic alpha herpes virus, Marek's disease virus of chickens, also encodes a functional ortholog of gga-miR-155 which was able to target some of the same target genes as gga-miR-155, including PU.1 [644, 645]. The possession of miR-155 orthologs in distantly related oncogenic herpes viruses strengthens the evidence that this miRNA is very important in lymphomagenesis. It also suggests that miR-155 may play an important role in herpes virus infection and

transformation. Additionally, the down-regualtion of PU.I, a transcription factor required for LMP1 transactivation, may provide a possible negative feedback mechanism whereby LMP1 up-regulation of miR-155 results in a reduction of LMP1 expression. However, miR-155 inhibiton in an LCL did not result in decreased EBNA2 or LMP1 protein expression [646].

The role miR-155 plays in HL is still an unsolved question, although clues are starting to emerge from miR-155 target identification studies. It might seem counter-intuitive that low expression of an oncogenic miRNA might be important in BL development but there is literature emerging which suggests that this may be the case. For example, activation induced deaminase (AID) is one of miR-155 targets which has been verified [647] and a mouse model where the miR-155 target site in AID was removed showed an increased half life of the AID mRNA, resulting in a high degree of Myc-Igh translocations [648]. Loss of miR-155 expression may therefore be relevant in the development of BL, which is characterised by a c-myc translocation to the IgH locus.

In summary, we have shown that miR-155 is an EBV regulated cellular miRNA which is expressed at a very high level in LCLs and HL cell lines. LMP1 is able to up-regulate miR-155 expression in BL cell lines but not to the levels seen in latency III BL lines, suggesting that LMP1 may be working in synergy with other EBV latent genes to up-regulate miR-155. The expression of miR-155 in BL is relatively low and this is the result of low transcription of BIC and not a block in the processing of BIC to miR-155 in BL cell lines.

4. Results Part II

Identifying cellular miRNAs involved in EBV-mediated B cell transformation

4.1 Introduction

In the previous chapter, it was shown that EBV infection of B cells causes the up-regulation of miR-155, a cellular miRNA known to be involved in lymphomagenesis. The next logical step to determine to what extent EBV modulates cellular miRNA expression during B cell transformation, was to perform an array of cellular miRNA expression. Many of the changes in miRNA expression will not be specific to EBV growth transformation, but will simply reflect changes in gene and miRNA expression associated with activation and proliferation of resting cells. B cells stimulated with CD40L and IL4 to generate proliferating B cell blasts are phenotypically very similar to EBV transformed blasts; they both show homotypic adhesions in culture, and express a similar repertoire of activation associated antigens. However, CD40L generated B cell blasts will only proliferate transiently in culture whereas EBV transformed B cell blasts may eventually produce an immortalised cell line [348, 649].

We aimed to compare the expression profiles of EBV generated B cell blasts and CD40L and IL4 stimulated B cell blasts, as a means of identifying miRNA expression changes which were not just associated with transient proliferation of resting cells, but were specific to the transformation of resting B cells by EBV. We hypothesised that these cellular miRNA and subsequent gene expression changes may predispose the LCL to the additional changes necessary for immortalisation, in contrast to the CD40L blasts. miRNA and cellular gene expression changes which facilitate the production of an immortalised cell line *in vitro* may also be relevant to the formation of EBV associated B cell tumours *in vivo*.

The aims of this chapter were to (i) identify the subset of miRNAs differentially expressed between EBV and CD40L blasts, (ii) characterise the expression of these miRNAs in a panel of EBV associated tumour cell lines and (iii) select one miRNA for target prediction and functional analysis.

4.2 Cellular miRNA expression profiling

To identify miRNAs potentially involved in the cellular transformation of resting B cells by EBV, we performed a Taqman QPCR miRNA array of 365 cellular miRNAs on the following samples: resting B cells, CD40L generated B cell blasts, EBV blasts and matched LCLs (figure 4.1). The Taqman array was only performed in biological duplicate for expense purposes but it was reasoned that the expression of miRNAs of interest could subsequently be validated on a larger sample set.

4.2.1 Cells used for miRNA profiling

The following experiment was performed in duplicate to obtain biological duplicate sample sets for the Taqman miRNA array: CD19 positive resting B cells were isolated from peripheral blood using CD19 dynabeads. B cells from 4 to 6 donors were pooled and a fraction was taken immediately for RNA extraction as a resting B cell sample (d0) and the B cell purity was confirmed by FACS staining for CD21 expression (figure 4.2). B cells from multiple donors were pooled to reduce the possibility of identifying miRNA expression differences which were donor specific and not typical of B cells, thus making the data more reproducible. The remaining B cells were either stimulated with 50ng/ml of soluble CD40L and 50ng/ml of IL4 to generate proliferating B cell blasts, or, infected with EBV at an m.o.i of 100, and both sets of blasts were harvested after 7 days. EBV infected B cell blasts were also grown for a further 5 weeks to produce an established LCL.

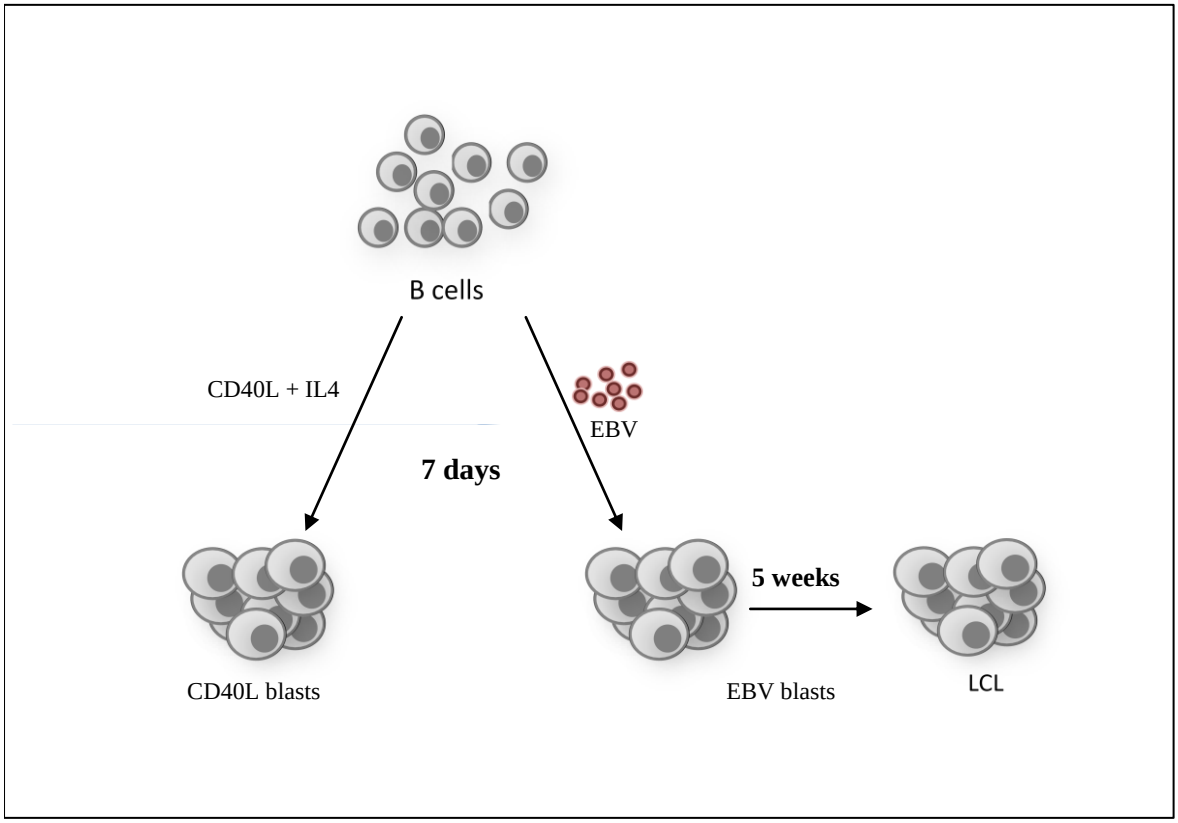


Figure 4.1: Diagram representing the experimental design of the Taqman miRNA array. Resting B cells were isolated from peripheral blood and either stimulated with CD40L and IL4 for 7 days or, infected with EBV at an m.o.i of 100 and harvested after 7 days or 6 weeks (LCL)

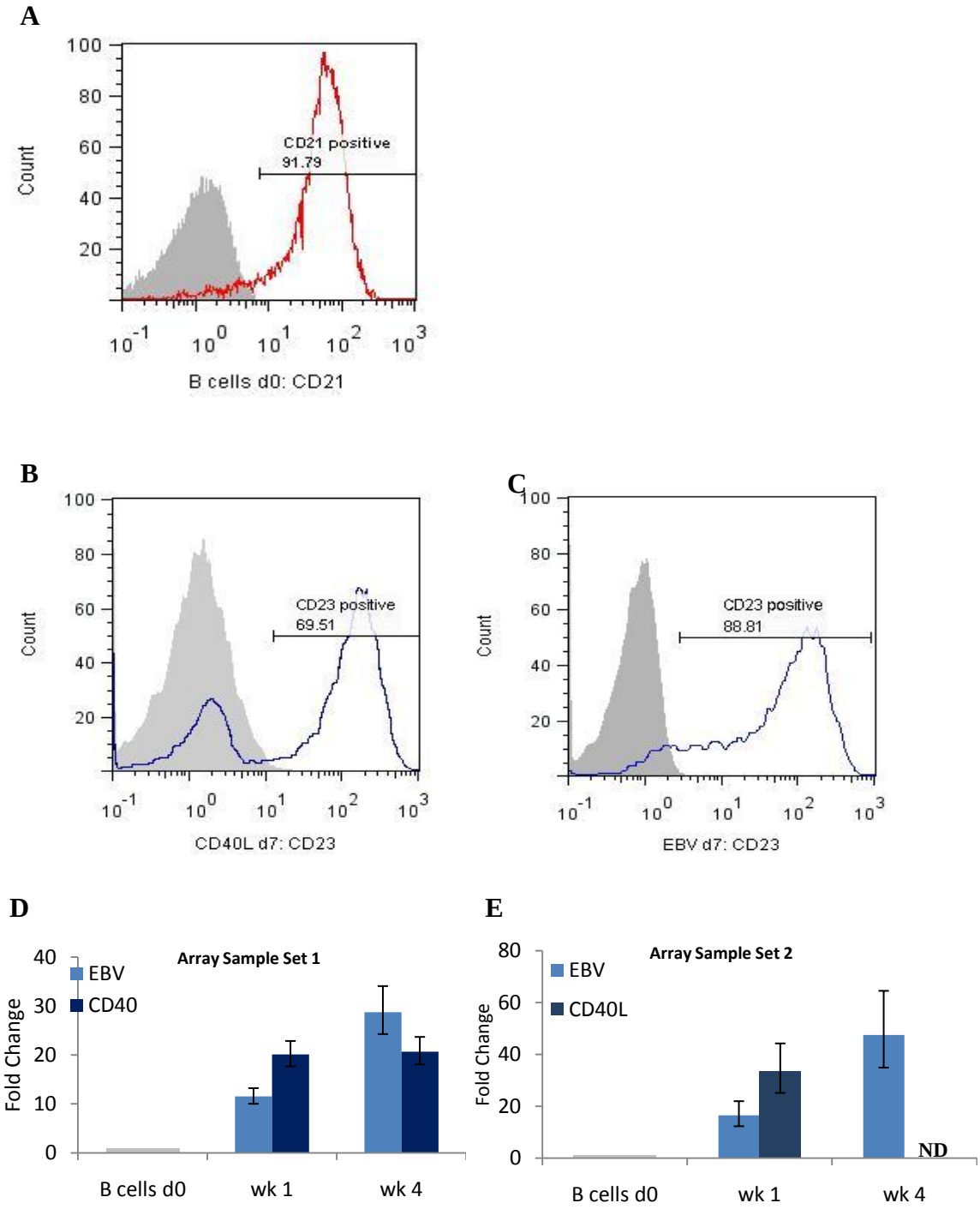


Figure 4.2: Activation and miRNA expression of miRNA array samples **A:** Flow cytometry data showing the purity of B cells isolated from peripheral blood after selection with $CD19^{+}$ dynabeads, determined by CD21 staining. Grey, filled histograms represent cells stained with a matched isotype control: **B and C:** Flow cytometry data showing activation of resting B cells 7 days after either stimulation with sCD40L and IL4 (**B**) or infection with EBV (**C**), determined by CD23 staining. **D and E**: QPCR for miR-155 in the two biologically distinct samples used for the Taqman miRNA array. Array sample set 2 does not contain CD40L blasts 4 weeks post-stimulation as cells did not survive (not determined(ND)). Error bars represent the range in fold change from the standard deviation of the ΔΔCt values.

CD40L blasts were generated using soluble trimeric CD40L to avoid contamination of the blast sample with CD40L secreting mouse L-cells, which are the standard method used to produce CD40L blasts. Mouse miRNAs are very similar, and in some instances identical in sequence to human miRNAs, therefore, contamination from mouse L-cells could produce false positives in the miRNA QPCR array.

Prior to the array analysis, activation of the B cells stimulated with sCD40L and IL4 or infected with EBV was verified by FACS for the B cell activation marker, CD23. Additionally, the expression of miR-155 was measured by QPCR in the biological duplicate array samples to ensure RNA quality, miRNA expression, and to further confirm activation of the B cells (figure 4.2).

4.2.2 Taqman miRNA array

The cellular miRNA expression profile of the following samples was determined on biological duplicates, prepared from pooled peripheral blood B cells at different time points: resting B cells (d0), EBV blasts, CD40L blasts and the LCL (6 weeks p.i). The miRNA expression of each sample was measured using a QPCR array; Taqman Array Human miRNA Panel version 1. The QPCR array measured the mature miRNA expression profile of 365 cellular miRNAs and the data was analysed using the comparative ΔΔCt method [609], the details of which are described fully in materials and methods section 2.9.2. Briefly, RNA was extracted from each sample using Ambion's mirVana kit to ensure the quantitative isolation of miRNAs and cellular mRNA. The RNA was reverse-transcribed in pools of stem loop primers specific for each of the 365 mature miRNAs and loaded into a microfluidics card where 365 cellular miRNAs and RNU48 and RNU 44 endogenous controls were assayed in singleplex QPCR reactions.

4.2.3 Taqman miRNA array fold change analysis

The miRNA expression data from the Taqman miRNA array was exported into Microsoft Excel for ΔΔCt analysis, and the fold changes of each miRNA in the EBV blasts, CD40L blasts and the LCL were calculated relative to the resting B cells. The aim of this analysis was to identify miRNAs which were differentially regulated between EBV blasts and CD40L blasts, relative to resting B cells. In addition, miRNAs differentially expressed between EBV blasts and the LCL were of interest as these miRNA expression changes may represent transformation specific changes only seen in established LCLs.

The array data was analysed using the following criteria: Firstly, for a miRNA to be classified as 'expressed' it required a ΔCt value of ≤12 (using RNU48 as an endogenous control). This was in line with the manufacture's recommendations because in this system, a ΔCt of 12 approximately corresponded to a Ct value of 38, the threshold for miRNA detection. Secondly, miRNAs which gave a ≥2 fold change, in the same direction, in biological duplicate samples, were classified as up or down-regulated. Thirdly, up-regulated miRNAs had to classify as 'expressed' after up-regulation and miRNAs which were down-regulated had to classify as 'expressed' before down-regulation in the resting B cells. Statistical analysis could not be performed on this data due to the limited number of replicates.

The miRNAs were classified accordingly and the results presented in figure 4.3. The majority of miRNAs (70%) were not expressed (dCt≤12) in any sample, which may reflect the tissue specific expression associated with many miRNAs [650]. A further 19% of the miRNAs assayed did not change in expression between any of the samples, thus, nearly 90% of the 365 miRNAs on the array were not involved in the transformation of resting B cells by EBV. The remaining miRNAs assayed did change expression in response to EBV infection and/or CD40 stimulation: 19 miRNAs (5%) were up-regulated in all samples, 7 (2%) were down-regulated

in all samples and 10 miRNAs (3%) were differentially regulated between the three samples (EBV blasts, CD40L blasts and LCLs. Tables 9-11). A further 6 miRNAs (miR-34a, -30e-5p, -335, -486, -650 and -151) gave inconsistent expression in biological duplicates (i.e. greater than 2 fold change in expression in only one of the biological duplicate samples), hence these miRNAs were classified as undetermined.

Of the 10 differentially regulated miRNAs, 7 showed differential expression between CD40L blasts and EBV blasts (miR-148a, -200b, -199a-3p, -28-5p, - 95, -31 and miR-15a). miR-15a was the only miRNA which showed differential expression between CD40L blasts and EBV day 7 blasts as well as between EBV blast and the LCLs. The remaining three miRNAs showed differential expression between EBV blasts and the LCL: miR-193a-3p, -132, and -125b (table 9).

4.2.4 Taqman miRNA array ΔCt analysis

The fold change in miRNA expression relative to resting B cells is informative for determining how a miRNA has changed in response to a specific stimulus, such as EBV infection or CD40 stimulation. However, it cannot give any indication of absolute expression, which may need to be taken into account when considering miRNA expression changes which are most likely to be functionally significant. For example: a miRNA which is up-regulated 20 fold by EBV infection may still be expressed at a lower level than another miRNA which has been down-regulated by 20 fold. To estimate the significance of results produced by the miRNA fold change analysis, an indication of absolute expression, such as the ΔCt analysis, was required.

The ΔCt values produced by the QPCR analysis are essentially the normalised raw miRNA expression data (ΔCt = Ct miRNA – Ct RNU48). The Taqman miRNA individual QPCR assays and arrays have been designed to produce comparable ΔCt values, allowing the ΔCt

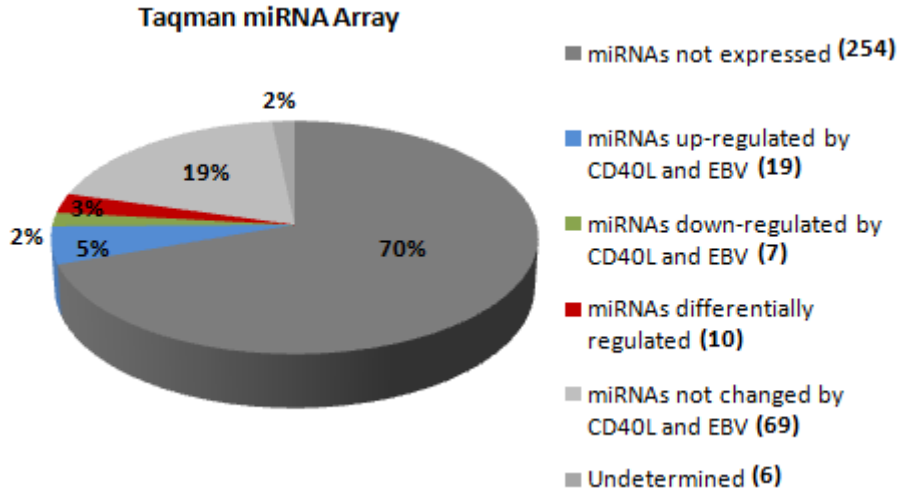


Figure 4.3: Pie chart representing the results from a Taqman miRNA array for 365 cellular miRNAs. Data represents duplicate biological samples from pooled peripheral blood B cells either harvested immediately after isolation (resting B cell sample), 7 days post-stimulation with CD40L and IL4, or 7 days and 6 weeks (LCL) post-infection with EBV. Data labels represent the percentage of miRNAs in each category, with the total number of miRNAs in each category indicated in brackets in the legend. miRNAs which were not expressed had a ΔCt value of ≥12 in all samples. miRNAs up or down-regulated by CD40L and EBV showed a ≥2 fold change in duplicate biological samples in CD40L blasts and both the EBV blasts and LCLs. Differentially regulated miRNAs showed consistent expression in biological duplicates and differential expression between the three samples: CD40L d7 blasts, EBV blasts and the LCLs. miRNAs classified as undetermined did not show consistent biological duplicates and therefore could not be classified.

	Fold Change			
miRNA	**CD40L d7**	**EBV d7**	**LCL**	
hsa-miR-148a	-1.4	11.0	63.3	CD40L d7 vs EBV d7
hsa-miR-200b	1.1	-66.6	-18.2	
hsa-miR-199a-3p	-1.9	-12.9	-25.9	
hsa-miR-28-5p	3.1	-2.1	-10.6	
hsa-miR-95	9.1	-2.4	-186	
hsa-miR-31	2.0	-72.7	-51.3	
hsa-miR-15a	2.0	-1.9	-6.9	EBV d7 vs LCL
hsa-miR-193a-3p	1.0	1.6	-5.4	
hsa-miR-132	14.0	12.0	-2.8	
hsa-miR-125b	-29.4	-42.5	-1.9	

Table 9: Fold change in expression of 10 miRNAs classified as differentially regulated between either CD40L d7 blasts and EBV d7 blasts or EBV blasts and LCLs. Fold change was calculated using the ΔΔCt method of analysis and was expressed relative to resting B cells. The fold change is the mean fold change in expression of duplicate biological replicates from a Taqman miRNA array. Red shading represents a ≥ 2 fold increase in expression relative to resting B cells and blue shading represents a ≥ 2fold decrease in expression.

	Fold Change		
miRNA	**CD40L d7**	**EBV d7**	**LCL**
hsa-miR-21	2071	2007	2897
hsa-miR-301	746	391	803
hsa-miR-18a	223	259	320
hsa-miR-9	166	59.4	362
hsa-let-7b	139	46.3	15.8
hsa-miR-20a	38.1	25.2	37.6
hsa-miR-210	14.6	24.3	15.2
hsa-miR-93	52.6	23.9	46.0
hsa-miR-365	4.9	23.0	61.7
hsa-miR-155	40.7	18.6	55.7
hsa-miR-130b	6.8	15.2	22.3
hsa-miR-20b	8.9	12.5	25.4
hsa-miR-17-5p	13.3	9.3	8.9
hsa-miR-92	9.6	7.0	13.2
hsa-miR-146a	3.1	6.0	15.6
hsa-miR-22	10.5	4.8	7.1
hsa-miR-146b	4.6	3.5	4.4
hsa-miR-19b	3.7	3.2	5.6
hsa-miR-330	2.2	2.4	3.4

Table 10: Fold change in expression of 19 miRNAs classified as up-regulated in both CD40L d7 blasts and EBV d7 blasts. Fold change was calculated using the ΔΔCt method of analysis and was expressed relative to resting B cells. The fold change is the mean fold change in expression of duplicate biological replicates from a Taqman miRNA array. Red shading represents a ≥ 2 fold increase in expression relative to resting B cells

	Fold Change		
miRNA	**CD40L d7**	**EBV d7**	**LCL**
hsa-miR-126*	-18.1	-150	-191
hsa-miR-451	-26.7	-131	-29.1
hsa-miR-126	-12.6	-17.6	-45.1
hsa-miR-223	-3.6	-9.0	-35.1
hsa-miR-26a	-2.9	-4.5	-10.1
hsa-miR-26b	-2.0	-3.3	-4.3
hsa-miR-195	-2.2	-2.7	-3.7

Table 11: Fold change in expression of 7 miRNAs classified as down-regulated in both CD40L d7 blasts and EBV d7 blasts. Fold change was calculated using the ΔΔCt method of analysis and was expressed relative to resting B cells. The Fold change is the mean fold change in expression of duplicate biological replicates from a Taqman miRNA array. Blue shading represents a ≤ 2fold decrease in expression.

values of different miRNAs to be compared directly. Expressing the miRNA array data as the ΔCt values alone allows the expression of one miRNA to be directly compared with another. To aid the visual interpretation of the data, the mean ΔCt values have been expressed as 12-mean ΔCt values. The endogenous control (RNU48) had a Ct value in of approximately 26 in every sample. The threshold of detection is set by the manufacturer as Ct38, therefore, the lowest detectable miRNA in this system will have a ΔCt value of 12 (Ct38-Ct26). Expressing the miRNA QPCR data as 12-ΔCt allows the lowest detectable miRNA expression to be set at 0, with an increase in 12-ΔCt corresponding to an increase in miRNA expression; this is visually easier to interpret in a graph than the raw ΔCt values.

Combining information from relative fold change and ΔCt analyses allows the potential functional significance of each miRNA expression change to be estimated. This may be important when only limited numbers of miRNAs can be selected for further analysis. Graphs which represent the 20 most abundantly expressed miRNAs in each of the four samples are shown in figure 4.4. A summary of the ΔCt analysis is presented in table 12, with the total number of miRNAs classified as 'expressed' being indicated for each sample. Further to this, a relative rank position was assigned to each of the differentially expressed miRNA with regard to the total miRNA expression in that sample. For example: the most abundantly expressed miRNA in a sample was ranked 1st. The mean ΔCt value for each miRNA is also included in brackets for comparison. It can be seen from this analysis that the CD40L and EBV blasts show very similar miRNA expression profiles in the top 20 abundantly expressed miRNAs and these include several oncomiRs such as miR-155, miR-21, miR-92 and miR-20a. Additionally, several miRNAs show very high expression in all samples, such as miR-142-3p, which although not significantly altered following EBV infection, may play an important role in B cell signalling processes.

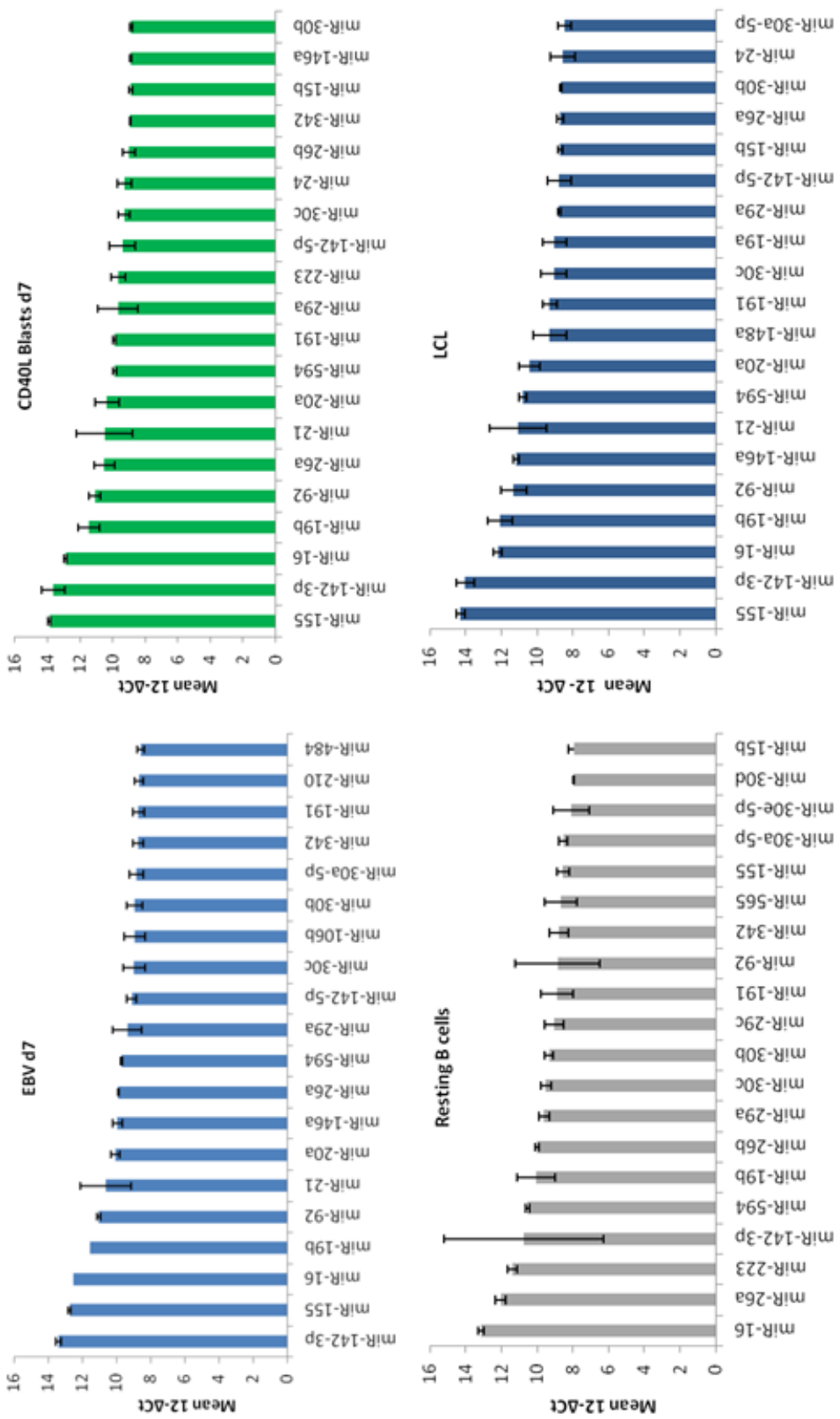


Figure 4.4: Mean ΔCt values of biological duplicate samples from a Taqman miRNA array for the 20 most abundantly expressed miRNAs in each array sample: EBV blasts 7 days pi; CD40L blasts 7 days post-stimulation; resting B cells and LCLs 6 weeks p.i. Data is expressed as 12-ΔCt, therefore the highest values correspond to the most abundantly expressed miRNAs. Error bars represent the range in fold change from the standard deviation of the ΔΔCt values in duplicate samples.

miRNA	Expression Resting B cells 114 total miRs (12-ΔCt)	Expression CD40L d7 blasts 131 total miRs (12-ΔCt)	Expression EBV d7 blasts. 122 total miRs (12-ΔCt)	Expression in LCL 121 total miRs (12-ΔCt)
miR-148a	63rd (3.4)	89th (2.9)	39th (6.6)	10th (9.3)
miR-200b	98th (1.2)	117th (1.4)	NE	NE
miR-199a-3p	107th (0.3)	NE	NE	NE
miR-28-5p	38th (6.2)	33rd (7.8)	58th (5.2)	84th (2.8)
miR-95	61st (3.9)	41st (7.0)	88th (2.6)	NE
miR-31	82nd (2.1)	86th (3.0)	NE	NE
miR-15a	29th (6.9)	34th (7.8)	46th (6.0)	61st (4.7)
miR-193a-3p	83rd (1.9)	105th (1.9)	87th (2.7)	NE
miR-132	97th (1.1)	61st (4.8)	67th (4.4)	NE
miR-125b	101rd (0.8)	NE	NE	121st (0.8)

Table 12: Summary of the ΔCt analysis (figure 4.4) for the 10 differentially regulated miRNAs from the Taqman miRNA array fold change analysis. Results are presented as the rank expression of each miRNA in either; resting B cells, CD40L blasts, EBV blasts 7 days pi and 6 weeks pi (LCL). The 12-ΔCt values for each miRNA are indicated in brackets. The total number of miRNAs classified as 'expressed' in each sample is listed in the heading columns. Any miRNAs with a 12- ΔCt value of ≤ 0 were classified as not expressed (NE).

4.2.5 Validation of fold change analysis

The Taqman miRNA array was only performed in duplicate biological samples. The purpose of the array was to identify transformation associated changes in miRNA expression, which would be confirmed on a larger set of replicate samples. The expression of all 10

differentially regulated miRNAs (table 9) was measured by QPCR in 5 biological replicate samples using individual miRNA QPCR assays for validation.

Although it would have been ideal to validate the Taqman miRNA array result using a different method from the array, neither solution hybridisation nor northern blot had the sensitivity of the QPCR assays, therefore, these alternative methods may not have been able to detect the miRNAs which needed to be validated.

There was an additional complication to the validation of the Taqman miRNA array. The array primers were designed based on the current version of the miRBase registry [513, 651]. After our Taqman miRNA was performed, a new version (v.10) of the miRBase registry was released. Unfortunately, this new release re-classified the sequences for many miRNAs on the array, including 4 of the 10 differentially regulated miRNAs we intended to validate (miR-31, miR-200b, miR-193a-3p and miR-199a-3p). Consequently, the individual Taqman assays used to validate 4/10 of the miRNAs from the array were not the same primers used in the original array. The significance of these primer changes is unclear and thus, the reproducibility of the array data is difficult to infer from these validations.

The miRNA validation experiments are expressed as 15-ΔCt as the Ct of RNU48 in this system was 23 (38-23=15). It is worth noting that the Taqman miRNA array produced slightly different Ct values than the individual Taqman miRNA assays used for the validations, (presumably due to the multiplex reverse-transcription step and the 1µl QPCR reaction

volume) and a ΔCt of 12 corresponded to a Ct value of 38 in the array system. Nevertheless, plotting the data as the 15-ΔCt values rather than fold change allowed all 5 biological replicate points to be displayed and the reproducibility of the data to be easily evaluated for each miRNA.

The 10 miRNAs which were assayed can be split into two groups according to their expressions in the original array: i) the 7 miRNAs which showed differential expressions between CD40L and EBV day 7 blasts (miR-148a, -200b, -199a-3p, -28-5p, -95, -31, -15a); ii) the 3 miRNAs which showed differential expression between EBV day 7 blasts and the LCLs (miR-193a-3p, -132, -125b). Whether or not a miRNA was validated was therefore determined by the significant difference in expression of each miRNA between the relevant samples, as determined by the student T test. A P value of ≤0.05 was considered significant.

Of the 7 miRNAs in group i), 4 miRNAs showed a significant difference in expression between CD40L day 7 and EBV day 7 blasts (P value ≤ 0.05), and these were: miR-148a, miR-28-5p, miR-31 and miR-95 (figure 4.5, A). Of the remaining 3 miRNAs from this group (miR-200b, miR-199a-3p and miR-15a), miR-200b and miR-199a-3p showed a significant difference in expression between CD40L blasts and the LCL (P= ≤ 0.05, figure 4.5 B); these two miRNAs were classified as differentially expressed between CD40L blasts and the LCL. The reasoning for continuing to investigate miR-200b and miR-199a-3p was that the expression difference in the EBV blasts 7 days pi may not have been large enough to be statistically significant, but it may still be functional significant; especially considering the continued down-regulation of these miRNAs in the LCL. Finally, miR-15a did not show a significant difference in expression between the CD40L blasts and either EBV time point so therefore was not further investigated (figure 4.5 C).

A

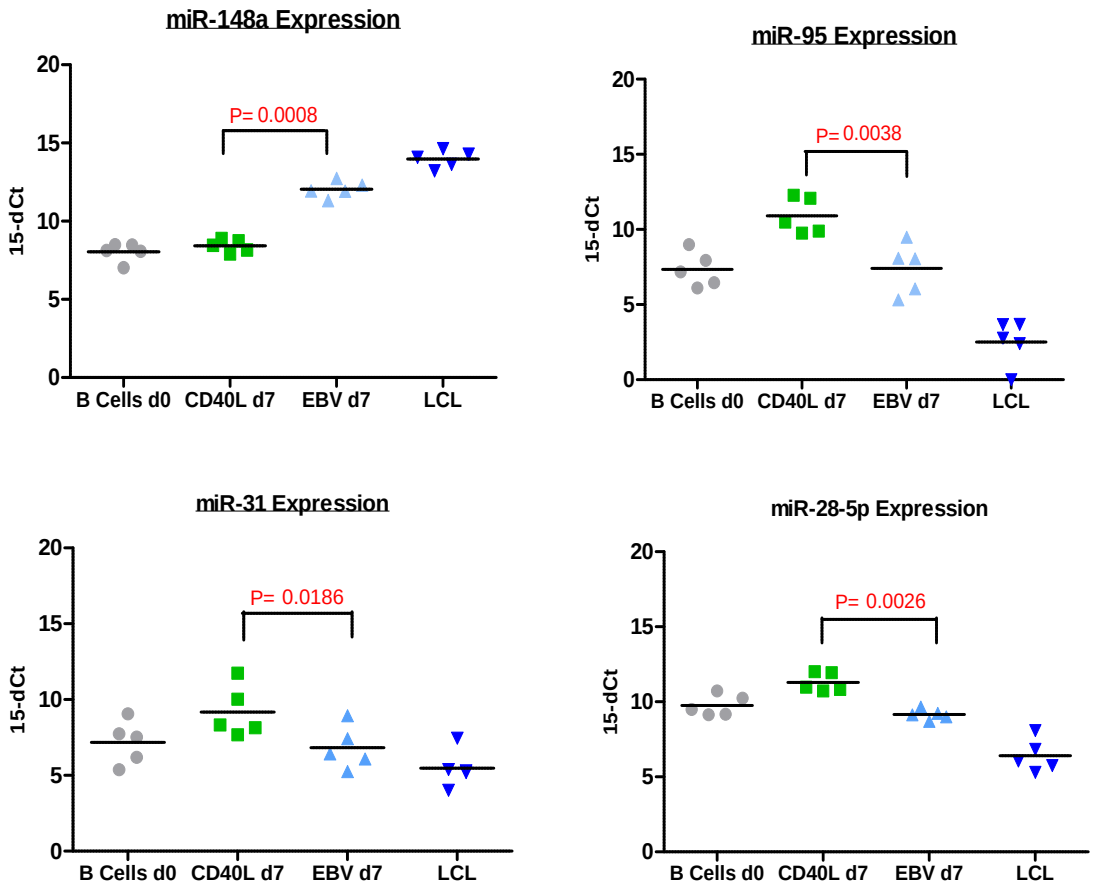


Figure 4.5: Taqman miRNA array validations. miRNA expression measured by QPCR in 5 biological replicate samples: resting B cells from peripheral blood (B cells d0); B cells stimulated with CD40L and IL4 for 7 days (CD40L d7); B cells infected with EBV at an m.o.i of 100 7 days pi (EBV d7) and 6 weeks pi (LCL). Data is expressed as 15 -ΔCt values (ΔCt represented by dCt in Y axis label). **A;** 4 miRNAs which showed a significant difference in expression between CD40L d7 blasts and EBV d7 blasts P= ≤ 0.05 on the Taqman array. P values are calculated using the student T test paired sample analysis between CD40L d7 and EBV d7 B cell blasts. Red P values indicate significant differences (P= ≤ 0.05) in expression.

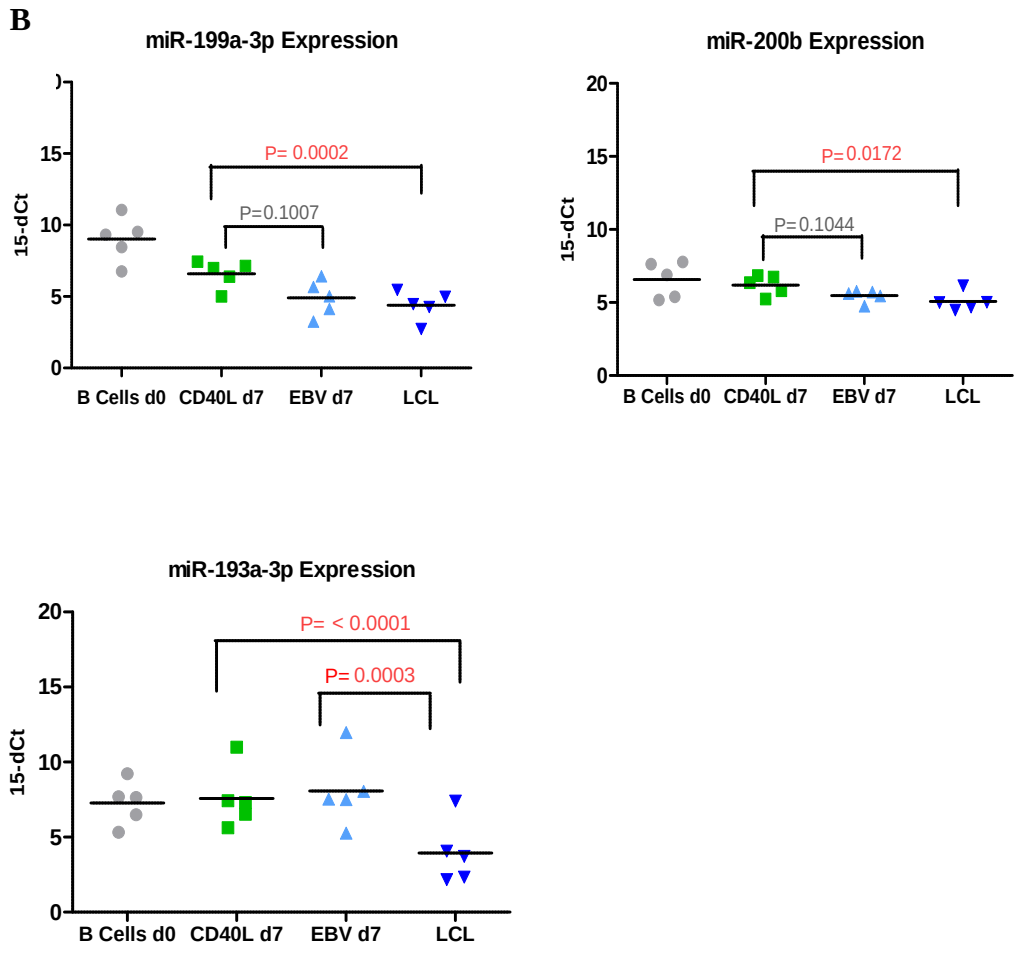


Figure 4.5 Taqman miRNA array validations continued. **B;** the 3 miRNAs which showed a significant difference in expression (P= ≤ 0.05) between CD40L blasts and the LCLs. P values were calculated using the student T test paired sample analysis. Grey P values indicate non-significant differences (P= ≥ 0.05) in expression whereas red P values indicate significant differences (P= ≤ 0.05) in expression.

C

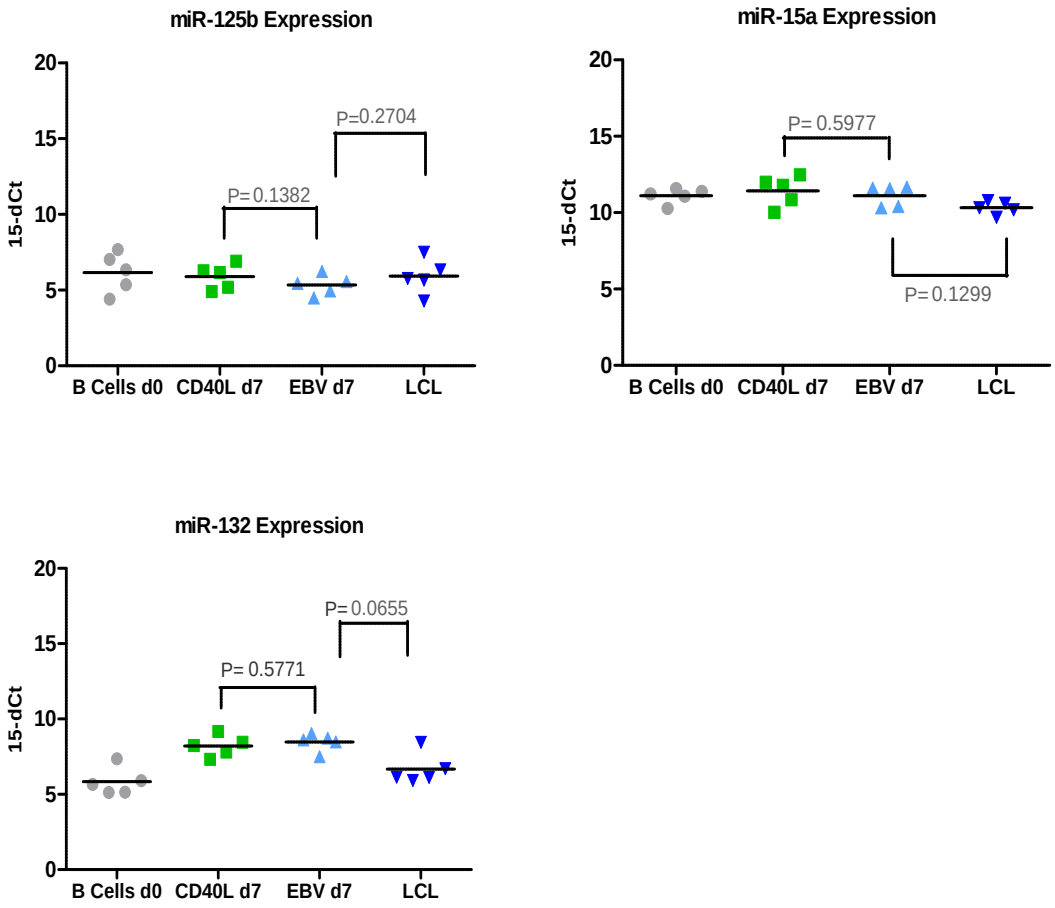


Figure 4.5 Taqman miRNA array validations continued. miRNAs differentially expressed between EBV d7 blasts and the LCL on the array. **C;** the 3 miRNAs which did not show a significant difference in expression between EBV day 7 blasts and the LCLs, nor CD40L blasts and either EBV infected time points. P values were calculated using the student T test paired sample analysis between. Grey P values indicate non-significant differences (P= ≥ 0.05) in expression.

miR-125b and miR-132 from group ii), plus miR-15a, did not show any significant difference in expression between the EBV day 7 blasts and the LCL (figure 4.5, C). However, miR-193a-3p did show a significant difference in expression between both EBV blast and CD40L blasts and the LCL and was therefore re-classified as a miRNA differentially expressed between CD40L day 7 blasts and the LCL (figure 4.4, B) for further study.

Collectively this data resulted in the successful validation of 7/10 miRNAs. Additionally, whilst miR-193a-3p did not validate the expression difference between EBV day 7 blasts and LCLs seen on the array, it did however, produce a significant difference in expression between CD40L blasts and LCLs. The change in miR-193a-3p classification may be due to the miRBase sequence modification and subsequent QPCR primer change, or it may simply reflect the reproducibility of the array. No statistical differences were observed for miR-125b, miR-132 and miR-15a, and these 3 miRNAs were consequently excluded from further analysis, as we could not demonstrate differential expression of these miRNAs upon validation.

4.3 Characterising the expression of differentially regulated miRNAs

4.3.1 miRNA expression in Mutu-BL

The 7 miRNAs which were differentially regulated between B cells stimulated with CD40L and IL4 and B cells infected with EBV (P value ≤ 0.01, figure 4.5 A and B) may be regulated by EBV latent gene expression during primary infection. To examine the possible regulation of these miRNAs by EBV latent gene expression we used QPCR to measure the miRNA expression in a panel of Mutu-BL clones. The EBV latent gene expression of the Mutu-BL clones was confirmed by western blot in section 3.3.1, figure 3.3.A. The miRNA expression data is expressed relative to the EBV loss clone. The most striking correlation between

miRNA expression and latency was seen with miR-95, miR-199a-3p and miR-28-5p (figure 4.6).

miR-95 and miR-199a-3p expression was lower in latency I Mutu-BL compared with EBV loss Mutu-BL and was down-regulated even further to un-detectable levels in the latency III clones, indicating that these two miRNAs are down-regulated by latency I gene expression, which appears to be further enhanced by a latency III pattern of EBV gene expression. miR-28-5p was specifically down-regulated in latency III Mutu-BL clones, a trend which has since been observed in a range of latency I and latency II BL cell lines [652].

The remaining 4 miRNAs did not show a convincing correlation with EBV latent gene expression in the Mutu-BL cell lines, however, it worth noting that this was a small selection of Mutu-BL clones and had a larger panel of Mutu-BL clones or additional latency I and latency III BL cell lines been analysed, trends may have been observed. miR-148a showed a slight increase in latency III Mutu-BL, but this was more pronounced in clone 95 (c95), and therefore may not be a characteristic of all latency III clones. miR-193a-3p showed variable expression over the clones and miR-200b and miR-31 showed consistent expression in all four Mutu-BL clones, however, this expression was lower than the expression of either miRNA in an LCL and was approaching the threshold for detectable expression ($\Delta Ct \geq 12$ in all samples); thus, is unlikely to be significant.

4.4 miRNA expression in HL analysis

We have identified a subset of miRNAs which may be involved in the transformation of resting B cells by EBV. The transforming capacities of EBV are intrinsically linked to its role

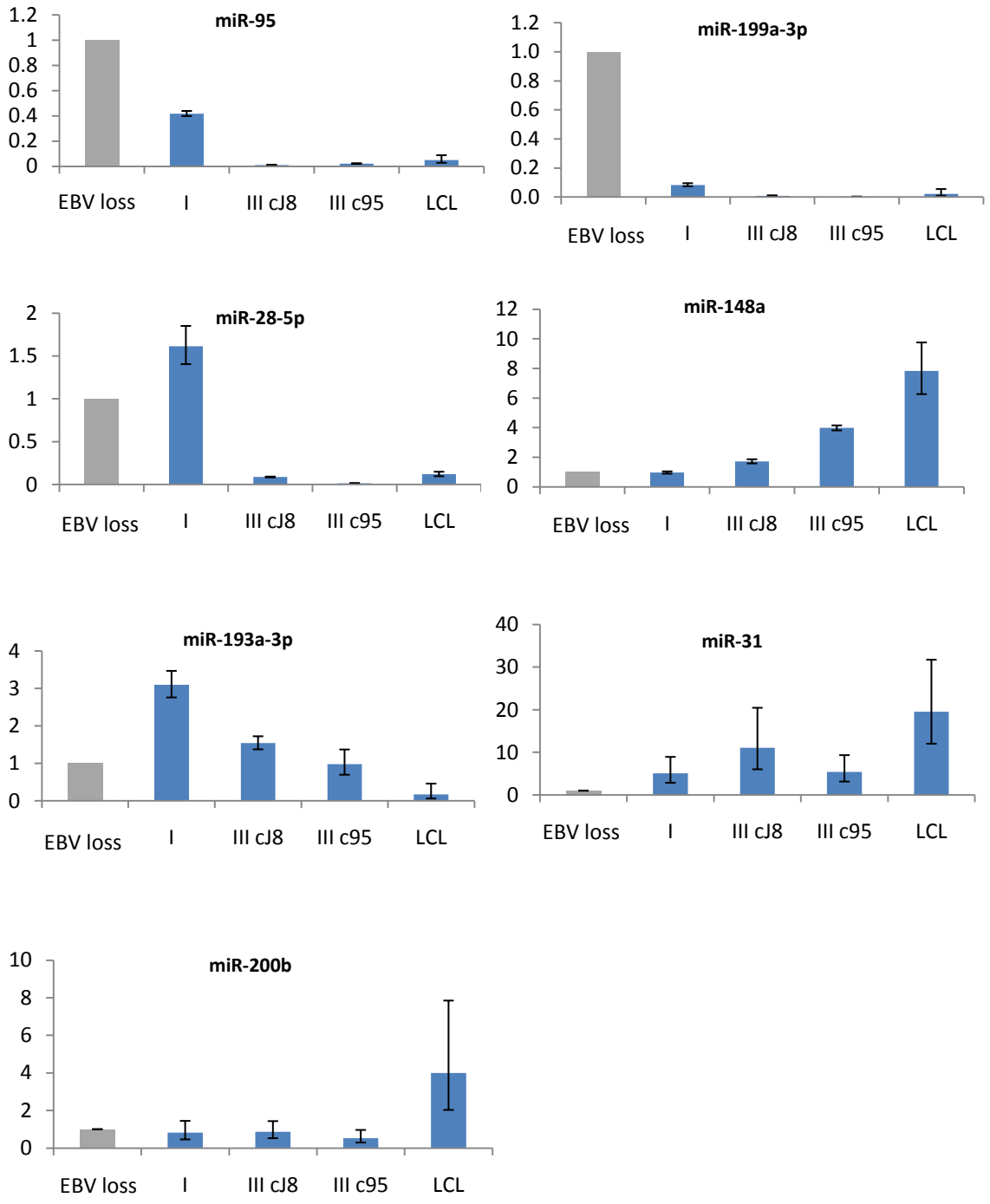


Figure 4.6: Relative QPCR expression data for 7 miRNAs in a panel of Mutu-BL clones expressing a range of EBV latent gene expression: EBV loss clone, a latency I clone (I), and two latency III clones (III cj8 and III c95). An LCL was included for comparison and data is expressed relative to the EBV loss Mutu clone. Error bars represent the range in fold change from the standard deviation of the ΔΔCt values

in lymphomagenesis so it is reasonable to hypothesise that miRNAs involved in B cell transformation may also be involved in EBV-mediated tumourogenesis.

Navarro et al [653] used QPCR to profile the expression of 157 miRNAs in the lymph nodes from 49 classic Hodgkin lymphoma patients (cHL) and 10 reactive lymph nodes (RLNs). 16/49 of the cHL tumours were EBV positive as determined by in situ hybridisation for EBER1 and EBER2 and QPCR for EBV BamH1 W. The paper [653] aimed to identify miRNA expression signatures associated with cHL, and any miRNAs whose expression also correlated with EBV status. The miRNA expression data could be used for comparison with our Taqman miRNA array expression data as a means of identifying miRNAs associated with EBV-mediated B cell transformation and cHL tumourogenesis.

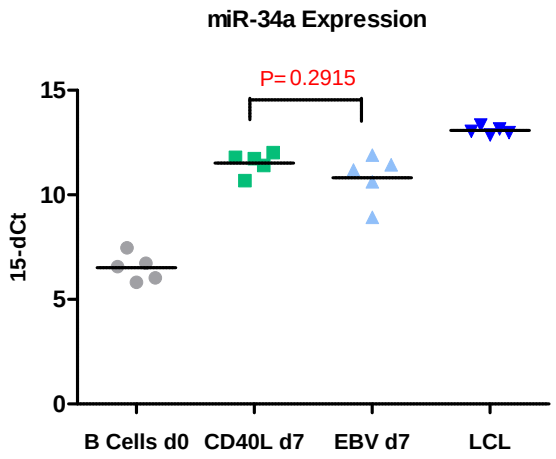
4.4.1 Identification of EBV regulated miRNAs involved in the development of HL

Our miRNA expression data from the 365 miRNAs profiled in resting B cells, CD40L blasts and EBV blasts (section 4.2.2) was compared with the expression data of 157 miRNAs in cHL and RLNs [653]. These comparisons revealed that 2/7 of our validated differentially regulated miRNAs were decreased in cHL relative to RLNs: miR-28-5p and miR-95.

4.4.2 miR-34a

The HL comparison identified miR-34a as significantly increased in cHL compared with RLNs. This miRNA was inconsistently expressed on our original Taqman miRNA array (section 4.2.2), hence its expression in EBV blasts and CD40L blasts was still unknown. We therefore decided to determine whether or not miR34a was differentially expressed between EBV and CD40L blasts, due to its differential regulation in cHL compared with RLNs. A miR-34a Taqman QPCR assay was used to confirm miR-34a expression in 5 biological replicates of resting B cells infected with EBV (day 7 and LCL) or stimulated with CD40L

A



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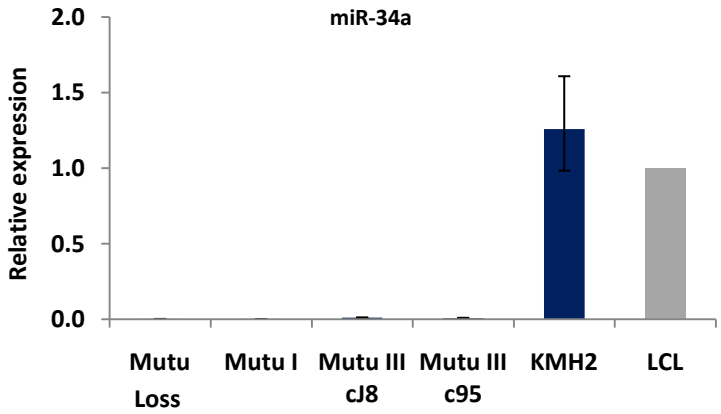


Figure 4.7 miR-34a array validation by QPCR and miR-34a expression in BL and HL cell lines. **A;** 5 biological replicates of resting B cells (B cells d0), B cells stimulated with CD40L 7 days post-stimulation, B cells infected with EBV 7 days pi (EBV d7) and 6 weeks pi (LCL). P values represent the results of a paired student T test comparing CD40L blasts and EBV d7 blasts. Data is expressed as 15-ΔCt. **B;** a panel of Mutu-BL clones expressing different forms of viral latency (EBV loss, latency I and two latency III clones cj8, c95) and a EBV negative HL cell line, KMH2. Data is expressed relative to an LCL. Error bars represent the range in fold change from the standard deviation of the ΔΔCt values.

and IL4 (figure 4.7) The data clearly show that miR-34a was up-regulated by CD40L stimulation as well as EBV infection, thus it was not an additional differentially regulated miRNA. Furthermore, we confirmed the high expression of miR-34a in HL by performing QPCR on the HL cell line KMH2, a small selection of Mutu-BL cell lines and an LCL for comparison.

Interestingly, miR-34a was expressed at very low levels in the Mutu-BL lines regardless of EBV status or latent gene expression but was very highly expressed in the HL line KMH2, further supporting the evidence that this miRNA is highly expressed in HL (figure 4.7);

miR-34a was therefore used as a positive control in the following HL cell line miRNA expression analysis.

4.4.3 miRNA expression in HL cell lines

To investigate the low expression of miR-28-5p and miR-95 in HL we used QPCR to measure the expression of both of these miRNAs in a panel of HL cell lines relative to resting B cells. The majority of the HL cell lines were EBV negative with the following two exceptions: L591 is an EBV positive HL cell line and expresses a latency III pattern of EBV gene expression and KMH2-EBV expresses a restricted pattern of latency II gene expression, with just EBNA1 and LMP2A expression [654]. Due to the difficulty in isolating the relatively few HRS tumour cells from a HL biopsy, there are only very few HL cell lines available and there are no HL cell lines which represent the latency II biopsy phenotype of EBV positive HL tumours. The HL cell lines may therefore not be representative of the EBV positive HL subset and the small number of cell lines available means that it is not wise to draw firm conclusions from miRNA expression data from such a limited panel of HL cell lines. Of the 7 miRNAs validated as differentially expressed between EBV and CD40L blasts, two (miR-199a-3p and

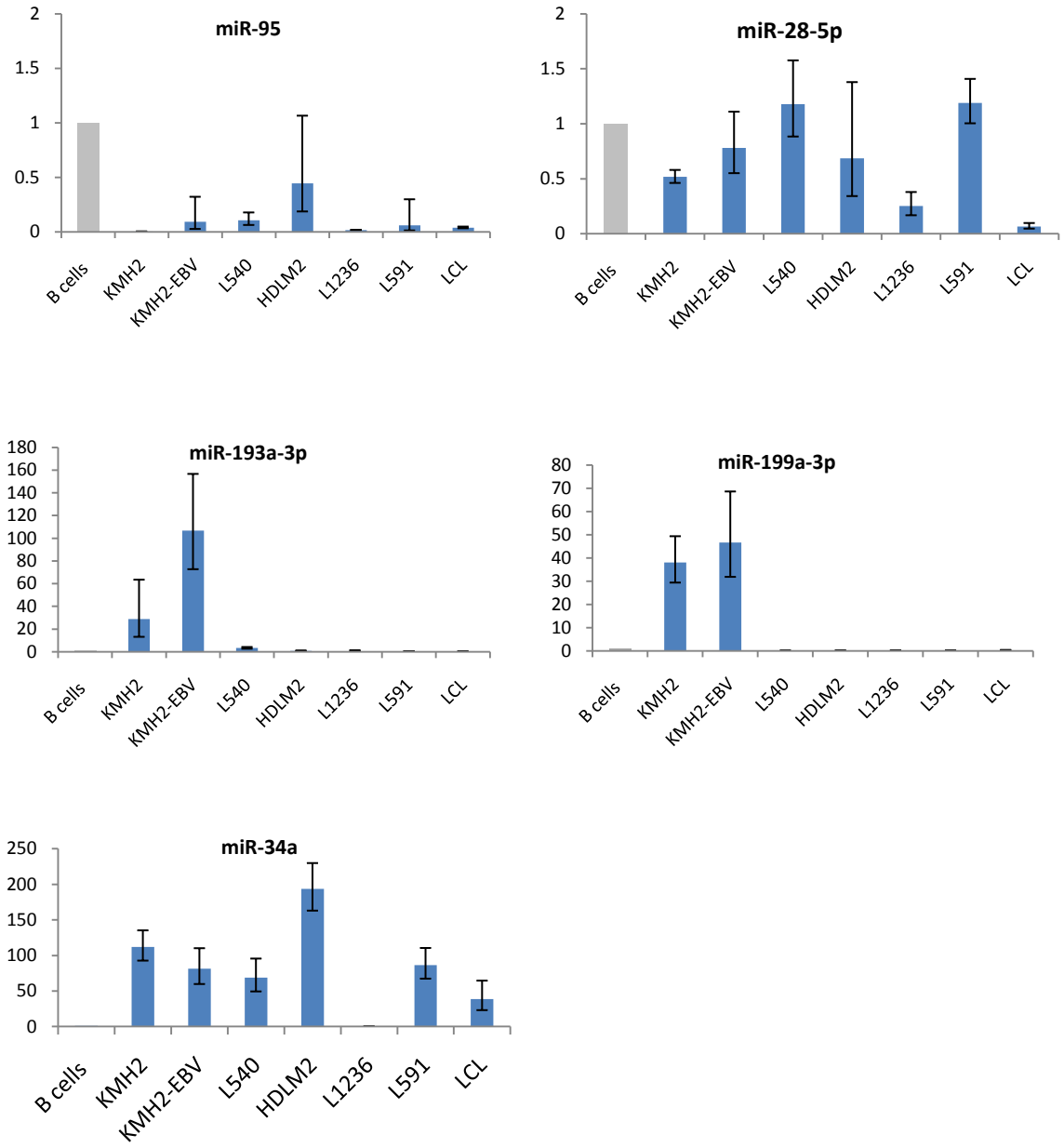


Figure 4.8 QPCR data showing the relative expression of 5 miRNAs in a panel of HL cell lines. Peripheral blood B cells and an LCL are included for comparison and the data is expressed relative to resting B cells. Error bars represent the range in fold change from the standard deviation of the ΔΔCt values

miR-193-3p) were not included in the published miRNA expression profiling of lymph nodes from cHL patients [653], therefore, we included miR-199a-3p and miR-193a-3p in our HL cell line miRNA expression profiling.

The miRNA expression in the panel of HL cell lines is expressed relative to resting B cells (figure 4.8). miR-34a was used as a positive control and showed high expression in all of the HL lines, with the exception of L1236; which may therefore have unusual miRNA expression for a HL cell line. miR-95 showed consistently lower expression in the HL cell lines than in resting B cells with only HDLM2 having a relative expression of greater than 0.1. The expression of miR-28-5p was variable across the cell lines with no obvious trend of down-regulation in the HL cell lines relative to resting B cells. All of the HL lines with the exception of L1236 showed relative miR-28-5p expression of ≥ 0.5. miR-199a-3p and miR-193a-3p both showed the same pattern of expression across the HL lines, with only the two KMH2 clones showing increased expression of these miRNAs relative to resting B cells and the remaining HL cell lines all showed decreased relative expression. miR-199a-3p showed very low relative expression in the HL lines, ranging from 0.008-0.08, which suggests that this miRNA may be down-regulated in HL relative to B cells. Conversely, miR-193a-3p showed variable expression in the HL cell lines, with relative expression ranging from 0.3-0.75, suggesting that it may not be down-regulate in HL relative to B cells.

In conclusion, miR-95 is a miRNA specifically down-regulated by EBV infection and not CD40L stimulation of B cells. We can confirm the reported low expression of miR-95 in cHL, suggesting that loss of expression of this miRNA may contribute to EBV-mediated transformation of B cells and the pathogenesis of cHL. However, we could not confirm the low expression of miR-28-5p in cHL using HL cell lines. The expression of miR-199a-3p and

miR-193a-3p was variable, nevertheless, the expression of miR-199a-3p showed that this miRNA is almost undetectable in HL cell lines and may therefore also be down-regulated in HL cell lines relative to B cells.

4.5 Identifying a miRNA for functional analysis

The aims of this chapter were to firstly identify miRNAs potentially involved in EBV-mediated transformation and then to determine what role, if any, these miRNAs play in EBV-mediated transformation of B cells and tumourogenesis. So far, we had identified a subset of 7 miRNAs differentially expressed during B cell transformation compared with mitogen activated B cells. Of these miRNAs, 4/7 (miR-148a, miR-95, miR-28-5p and miR-31, (figure 4.5 A)) showed a significant difference in expression at the early day 7 time points and represented the best candidates for functional analysis.

We selected miR-148a for functional analysis because this miRNA was the only miRNA to be up-regulated by EBV infection and not CD40L stimulation of resting B cells. miR-148a was also up-regulated to the 10th most abundantly expressed miRNA in an LCL, strongly suggesting a function role for this miRNA in LCLs. miRNAs often have fine tuning roles in gene expression, making target gene validation at the protein level difficult to confirm. By selecting a miRNA with very high expression in an LCL we hoped to increase the probability of identifying target genes which would show detectable changes in protein expression following inhibition of the very high, endogenous expression of miR-148a in LCLs.

4.5.1 Further characterising miR-148a expression

It was important to characterise the expression of miR-148a prior to functional investigation as information regarding the expression of this miRNA could aid in the identification of

functionally relevant target genes for validation. There was little information regarding the expression or function of miR-148a in the literature and what information was available was mainly in epithelial cells and not obviously relevant to B cells or EBV-mediated B cell transformation. High miR-148a expression had been demonstrated in human livers where miR-148a was found to repress the transcription factor PXR [655], and the down-regulation of miR-148a by DNA methylation had also been linked to cancer metastasis [656]. Interestingly, miR-148a had been shown to down-regulate DNMT3b in HeLa cells through a binding site in the coding region of the gene rather than the 3'UTR. This form of repression is not specific to miR-148a and evidence is accumulating which suggests that miRNAs can also mediate translation repression through binding to the 5'UTR and the coding regions of target genes [477, 657, 658]; contributing to the complexity of miRNA target identification. Additionally, it has been shown that miR-148a and miR-21 are up-regulated in the $CD4^+$ T cells of lupus patients, where the two miRNAs cooperate to cause the down-regulation of DNMT1 protein expression, and consequently increase hypomethylation of the T cells [659].

From our previous data, we observed an up-regulation of miR-148a by EBV infection of resting B cells resulted in an approximately 10 fold increase in miR-148a expression by 7 days p.i which was further increased by week 6 (LCL) to an approximately 60 fold increase. To determine when miR-148a was up-regulated following EBV infection, a more extensive time course assay was performed with B cells harvested 2,3,4,5,6 and 14 days p.i. miR-148a was not up-regulated until approximately 4 days p.i (figure 4.9). The delay in miR-148a up-regulation suggested a possible role for the EBV latent genes whose expression is also delayed p,i, such as LMP1 or LMP2, in the up-regulation of this miRNA.

The expression of miR-148a in the EBV negative Mutu-BL cell line was only 8 fold less than the miR-148a expression in an LCL. Therefore, Mutu-BL expressed considerably more miR-

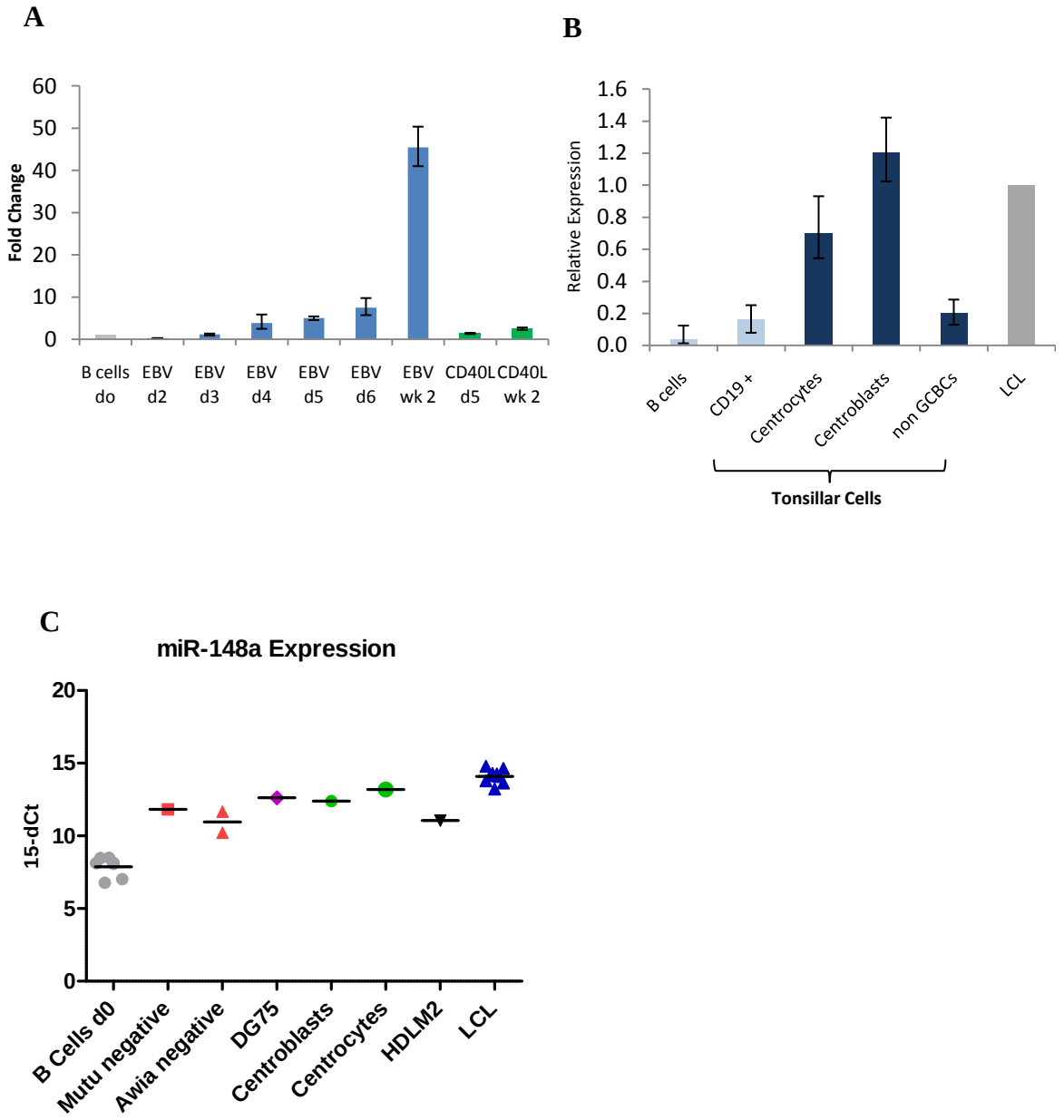


Figure 4.9 miR-148a expression by QPCR in a range of cell lines and tonsillar cells. **A;** Fold change in miR-148a expression relative to resting B cells (d0) post EBV infection or CD40L and IL4 stimulation at corresponding days (d) p.i. **B;** miR-148a expression in tonsillar B cells sorted with $CD19^{+}$ dynabeads or by FACS for the two GC B cell subsets centrocytes and centroblasts and the remaining GC B cells depleted cells. An LCL and peripheral blood B cells (B cells) were included for comparison. Data is expressed relative to the LCL. Error bars represent the range in fold change from the standard deviation of the ΔΔCt values. **C;** Comparison of miR-148a expression in different B cell subsets and cell lines including: three EBV negative BL cell lines Mutu, Awia and DG75; One HL cell line HDLM2; Two GCBC subsets, centrocytes and centroblasts. Data is expressed as15-ΔCt (15-dCt).

148a than resting B cells where miR-148a expression was approximately 40-60 fold less than an LCL (summarised in figure 4.9 C). Since BL tumour lines have a germinal centre phenotype we wanted to know what the expression of miR-148a was in germinal centre B cells compared with resting B cells. QPCR for miR-148a in the centrocytes, centroblasts and $CD19^+$ B cells from a tonsil revealed that miR-148a expression was high in both the GCBC-subsets, with the expression in the centroblasts being greater than that in an LCL (figure 4.9).

The high expression of miR-148a observed in GC B cell subsets led us to analyse a panel of BL latency I cell lines and a panel of HL lines to determine whether this high expression was characteristic of all cells with a GC B cell phenotype. The results in figure 4.10 show that miR-148a expression varies across the cell lines with the majority of the cell lines showing less than 10% of the miR-148a expression seen in an LCL. This suggests that the high miR-148a expression seen in Mutu-BL clones may not be characteristic of all BL and HL cell lines with a GC B cell phenotype, although analysis of miR-148a expression in a wider panel of BL cell lines would be required to confirm this.

NK/T cell lymphoma is another EBV associated lymphoid malignancy, so to fully characterise the association of miR-148a with EBV transformation and tumourogenesis we analysed a panel of EBV positive (SNK6, SNK10 and SNK16) NK cell lines and an EBV negative NK cell line (NKL) in comparison with an LCL. Primary NK cells isolated by negative selection from peripheral blood were also included for comparison. The results presented in figure 4.10 show that the expression of miR-148a in primary NK cells was very low and similar to the expression of miR-148a in resting B cells. miR-148a was almost undetectable in all of the NK cell lines indicating that the up-regulation of this miRNA is not

involved in the NK lymphoma and EBV latent gene expression does not up-regulate miR-148a in an NK cell background.

miRNA expression is very stable in paraffin embedded tissue [551] hence the RNA extracted from paraffin embedded tumour samples can be used to assess miRNA expression. An LCL is phenotypically very similar to PTLD tumour cells which also usually express a latency III pattern of EBV gene expression. RNA from two PTLD, two HL and one non-Hodgkin lymphoma (NHL) paraffin embedded tumour biopsies was extracted and used for QPCR measurement of miR-148a expression. All of the tumour biopsies showed detectable miR-148a expression with a range of 10-30% of the expression seen in an LCL.

4.6 miR-148a primary target gene prediction

The function of miR-148a, like most miRNAs, is still unknown. There are a few validated gene targets for miR-148a in the literature, including: DNMT3b [478], DNMT1[659], HLA-G[660], TGIF2 [656] and Pregnane X receptor (PXR) [655]. However, the role of miR-148a during the transformation of resting B cells by EBV or its role in GC B cells remains unknown. To address this question we sought to identify primary miR-148a targets predicted by miRNA target prediction programs. These targets could then be validated using miR-148a inhibitors in an LCL to determine whether or not the predicted genes are repressed by the high expression of miR-148a in an LCL.

4.6.1 Primary target identification using miRNA target prediction programs

The binding of a miRNA to a target mRNA is a complicated process which is still not fully understood. Computer programs designed to predict miRNA targets can only produce a list of

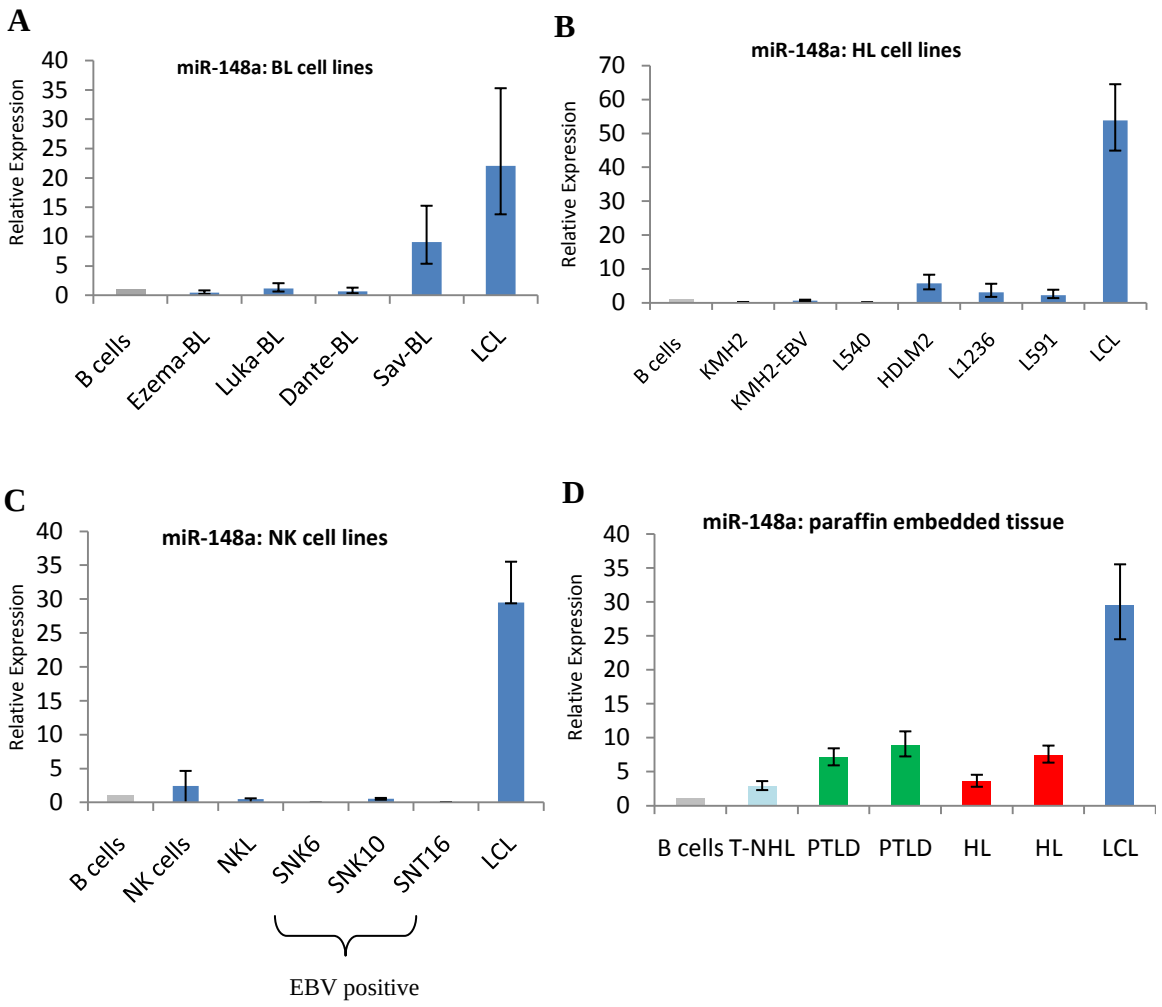


Figure 4.10 QPCR expression data for miR-148a in panels of cell lines and formalin fixed tissue samples. Data is expressed relative to resting B cells. **A;** miR-148a expression in a panel of latency I BL cell lines. **B;** miR-148a expression in a panel of EBV negative HL cell lines with the exception of L591 which expresses a latency III pattern of EBV gene expression and KMH2-EBV which expresses EBNA1 and LMP2A **C;** miR-148a expression in a panel of NK tumour lines. Primary NK cells isolated from peripheral blood are included for comparison. NKL is an EBV negative NK cell line and SNK6, SNK10 and SNT16 are all EBV positive and express a latency II pattern of EBV gene expression. Error bars represent the range in fold change from the standard deviation of the ΔΔCt values.

best estimate targets based on the most current and limited knowledge. Each miRNA target prediction program uses differing parameters and places more or less emphasis on certain parameters than others. Consequently, each miRNA target prediction program produces a different list of predicted miRNA targets with a presumably high rate of false positives and negatives. Theoretically, combining the data from multiple miRNA target prediction programs will enable the identification of miRNA target genes with the highest probability of being valid.

Three miRNA target prediction programs with a database of miRNAs and their corresponding predicted gene targets available for download were: miRanda [661], Target Scan [492, 502] and microT [662]. The predicted gene targets for miR-148a were downloaded from these programs and entered into a Microsoft Access database for comparison.

The degree of overlap between the three miRNA target prediction programs is represented in figure 4.11. There were 183 genes predicted by all three programs and these are listed in table 13.

4.6.2 Identifying the most probable primary targets

To identify predicted miR-148a targets for validation we further reduced the number of predicted miR-148a targets to focus on just those with the highest miR-148a binding scores in each program. The three miR-148a target gene databases were sorted based on the individual scoring system of that database. A comparison was then performed between the genes with the top 10% highest score values from each database (i.e. the best predicted matches) and 2 genes were identified as common to all three databases. A comparison was also performed between the top 20% of genes with the highest scores to generate a list of 12 common genes. (figure 4.12 and table 14).

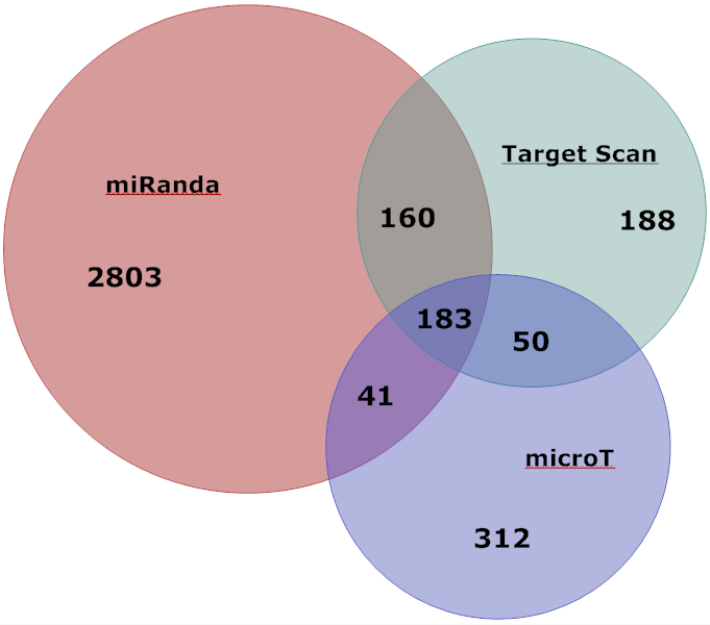


Figure 4.11: Venn diagram representing the predicted miR-148a target genes from three independent miRNA target prediction programs and the overlap between them.

Gene Name	Score
DCP2	26.96
USP6	26.11
MEOX2	25.63
BCL2L11	24.63
C18orf25	23.93
B4GALT5	23.04
OSBPL11	22.84
RBM24	22.09
INOC1	22.02
CPEB4	21.58
CDC2L6	21.41
MTF1	21.27
TNRC6A	21.07
MAFB	20.91
ATP11A	20.65
MLL	20.63
LEPROTL1	20.04
EIF2C1	19.97
C1orf144	19.33
USP32	19.1
NRP1	19.1
NPTX1	19.08
OTX2	18.79
CAND1	18.7
LASS6	18.51
SH3PXD2A	18.18
BCL11A	17.84
MAF	17.7
ARFIP1	17.7
INHBB	17.7
SMS	17.7
FMR1	17.7
MITF	17.6
MXD1	17.29
EIF2C4	16.95
ELF5	16.95
MIER1	16.88
FXR1	16.3
BTBD3	16.3

Gene Name	Score
MAP3K9	16.3
HOXC8	16.3
BACH2	16.3
NHS	16.3
CNTN4	16.3
MMD	16.3
OTUD4	16.3
YWHAB	16.3
COL2A1	16.3
ADAMTS5	16.09
CDK5R1	15.69
CCKBR	15.65
ZDHHC17	15.51
ITGA5	15.11
WDR47	15.11
C5orf30	15.11
ATP6AP2	15.07
LRP2	14.91
MLLT10	14.91
USP48	14.91
ELAVL2	14.91
QKI	14.91
ERRFI1	14.91
ATXN1	14.91
CANX	14.91
BAI3	14.91
MED12L	14.91
ATXN7L1	14.91
NCAM1	14.91
BRPF1	14.91
CUL5	14.91
PPP1CB	14.91
DNMT1	14.91
TGIF2	14.22
SNN	14.16
NFAT5	13.63
SYNJ1	13.52
SFRS11	13.51
ARHGEF12	13.51

Gene Name	Score
ESRRG	13.51
GPATCH8	13.51
DEDD	13.51
TOMM70A	13.51
TANC1	13.51
CCDC6	13.51
MNT	13.51
PNPLA6	13.51
JARID2	13.51
SRPK2	13.51
MRAS	13.5
DYRK1A	13.42
CXorf23	13.27
ROCK1	13.21
SMAD2	13.17
MTMR9	12.73
DDAH1	12.72
DLG2	12.72
PIGA	12.32
WNT1	12.32
ZADH2	12.32
SNF1LK	12.11
ERBB3	12.11
ALS2CR2	12.11
FBXL19	12.11
RAB34	12.11
GTF2H1	12.11
SLC2A1	12.11
PIK3R3	12.11
STARD13	12.11
DNAJB12	12.11
DMXL1	12.11
TMEM9B	12.11
SESTD1	12.11
ASPH	12.11
NRAS	12.11
WDR20	12.11
ROBO1	11.44
PDE4D	11.43

Table 13: List of 183 genes predicted to be miR-148a targets by three target prediction programs: miRanda, Target Scan and microT. Score values form the miRanda program are included for comparison.

Gene Name	Score
MYST2	11.36
ACVR2B	11.34
PITPNM2	11.32
ARRDC3	10.99
EPAS1	10.78
MTA2	10.72
HMGB3	10.72
YTHDC2	10.72
CDC14A	10.72
DYRK1B	10.72
PRKAG2	10.72
HECW2	10.72
EIF4E3	10.72
ARL6IP1	10.72
COL4A1	10.72
GADD45A	10.72
FBXO28	10.55
ZCCHC2	10.2
MARCH3	9.97
XPO4	9.86
RAB14	9.66
SIX4	9.66
DENND4C	9.66
POU3F2	9.66
LYSMD2	9.32
ABCD3	9.32
DMPK	9.32
SAPS1	9.32
CDK6	9.32
PPP1R9B	9.32
RIPK5	9.32
RCC2	9.32
MTMR14	9.32
GAPVD1	9.32
ARF4	9.32
NDP	9.32
AP4E1	9.27
SLC24A3	9.22
CAMK2A	8.83

Gene Name	Score
USP33	8.78
BAZ2A	8.78
WASL	8.78
PLAA	8.78
LYSMD3	8.74
SGCB	8.74
PHF20	8.72
CSF1	8.57
KIAA1045	8.53
MAP3K4	8.44
MGAT4A	8.34
PRNP	8.13
ITGB8	7.94
ANKRD13A	7.93
NFIX	7.93
C1GALT1	7.93
KLHL18	7.93
TEK	7.93
TMEM54	7.93
LRRC41	7.93
SPIRE1	7.93
LIN28B	7.93
ST18	7.86
TEAD1	7.72
EPS15	7.45
PRICKLE2	7.45
SNX27	7.45

Table 13: Continued.

A: Top 10% analysis: **B: Top 20% analysis:**

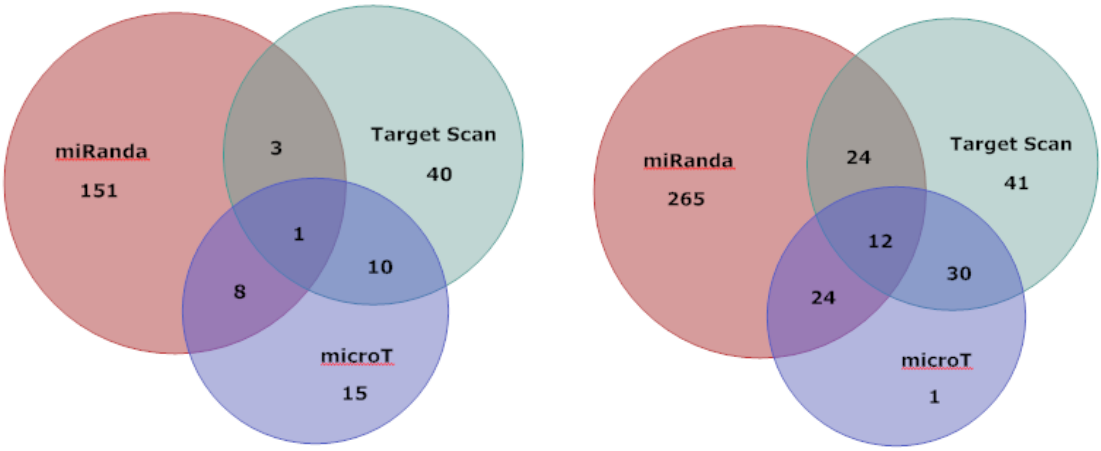


Figure 4.12: Venn diagrams representing the results of a comparison between **A;** the top 10% and **B;** the top 20% of genes with the highest score values predicted to be targeted by miR-148a by three independent target prediction programs

Gene Name	Aka	Function
LEPROT1 leptin receptor overlapping		Putative protein. Little is known
BCL2L11	Bim	Pro-apoptotic member of the BCL2 family of proteins.
USP6 Ubiquitin specific peptidase 6	HRP1, TRE2 TRE17	Oncogene. Isopeptidase whose expression in normal tissue is testis specific but is expressed in a number of cancer cell lines. Initiates tumourogenesis by inducing the production of matrix metalloproteinases [663, 664]
OSBPL11 Oxyterol binding protein		Little is known. Widely expressed.
MAFB Transcription factor MAFB	KRML	Acts as a transcriptional activator or repressor and is involved in erythroid differentiation [665]. Can act as a tumour suppressor gene [666].
MLL Myeloid lineage leukemia	ALL1, HRX	Histone methyltransferase that plays an essential role in early development and hematopoiesis. Chromosomal aberrations involving MLL are a cause of acute leukemias [667, 668].
EIF2C1 Eukaryotic translation initiation factor 2C1	AGO1	Required for RNAi and gene transcription silencing [669, 670].
USP32 Ubiquitin specific protein 32		Little is known. Related to USP6
FMR1 Fragile X mental retardation 1		RNA binding protein involved in intracellular RNA transport and the regulation of translation. Highly expressed in several cell types including lymphocytes [671, 672].
EIF2C4 Eukaryotic translation inititation factor 4	AGO4	Required for RNAi. Also required for the replication of the human hepatocyte delta virus.
NHS Nance Horan Syndrome	`	Unknown. May be involved in eye, tooth and brain development
CNTN4 Contactin 4	BIG-2	Mediates cell surface interactions during nervous system development

Table 14: Genes with the top 20% (top 10%) score values from three miRNA target prediction programs (miRanda, Target Scan and MicroT) were compared and those predicted by all three programs are listed with a brief description of known function.

4.7 Validation of predicted miR-148a primary targets

As discussed in section 4.6.1, one of the caveats of using miRNA target prediction programs is their lack of specificity. Furthermore, these programs cannot identify target genes relevant to the cell background or condition of interest. 3'UTR reporter assays could verify the existence of a miR-148a binding site in the 3'UTR of the predicted target genes. However, the regulation of these genes in an LCL would still need to be validated. For this reason, we decided to validate the repression of predicted miR-148a target genes at the protein level following inhibition of miR-148a in an LCL. This would quickly identify whether the protein expression of the gene of interest was repressed in an LCL by the endogenous level of miR-148a expression.

4.7.1 Creating a miR-148a responsive luciferase reporter

To validate the efficacy of commercially available miR-148a inhibitors in an LCL, we created a luciferase reporter containing a miR-148a binding site with 100% complimentarity to miR 148a inserted into the 3'UTR of the luciferase gene. miR-148a repression of luciferase could then be measured in an LCL in the presence or absence of miR-148a inhibitors 36 hours after co-transfection. Luciferase expression was normalised to β-galactosidase (β-gal) expression after an equal amount of both luciferase and β gal plasmids were co-transfected into each sample.

An oligonucleotide with 100% complimentary to miR-148a was designed using miRBase (version 14) for the miR-148a sequence and synthesised with *Spe*1 and *Hind*III sticky ends for ligation into the pmiR-Report vector. Full details and sequences are described in section 2.20 and simplified diagrams of the luciferase and luciferase-148a vectors are shown in figure 4.13.

4.7.2 Validation of miR-148a inhibitors using a miR-148a- luciferase reporter

miRNA inhibitors are small RNAs with a complimentary sequence to the target miRNA. They inhibit the function of the target miRNA by binding to it and preventing the miRNA from binding to mRNA target genes, thereby inhibiting the function of the miRNA. These inhibitors are O-methylated to prevent degradation by the cell and are stable for 48 hours [456].

Commercially available miR-148a inhibitors were transiently transfected into an LCL and the cells were harvested for RNA extraction 36 hours post-transfection. The transfection efficiency of the miR-148a inhibitor could not be measured directly although it could be estimated by the efficiency of transfection of a fluorescently labelled oligonucleotide (siGLo). The transfection efficiency of siGLo as determined by flow cytometry (figure 4.14) was consistently between 95-98%, indicating that the transfection efficiency of the LCL with a small RNA was very good.

The luciferase expression of the luciferase-148a reporter was approximately 60% of that seen in the control luciferase reporter (figure 4.14). The luciferase expression in luciferase-148a could be restored to the same levels as the control reporter after 2μM of a miR-148a inhibitor was transfected. The transfection of a control (scrambled) miRNA inhibitor had no effect on the luciferase expression from the luciferase-148a reporter in an LCL, indicating that the miR-148a inhibitor was efficiently inhibiting the function of miR-148a.

4.7.3 DNMT1 protein expression following miR-148a inhibition

DNMT1 is a published gene target of miR-148a and its expression was shown to be down-regulated by ectopic miR-148a expression in Jurkat cells [659], primary $CD4^+$ T cells [659] and cholangiocytes [673]. To determine whether DNMT1 was regulated by miR-148a in an LCL, a western blot for DNMT1 protein expression was performed 36 hours post-transfection

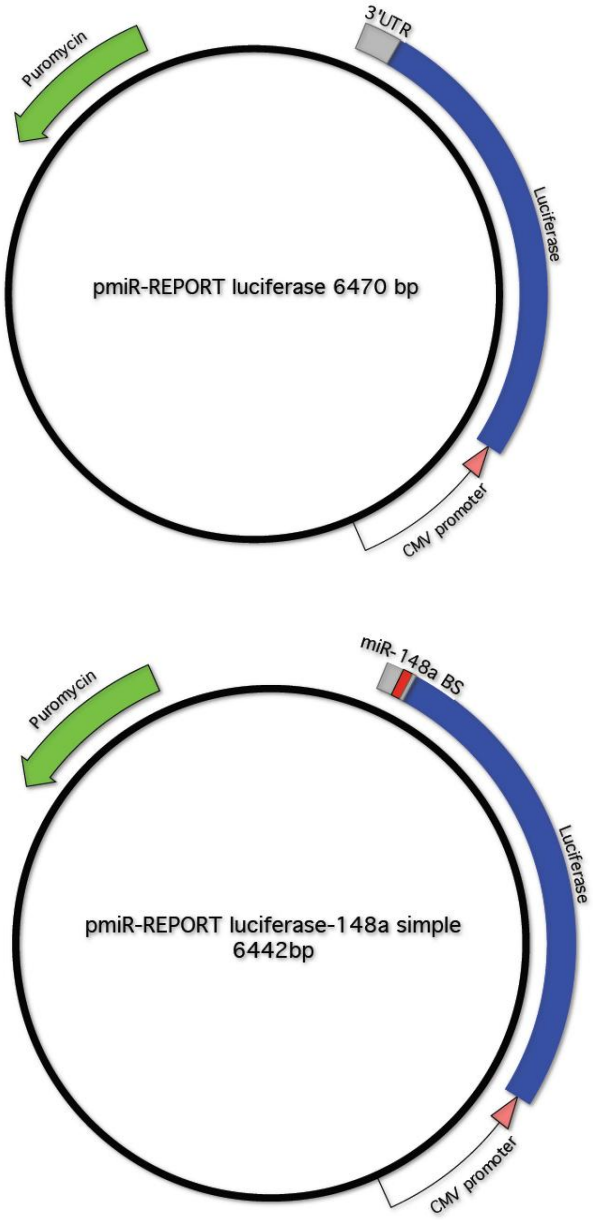
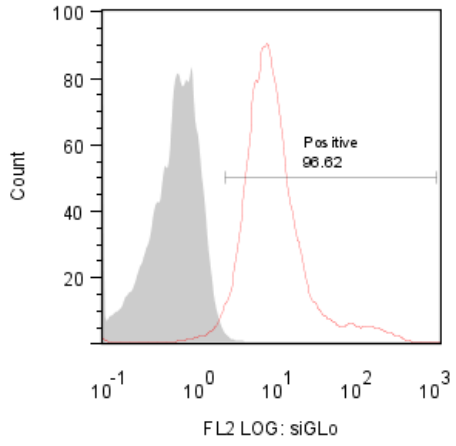


Figure 4.13: Simplified maps of the pmiR-REPORT vectors created to validate the efficacy of miR-148a inhibitors. **A;** the non-modified luciferase reporter which was used as a negative control in transfection experiments. **B;** The luciferase vector with a miR-148a binding site with 100% complimentarity to miR-148a inserted into the 3'UTR of the luciferase gene (luciferase-148a). This binding site made the luciferase susceptible to miR-148a mediated mRNA degradation/translational inhibition, reducing the luciferase expression from the plasmid in the presence of functional miR-148a.

A



B

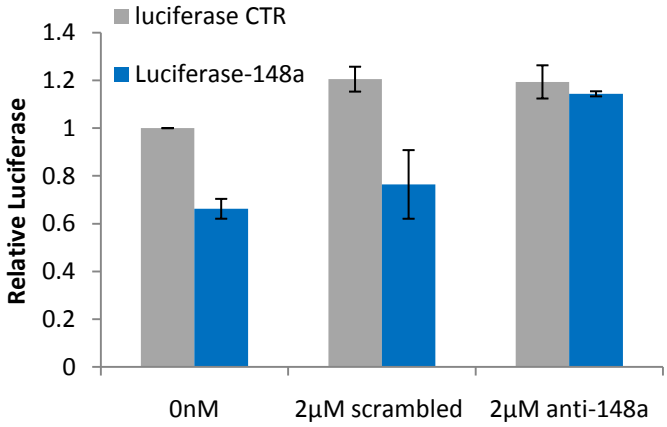


Figure 4.14 A; Luciferase reporter assays demonstrating the efficacy of miR-148a inhibitors. Flow cytometry data showing transfection efficiency of a small fluorescence labelled oligo (siGLo) measured 24 hours post transfection into an LCL via electroporation. Grey, filled histogram represents a no-RNA transfected LCL control. **B;** relative luciferase expression in an LCL transfected with either a luciferase reporter (luciferase CTR) or a luciferase reporter containing a miR-148a binding site in the 3'UTR of the luciferase gene (luciferase-148a). The luciferase CTR or Lucifease-148a plasmids were co-transfected with a β-gal plasmid, which was used for normalisation and 0nM or 2nM of a commercially available miR-148a inhibitors (anti-miR-148a) or scrambled inhibitors (scrambled). Cells were harvested 24 hours post-transfection and luciferase and β-gal expression measured using a lunomitor. Data is expressed relative to the Luciferase CTR sample transfected with 0nM of miR-148a or control inhibitors. Error bars represent the standard deviation of triplicate assays.

with 2µM of a miR-148a inhibitor. The protein expression of DNMT1 and calregulin were measured by western blot in triplicate biological replicate samples of an LCL 36 hours post transfection of a control scrambled RNA or miR-148a inhibitor. However, the results in figure 4.15 show that there was no change in DNMT1 protein expression in any of the samples, indicating that miR-148a inhibition in an LCL does not affect DNMT1 protein expression.

4.7.4 Bim protein expression following miR-148a inhibition

Bim (BCL2LII) is a pro-apoptotic member of the BCL2 family of proteins and is known to be down-regulated by EBV during B cell transformation [136]. Every form of analysis performed on the miR-148a target prediction data produced Bim as a candidate miR-148a target. For this reason, we decided to transfect commercially available miR-148a inhibitors into LCLs (Bim negative) to measure the Bim protein expression by western blot.

2µM of miR-148a inhibitors were transfected via electroporation into an LCL and the Bim protein detected by western blotting. DG75 was included in the blot as a positive control for Bim expression as it has high expression of Bim due to a mutation in the Bax gene [674], which allows the tolerance of high Bim expression. The results show that miR-148a inhibition in an LCL does not result in any detectable increase in Bim expression (figure 4.15) which was not unexpected because Bim is transcriptionally and epigenetically silenced in an LCL [136, 675]. Therefore, translational repression by a miRNA was unlikely to be a significant factor in Bim expression in an LCL. However, the repression of Bim protein by miR-148a may still be important in the early stages of B cell transformation, before Bim expression has been repressed transcriptionally and epigenetically.

4.7.5 miR-148a over-expression in DG75-BL

DG75-BL has high expression of Bim, therefore the effect of miR-148a over-expression on Bim protein expression can easily be measured by western blot in this cell line. To over-express miR-148a in DG75 we used commercially available miR-148a mimics. These mimics are the pre-miR-148a precursors, which are processed by Dicer-1 in the cytoplasm of the cell and incorporated into the RISC complex as a functional miR-148a.

2µM of commercially available miR-148a mimics were transfected into DG75 via electroporation and the protein was harvested 36 hours post transfection for western blot. Bim and DNMT1 protein expression was measured by western blot. DNMT1 was included out of interest as a published miR-148a target gene. Bim and DNMT1 protein was consistently expressed across the control mimic and miR-148a mimic transfected samples. This again shows no effect of miR-148a on DNMT1 protein expression in a B cell background and also suggests that increased miR-148a expression did not affect the high Bim expression in DG75-BL. The lack of Bim protein repression seen after miR-148a transfection could be due to the relatively high endogenous expression of miR-148a in DG75 (figure 4.15). Alternatively, miR-148a may not be a significant regulatory factor for Bim expression in an established LCL.

4.8 Multiple miRNA gene target analysis

miRNA mediated gene repression is known to be more effective when there are multiple miRNA binding sites, preferably in close proximity [441]. These miRNA binding sites can be for the same miRNA or for different miRNAs. It is thought that the enhanced repression caused by multiple miRNA binding sites allows a gene to have high sensitivity to a particular miRNA only in certain environments or tissues where other specific miRNAs are also expressed. It is this cooperative repression which raised two questions: (i) are any of the

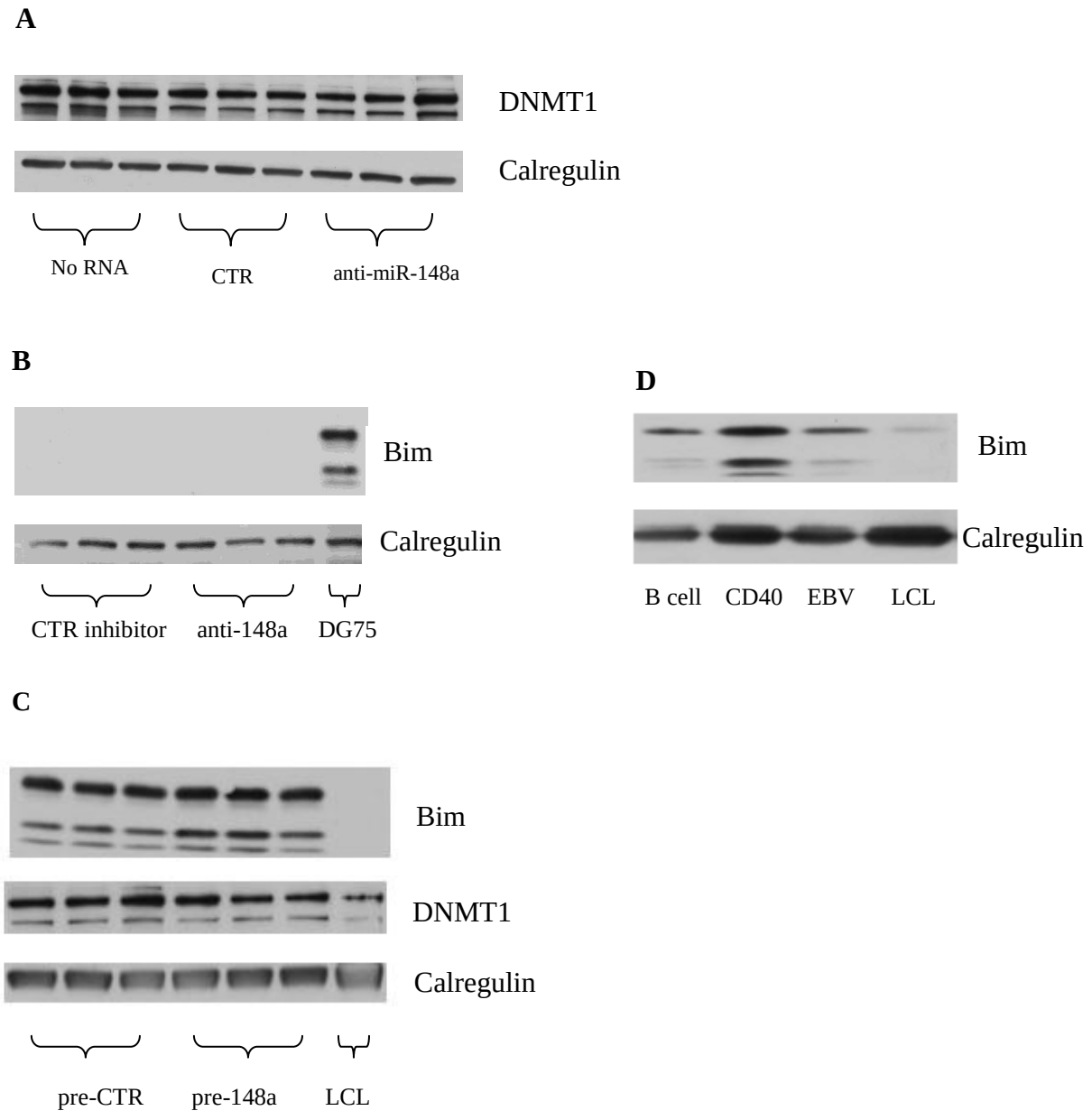


Figure 4.15: Western blot for Bim and DNMT1 following miR-148a inhibition or ectopic miR-148a expression. Samples were collected in biological triplicate. **A;** DNMT1 and calregulin protein expression by western blot in triplicate replicates of an LCL transfected by electroporation with; no RNA, 2µM control inhibitor (CTR inhibitor) or 2µM of a miR-148a inhibitor (anti-miR-148a). **B;** 2µM of miR-148a inhibitor (anti-148a) or control inhibitors (anti-CTR) were transfected into an LCL via electroporation and protein harvested 36 hours post transfection. DG75 was included as a Bim positive control. **C**; 2µM of pre-miR-148a (pre-148a) or control pre-miRNAs (pre-CTR) were transfected into DG75 via electroporation and protein was harvested 36 hours post-transfection. D; B cells, B cells stimulated with CD40L (CD40) or infected with EBV at 7 days p.i (EBV) or 6 weeks p.i (LCL) were probed from Bim and calregulin.

miRNAs identified as up-regulated by EBV and CD40L in the Taqman miRNA array also predicted to target any of the 183 miR-148a target genes listed in table 13? And (ii) could the lack of Bim protein repression observed following miR-148a over-expression in DG75-BL be due to the need for multiple miRNA repression?

From the Taqman miRNA array data, there were 19 miRNAs up-regulated by EBV and CD40L (table 10) which was a very large data set to compare. Not all of these miRNAs had predicted target genes in all three target prediction programs, so to simplify the data analysis, all of the data comparisons were performed using the data from just one miRNA target prediction program, Target Scan. Target scan had predictions for all 19 up-regulated miRNAs and was arguably the most accurate miRNA target prediction program available [491].

The predicted target genes for the 19 up-regulated miRNAs were compared with each other to identify genes targeted by all/some of them. In order to achieve this, a computer program was specially written to cross analyse the data sets. The primary function of the program was to iterate through each of the predicted target genes from each of the 19 miRNAs to identify commanality of the genes regarding the predicted miRNA regulation. The results from the 7 differentially regulated miRNAs and the 6 down-regulated miRNAs are included for comparison (tables 15-17)To generate a list of the predicted miR-148a target genes which were also predicted to be targeted by the other cellular miRNAs up-regulated during B cell transformation, a comparison between the data in table 15 and the 183 genes predicted to be miR-148a targets listed in table 13 was made. We focused the analysis on genes which were predicted to be targeted by ≥ 50% (9 or more miRNAs) of the miRNAs up-regulated during EBV infection of B cells. From the list of 183 miR-148a predicted target genes (table 13), 10 genes were found to also be predicted targets of 9 or more miRNAs up-regulated by EBV infection (table 18). This list of genes is varied, however, there are two RNA binding proteins,

Number of up-regulated miRNAs compared	Number of genes predicted
19/19	0
18/19	0
17/19	0
16/19	0
15/19	0
14/19	0
13/19	1
12/19	2
11/19	12
10/19	28
9/19	66
8/19	139
7/19	257
6/19	415
5/19	675
4/19	1208
3/19	1523
2/19	2169

Number of differentially regulated miRNAs compared	Number of genes predicted
7/7	0
6/7	0
5/7	1
4/7	14
3/7	80
2/7	484

Number of down-regulated miRNAs compared	Number of genes predicted
7/7	0
6/7	0
5/7	0
4/7	12
3/7	140
2/7	672

Tables 15-17: Results from a multiple miRNA target prediction analysis. miRNAs whose expression changed in response to EBV infection of resting B cells were analysed. Predicted target genes for miRNAs up-regulated or down-regulated following EBV infection, or, differentially regulated between EBV and CD40L blasts in a Taqman miRNA were obtained using Target Scan. The predicted gene targets for all miRNAs in each category were compared using a custom designed computer program. The numbers of genes predicted to be targeted by miRNAs in each category are indicated.

Gene Name	Aka	Function
BCL2L11	Bim	Pro-apoptotic gene
ATP11A		ATPase, little known.
ATXN1 Ataxin 1		Binds RNA. May be involved in RNA metabolism, has been shown to bind ubiquitin binding proteins [676]
ATXN7L1 Ataxin like protein		Little known.
FXR1 Fragile X related syndrome related protein		RNA binding protein. Binds FRM1 (table 3) and FXR2. May regulate the transport and translation of certain mRNAs.[677, 678]
ITGB8 Integrin beta 8		Receptor for fibronectin [679]
MTF1 Metal regulatory transcription factor 1		Activates the metallothionein I promoter [680].
MXD1 MAX dimerisation protein		Little known
NFAT5 Nuclear factor of activated T cells 5	TonEBP	Transcription factor involved in the induction of gene expression. Induced by osmotic stress [681, 682].
SH3PXD2A		Required for podosome formation, degradation of the extracellular matrix, and for the invasiveness of some cancer cells. Found in several cancer cell lines, particularly invasive breast cancer cell lines and melanomas [683, 684].

Table 18: Genes predicted by target scan to be targeted by 9 or more of the 19 miRNAs up-regulated by EBV infection of resting B cells and miR-148a. The list of genes predicted to be targeted by 9 or more of the up-regulated miRNAs were compared with the 183 genes predicted to be miR-148a targets by three target prediction programs (table 13). The common genes are listed in this table with brief known function.

consistent with previous miR-148a target prediction analyses which have suggested a possible role for miR-148a in the regulation of RNAi and mRNA transport.

Interestingly, Bim was one of the 10 genes predicted to be targeted by ≥ 50% of the miRNAs up-regulated during B cell transformation, and by miR-148a. This suggests that a large number of miRNAs expressed in B cells blasts are able to cooperate to regulate the expression of Bim. Therefore, the over-expression or inhibition of only one of the many miRNAs predicted to down-regulate Bim, may not be sufficient to cause a detectable change in Bim protein expression; offering a potential explanation for the lack of Bim protein repression we observed following miR-148a transfection in DG75-BL. This would be consistent with miR-148a having a fine tuning role in Bim protein expression. Alternatively, miR-148a may only significantly affect the expression of Bim protein in primary B cells and early in the transformation process, when the expressions of the other Bim regulatory miRNAs are lower. This could be tested using combinations of miRNA mimics for transfection into DG75. Alternatively, combinations of miRNA sponges, or simply over-expressing the 3'UTR of Bim could be used to sequester the multiple miRNAs up-regulated in early EBV blasts to examine the role of these miRNA in the regulation of Bim protein expression following EBV infection of resting B cells. Over-expression of multiple miRNAs using miRNA mimics cannot be used to repress Bim expression in LCLs as Bim protein expression is very low in EBV blasts 7 days and 6 weeks p.i (figure 4.15)

Discussion II

(a) Cellular miRNA expression profiling

The comparison between EBV infected and CD40L and IL4 stimulated B cell blasts has proved to be an interesting model which has allowed the identification of a novel set of 7 miRNAs whose expression changes are specific to EBV-mediated B cell transformation. The miRNA expression profiling by QPCR was, and remains, the most sensitive and specific technique available for quantitating miRNA expression. The miRNA array agreed with the limited miRNA expression data which was available at the time, demonstrating that miR-155 [603, 640], miR-146a [629, 685] and miR-21 [603] were all up-regulated following EBV infection, suggesting that this technique was reliable. In addition to the oncogenic miRNAs -155 and -21, five of the six oncogenic miR-17-92 cluster were also up-regulated by both EBV infection and CD40L stimulation, suggesting that activation and proliferation of resting B cells involves the up-regulation of at least 7 oncogenic miRNAs; a corresponding down-regulation of tumour suppressor genes would be predicted to result from the up-regulation of multiple oncogenic miRNAs, which may contribute to the continuously proliferating, apoptotic resistant phenotype of LCLs. It would be interesting to determine whether the any of the oncogenic miRNAs were also up-regulated by alternate patterns of EBV latency, such as latency I, so the contribution of these miRNAs to EBV associated malignancies (which rarely express a latency III pattern of EBV gene expression) could be evaluated.

The miRNA QPCR array was only performed in duplicate; therefore, the reproducibility of the array was not as thorough as it would have been had we performed the array in the standard triplicate samples. With hind-sight it would have been more constructive to perform

the array in triplicate on just the resting B cells, EBV and CD40L day 7 blasts, leaving out the LCL for further analysis as very few differences between EBV blasts at day 7 and the LCL were identified.

To further confirm the miRNA array data we performed QPCR for individual miRNA expression in 5 biological replicates of peripheral blood B cells either infected with EBV or stimulated with CD40L and IL4. It was not ideal to use the same method of measurement for the validation experiments as was used for the array but miRNA QPCR was the only technique at the time which was sensitive enough to measure a variety of miRNAs with such differing expression levels (as indicated by the ΔCt analysis). Furthermore, since the original arrays were performed in duplicate, the analysis of 3 additional biological replicates served to confirm the biological reproducibility of the original data set. Of the 10 differentially regulated miRNAs identified, 4 (miR148a, miR-95, miR-28-5p and miR-31) showed a significant difference in expression between EBV and CD40L blasts in 5 biological replicates. These miRNAs were the most interesting candidates for further study. There were a further 3 miRNAs which showed a significant difference in expression between the LCL and the CD40L blasts (miR-199a-3p, miR-193a-3p and miR-200b), which were also included for further investigation as these miRNAs may also be involved in transformation, even though the differences seen by 7 days p.i were not large enough to be significant. Ideally we would have included a CD40L blast at 6 weeks post-stimulation for comparison with the LCL but CD40L blasts generated with soluble CD40L stop proliferating after approximately 4-6 weeks and would therefore no longer serve as a good control for a proliferating and activated LCL.

The ΔCt analysis was very informative and showed why miR-155, miR-21 and miR-146a were some of the first miRNAs identified in an LCL as these miRNAs are very abundantly expressed in LCLs. This analysis also revealed that miR-148a was up-regulated from the 64th

most abundantly expressed miRNA in B cells to the 10th most abundantly expressed miRNA in an LCL, strongly suggesting a functional role for this miRNA in an LCL. The other 6 differentially regulated miRNAs were all down-regulated following EBV infection and the ΔCt analysis showed that none of these miRNAs were in the top 20 abundantly expressed miRNAs in a resting B cell. Current knowledge of miRNAs doesn't allow us to say what ΔCt value corresponds to a biologically relevant level of miRNA expression, and it could be that all the miRNAs detected in the array were at biologically functional levels. However, for our functional studies it was logical to pick the most abundantly expressed miRNA for analysis as the changes in gene expression caused by this miRNA were most likely to be detectable.

(b) miRNA expression in BL and HL

Three of the 7 differentially miRNAs showed a clear correlation with a latency III pattern of EBV latent gene expression in Mutu-BL cell lines (miR-28-5p, miR-95 and miR-199a-3p). Consistent with our findings, miR-28-5p was identified in an array by Cameron et al as down-regulated in latency III Mutu-BL cell lines [652]. However, Cameron et al also showed that miR-34a was up-regulated in latency III Mutu-BL, which is in contrast to our data (figure 4.7). miRNAs -95 and -199a-3p also showed a down-regulation in latency I Mutu-BL clones, suggesting that these miRNAs may be down-regulated as a result of EBNA1, the EBERS or possibly BART miRNAs.

The remaining miRNAs, including miR-148a, did not show a correlation with EBV latent gene expression and this may be due to the relatively high expression of these miRNAs in the Mutu-BL negative clone. For example, miR-148a expression in Mutu-BL negative was only 8 fold less than that of an LCL, already much higher than the expression seen in resting B cells (summarised figure 4.9). EBV latent gene expression may not have a substantial effect on miR-148a expression in a cell background where its expression is already elevated. Therefore,

a lack of correlation with EBV latent gene expression in Mutu-BL cannot exclude the possibility that these miRNAs are regulated by EBV latent gene expression during the transformation of resting B cells. Furthermore, the expression of c-Myc has been shown to have a significant effect on miRNA expression [542], thus the miRNA expression of BL cell lines would be expected to differ significantly from resting B cells. Therefore, EBV latent gene expression in a cell background where c-Myc expression is high would be unlikely to always cause the same changes in miRNA expression as those observed following EBV infection of resting B cells.

The miRNA expression profiling of RLNs and the lymph nodes from patients with cHL by Navarro et al [653] provided a unique opportunity to identify miRNAs involved in the transformation of resting B cells by EBV which are also associated with cHL. A comparison between RLNs and cHL is complimentary to the comparison we had used between CD40L blasts and EBV blasts and proved a useful data set for comparison. The comparison revealed that miR-95 and miR-28-5p, which we had already shown to be down-regulated in latency III Mutu-BL, were also down-regulated in cHL compared with RLNs. This is an interesting result and is supporting the hypothesis that these miRNAs are involved in B cell transformation, although no targets for these miRNAs have been reported therefore it is not possible to speculate what the role of these miRNAs may be.

miR-34a was one of two miRNAs up-regulated in cHL compared with RLNs and this miRNA was not consistently expressed in our biological duplicate Taqman array samples. To clarify the expression of miR-34a in EBV infected and CD40L blasts, the expression of miR-34a was measured in 5 biological replicate samples. The QPCR data clearly showed that miR-34a was in fact up-regulated by both EBV and CD40L and was therefore eliminated from further investigation. We measured the expression of miR-34a in a panel of HL cell lines to

determine whether these cell lines were a good model for miRNA expression in HL. miR-34a expression was very high, with the exception of L1236. In general, therefore, the HL cell lines appeared to be in agreement with Navarro et al's data on biopsy tissues, so we continued with our miRNA expression analysis. The low expression of miR-34a expression in the BL cell line may be due to high cMyc expression, as miR-34a was reported in a microarray to be down-regulated by cMyc [542].

miR-95 showed consistently lower expression in the HL cell lines relative to resting B cells which agreed with the data produced by Navarro et al, however, miR-28-5p expression was variable, with two of the six HL cell lines showing higher miR-28-5p expression than resting B cells. The HL cell line data cannot be expected to exactly correlate with data collected from the lymph nodes from patients, consequently, the miR-28-5p result could be a discrepancy between tissue samples and cell lines, or it could be that miR-28-5p is not consistently down-regulated in cHL.

miR-199a-3p and miR-193a-3p were not included in the published QPCR array of RLNs and the lymph nodes from patients with cHL, therefore, we included them in our HL cell line analysis. These two miRNAs both showed decreased expression in 4/6 of the HL cell lines and increased expression in the two HL cell lines, KMH2 and KMH2-EBV. This result is unusual and implies that the KMH2 cell line has a different miRNA expression profile from the other HL cell lines. This data suggests that miR-199a-3p and miR-193a-3p may also be down-regulated in HL, however, this would need to be confirmed using in-situ-hybridisation for these miRNAs in HL biopsy material. Interestingly, miR-199a-3p has recently been identified an anti-viral associated miRNA in a mouse model system examining the effects of herpesvirus infection on host cellular miRNAs. The down-regulation of miR-199a-3p was also confirmed in human cells post-infection with CMV and miR-199a-3p was shown to

affect ERK/MAPK and PI3K/AKT signalling pathways [686]. This indicates that the modulation of this miRNA by EBV is likely to also be a method employed by EBV to evade the host anti-viral response. The down-regulation of miR-199a-3p in EBV positive tumour cell lines may therefore reflect a down-modulation by EBV, however, the low expression of this miRNA in EBV negative tumour cell lines, such as the HL cell lines, is difficult to explain. It may be that these tumour cells are currently, or have previously, been infected with a virus or this miRNA has a tumour-suppressor function in addition to an anti-viral function.

(c) Characterising miR-148a expression

The only miRNA to be up-regulated by EBV and not CD40L was miR-148a and its expression was up-regulated to the 10th most abundantly expressed miRNA in an LCL. There was very little information regarding the expression or function of miR-148a in the literature, particularly in relation to B cells or EBV. For these reasons we wanted to further characterise the expression of this miRNA as a prelude to functional analysis. miR-148a was not up-regulated in B cells until approximately 4 days p.i, suggesting that it may be up-regulated by a latent gene which is not expressed immediately, such as LMP1 or LMP2. Alternatively, pri-miR-148a transcription is up-regulated early in transformation and the processing of pri-miR-148a is delayed until 4 days p.i through an unknown mechanism.

We showed that miR-148a was highly expressed in GC B cells (centrocytes and centroblasts) suggesting that miR-148a may be involved in B cell differentiation. However, miR-148a expression profiling of cell lines revealed that miR-148a expression in BL and HL cell lines was variable; therefore miR-148a expression is not high in all cell lines with a GC B cell phenotype. miR-148a expression in a panel of four NK cell lines and a primary NK sample was very low, regardless of EBV status, suggesting that miR-148a expression is not up-regulated in EBV associated NK lymphomas.

Using RNA isolated from paraffin embedded tissue we were able to show miR-148a expression in two PTLD, two HL and one T-NHL tissue samples. miR-148a expression ranged from 10-30% of the expression seen in an LCL, with the two PTLD samples having the highest miR-148a expression. Considering PTLD so closely resembles the phenotype of an LCL, the expression of miR-148a would be predicted to be high in these tumours. However, we did not have access to a control tissue sample, such as tonsil section, so it is difficult to interpret this data. Additionally, the PTLD and HL tumour samples will contain a large percentage of reactive lymphocyte infiltrate, therefore the tumour cells themselves will only comprise a small fraction of the RNA extracted from these tumour samples. Thus, a miR-148a expression of 10-30% of that seen in an LCL may represent a significant expression of miR-148a in the tumour cells or a moderate expression of miR-148a in the reactive infiltrate. It is worth noting here that we observed a decrease in miR-148a expression in one late passage LCL (>100 passages) to approximately two fold greater expression than the resting B cells. This result needs to be repeated but it may suggest that miR-148a expression may be down-regulated as LCLs become an immortalised cell line with an increased tumourogenic phenotype; PTLD tumour cells may therefore not necessarily be expected to have high expression of miR-148a. Alternatively, expression of miR-148a is important for the initiation of transformation but is not essential for its maintenance as other epigenetic and genetic changes take ove the regulation of Bim in these cells [136, 675].

The HL tissue samples had higher miR-148a expression than the HL cell lines, which was not expected and may reflect the flaw in the experiment (no correct negative control) or it may indicate that miR-148a is highly expressed in the reactive infiltrate of HL biopsy material. In-situ-hybridisation for miR-148a expression on HL tissue samples would establish the expression of miR-148a in HL tissue. Collectively, the further characterisation of miR-148a

has demonstrated that miR-148a may be involved in B cell differentiation, however, miR-148a expression profiling does not suggest a role for the up-regulation of this miRNA in lymphomagenesis, except possibly in PLTD.

(d) miR-148a primary target prediction

To identify gene targets of miR-148a we used a combination of three miRNA target prediction programs: miRanda, Target Scan and microT. We have shown that the overlap of predicted targets genes is small, with only 183 genes predicted to be miR-148a targets by all three programs. This highlights the limitations of the miRNA target prediction programs as they produce a large number of false positives and negatives.

To identify a small subset of the most probable miR-148a target genes we used the scoring system of each miRNA target prediction program to generate a list of miR-148a targets within the top 10% or 20% score values in each program. 12 genes were identified as miR-148a targets with a top 20% scored value in all three programs and these genes included three RNA associated proteins: AGO1, AGO2 and FMR1; and two ubiquitin specific proteins, USP6 and USP32. It is reasonable to speculate that miR-148a may therefore be involved in the control of post-transcriptional gene expression, such as RNAi and possibly mRNA stability.

The repression of a target gene by a miRNA is often a process which involves a number of different miRNAs as multiple miRNAs have been shown to have an additive effect in translational repression [499]. To investigate which miR-148a gene targets were also predicted to be gene targets of the other 19 miRNAs identified in the Taqman miRNA array as up-regulated by EBV infection, we compared the predicted miRNA targets of all 19 miRNAs. To simplify the analysis and because not all miRNAs had predicted gene targets in all three target prediction programs, the analysis was performed using the gene lists form just

Target Scan. This analysis revealed that no genes were predicted to be targeted by >13/19 of the up-regulated miRNAs. Bim was again identified and was predicted to be targeted by 9/19 of the miRNAs up-regulated in B cells following EBV infection. This multiple miRNA target prediction is flawed in the respect that we compared 19 miRNAs whose expression had not been validated as up-regulated by EBV. However, by comparing every combination of 19/19 miRNAs, down to 2/19 miRNAs, no combination of miRNA comparisons were missed or discounted. There will be a high number of false positives with this type of data analysis, but as with the other forms of analysis, when compared with multiple sources of information this data can still be informative.

Despite Bim being a candidate miR-148a target we could not show any change in Bim expression following transfection of miR-148a inhibitors or mimics into an LCL or DG75 respectively. It is not surprising that miR-148a inhibition in an LCL did not result in the re-expression of Bim protein as Bim is reported to be transcriptionally and epigenetically down-regulated in these cells, therefore, translational repression by one miRNA will be unlikely to have a dominant effect [687]. However, there was no detectable increase in DNMT1 protein expression following miR-148a inhibition either. It is possible that miR-148a is not a significant factor determining DNMT1 expression in LCLs, but it also raises the possibility that the miR-148a inhibitors were not sufficient to repress miR-148a function. The luciferase reporter which was used to validate the efficacy of the miR-148a inhibitors in an LCL was an artificial system which contained a miR-148a binding site with 100% complimentarity to miR-148a, rather than the typical imperfect base pairing seen in miRNA:mRNA interactions. Thus, the miR-148a reporter may have been more sensitive to miR-148a inhibition than authentic miR-148a target genes. Alternative methods of miR-148a inhibtioin may therefore

be required for future investigation into miR-148a function, such as miRNA sponges, as discussed in section 4.8.

The over-expression of miR-148a in DG75-BL using miR-148a mimics served to validate Bim as a miR-148a target gene. The lack of Bim repression following miR-148a transfection could be due the high miR-148a expression in DG75-BL, with similar miR-148a expression levels to those seen in centrocytes (figure 4.9). Further increasing miR-148a expression may not have any effect on an already saturated miR-148a binding site in the 3'UTR of Bim. This is also supported by the lack of DNMT1 protein repression observed in DG75-BL after miR-148a transfection. Alternatively, Bim may not be a genuine miR-148a target gene as the miR-148a binding sites predicted in the 3'UTR of Bim have not been validated experimentally using luciferase reporters.

The multiple miRNA target prediction analysis provided an alternative explanation for the lack of Bim repression following miR-148a transfection as many miRNAs were also predicted to target Bim; thus, Bim regulation by a single miRNA may only be significant in the context of the total miRNA expression (cell environment). The multiple miRNA target prediction analysis also demonstrated the difficulty in identifying biologically relevant miRNA targets. Every bioinformatics analysis performed identified Bim as a candidate miR-148a target gene, however, the regulation of Bim by miR-148a was unlikely to be a significant factor in EBV-mediated transformation for the reasons previously discussed. miRNA inhibition and over expression studies coupled with proteomic analysis of gene expression in a relevant cell type is the ideal approach to identifying relevant miRNA gene targets. Additionally, established cell lines may not be the correct model for determining the post-transcriptional regulation of Bim, as these lines may already have established mechanisms for maintaining the expression of Bim. It may be that miR-148a regulation of

Bim is only relevant during the first few weeks of transformation, or during B cell differentiation, and to test this miR-148a over-expression and inhibition in primary B cells would be necessary.

In summary, the results in this chapter suggest that EBV does modulate the host cellular miRNA expression during B cell transformation and a subset of these miRNAs also show down-regulation in EBV associated lymphomas, and may therefore, be significant to transformation and EBV-mediated lymphomagenesis. miR-148a was identified as the only miRNA to be up-regulated by EBV and not CD40L stimulation of B cells and this miRNA may also be involved in B cell differentiation due to its high expression in GC B cells. The function of miR-148a remains to be elucidated; however, miR-148a target prediction analysis suggests a potential role for miR-148a in the modulation of RNAi.

5. Results Part III

Cellular gene expression profiling of EBV and CD40L blasts

5.1 Introduction

In the previous chapter, the miRNA expression profiles of EBV blasts and CD40L blasts were compared. This comparison allowed the identification of cellular miRNAs whose expression changes were associated with EBV-mediated B cell transformation rather than transient activation and proliferation of a resting B cell; however, the role of these cellular miRNAs during B cell transformation is still undetermined.

Cellular gene expression profiling of EBV blasts and CD40L blasts would enable the identification of genes whose expression changes are specifically associated with transformation, which could be used for two purposes: firstly, for comparison with the miRNA expression data from the previous chapter, to identify miRNA target genes regulated by EBV. This would enable the contribution of miRNAs to the cellular gene expression profile in transformed and cytokine stimulated B cells to be estimated; secondly, to produce a novel list of genes whose expression changes may be required for EBV-mediated B cell transformation. Investigation into the function of these cellular genes during B cell transformation will provide a greater understanding of the mechanism of EBV-mediated B cell transformation.

5.2 Cellular gene expression profiling

To identify cellular genes which may be involved in the transformation of resting B cells by EBV, an Affymetrix exon array was performed as described in section 2.11. The miRNA

array data showed few differences between EBV blasts and LCLs, therefore we excluded the LCL from the cellular gene expression profiling. The array was performed in biological triplicate on resting B cells, EBV and CD40L blasts 7 days p.i. One of the three biological replicates was the same sample set used in the Taqman miRNA array. EBV infection of the EBV blasts was confirmed by QPCR for the EBV Cp promoter (figure 5.1).

The microarray data was analysed to produce two different data sets (i and ii) which could be used for separated purposes: (i) gene expression changes relative to resting B cells. This analysis was comparable to the miRNA array analysis which provided information on the gene expression in the B cells 7 days following EBV infection or CD40L stimulation; thus, for miRNA array comparisons, these gene lists were predominantly used. (ii) A direct comparison between EBV and CD40L blasts. This analysis was used to identify a small subset of genes which showed a statistically significant difference in expression between the two different lymphoblasts. These genes therefore represented the genes with the largest expression difference between the two blasts, making them the best candidates for functional analysis.

5.2.1 SAM analysis of CD40L blasts and EBV blasts relative to resting B cells

Significance analysis of microarrays (SAM) [613] was performed on the microarray data by bioinformatician Wenbin Wei, as described in section 2.11.2. Briefly, genes were assigned a score based on the change in gene expression relative to the standard deviation of repeated measurements. This allowed an estimate to be made of the percentage of genes identified by chance; termed the false discovery rate (FDR). Stringency can be set according to preference and for this analysis we chose highly stringent conditions (minimum fold change of 1.5 and a FDR of ≤ 0.05), to reduce the identification of false positive genes.

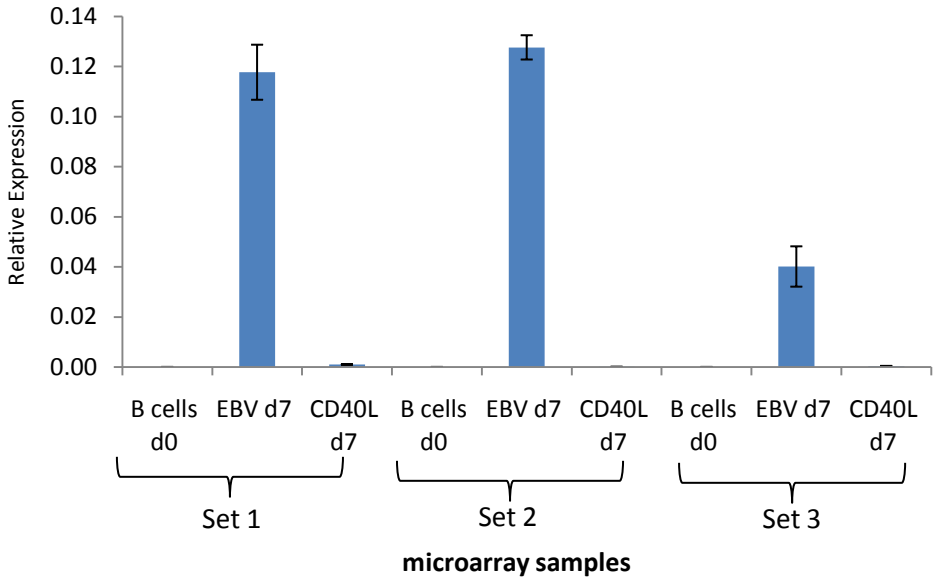


Figure 5.1: EBV promoter usage in the microarray samples. Cp expression by QPCR in three biological replicates of resting B cells (B cells d0) either infected with EBV or stimulated with CD40L and IL4 and harvested after 7 days. Data is expressed relative to GAPDH (assigned a value of 1) and error bars indicate the standard deviation of the QPCR assay.

SAM analysis comparing either EBV blasts or CD40L blasts with resting B cells revealed a large number of genes which showed a significant (≥1.5 fold, ≤ 0.05 FDR) expression change following EBV infection or CD40L stimulation of resting B cells. Relative to resting B cells, 4123 and 2545 genes were up-regulated by EBV infection or CD40L stimulation respectively, and 887 and 330 genes were down-regulated by EBV infection or CD40L stimulation respectively. The results of the SAM analysis are summarised in table 19. The degree of overlap between the gene expression changes in EBV and CD40L blasts are represented by Venn diagrams in figure 5.2. The majority of the gene expression changes in the CD40L blasts also occurred in the EBV blasts (83%), illustrating the high degree of similarity in the gene expression profiles of these two blasts.

5.2.2 SAM analysis of CD40L and EBV blasts

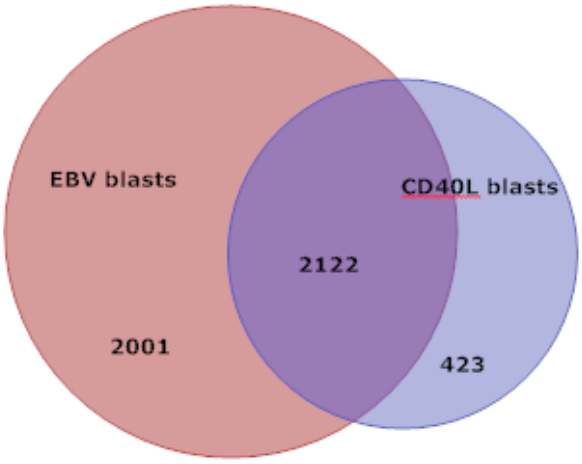
SAM analysis directly comparing EBV day 7 blasts with CD40L day 7 blasts produced a list of 61 genes whose expression was significantly different between EBV blasts and CD40L blasts (≥ 1.5 fold change, ≤ 0.05 FDR). The data is expressed as a heat map where red indicates increased relative expression and blue indicates decreased relative expression (figure 5.3). The relative expression of the resting B cells was also included in the heat map for comparison. The 61 genes differentially expressed between EBV and CD40L are also listed, along with fold change, in table 20.

5.3 Comparative analysis of the miRNA array and cellular gene microarray

The miRNA and gene expression profiles of CD40L and EBV blasts have been determined using the two arrays systems. The question still remains: are any of the genes differentially expressed between EBV and CD40L blasts regulated by the differentially expressed miRNAs in these two types of lymphoblasts? Answering this question would enable us to determine the

A

Up-regulated Gene Sets



B

Down-regulated Gene Sets

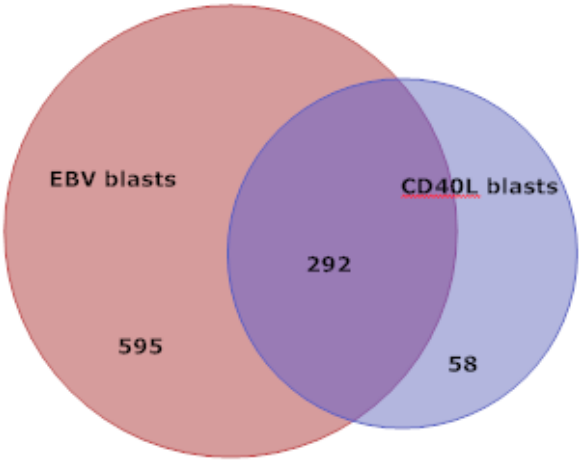


Figure 5.2: Venn diagrams representing genes **A;** up-regulated and **B;** down-regulated by EBV and CD40L day 7 blasts relative to resting B cells in a microarray.

	Relative to resting B cells	
Data Set	Number of genes up regulated	Number of genes down-regulated
EBV d7 blasts	4123	887
CD40L blasts	2545	350
Common Genes*	2122	292

Table 19: Summarising the gene expression changes in the CD40L vs EBV array. Genes up or down-regulated by EBV and CD40L blasts relative to resting B cells (>1.5 fold, FDR 0.05).

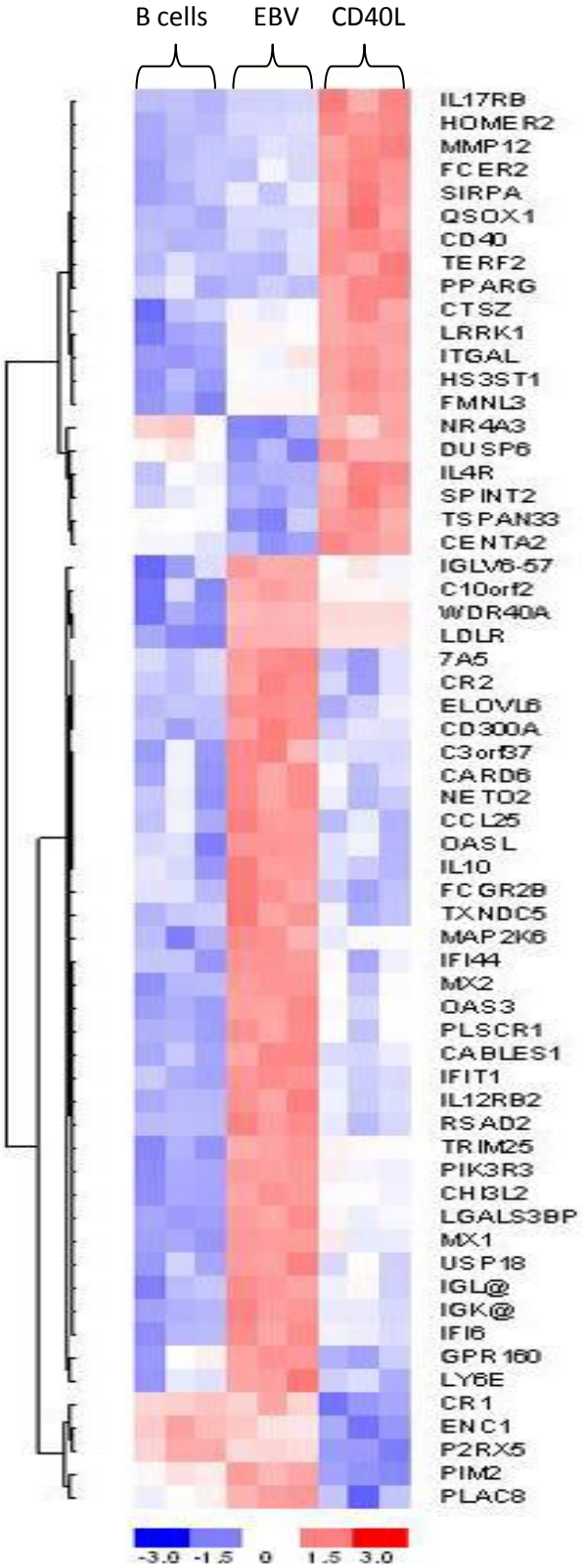


Figure 5.3: Heat map generated by unsupervised SAM analysis comparing EBV day 7 blasts and CD40L day 7 blasts. Shown are the 61 genes which had >1.5 fold and <0.05 FDR differences in gene expression between EBV and CD40L day 7 blasts. Resting B cells are included for visual comparison but were not included in the unsupervised SAM analysis. The colour of each block represents the expression of a gene relative to the average expression of that gene across all samples, with red indicating high relative expression and blue indicating low relative expression.

Gene Symbol	Fold Change CD40 blasts/EBV blasts	Probability of false positivity
NR4A3	12.63	0.00
IL17RB	9.76	2.90
QSOX1	8.69	4.08
DUSP6	8.20	0.00
MMP12	7.85	0.00
FCER2	7.19	0.00
IL4R	6.87	0.00
TSPAN33	5.99	2.90
HOMER2	5.98	0.00
SPINT2	4.89	0.00
SIRPA	4.29	4.37
HS3ST1	3.83	0.00
ITGAL	3.13	4.37
CENTA2	2.90	0.00
CTSZ	2.50	4.37
CD40	2.42	0.00
TERF2	2.09	4.37
PPARG	1.98	4.08
FMNL3	1.93	2.90
LRRK1	1.69	2.90
C10orf2	-1.51	4.08
WDR40A	-1.69	2.90
LDLR	-1.93	0.00
TRIM25	-2.11	0.00
OASL	-2.13	4.08
PIK3R3	-2.24	0.00
IGL@	-2.40	4.37
IGK@	-2.46	0.00
C3orf37	-2.56	4.37
CARD6	-2.57	4.08
FCGR2B	-2.61	0.00
LGALS3BP	-2.77	0.00
ENC1	-2.81	4.08
ELOVL6	-2.92	0.00
GPR160	-3.09	0.00
LY6E	-3.23	2.90
USP18	-3.43	2.90
IGLV6-57	-3.54	4.08
PIM2	-3.54	0.00
P2RX5	-3.60	0.00
TXNDC5	-3.90	4.08
NETO2	-3.98	0.00

Gene Symbol	Fold Change CD40 blasts/EBV blasts	Probability of false positivity
OAS3	-4.06	4.08
IL10	-4.07	0.00
MAP2K6	-4.09	4.08
MX2	-4.32	2.90
PLSCR1	-4.45	4.08
CCL25	-4.50	0.00
MX1	-4.74	0.00
CR1	-5.03	2.90
CD300A	-5.71	0.00
IL12RB2	-6.26	0.00
CABLES1	-6.34	0.00
IFI6	-6.84	0.00
RSAD2	-7.16	0.00
7A5	-7.17	0.00
PLAC8	-8.21	4.08
CHI3L2	-8.88	0.00
IFI44	-9.52	4.08
CR2	-13.94	0.00
IFIT1	-19.37	0.00

Table 20: Table of genes identified by unpaired SAM analysis as differentially regulated between EBV and CD40L blasts with a fold change >1.5 and a FDR of <0.05

extent to which cellular miRNAs are contributing to the cellular gene expression changes unique to EBV transformed B cells. However, there are caveats to this type of analysis. Firstly, miRNAs predominantly repress gene expression by translational repression, therefore most primary miRNA target genes will not be identified on a microarray. Secondly, miRNA induced mRNA degradation often produces small changes in transcript expression, which may be missed by the stringent conditions of our SAM analysis. Thirdly, miRNA target prediction programs will be required for the data comparisons which, as discussed in the previous chapter, produce a large number of false positives and negatives, increasing the complexity of the analysis. Nevertheless, these comparisons have been successful in previous reports [160, 524, 534, 640, 688] and may allow the identification of novel miRNA targets relevant to the transformation of resting B cells by EBV.

We began with a comparison of the microarray data and the miR-148a target prediction analysis in chapter 4.6.1, to identify miR-148a target genes down-regulated during B cell transformation. We then progressed to a comparison between the global miRNA array data and the microarray data, with an emphasis on differentially regulated genes and miRNAs.

5.3.1 Comparing miR-148a target predictions with the microarray

miR-148a was identified as significantly up-regulated by EBV infection but not by CD40L stimulation of resting B cells. Previously, (section 4.6.1) 183 target genes for miR-148a were predicted by three target prediction programs. A comparison between the cellular gene expression profile of EBV infected and CD40L stimulated B cells and the 183 miR-148a predicted target genes may identify primary miR-148a gene targets regulated by miR-148a during B cell transformation. Gene expression changes relative to resting B cells formed the majority of the comparisons as this form of microarray analysis was the most comparable to the miRNA array analysis.

Primary miR-148a targets whose expression was regulated by miR-148a during EBV-mediated transformation of resting B cells would be expected to be down-regulated following EBV infection but not after CD40L stimulation. The miR-148a target prediction and cellular gene array data comparison is summarised in table 21. There were 16/750 (2.1%) genes down-regulated by EBV compared to resting B cells in the cellular gene expression array which were also predicted to be miR-148a targets by the three miRNA target prediction programs. Although, 4/16 of these genes were also down-regulated by CD40L, therefore, regulation of these genes by miR-148a was unlikely to be responsible for the expression changes observed in these four genes (table 22, highlighted in grey).

BCL2L11 (Bim) and MAFB were 2/12 genes down-regulated by only EBV infection of resting B cells (table 22) and they were also predicted to be in the top 10% and 20% score values for miR-148a predicted target genes respectively. However, we could not show any regulation of Bim protein expression in an established LCL in the previous chapter, suggesting that this type of data analysis needs to be treated with caution.

The significance of 2.1% of the genes down-regulated by EBV being miR-148a predicted targets is questionable considering 6/161 (3.7%) genes down-regulated by CD40L were also predicted to be miR-148a targets by the three miRNA target prediction programs (SNF1LK, ARRDC3, PRNP, MYST2, SNN and HMGB3). Additionally, 31/4018 (0.8%) genes up-regulated by EBV compared with resting B cells were predicted to be miR-148a targets and 8/1767 (0.45%) genes up-regulated by CD40L compared with resting B cells were predicted to be miR-148a targets.

Microarray Analysis	Number of miR-148a targets	miR-148a targets: percentage of total
Down-regulated - EBV vs d0 B cells	16/750	2.1
Down-regulated - CD40L vs d0 B cells	6/161	3.7
Up-regulated - EBV vs d0 B cells	31/4018	0.8
Up-regulated - CD40L vs d0 B cells	8/1767	0.45
Up-regulated - EBV vs CD40L	1/41	2.4
Down-regulated - EBV vs CD40L	0/20	0

Table 21: A data comparison between genes altered by EBV infection or CD40L stimulation of resting B cells and miR-148a predicted target genes. 6 different analyses were performed on the microarray data, comparing EBV or CD40L blasts with either resting B cells or directly with each other. A list of 183 genes predicted to be miR-148a targets by 3 independent target prediction programs (table 13 chapter 4) was compared with the gene lists produced by each microarray analysis. The percentage of predicted miR-148a targets from each microarray analysis is also displayed.

Gene Symbol	Fold Change EBV vs d0 B cells
PRNP	-3.87
BCL2L11	-3.75
ARRDC3	-2.74
MMD	-2.56
BACH2	-2.54
SNN	-2.41
CDC14A	-2.13
MYST2	-2.06
PHF20	-2.03
JARID2	-1.97
DYRK1A	-1.94
BAZ2A	-1.88
SLC2A1	-1.85
SESTD1	-1.82
CAMK2A	-1.72
MAFB	-1.51

Table 22: 16 genes predicted to be miR-148a targets from 3 independent target prediction programs (table 13) which were also identified on a transcriptional array as down-regulated by EBV relative to d0 resting B cells. Genes also down-regulated by CD40L

5.3.1 miRNA array target prediction

To compare the global miRNA and gene expression arrays, the predicted cellular gene targets for each miRNA were obtained; the miRNA predicted gene target list was then directly compared with the microarray data (data analysis and results are summarised in figure 5.4). The miRNA target prediction program with the least stringent target prediction conditions (miRanda, Sanger v5) was used to reduce the number of false negatives. False positives were less of a concern in this analysis as we were only focusing our data analysis on 61 genes, therefore, the number of false positives would be small enough for removal in future validation experiments. For the differentially expressed miRNA and gene array comparison, gene targets for the 4 miRNAs whose expression was differentially expressed between CD40L blasts and EBV day 7 blasts (miR-148a, miR-95, miR-31, miR-28-5p,) were generated using the miRanda target prediction program, and the data was used for comparison with the EBV and CD40L blasts cellular gene expression microarray data.

5.3.3 miRNA and cellular gene expression array comparisons

Initially, we compared the list of genes from the array which were either commonly up or down-regulated by EBV and CD40L, with the miRNAs which were commonly down or up-regulated by EBV and CD40L. The predicted genes for the 19 miRNAs up-regulated by both CD40L and EBV blasts were compared with the 109 genes down-regulated by both CD40L and EBV blasts; all of the 19 up-regulated miRNAs were predicted to target at least one of 31/109 down-regulated genes.

The predicted gene targets of the 7 miRNAs down-regulated by both EBV and CD40L were then compared with the list of 1510 genes up-regulated by both EBV and CD40L; all 7 of the

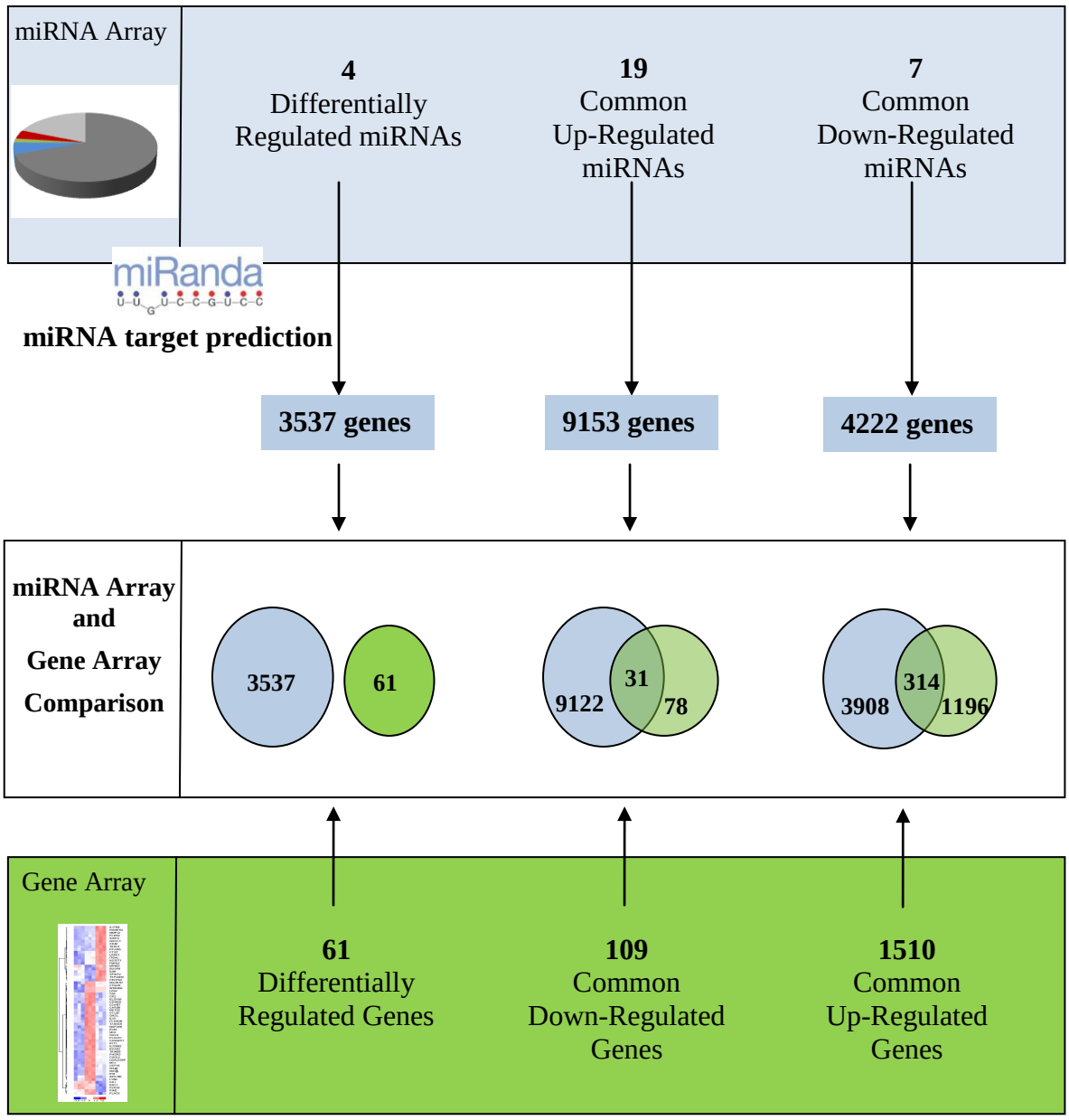


Figure 5.4: Diagram representing the miRNA and gene expression array data comparison. miRNAs of interest had their gene targets predicted using the miRanda target prediction algorithm. These predicted miRNA gene targets were then compared with the cellular gene expression array data, represented in a Venn diagram where blue represents miRNA predicted gene targets and green represents genes from the gene expression array.

miRNAs were predicted to target 314/1510 of the genes up-regulated on the microarray by both EBV and CD40L.

Next we compared the 61 genes differentially expressed between EBV and CD40L blasts with the miRNA array data. The 61 genes differentially expressed between EBV and CD40L blasts were split into two groups: those with relatively higher expression in EBV blasts (41genes), and those with a relative lower expression in EBV blasts, compared with CD40L blasts (20 genes). Predicted gene targets for the only up-regulated miRNA (miR-148a) were compared with the list of differentially regulated genes which showed lower relative expression in the EBV blasts; there were no common genes. There were 3 miRNAs from the miRNA array which showed a significant down-regulation in EBV day 7 blasts compared with CD40L day 7 blasts and these were: miR-95, miR-28-5p and miR-31. The predicted gene targets for these three miRNAs were compared with the list of differentially regulated genes which showed relatively higher expression in the EBV blasts. Again, there were no common genes, indicating that none of the differentially regulated miRNAs regulated the level of transcripts of any of the differentially regulated genes.

The lack of correlation predicted between the differentially regulated genes and miRNAs may be due to the small number of genes and miRNAs involved in the comparison. In contrast, the comparisons between the commonly regulated genes and miRNAs identified a number of predicted miRNA:gene target regulations. This positive result may be due to the large number of miRNAs in the commonly up-regulated comparison (19 miRNAs) and the large number of genes in the commonly down-regulated miRNA comparison (1510), substantially increasing the probability of producing a positive result. Nevertheless, the miRNA and gene expression array comparison identified possible gene targets for EBV regulated miRNAs. However, our research was focused on the differentially expressed genes and miRNAs, therefore we did not

investigate the correlation between the commonly regulated genes and miRNAs any further; our research subsequently focused on the genes which were identified on the microarray as differentially expressed between EBV and CD40L blasts.

5.4 Analysis of EBV and CD40L blast transcription microarray

5.4.1 Kegg pathway analysis

To understand the large number of gene expression changes produced by microarrays, a number of programs have been designed to analyse the gene expression changes in the context of known signalling pathways or diseases. Kegg (Kyoto encyclopaedia of genes and genomes) pathway analysis [689] is one such tool available for this type of analysis. The genes which were significantly up or down-regulated following EBV infection or CD40L stimulation, relative to resting B cells, were entered into the Kegg analysis tool. The genes were then compared with the Homo sapiens reference database and the Kegg pathway analysis tool produced a list of cellular signalling pathways which the genes altered in response to EBV infection or CD40L stimulation could be grouped into.

The TLR7 signalling pathway was predicted to be activated by EBV infection and not CD40L stimulation, with the following genes up-regulated by EBV infection and not CD40L stimulation: IFNα/β receptor, TLR7/8, IRAK4, IRF7, IFNα; these data are summarised in figure 5.5. Closer examination of the array data revealed that TLR7 was also up-regulated by CD40L stimulation in 2/3 of the samples, although to a lesser extent than in EBV blasts. A literature search revealed that EBV manipulates the TLR7 signalling pathway following infection of primary B cells [637], which highlights the value of this type of analysis tool. Table 23 summarises the Kegg pathway analysis and lists the three most significantly altered

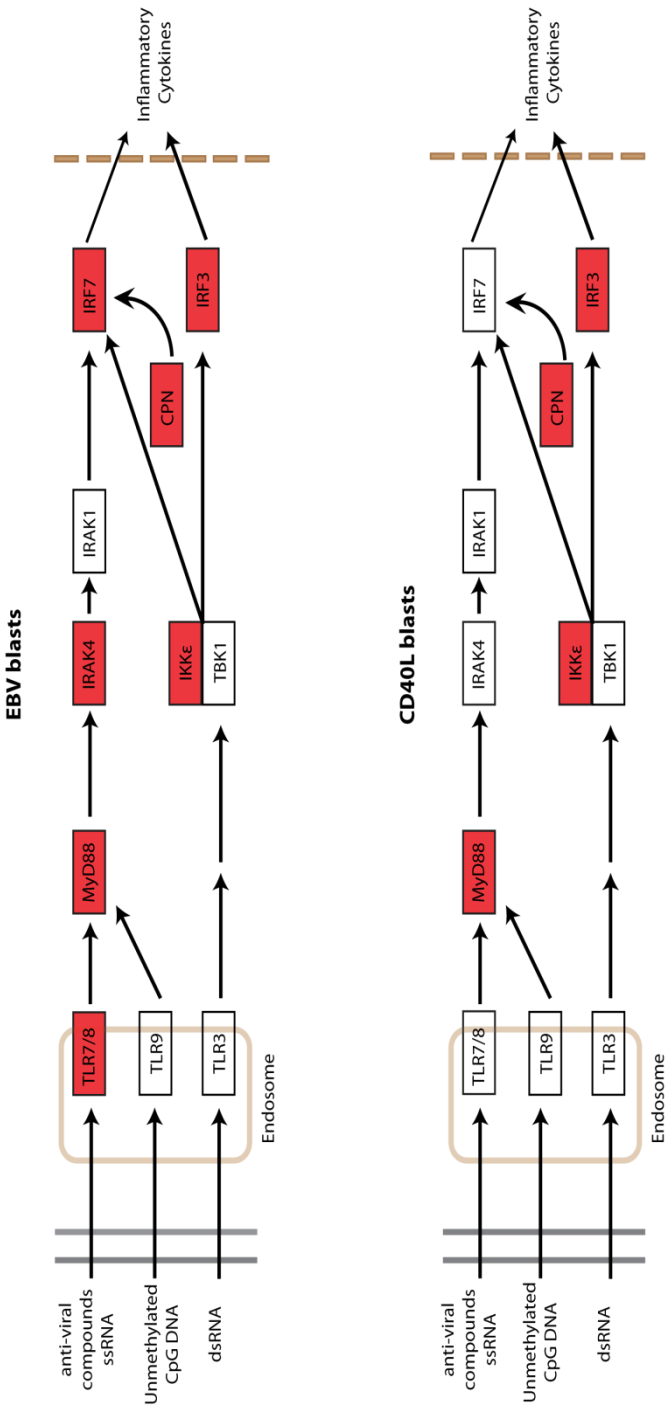


Figure 5.5: Diagram representing TLR7 signalling in EBV blasts and CD40L blasts, adapted from Kegg pathway analysis Genes up-regulated in a microarray in these blasts relative to resting B cells are highlighted in red.

Kegg Pathway	EBV blasts Number of genes Up (↑) or Down (↓)	CD40L blasts Number of genes Up (↑) or Down (↓)	EBV blasts Selected genes Up (↑) or Down (↓)	
TLR7/8 signalling	↑7 ↓0	↑4 ↓0	TLR7/8 IRAK4 IRF7	
Apoptosis	↑31 ↓4	↑16 ↓2	Fas TNFα MyD88 IRAK FLIP Bid Apaf-1 IAP CASP10 CASP3 DFF45 BCL2 BCL-XL Bax ATM	CASP8 NF-κB
MAPK signalling	↑25 ↓15	↑16 ↓4	FAS TNF ASK1, MKK6 p38, MEF2C CASF MEF2C PP2CB	CD14 TRAF6 TAB2 MEKK2B PP2CA RSK2 FGF G12 RafB

Table 23: Summary of the Kegg pathway analysis. Genes up or down-regulated by EBV and CD40L relative to resting B cells were entered into the Kegg pathways analysis program. The top three pathways predicted to be modulated by EBV are included with the number of genes up or down-regulated in each pathway. Genes which were up or down-regulated on the microarray by only EBV blasts (not CD40L blasts) are listed. Red genes were up-regualted by EBV and green genes were down-regulated by EBV.

pathways predicted by the Kegg pathway analysis. The genes whose expression altered in only the EBV blasts are listed for each of the three pathways, as well as the number of genes altered in each pathway.

This analysis has revealed that CD40L and EBV blasts both modulate the expression of genes in the same pathways, perhaps unsurprisingly considering the similarity of the gene expression profiles of these two lymphoblasts. EBV may be predicted to modulate a larger number of target genes in the apoptosis and MAPK signalling pathways which may result in enhanced MAPK signalling and apoptosis resistance of LCLs. However, the functional significance of these gene expression changes requires investigation before conclusions can be drawn.

5.4.2 Tumour suppressor gene analysis

The process of EBV-mediated B cell transformation is distinct from the process of immortalisation required to produce an immortalised LCL. This suggests that additional cellular gene expression changes are required following transformation to produce an immortalised LCL. Immortalised LCLs, unlike early passage LCLs, show a tumourogenic phenotype with the ability to form tumours in nude mice [348], suggesting that the process of immortalisation is partially comparable to lymphomagenesis. CD40L blasts do not obtain all the necessary gene expression changes to produce an immortalised cell line, suggesting that the cellular gene expression changes specific to EBV induced transformation may predispose B cells to the additional gene expression changes required for cellular immortalisation. Therefore, it is reasonable to propose that some of the cellular genes specific to transformation will also be candidate tumours suppressor genes (TSGs) or oncogenes.

An analysis was performed to identify whether any of the genes specifically down-regulated by EBV were candidate TSGs. A TSG database was created within our department by Professor Ciaran Woodman. The TSG database was compiled using a PUBMED literature search for the terms: tumor, tumour and suppressor. All abstracts were inspected but only genes which showed unambiguous evidence of tumour suppressor activity in cell lines or animal models were entered into the database. All gene symbols were then harmonised to those on the NCBI database to allow comparison with affymetrix gene expression data (which uses NCBI databases gene symbols). Over 800 genes were identified in the literature as having expressed tumour suppressor function and these constituted the TSG database.

A broad comparison between the tumour suppressor database and the microarray data was performed which included all 595 genes which were down-regulated by EBV and not by CD40L, relative to resting B cells; 45 TSGs were identified (table 24).

Of the 61 genes differentially expressed between EBV and CD40L blasts, six genes were specifically down-regulated in EBV blasts compared with CD40L blasts: NR4A3, DUSP6, IL4R, SPINT2, TSPAN33 and CENTA2. Three of these genes were identified as candidate TSGs: NR4A3, DUSP6 and SPINT2 (highlighted in yellow, table 24). The function and known tumour suppressor activity of these three genes are listed in table 25.

NR4A1 and NR4A3 were shown to exhibit tumour suppressor function in a mouse knockout model but only in double-knockout mice where both genes were deleted [690]. The tumour suppressor activity of NR4A3 may therefore depend on the low expression of NR4A1; it was therefore important to determine the expression of both of these TSGs.

TSG	Location
EGR1	5q23-q31
SMAD3	18q21.1
BACH2	6q14.3-q16.1
BCL2L11	2q13
DUSP6	12q22-q23
GGNBP2	17q12
NR4A1	12q13
SPINT2	13q22.2-13q22.3
ATF3	1q32.3
BEX2	Xq22
CDKN1A	6p21.31
CREBBP	16p13.3
CYLD	16q12-q13
DDX3X	Xp11.3-p11.23
EEF1A1	6q14.1
FBXL10	12q24.31
HBP1	7q22-q31
NR4A3	9q22
PAQR3	4q21.21
PFDN5	12q12
PMAIP1	18q21.32
RAB37	17q25.1
RANBP2	2q12.3
RARA	17q21
RPS14	5q31-q33
RPS6KA6	Xq21
SIRT1	10q21.3
SKI	1q22-q24
SOCS3	17q25.3
TAGLN	11q23.2
THBS1	6q27
TLE1	15q22

TSG	Location
TLE4	9q21.31
TP53BP2	1q42.1
ZEB1	10p11.2
ARID4A	14q23.1
BCLAF1	6q22-q23
CASP8	2q33-q34
DIRAS1	19p13.3
EP300	22q13.2
FOXP1	3p14.1
GLTSCR2	19q13.3
MAP3K8	10p11.23
PLK3	1p34.1
PTGER4	5p13.1
XRCC6	22q13.2-q13.31

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Table 24: Results of the TSG analysis**.** Genes down-regulated by EBV (FDR 0.05) and not CD40L blasts relative to resting B cells were compared with a list of genes compiled from the literature as having shown TGS function in some cell background. Included in the table is data from previous arrays performed in the department where germinal centre B cells were either infected with EBV or transfected with LMP1. Genes highlighted in yellow are classed as differentially expressed between EBV and CD40L blasts (FDR 0.05%, >1.5 fold) and can been seen on the heat map in figure 5.3

Potential TSG	Alias	Function	Tumour Association
DUSP6	MKP3 PYST1	A protein tyrosine phosphatase which inactivates MAP kinases with a specificity for the ERK family.	A candidate TSG for pancreatic cancer[617, 691, 692]
SPINT2	HAI-2 KOP	Membrane protein which acts as an inhibitor of hepatocyte growth factor activator.	Acts as a TSG through its inhibition of hepatocyte growth factor activator in papillary and clear cell renal carcinoma[693] and paediatric medullablastoma.[694]
NR4A3	Nor-1 MINOR CHN	Is part of the Nur77 (NR4A1) family of nuclear steroid receptors and NR4A3 shows very strong homology with NR4A1[695]. NR4A3 has been linked with NR4A1 and Bim as mediators of negative selection of CD4 and CD8 T cells[696] It may be involved in the response to forskolin or TPA. Binds to the B1A response element. Two isoforms exist, α and β.	Abrogation of NR4A1 and NR4A3 in mice led to the rapid development of AML[690]
NR4A1	Nur77	This orphan nuclear receptor can act as a proapoptotic or antiapoptotic protein. NR4A1 can bind Bcl2 and convert it from a cytoprotective to a cytodestructive protein [697]. NR4A1 been shown to bind EBNA2 in fibroblasts which prevents NR4A1 mediated apoptosis [698].	Abrogation of NR4A1 and NR4A3 in mice led to the rapid development of AML[690]

Table 25: Candidate TSGs with brief known function. Three TSGs down-regulated by EBV infection but not CD40L stimulation of resting B cells are listed with brief known function (DUSP6, SPINT2 and NR4A3). The additional TSG NR4A1 is included because the tumours suppressor function of NR4A3 can depend on loss of NR4A1 expression.

5.5 Microarray validations

To validate the differential expression of genes from the microarray we used custom designed Taqman microfluidic cards. These cards allowed singleplex QPCR to be performed for a large number of genes (45 genes), plus three endogenous controls for normalisation: B2M, GAPDH and PPIA. All Taqman QPCR assays were selected (where possible) to be specific for only the gene isotype identified on the microarray. The three biological triplicate microarray sample sets and the matched LCL from each biological triplicate were used for the validation experiments. The RNA was reverse transcribed using random hexamers and loaded into the microfluidic card for QPCR analysis (outlined in section 2.10.4) The data was analysed using the ΔΔCt method of analysis [609] and the endogenous control, B2M, was used for normalisation as it was the most consistently expressed of the three endogenous controls.

5.5.1 Selecting genes for validation

Two genes were included in the array validations as positive controls since their expression changes following EBV infection were already established: Bim is down-regulated by EBV infection of resting B cells [687, 699] and was included as a positive control for down-regulation of genes post-EBV infection. BLIMP1 is involved in B cell differentiation and is known to be up-regulated following EBV infection of resting B cells [700, 701], and was therefore included as a positive control for up-regulated genes post-EBV infection.

The three differentially expressed candidate TSGs identified in the previous analysis were chosen for validation, plus the additional TSG, NR4A1. The remaining 41 genes chosen for validation were also from the list of 61 differentially regulated genes and were included for a collaborative project.

5.5.2 Microarray QPCR validation

The results of the microarray validation for all 45 genes are represented in a heat map (figure 5.6) using the ΔCt values as the data output. Blue indicates a lower level of relative expression and red indicates a higher level of expression. The QPCR data for the 4 TSGs and the two positive controls are presented in figure 5.7. The data is plotted as 20-ΔCt values to allow individual data points, and therefore the reproducibility of the data, to be visualised. The P values were calculated using a paired analysis of the student T test comparing the EBV and CD40L day 7 blasts. The 3 TSGs differentially expressed on the microarray (NR4A3, DUSP6 and SPINT2) produced a significant ($P= \leq 0.01$) difference in expression between EBV and CD40L blasts. The two additional positive control genes (Bim (BCL2L11) and BLIMP1 (PRDM1)) both showed the expected changes in gene expression, indicating that the microfluidic cards were producing accurate data.

Validation of NR4A3, NR4A1, DUSP6 and SPINT2 expression at the protein level was attempted using western blot but the antibodies for these genes were not sensitive enough to detect endogenous protein level and can therefore only be used for over-expression studies.

5.6 Further analysis of TSG expression and regulation by EBV

To further understand the possible functional significance of the down-regulation of DUSP6, SPINT2 and NR4A3, we sought to characterise the expression of these three TSGs in more detail and investigate their down-regulation by EBV.

5.6.1 TSG expression in B cells following LMP1 and gp42 knockout-EBV infection

The LMP1 knockout virus is a non-transforming virus which causes transient activation and proliferation of resting B cells for approximately 10 days before the cells stop proliferating

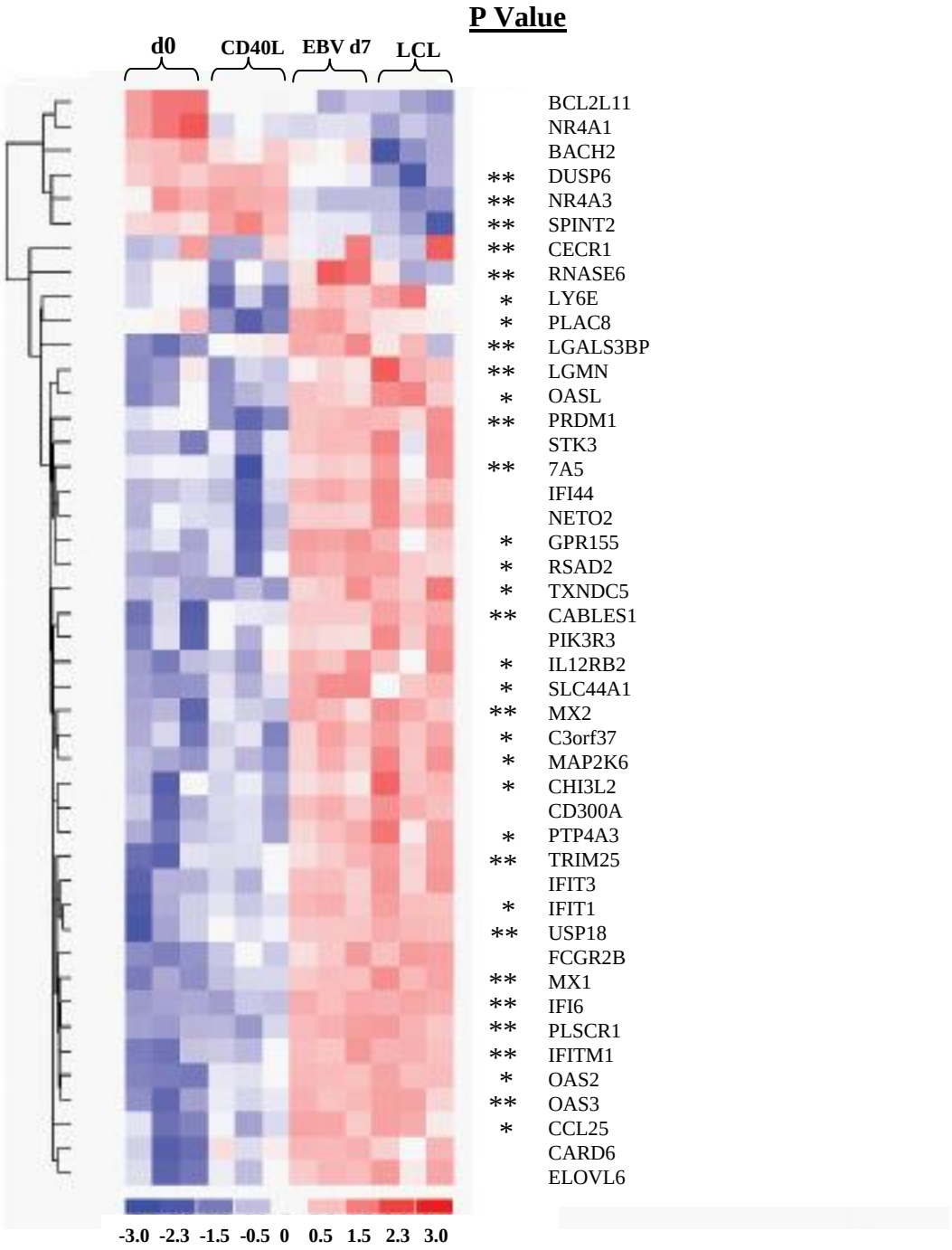


Figure 5.6: Heat map representing the microarray validation by QPCR of 45 genes differentially expressed between EBV blasts and CD40L blasts. Data is expressed as relative ΔCt values where red represents a relative increase in expression and blue represents a relative decrease in expression. P values were calculated using the paired student T test, comparing CD40L d7 blasts and EBV d7 blasts. Genes marked with * had a P value of ≤ 0.05 and genes marked with ** had a P values of ≤ 0.01 to three decimal places.

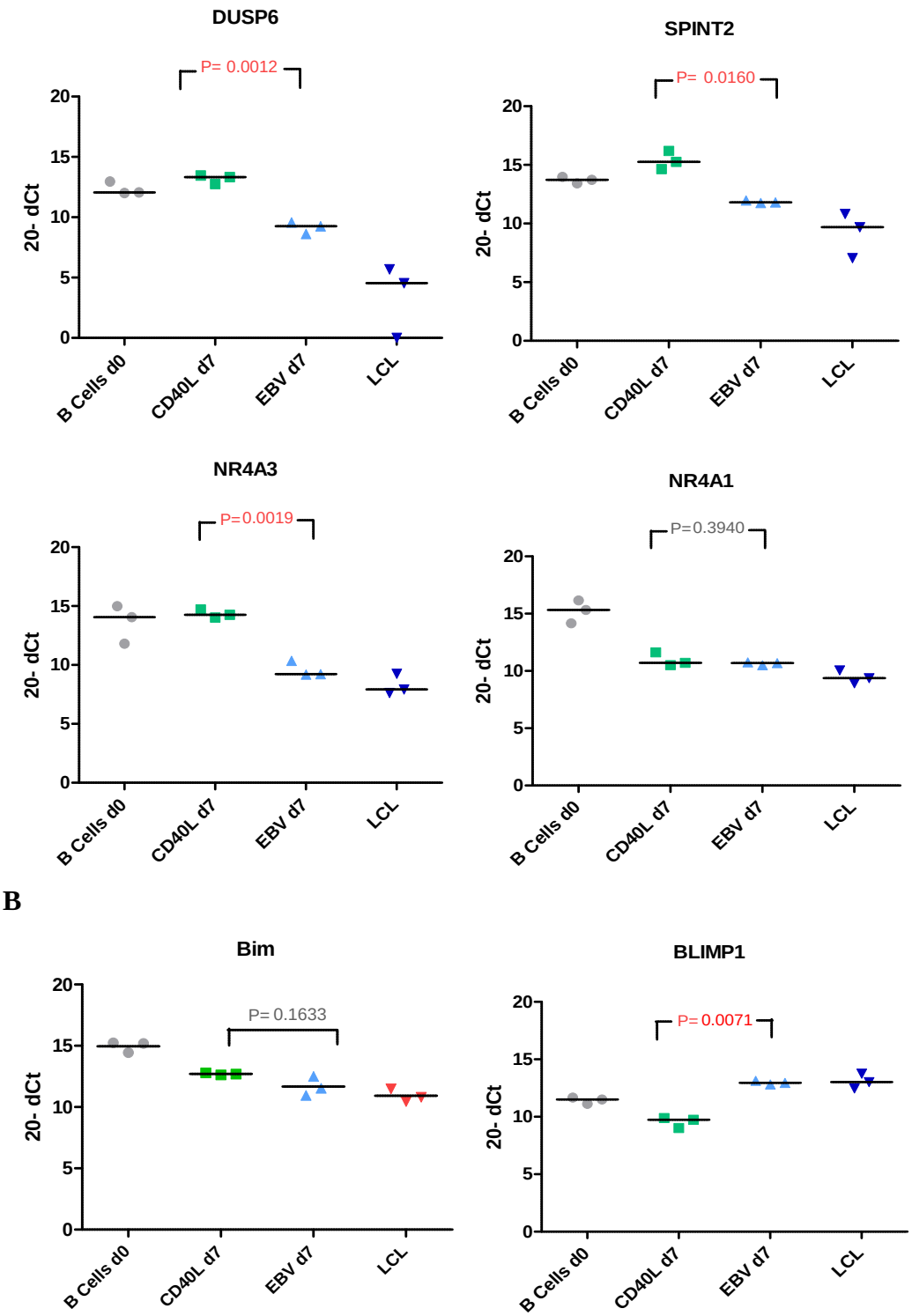


Figure 5.7: Microarray validations of TSGs by QPCR. **A;** 4 candidate TSGs indentified on the microarray as down-regulated by EBV and not CD40L. **B;** QPCR positive controls, Bim and BLIMP1. P values represent the result of a paired student T test comparing CD40L and EBV blasts.

and eventually die. If DUSP6, SPINT2 and NR4A3 are involved in the transformation of resting B cells, then it would be informative to determine whether or not they are also down-regulated by a non-transforming mutant LMP1KO EBV. To control for the effect of virus binding in the absence of EBV internalisation and expression, we used a gp42 knockout virus (described in section 3.3.5).

B cells were positively selected from two donors and pooled prior to infections, and $7x10^6$ B cells were used immediately for RNA extraction to serve as the d0 time point. The wtEBV, LMP1KO and Δgp42 viruses were titred (section 2.3.3) and resting B cells were infected at an m.o.i of 100 for each virus; cells were harvested 7 days pi. The experiment was repeated twice to produce biological triplicate samples sets.

QPCR for EBV promoter usage (Wp and Cp) and LMP1 expression was performed on all samples and demonstrated that there was no EBV promoter usage or LMP1 expression in Δgp42 virus infected cells. LMP1KO infected B cells showed both Cp and Wp transcripts but no LMP1 expression, whereas the wtEBV infected B cells showed expression of both promoter transcripts and LMP1, indicating that all the viruses were functioning as expected (figure 5.8).

The RNA from triplicate biological replicates of: resting B cells (d0), wtEBV infected, LMP1KO infected and Δgp42 infected B cells, was reverse transcribed using random hexamers and loaded into the custom designed Taqman microfluidic cards for QPCR analysis. SPINT2 only showed down-regulation with wtEBV and LMP1KO EBV infections. Conversely, the results show that B cells infected with all viruses showed down-regulation of DUSP6, NR4A3, and NR4A1. Additionally, Bim and BLIMP1 also showed a down-regulation and up-regulation following Δgp42 virus infection respectively. It is not possible to

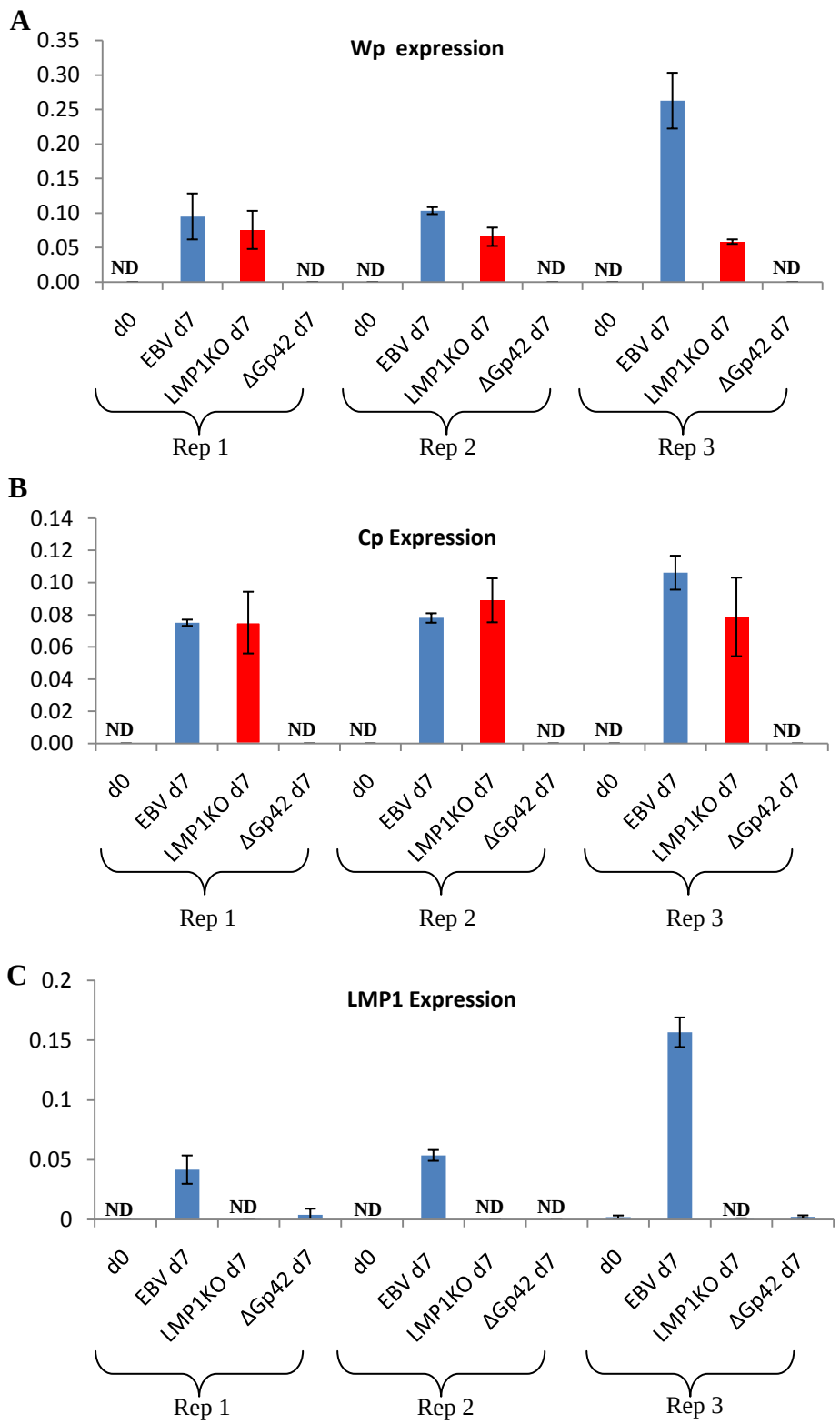


Figure 5.8: EBV promoter and latent gene expression by QPCR in B cells infected with wtEBV, LMP1KO or Δgp42 EBV. **A;** Wp. **B;** Cp, **C;** L MP1 expression in resting B cells (d0) infected with either wt EBV, LMP1KO or a gp42KO virus. ND represents samples whose expression was not-determined. Data is expressed relative to an appropriate control. Error bars represent the standard deviation of the QPCR.

draw conclusions about the non-transforming capacities of the LMP1KO virus when the Δgp42 virus infected cells also showed down-regulation of 3/4 of the TSGs.

The statistical significance of the gene expression changes observed in B cells following infection with Δgp42 virus are indicated on each graph (figure 5.9), with 3/6 of the genes showing statistically significant changes in gene expression in the Δgp42 infected B cells; indicating that the Δgp42 virus can in some instances can produce the same effect on gene expression as wtEBV.

The data presented in figure 5.9 were unexpected, and suggested that several EBV induced changes, which were not induced in CD40L blasts, may be initiated by virus binding alone. We therefore asked whether this phenomenon was restricted to EBV-regulated TSGs, or was more widespread. To ascertain whether the Δgp42 EBV produced the same changes in gene expression as the wtEBV in a wider number of genes, the QPCR data for the remaining 41 genes was analysed. An additional 27 genes showed a change in expression post-infection with Δgp42 virus. All but two (PLAC6 and C3orf37) of the additional 27 gene expression changes produced by Δgp42 virus mirrored those caused by the wtEBV. A heat map representing the ΔCt values produced from this QPCR data is presented in figure 5.10. It is clear from this data that the Δgp42 virus does not produce gene expression changes to the same extent as the wt or LMP1KO EBV, however, the gene expression changes are great enough to prevent the LMP1KO data from being significantly greater, therefore firm conclusions from this data cannot be drawn.

The resting B cells and Δgp42 infected B cells produce the most varied QPCR data, presumably due to the heterogeneous condition of the cells; despite this, 11/45 genes assayed

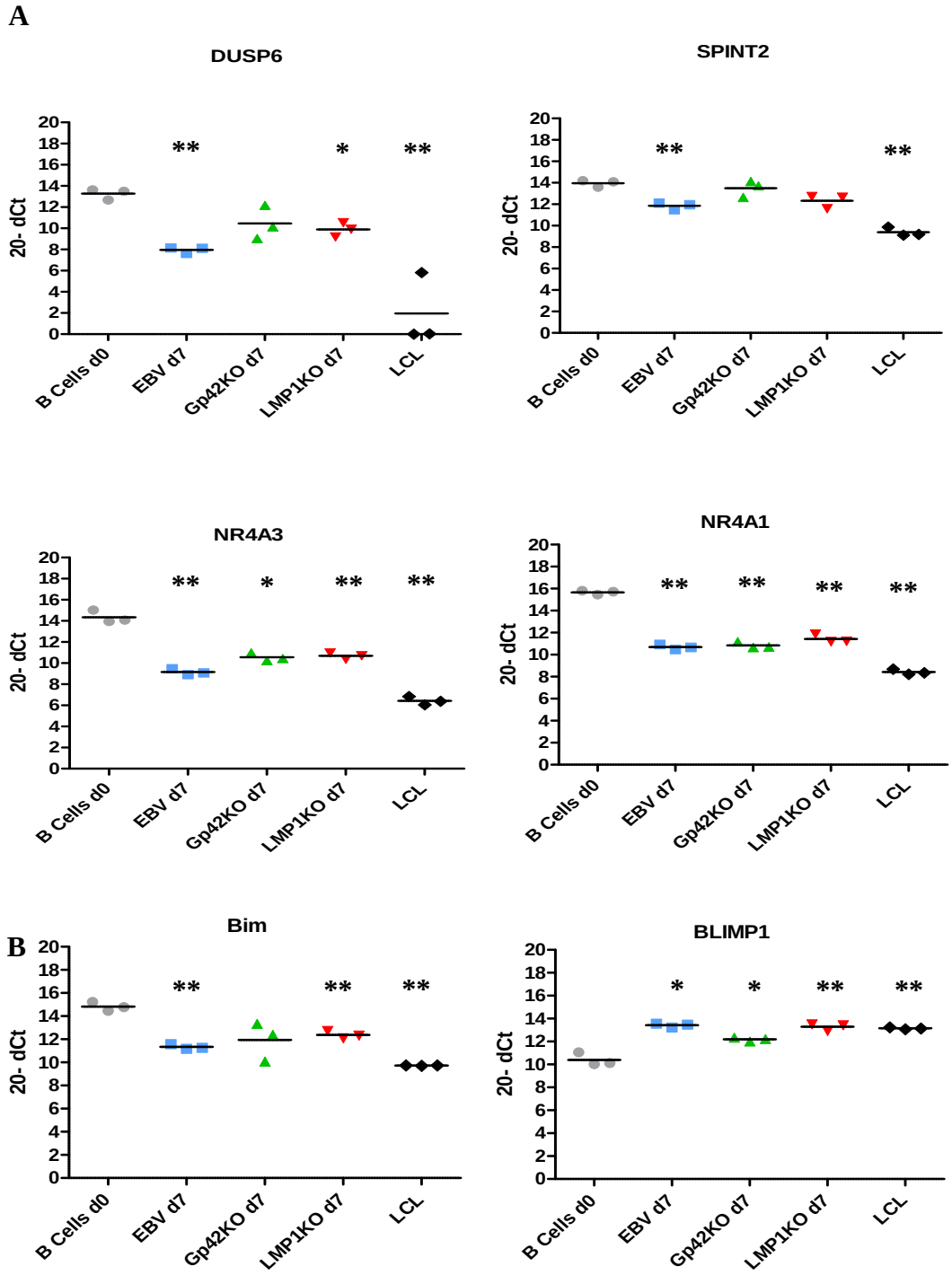


Figure 5.9: TSG expression by QPCR in B cells infected with wtEBV, LMP1KO or Δgp42 EBV. QPCR data was collected using custom designed Taqman microfluidic cards. Gene expression was measured in resting B cells isolated from peripheral blood (B cells d0) and B cells infected with the following viruses: wtEBV at 7 days p.i (EBV d7) and 6 weeks p.i (LCL), gp42 knockout virus (gp42KO d7) harvested 7 days p.i and an LMP1 knockout virus 7 days p.i (LMP1KO d7). **A;** 4 candidate TSGs and **B;** two positive controls. The statistical significance of the difference in gene expression between the virus infecting cells and the resting B cells was calculated using the student T test, paired analysis; P values of ≤ 0.05 or ≤ 0.01 are represented by * and ** respectively.

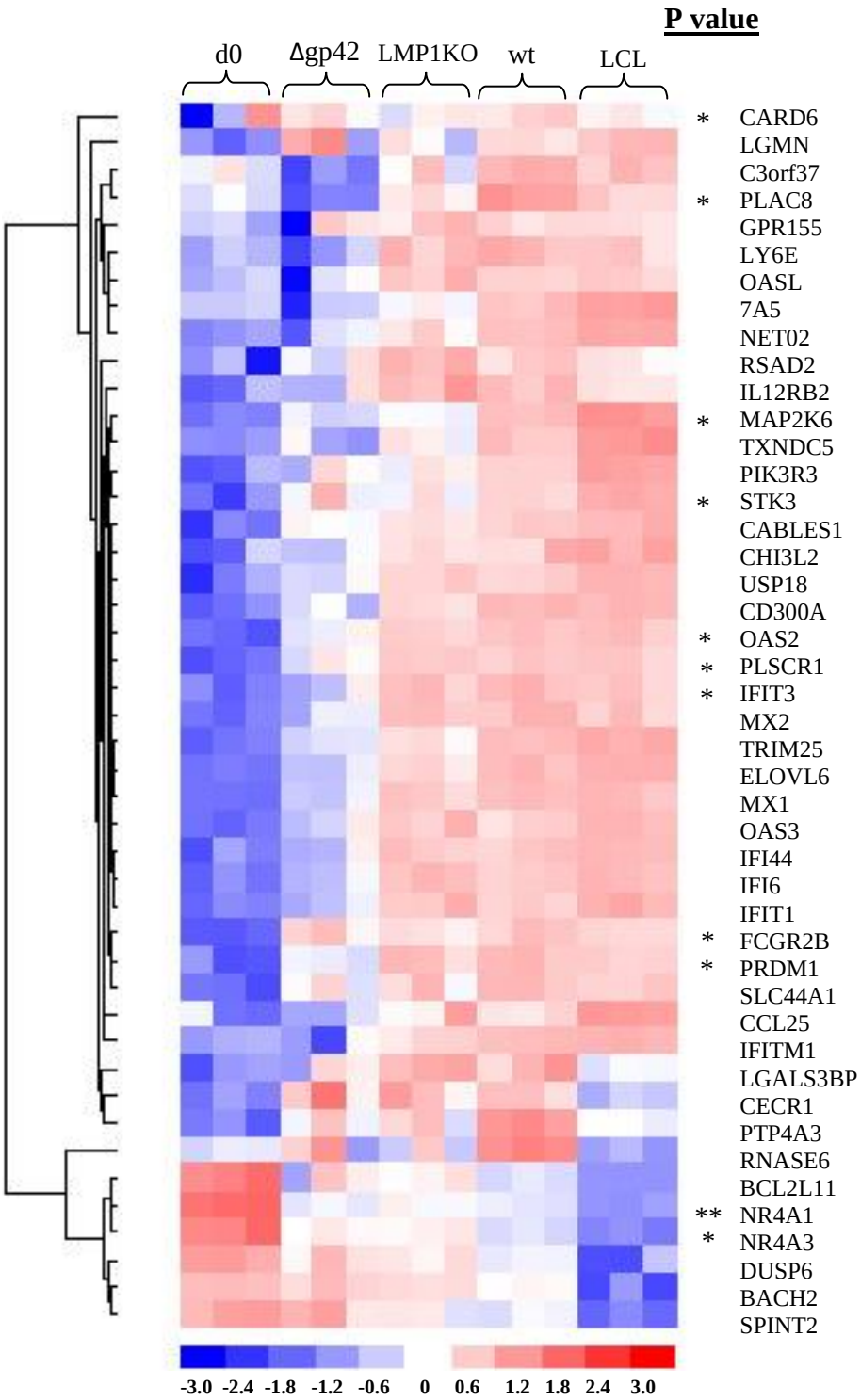


Figure 5.10: Heat map representing the results of a QPCR assay on biological triplicate samples of B cells (d0) isolated from peripheral blood and infected with either wtEBV 7 days p.i (wt) and 6 weeks p.i (LCL), Δgp42 EBV (Δgp42) and LMP1KO EBV (LMP1KO). Data is expressed as relative ΔCt values where red represents a relative increase in expression and blue represents a relative decrease in expression. P values represent the results of a paired student T test comparing resting B cells with Δgp42 EBV infected cells. Genes marked with * had a P value of ≤ 0.05 and genes marked with ** had a P values of ≤ 0.01.

showed a significant difference in expression between resting B cells and the Δgp42 infected B cells by the student T test. Collectively, this suggests that a significant subset of genes differentially expressed between EBV and CD40L are also affected by virus binding alone, but expression is maintained (or even amplified) in LCLs, indicating additional mechanisms for maintaining differential gene expression.

5.6.2 TSG expression in malignant cell lines

The expression of the three differentially regulated TSGs, DUSP6, SPINT2 and NR4A3, was measured by QPCR in a limited panel of BL cell lines expressing the latency state of the original tumour biopsy; latency I. An EBV negative BL cell line (DG75) and an LCL were included for comparison. Data is expressed relative to resting B cells. The panel of BL cell lines represents only a limited number of cell lines for analysis but were well characterised latency I BL cell lines available within our group, the gene expression data from this small panel of cell lines cannot therefore be classified as representative of all BL cell lines, a wider panel would be required to draw those conclusions. The data shows that DUSP6 and NR4A3 were both expressed at very low levels in all BL cell lines. DUSP6 expression was elevated in Dante when compared to the other BL cell lines; however, this expression was still only 20% of that seen in the resting B cells. SPINT2 had variable expression across the cell lines, with relative expression ranging from LCL-like levels of 0.01 in Ezema, to expression 8 fold greater than resting B cells in Sav (figure 5.11). The SPINT2 expression data suggests that the down-regulation of SPINT2 during EBV infection is not relevant to the development of BL, whereas the down-regulation of NR4A3 and DUSP6 may contribute to the malignant phenotype of BL cells.

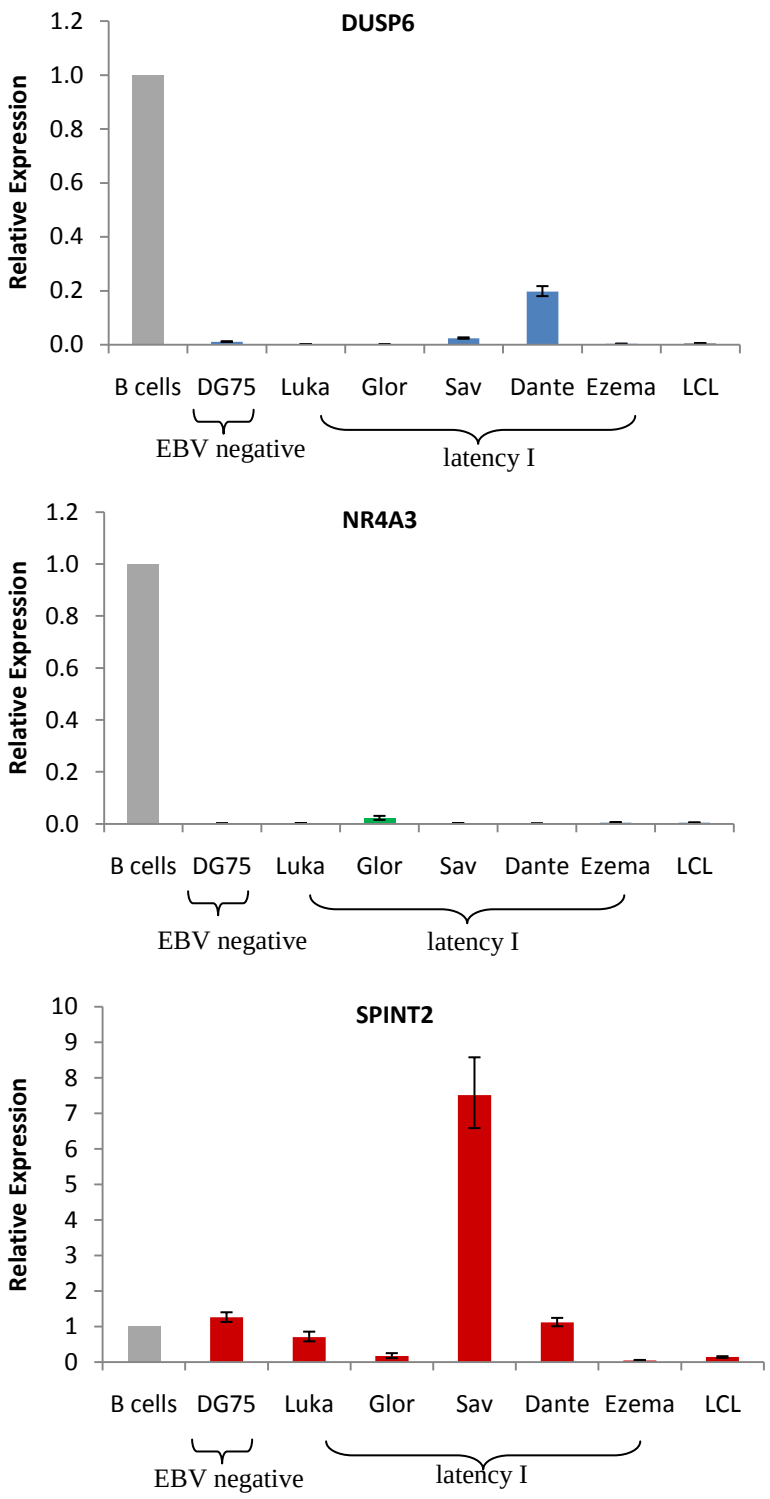


Figure 5.11: TSG expression by QPCR in BL cell lines. **A;** DUSP6, **B;** NR4A3 and **C;** SPINT2 in a panel of BL cell lines and an LCL. Data is expressed relative to resting B cells and error bars represent the standard deviation of duplicate QPCR samples.

5.7 Functional consequences of re-expression of DUSP6 in LCLs

DUSP6 is a tyrosine phosphatase which specifically targets the ERK/MAPK pathway by removing ERK phosphorylation and therefore preventing ERK activation [702, 703]. The down-regulation of DUSP6 may therefore be required for constitutive or enhanced activation of MAPK signalling, which is associated with many different cancers [704]. Re-expression of DUSP6 in pancreatic cancer cell lines resulted in loss of proliferation and an accumulation of cells in the sub G1-phase of the cell-cycle [617]. We therefore sought to investigate the effect of ectopic DUSP6 re-expression on the proliferation of LCLs.

5.7.1 Creating a DUSP6 expressing lentivirus

To investigate the effect of DUSP6 re-expression in LCLs we created a tetracycline inducible, DUSP6 expressing lentivirus. It was important to use an inducible lentivirus system as the re-expression of a candidate tumour suppressor gene may cause apoptosis or prevent proliferation of the LCLs before functional assays can be performed. We chose to use a tetracycline inducible lentivirus system kindly donated by Marco Herold [616] for production of the DUSP6 lentivirus. Details of the cloning strategy are in chapter 2 section 2.21. Briefly, PCR was performed to amplify DUSP6 from a Topo-DUSP6-His expression vector (donated by Toru Furukawa [617]). The PCR fragment was ligated into an intermediate vector downstream of a tetracycline-inducible promoter. DUSP6 was then excised from the intermediate vector, along with the tetracycline inducible promoter, and ligated into the final vector. The final vector therefore contained DUSP6 under the control of a tetracycline inducible promoter (TREX-P); it also contained eGFP and tetracycline repressor protein (hTetR), constitutively expressed from a ubiquitin promoter. A simplified map of the final DSUP6 lentiviral vector is shown in figure 5.12.

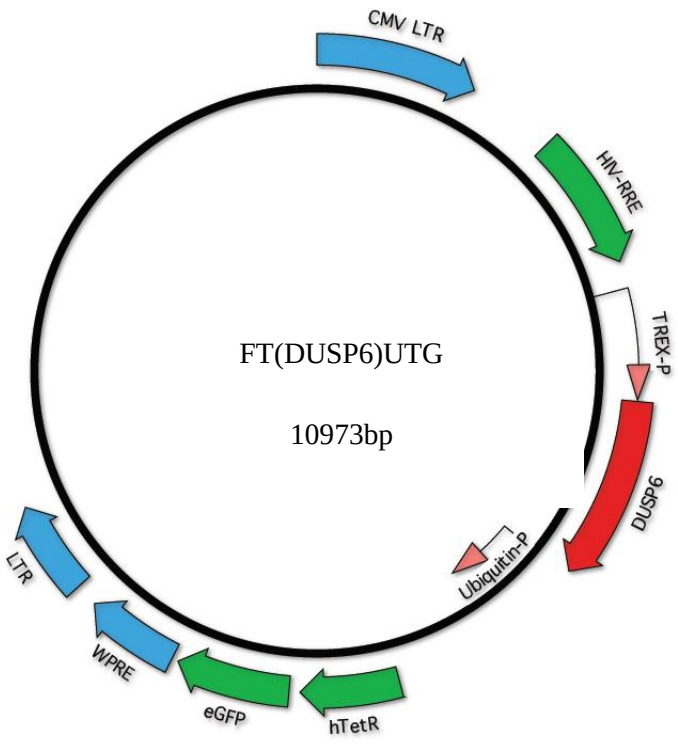
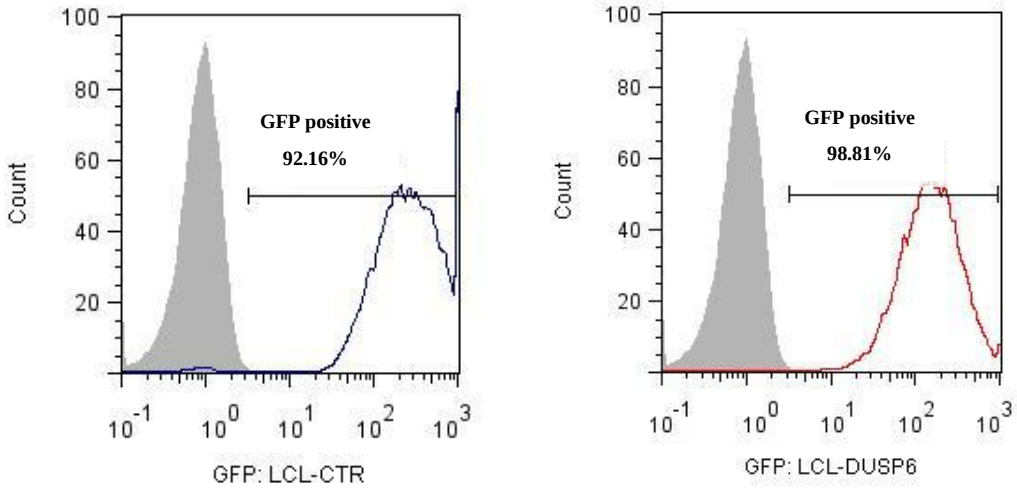


Figure 5.12 Simplified schematic map of the DUSP6 lentiviral vector, FT(DUSP6)UTG. The FT(DUSP6)UTG lentivirus was used to generate stable a LCL-DUSP6 cell line with tetracycline inducible DUSP6 expression. DUSP6 expression was controlled by a tetracycline promoter (TREX-P) while eGFP and the tetracycline repressor protein (hTetR) were constitutively expressed from the ubiquitin promoter (ubiquitin-P).

A



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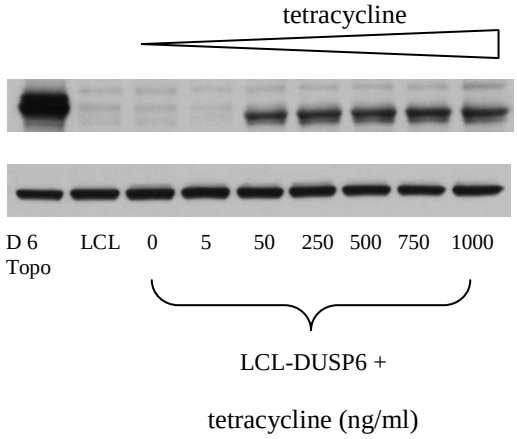

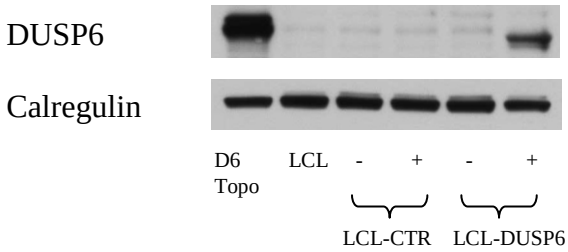

C

Figure 5.13: Western blot and flow cytometry data showing DUSP6 lentivirus transduction and gene expression in a LCL. **A;** Flow cytometry data showing the GFP positivity of an LCL transduced with either a CTR-lentivirus or a DUSP6, GFP positive lentivirus. **B;** Western blot for DUSP6 and calregulin expression in 293 cells transfected with a HIS-tagged DUSP6 topo vector (D6 Topo), the parental LCL and LCL-DUSP6 harvested 48 hours after culture with increasing concentrations of tetracycline to induce DUSP6 expression. **C;** 293 cells transfected with a HIS-tagged DUSP6 topo vector (D6 Topo), the parental LCL and LCL-CTR and LCL-DUSP6 harvested 48 hours after culture with either no tetracycline (-) or with 0.5µg/ml of tetracycline.

5.7.2 Validating the DUSP6 lentivirus

To test the tetracycline inducible promoter of the DUSP6 lentivirus in an LCL, $5x10^5$ LCLs were transduced with either a DUSP6 lentivirus or a control empty vector lentivirus (CTR), and the cells were sorted for GFP. GFP positive cells were then cultured to produce stable cell lines; the cell lines were designated LCL-DUSP6 and LCL-CTR (figure 5.13, A). LCL-DUSP6 and LCL-CTR were plated out to $5x10^6$ cells per well of a 6 well plate and cultured

for 48 hours at a range of concentrations of tetracycline from 0-1µg/ml. Cells were harvested for protein and western blots were performed for DUSP6 and calregulin. DUSP6 expression was induced with 50ng/ml of tetracycline, however, DUSP6 expression apparently did not increase in a dose dependent manner with further increases in the concentration of tetracycline. This indicates that the tetracycline inducible promoter was maximally active in 50ng/ml of tetracycline, and that a dose responsive increase in DSUP6 expression may be seen at concentrations ranging from 5ng/ml-50ng/ml.

To demonstrate that the control lentivirus did not express DUSP6, LCL-CTR and LCL-DUSP6 were culture for 48 hours in the presence or absence of 0.5µg/ml of tetracycline. Cells were harvested for protein and western blot was performed for DUSP6 and calregulin expression. DUSP6 was only expressed in the induced LCL-DUSP6 expressing cell line.

5.7.3 DUSP6 expression and ERK1/2 phosphorylation in an LCL

DUSP6 has been shown to specifically de-phosphorylate and inactivate ERK1/2 in cultured cells [702]. Ectopic DUSP6 expression in pancreatic cancer cell lines, which contain a gain-of-function mutation in KRAS and therefore have constitutive ERK phosphorylation, is associated with loss of ERK phosphorylation. The re-expression of DUSP6 in an LCL would therefore be predicted to prevent ERK phosphorylation in a LCL. Stimulation of a LCL with

the phorbol ester 12-O-tetradeconoylphorbal-13-acetate (TPA) has been shown to induce ERK phosphorylation [705], we investigated the effect of DSUP6 expression on TPA induced ERK phosphorylation in a LCL.

LCL-CTR and LCL-DUSP6 were cultured in the presence and absence of 0.1µg/ml of tetracycline for 48 hours to induce DUSP6 expression. Cells were then stimulated with 20µg/ml of TPA or 1µl of DMSO as a negative control and the cells were harvested after 24 hours for western blot. The blots were probed for total ERK (ERK1/2), phospho-ERK, DUSP6 and calregulin. The data shows no reduction in TPA induced ERK-phosphorylation in the presence of DUSP6 in a LCL (figure 5.14). DUSP6 mediated de-phosphorylation of ERK1/2 may serve to dampen the ERK signalling by slowing the rate of ERK activation rather than preventing it entirely. Measuring ERK phosphorylation 24 hours post-TPA stimulation may therefore not detect any decrease in phosphorylation. It is also possible that LCLs have several mechanisms to maintain ERK phosphorylation and DUSP6 re-expression in established LCLs may therefore not have any effect on ERK1/2 phosphorylation.

5.7.4 The effect of DUSP6 expression on LCL proliferation

The tumour suppressor activity of DUSP6 in pancreatic cancer cell lines was partly ascribed to its ability to reduce cell proliferation upon re-expression. Pancreatic cancer cell lines were unable to complete the cell-cycle following DUSP6 expression, with cells being held in the sub-G1 fraction [617]. To determine the effect of DUSP6 re-expression on LCL proliferation, LCL-DUSP6 and LCL-CTR were cultured in the presence and absence of 0.5µg/ml of tetracycline and cell proliferation was determined by cell counting using a trypan blue exclusion assay. The parental LCL (LCL A4) cells were included as an additional control for the effect of lentivirus transduction, and were cultured in tetracycline free media. Cells were seeded at 0.2 $x10^6$ cells per ml in a total of 5ml of media and multiple aliquots were counted

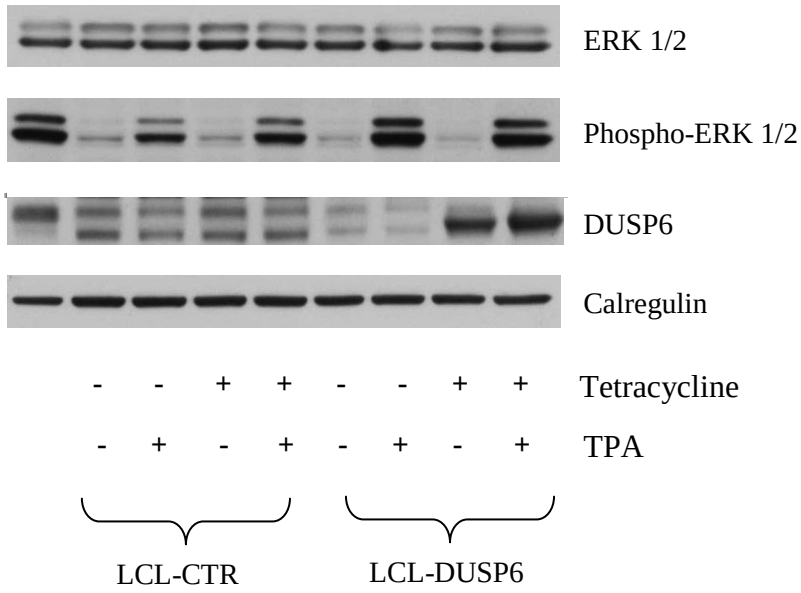

Figure 5.14: Western blot analysis of ERK phosphorylation following TPA stimulation of LCLs in the presence or absence of DUSP6. Stable cell lines were produced following the transduction of an LCL with a tetracycline inducible empty vector (LCL-CTR) or DUSP6 (LCL-DUSP6) expressing lentivirus. The LCLs were then cultured in the presence or absence of tetracycline for 48 hours prior to stimulation with TPA to induce ERK phosphorylation. LCLs were harvested 24 hours post-TPA stimulation for western blot analysis. Blots were probed for total ERK (ERK 1/2), phospho-ERK, DUSP6 and calregulin as a loading control.

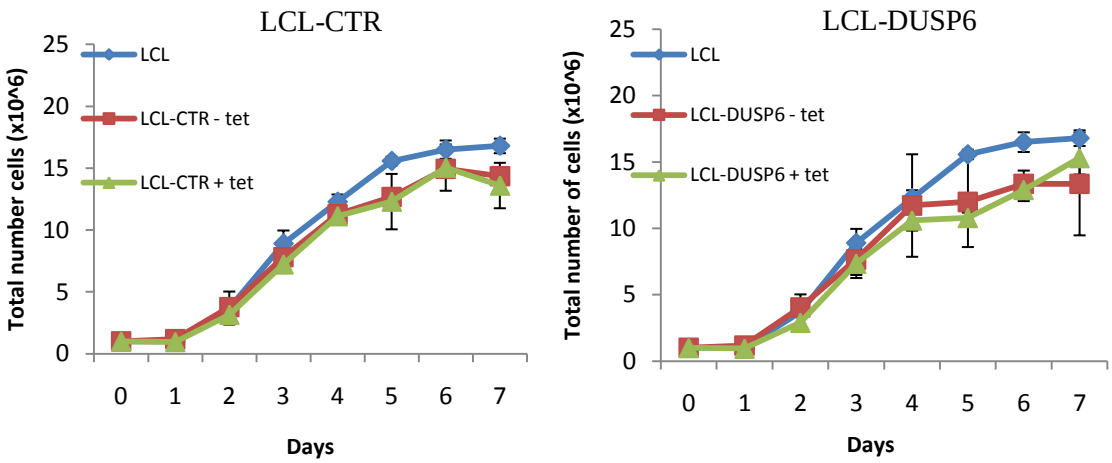

Figure 5.15: Trypan blue exclusion assay following DSUP6 re-expression in a LCL. Graphs represent the total number of live cells counted in a culture over 7 days. LCL-DUSP6 and LCL-CTR were in cultured in the presence (+ tet) or absence (- tet) of 0.5µg/ml of tetracycline and the cells. The parental LCL was cultured in the absence of tetracycline as a control.

daily for 7 days. There was no apparent difference in LCL proliferation in the presence of DUSP6. The proliferation of both the induced and un-induced LCL-CTR and LCL-DUSP6 cell lines was the same (figure 5.15). The parental LCL may have slightly increased rate of proliferation than the LCL-CTR and LCL-DSUP6 cell lines; however, this was variable in repeat assays and may not be significant.

A complementary and more sensitive assay to measure LCL proliferation was also performed using flow cytometry. GFP positive LCL-CTR and LCL-DUSP6 cells were counted and mixed at a 1:1 ratio with the GFP negative parental LCL cell line; the cell lines were cultured in the presence or absence of 0.5µg/ml of tetracycline for 7 days. GFP was measured by flow cytometry after 7 days to determine whether the GFP positive LCL-DUSP6 cells were out-competed by the GFP negative LCL due to a decreased rate of proliferation. The results presented in figure 5.16 indicate that neither the control, nor the DUSP6 expressing LCLs were out-competed by the GFP negative LCLs in the presence or absence of tetracycline. Collectively these results suggest that DUSP6 re-expression does not affect the proliferation of established LCLs.

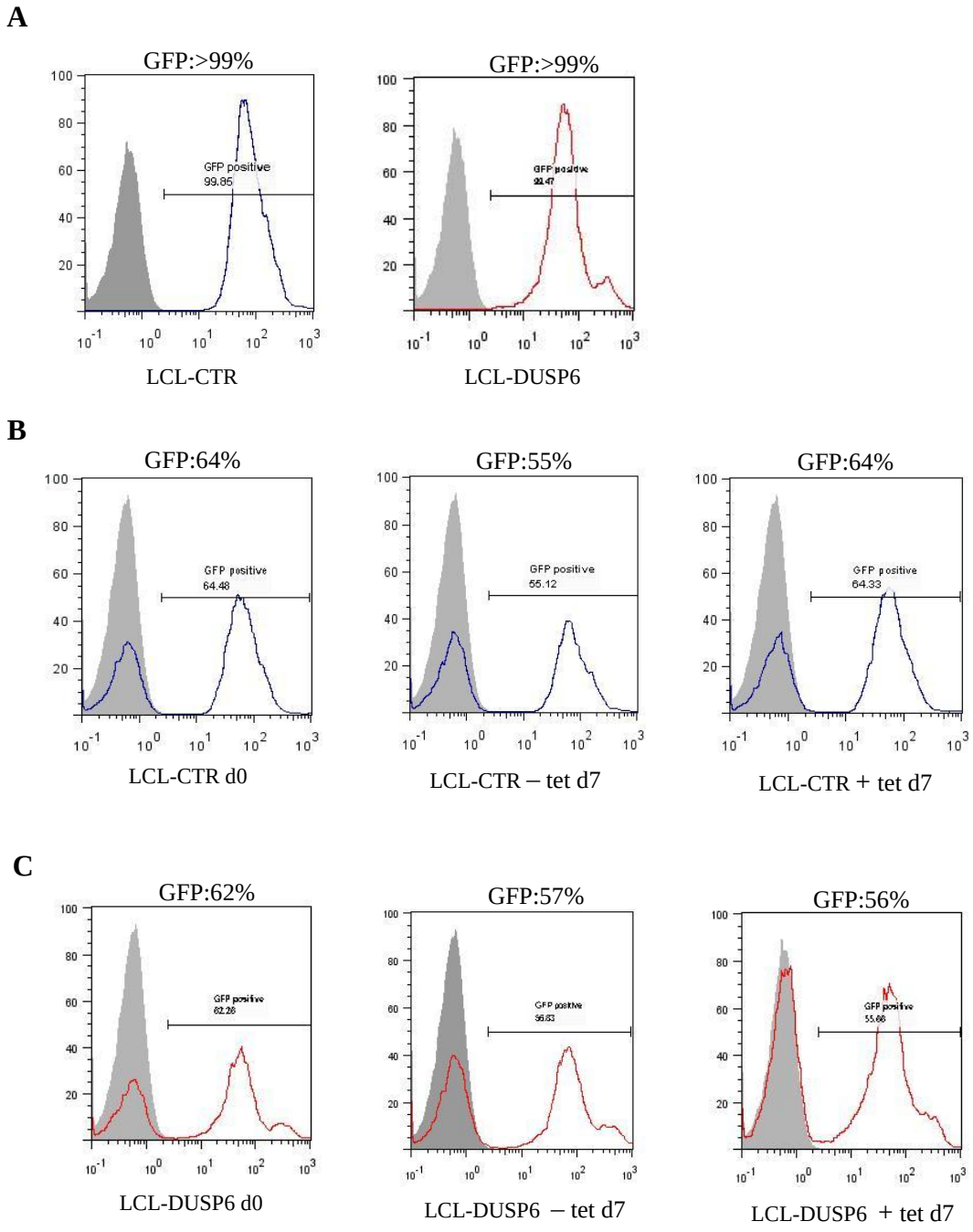

Figure 5.16: Cell growth assay examining GFP expression by flow cytometry in stable LCL-DUSP6 or LCL-CTR cell lines either induced or un-induced with tetracycline. LCLs were transduced with FT(DUSP6)UTG or a control empty vector plasmid FT(empty)UTG to generate LCL-DUSP6 and LCL-CTR cell lines respectively. **A;** LCL-CTR and LCL-DUSP6 GFP positive cell lines prior to experiment. **B;** LCL-CTR or **C;** LCL-DUSP6 cells were mixed with GFP negative LCLs at a ratio of 1:1 (d0). Cells were cultured with or without tetracycline and GFP was measured after 7 days as a measure of cell proliferation.

Discussion III

The aims of this chapter were twofold: firstly, we aimed to identify transcriptional changes which were associated with EBV-mediated B cell transformation; secondly, we sought to determine whether there was any correlation between the expression of the cellular miRNAs profiled in the previous chapter, and the levels of cellular gene transcripts; whilst from what we know of miRNA mode of action, we would not necessarily expect good correlations with target transcripts, there are many examples in the literature where this approach has yielded results.

(a) miRNA and cellular gene expression comparison

(i) miR-148a target prediction analysis and microarray comparison: The miRNA target prediction analysis revealed 12 miR-148a predicted target genes specifically down-regulated by EBV infection and not CD40L stimulation of resting B cells. However, we demonstrated the transcriptional down-regulation of Bim by CD40L (figure 5.8), and in the previous chapter, miR-148a expression was shown not to affect Bim expression in an established LCL. This emphasises the caution which is required when comparing mRNA microarray and miRNA expression data as many false positive miRNA targets can easily be identified due to the limitations of microarray analysis and miRNA target prediction programs.

Furthermore, there were a similar percentage of miR-148a target genes up and down-regulated in both CD40L blasts and EBV blasts, and there was a lack of enrichment of miR-148a targets genes in the EBV down-regulated gene set. This implied that the target prediction analysis was producing a significant percentage of false positive in every analysis; for this reason, we did not pursue this analysis further.

(ii) miRNA array and microarray data comparative analysis: We have shown that expression of the 61 differentially regulated genes indentified in the microarray comparing EBV and CD40L blasts were not predicted to be targeted by any of the 3 miRNAs which showed differential expression between EBV and CD40L blasts 7 days p.i/stimulation. As discussed previously, miRNAs negatively regulate gene expression predominantly through translational repression, therefore, many of the gene targets of these miRNAs will not be detected on a microarray, which may explain the lack of correlation seen between the two array sets. Additionally, comparing miRNA and gene expression data using bioinformatic prediction of miRNA targets has several caveats, including (i) an assumption that an up-regulation in miRNA expression will correlate with a decrease in gene expression, which is not always the case [480-482] (discussed in section 1.14), and (ii) miRNA target prediction programs only scan the 3'UTR of gene targets, therefore, any miRNA target sites in 5'UTRs or protein coding regions will be discounted [477, 478]. Nevertheless, miRNA and mRNA expression data has been successfully correlated in previous studies [160, 524, 534, 640, 688], despite the high number of false positive and negative results inevitably produced from such comparisons.

In contrast, all of the miRNAs commonly up or down-regulated by EBV or CD40L stimulation were predicted to target > 20% of the corresponding genes up or down-regulated on the microarray. This suggests that the lack of correlation between the differentially regulated miRNAs and the differentially regulated genes may be a valid result. However, the previous miRNA analysis comparing miR-148a predicted target genes and the microarray data would suggest that these comparisons are likely to produce a number of false positives; indicating that the lack of false positives in this instance may be due to the small number of differentially regulated genes.

The commonly up or down-regulated miRNAs were predicted to target one or more gene target from the array. This data could have been further refined using multiple miRNA target prediction programs to generate a list of the predicted miRNA gene targets which were the most probable. This refined gene list could then have been used as a basis for miRNA target validation experiments. However, our investigation was focused on the genes and miRNAs which were differentially expressed between EBV and CD40L blasts, therefore we did not continue with this line of investigation.

(b) Cellular gene expression in EBV and CD40L blasts

We had hypothesised that CD40L blasts and EBV blasts would show very similar gene expression changes relative to resting B cells; this was confirmed by the two data analyses which independently compared EBV and CD40L blasts directly, or with resting B cells.

When analysing the gene expression data relative to resting B cells, EBV blasts showed approximately twice the number of gene expression changes of the CD40L blasts. Therefore, there were many gene expression changes (>2000) which occurred in EBV blasts relative to resting B cells which were not classified as significant in the CD40L blasts. However, SAM analysis comparing the CD40L and EBV blasts directly, identified only 61 genes which were significantly differentially expressed between the two types of lymphoblasts. This indicates that the majority of the 2000 plus gene expression changes detected in EBV blasts relative to resting B cells also occurred in the CD40L blasts, but to a lesser extent.

Consequently, many of the gene expression changes in the CD40L blasts relative to resting B cells, were not great enough to be classified as significant by the stringent conditions (>1.5 fold, q value 0.05) set for the SAM analysis, although they were large enough to prevent the

gene expression difference from being statistically significant when a direct comparison between the EBV and CD40L blasts was performed.

The EBV:CD40L blasts gene expression comparison highlights the effectiveness of the CD40L blast control, as without it, we would have needed to identify genes involved in EBV-mediated B cell transformation from the nearly 5000 gene expression changes observed when comparing EBV blasts with resting B cells.

(c) Tumour suppressor gene analysis and validations

Using a database compiled through data-mining we have identified three candidate TSGs specifically down-regulated in EBV blasts and not in CD40L blasts; DUSP6, NR4A3 and SPINT2. We have validated the differential expression of these TSGs using QPCR and have shown that they are further down-regulated in the LCL. QPCR analysis also revealed that DUSP6 and SPINT2 are slightly up-regulated in CD40L blasts relative to resting B cells, enhancing the difference seen in a direct comparison between EBV and CD40L blasts.

The Kegg pathway analysis was a useful tool for evaluating the gene expression changes in the EBV and CD40L blasts relative to resting B cells. The up-regulation of the TLR7 pathway was the most striking difference between EBV and CD40L blasts and it is perhaps not surprising that virally infected B cells show a stronger TLR activation than mitogen stimulated B cells. Nevertheless, TLR stimulation has been shown to enhance the proliferation [706] and transformation [707] of naïve B cells, suggesting that the enhanced activation of this pathway be EBV may facilitate the transformation process. However, since the up-regulation of TLR7 signalling by EBV was already published, we did not investigate this observation further.

The Kegg pathway analysis also identified apoptosis and MAPK related pathways activated by EBV blasts which may contribute to the transformed phenotype of LCLs. Interestingly, Nur77 (NR4A1) was listed as one gene down-regulated by both EBV and CD40L in the MAPK pathway. NR4A1 is a pro-apoptotic candidate TSG [690, 696], which often functions in conjunction with NR4A3. It has been reported that NR4A1 and NR4A3 can be down-regulated by MAPK signalling [708], suggesting a possible relationship between activated MAPK signalling and the down-regulation of these pro-apoptotic TSGs during B cell transformation. Furthermore, the down-regulation of the TSGs SPINT2 and DUSP6 has been reported to result in increased MAPK signalling [693, 709]; suggesting that not only is the MAPK signalling pathway important for B cell transformation, but also that the three TSGs down-regulated by EBV may be functionally linked.

The protein expression of these TSGs cannot be measured using western blot as commercially available antibodies are not sensitive enough to detect endogenous protein expression. The use of these antibodies in different applications such as immuno-fluorescence or the development of new antibodies for these proteins will be required to validate the down-regulation of the TSGs at the protein level. The transcriptional data alone was convincing enough for us to continue our investigation into the genes, although protein validation will be required for future work.

(d) Tumour suppressor gene expression data

To determine the importance of candidate TSG down-regulation in the transformation process, the expression of the three TSGs was measured in B cells infected with the non-transforming LMP1KO virus and a Δgp42 virus to control for the effect of virus binding. It was hoped that this expression data would reveal whether the TSGs were down-regulated by

LMP1, and whether the TSG down-regulation occurred in B cells which were not growth transformed.

After confirming the absence of LMP1 expression in LMP1KO virus infected B cells and the absence of any EBV promoter usage in Δgp42 virus infected B cells, we showed that the non-transforming LMP1KO and Δgp42 viruses also down-regulated the expression of NR4A3, DUSP6 and NR4A1. This data is comparable to the up-regulation of miR-155 observed following Δgp42 virus infection of B cells in chapter 3. The up-regulation of miR-155 by Δgp42 virus was interpreted as a response to B cell activation caused by virus binding as miR-155 was known to be up-regulated in activated B cells. This interpretation may extend to the 31 genes whose expression was also altered following EBV binding.

In total, 31/45 differentially regulated validated from the microarray also showed altered expression following infection with Δgp42 virus. All gene expression changes mirrored those of the wtEBV; indicating that virus binding alone can stimulate gene expression changes. The LCL expression data demonstrated that these gene expression changes are maintained in the LCL, suggesting that they are important for B cell transformation. Consistent with this, EBV binding via gp350 to CD21 has been reported to produce changes in cellular gene expression, including IL6 production and NF-κB activation [337, 710-713]. CD21 activation has also been shown to rescue GC B cell from apoptosis [714], suggesting that the signalling mediated by CD21 activation can have significant effect on B cell gene expression.

For EBV to successfully transform a resting B cell it may need to rapidly initiate the gene expression changes necessary for transformation, to prime the resting B cell for activation and transcription of EBV latent genes necessary for EBV-mediated B cell transformation. Virus binding and subsequent CD21 mediated signalling may be a method by which EBV initiates

the cellular gene expression changes essential for transformation, prior to entering the cell. Therefore, the gene expression changes observed in resting B cells infected with Δgp42 virus may be required for successful EBV-mediated B cell transformation, an observation which warrants further investigation.

Whilst we were surprised by the number of differentially regulated genes which were modulated by EBV binding alone, the fact that these changes continued to be effected in LCLs long after the EBV/CD21 complex-mediated signals had expired, suggested that the changes were likely to be important for transformation. We therefore asked whether these genes were similarly modulated in EBV associated malignancies. The expression of the three TSGs was measured in a panel of latency I BL cell lines where we demonstrated that the expression of DUSP6 and NR4A3 were almost undetectable, which is consistent with a tumour suppressor function for these genes. The expression of SPINT2 was less consistent, with expression greater than resting B cells in the majority of the BL cell lines. This suggests that the down-regulation of SPINT2 in resting B cells transformation is not necessary for BL development. SPINT2 may be up-regulated by the high cMyc expression in BL or may only be down-regulated in tumours expressing a less restricted pattern of EBV latency. It is also possible that the down-regulation of SPINT2 may be involved in EBV-mediated transformation of resting B cells but not EBV associated lymphomagenesis. Further characterisation of SPINT2 expression in tumours of EBV associated lymphomas will provide insight into the tumour suppressor function of this gene in relation to EBV.

(e) Re-expression of DUSP6 in an LCL

DUSP6 is a phosphatase which specifically inactivates ERK1/2 in the MAPK pathway, thereby inhibiting MAPK signalling [702]. The re-expression of DUSP6 in pancreatic cell lines was shown to induce cell-cycle arrest and sensitise the cells to apoptosis [617]. To

examine the role of DUSP6 down-regulation in EBV-mediated growth transformation, a lentivirus expressing DUSP6 under the control of a tetracycline responsive promoter was successfully created. DUSP6 expression was induced upon the addition of tetracycline but the induction did not appear to be dose responsive, although it is possible that the dose responsive induction of DUSP6 at lower concentrations of tetracycline is occurring but it cannot be seen with the low sensitivity DUSP6 antibody. This poses a potential disadvantage for future work using this system as re-expression of DUSP6 to the physiological levels seen in resting B cells will not be possible.

Whilst we had concerns about the physiological relevance of the over-expressed DUSP6, we considered that it would be useful to first demonstrate an effect of DUSP6 on LCL phenotype, and then validate such effects by reducing the tetracycline concentration to limit the amount of DUSP6 expressed. However, induction of DUSP6 expression in LCL-DUSP6 did not affect LCL proliferation, in contrast to the affect of DUSP6 re-expression in pancreatic cancer cell lines [617]. This difference may represent the difference in cell systems used. MAPK signalling is constitutively active in the majority of pancreatic cancer cell lines due to a gain-of-function mutation in KRAS. ERK phosphorylation in this cell system is therefore central for the tumourogenic phenotype of these cells. LCLs appear to have low basal levels of ERK phosphorylation (figure 5.14) indicating that constitutive ERK1/2 activation may not be as important in these cell lines. Alternatively, LCLs may modulate alternative pathways which enable MAPK signalling, introducing a level of redundancy in the MAPK pathway which does not exist in pancreatic cell lines; therefore, DUSP6 re-expression in an LCL may not result in a loss of ERK phosphorylation.

Preliminary data investigating the effect of DUSP6 re-expression on ERK phosphorylation revealed that DUSP6 cannot reduce ERK phosphorylation 24 hours post-stimulation with

TPA, supporting the hypothesis that DUSP6 re-expressing in an LCL may not prevent ERK phosphorylation. A time course assay investigating the effect of DUSP6 re-expression on the rate of ERK-phosphorylation might determine the extent to which DUSP6 can modulate the activation of this pathway in a LCL.

The down-regulation of DUSP6 during B cell transformation may be necessary during early transformation to stimulate B cell proliferation and protect from apoptosis, but may not be essential in an established LCL. An investigation into the effect of DUSP6 re-expression in early EBV blasts (7 days p.i) may provide greater insight into the role of this gene during B cell transformation. This point is also relevant to the investigation of NR4A3 and SPINT2, and future investigation into the function of these candidate TSGs will focus on EBV blasts and early passage LCLs.

There have been conflicting reports regarding the requirement of ERK1/2 activation during EBV-mediated transformation [169, 715], although these have used MAPK inhibitors with differing specificities, so the role of MAPK signalling during B cell transformation has not been definitively shown. Fenton et al [715] showed that MAPK inhibitors prevent phorbol ester induced BZLF1 expression, suggesting that the ERK1/2 signalling pathway is involved in the control of viral latency. Modulation of the MAPK pathway by EBV may therefore be required to enable the virus to regulate viral latency rather than B cell proliferation and apoptosis resistance. If so, re-expression of DUSP6 in EBV transformed B cells might render them insensitive to certain triggers of the lytic cycle. This could be examined more carefully in the Akata-BL cell line which can efficiently be induced into lytic cycle by different triggers, including BCR-ligation or phorbal ester treatment [246, 716, 717].

In summary, we have identified a subset of 61 genes specifically modulated by EBV infection and not CD40L stimulation of resting B cells. Data analysis revealed that none of the

differentially regulated genes were predicted to be targeted by any of the differentially regulated miRNAs. However, the cellular miRNAs commonly up or down-regulated by EBV and CD40L were predicted to target corresponding cellular genes on the microarray commonly down or up-regulated by EBV and CD40L; therefore, future investigation into the contribution of cellular miRNAs to EBV-mediated B cell transformation may focus on the genes and miRNAs which are commonly regulated by EBV and CD40L.

The expression of 45/61 of the differentially regulated genes was successfully validated and included the expression of 3 candidate tumour suppressor genes down-regulated following EBV infection. Two of these candidate tumour suppressor genes showed low expression in a panel of BL cell lines, consistent with a tumour suppressor function in BL tumours. An inducible lentivirus system has enabled the re-expression of these TSGs to be investigated in EBV infected B cells. DUSP6 re-expression in an LCL did not affect LCL proliferation and highlighted a possible flaw of investing the function of these TSGs in established LCLs. Future investigation into the function of the three TSGs in EBV-mediated B cell transformation will therefore focus on early stage EBV blasts rather than established LCLs.

6. Conclusions and Future Work

The transformation of resting B cells by EBV requires the modulation of cellular gene expression, in some instances these gene expression changes may also contribute to the development of EBV associated malignancies. The aim of this work was to investigate the role of cellular miRNAs in EBV induced transformation and oncogenesis.

(a) The regulation of miR-155 by EBV

The precursor of the oncogenic miR-155 (BIC) was reportedly up-regulated in HL [612] and miR-155 expression was shown to be elevated in LCLs [374, 603]. We demonstrated that miR-155 expression was up-regulated by a latency III pattern of viral gene expression and by induced expression of the viral oncogene LMP1. The investigation into the regulation of miR-155 by EBV, involved the re-analysis and clarification of a previously published result which apparently demonstrated a processing defect in BL cell lines [375]. Using newly available miRNA QPCR assays, we were able to demonstrate that the data in this previous report was misinterpreted largely as a result of the limited sensitivity of northern blotting, the only technique previously available to measure miRNA expression; emphasising the caution with which the literature at that time needed to be interpreted.

The role of miR-155 in EBV induced cellular transformation was an area of much interest in the field, with many reports published regarding the potential role of this miRNA in EBV induced cellular transformation and oncogenesis, prompting our research to focus on the identification of novel miRNAs modulated by EBV. However, it was not until recently that the definitive result was published which demonstrated that miR-155 was essential for LCL growth, as miR-155 inhibition in an LCL has been shown to cause cell-cycle arrest and spontaneous apoptosis [646]. The cellular gene targets of miR-155 during transformation have

still not been identified and miR-155 inhibition was shown not to affect LMP1 or EBNA2 protein expression [646], therefore, the mechanism by which miR-155 promotes EBV mediated transformation remains to be elucidated. The dependence of LCLs on miR-155 expression demonstrates the importance of miR-155 in B cell transformation and highlights a possible weakness in the criteria which we applied to identify novel transformation associated miRNAs as miR-155 is strongly up-regulated in both EBV and CD40L blasts. Although not tested experimentally, we would predict that proliferation of CD40 blasts would also be impaired by inhibition of miR155.

(b) Cellular miRNAs modulated by EBV during transformation

It is clear from our results that EBV does modulate the cellular miRNA expression of B cells during the process of B cell transformation. These expression changes include the up-regulation of seven oncogenic miRNAs , including miR-17-92 cluster, miR-21 and miR-155. Interestingly, all of the aforementioned oncogenic miRNAs are also up-regulated in proliferating B cell blasts, which can only transiently proliferate in culture; therefore the up-regulation of these miRNAs is not sufficient to induce continuous proliferation or transformation of B cells. This led us to focus on the miRNAs which were differentially expressed between EBV and CD40L blasts, reasoning that these miRNA and gene expression changes may be key to the transformation process. However, in retrospect, this view may have been too simplistic for a number of reasons:

Firstly, miRNAs are known to target genes for degradation in a coordinated manner [499]; therefore, it is likely that the simultaneous up-regulation of 7 oncogenic miRNAs will contribute to EBV-mediated cellular transformation and oncogenesis even though they are regulated by both EBV and CD40L. Indeed, our own multiple target prediction analysis revealed that 68 genes were predicted to be targeted by 9 or more of the 19 miRNAs up-

regulated by EBV infection. Thus, the total miRNA expression profile of a cell is important when considering the consequences of miRNA gene expression changes in response to EBV infection. The multiple miRNA target prediction analysis has also provided a possible explanation for the lack of Bim protein repression we observed following transfection of miR-148a alone into DG75 as multiple miRNA binding sites functioning in cooperation may be required for Bim repression. This analysis has also raised the interesting possibility that 10 miRNAs up-regulated following EBV infection of B cells (including miR-148a) may negatively regulate Bim protein expression early following EBV infection, prior to previously described EBV-mediated transcriptional and epigenetic down-regulation (figure 6.1). The multiple mechanisms employed by EBV to control the expression of Bim indicate that Bim mediated down-regulation is very important for transformation and the repression of Bim protein expression early post-infection by cellular miRNAs may aid a transforming B cell escape apoptosis before more efficient and stable down-regulation, mediated by EBV latent genes, can occur.

Secondly, during the course of this work it was reported that under specific culture conditions, CD40L generated B cell blasts can proliferate indefinitely in culture to produce an immortalised cell line [718]. That report raises the possibility that the miRNA and gene expression changes induced by CD40L and IL4 can, under certain conditions, produce B cell blasts which are phenotypically indistinguishable from an LCL. Consequently, the gene and miRNA expression changes induced by both EBV and CD40L blasts may also be important for cellular transformation. Nevertheless, it is still the case that in our experiments we were comparing two types of blasts; one which was destined to stop proliferating within weeks, and a second which would become transformed.

The miRNA array and microarray comparison did not identify any cellular genes which were predicted to be regulated by any of the differentially regulated miRNAs. This is most likely due to the small number of selected genes and miRNAs involved in the analysis. Nevertheless, the analysis of the two array sets did identify possible miRNA target genes which were modulated in response to both CD40L stimulation and EBV infection. Considering the importance of miR-155 in LCL proliferation and survival, future work aimed at elucidating the role of cellular miRNAs in EBV-mediated transformation could include the cellular genes and miRNAs regulated by both stimuli. It would be interesting to use the multiple miRNA target prediction analysis to identify cellular gene targets predicted by the microarray comparison, which are also predicted to be regulated by more than one EBV regulated miRNA. This would reduce the number of false positive identifications produced by the miRNA and gene expression array comparisons, making the identification of genuine miRNA target genes more probable.

The miRNA array identified 7 miRNAs which were differentially expressed between EBV and CD40L blasts. Further characterisation of the expression of the differentially regulated miRNAs revealed a down-regulation of miR-95 and miR-199a-3p in the latency I Mutu-BL clone relative to the EBV loss Mutu-BL clone. This is an interesting result because latency I BL cell lines are more resistant to apoptosis than the EBV loss clones [213, 330], although extensive gene expression profiling of EBV positive and EBV loss BL clones could not identify any cellular gene expression changes common to all BL clones (unpublished results, Boyce, A). The down-regulation of miR-95 and miR-199a-3p in EBV positive clones could be confirmed on a greater number of BL cell lines. If miR-95 and miR-199a-3p are down-regulated in EBV positive BL clones, this would provide a novel opportunity to identify genes which confer the apoptosis resistant phenotype to EBV positive clones, which are perhaps

modulated post-transcriptionally. To test the effect of these miRNAs on the proliferation and apoptosis sensitivity of BL cell lines, a lentivirus system could be used to re-express the miRNAs in EBV positive BL clones. Similarly, miRNA function could be inhibited in the EBV loss BL clones following transduction with a lentivirus expressing a miRNA sponge [719].

(c) EBV-mediated down-regulation of tumour suppressor genes

Cellular gene expression profiling of CD40L and EBV blasts identified 61 genes differentially expressed between the two types of blast. Perhaps unsurprisingly, data mining revealed that 13 of these genes were associated with the immune response, such as interferon stimulated genes and known antiviral genes. This suggests that a significant number of the genes which are differentially expressed between EBV and CD40L may not be aiding transformation but simply reflecting the host antiviral response triggered by EBV infection. Consistent with this observation, the differentially regulated miRNA, miR-199a-3p, has recently been identified as a broadly antiviral miRNA [686]. The modulation of approximately half of the differentially regulated genes following infection with a Δgp42 virus may also be partially explained by the fact that some of the antiviral genes are responding to virus binding. Nevertheless, the gene expression changes induced by virus binding alone are maintained in an LCL, indicating that they could still be involved in EBV-mediated growth transformation. Signalling through CD21 has been shown to induce cellular gene expression changes in B cells, including NF-κB activation and IL6 production [710, 713], which may account for some of the gene expression changes observed following virus binding. However, NF-κB is also activated by CD40 signalling [720], therefore NF-κB activation alone cannot account for the gene expression changes induced by EBV binding. The role of virus binding could be investigated using agonistic CD21 antibodies which cause

signalling through CD21. Additionally, it would be interesting to determine whether the same gene expression changes, such as tumour suppressor gene (TSG) down-regulation, could be induced through CD21 stimulation of CD40L blasts, and what effect this would have on proliferation and cell survival.

Three of the six genes specifically down-regulated following EBV infection and not CD40L stimulation of resting B cells were candidate TSGs. The function of the three candidate TSGs down-regulated by EBV will continue to be investigated using the tetracycline inducible lentivirus system. However, for reasons already articulated, future work will not focus exclusively on the differentially regulated genes and will aim to incorporate the information from the global gene expression changes observed following EBV infection. For example, the expression and function of the candidate tumour suppressor gene NR4A1, which is down-regulated following both EBV infection and CD40L stimulation, will be investigated in conjunction with NR4A3, as the down-regulation of both of these genes has been reported to be required for tumourogenesis [690].

Both NR4A1 and NR4A3 are members of the NR4A family of orphan nuclear receptors [695]. Analysis of the crystal structure of another member of the NR4A family, NR4A2, revealed that the ligand binding domain of NR4A2 was unlikely to bind ligand, indicating that the NR4A family of receptors may act as real orphan receptors and function independently of ligand binding [721]. The function of the NR4A receptors is believed to be mediated, at least in-part, via binding to DNA sequences up-stream of gene transcriptional start sites. Cloning of NR4A3 revealed that its DNA and ligand binding domains contain amino acid sequences which are homologous to those of NR4A1, suggesting that these two receptors may function in a similar manner [722]. This is supported by the observation that transgenic mice with

mutations in either NR4A1 or NR4A3 show no phenotype, however, double knockout mice develop AML, suggesting a redundancy in function between these two receptors [690, 723].

It has been demonstrated that NR4A1 and NR4A3 both translocate from the nucleus to the mitochondria following stimulation with ionomycin to induce apoptosis [724]. Additionally, over-expression of NR4A1 or NR4A3 causes apoptosis of T cells, suggesting that these two receptors modulate the apoptosis pathways in T cells [723]. The mechanism by which NR4A1 and NR4A3 induce apoptosis in T cells was shown to involve the translocation of these two receptors to the mitochondria where they associate with Bcl2, causing exposure of the Bcl2-BH3 domain, converting Bcl2 from an anti-apoptotic protein to a pro-apoptotic protein [696, 697, 725]. Bim is an antagonist of Bcl2 [726] and it has been hypothesised that NR4A1 and NR4A3 both function in conjunction with Bim to modulate apoptosis in T cells undergoing negative selection *in vivo* [696].

The role of NR4A3 and NR4A1 down-regulation by EBV could be hypothesised to provide an additional mechanism exploited by the virus to evade apoptosis, which may even compliment the down-regulation of Bim observed in EBV transformed cells. LMP1 has been shown to induce Bcl-2 expression during transformation [727, 728] and EBV selectively down-regulates Bim, an antagonist of Bcl-2, suggesting that maintaining Bcl-2 expression is important for EBV transformation. If both NR4A1 and NR4A3 are able to convert Bcl-2 to a pro-apoptotic gene in B cells then it would be reasonable to predict that the down-regualtion of these two genes is also important for transformation, to maintain expression of functional, anti-apoptotic Bcl-2 (figure 6.2). In additional to the transcriptional down-regulation of NR4A3 and NR4A1 we have demonstrated following EBV infection, it has been reported that EBNA2 can directly bind NR4A1 in fibroblast and kidney cell lines, preventing NR4A1 nuclear export to the mitochondria and consequently NR4A1 mediated apoptosis [698]. It

would be interesting to determine whether EBNA2 also binds NR4A3 and whether EBNA2 sequestration of both genes occurs in B cells. The sequestration of NR4A1 by EBNA2 suggests that loss of function and expression of NR4A1 and NR4A3 may be an important strategy employed by EBV to inhibit apoptosis during B cell transformation. The re-expression of NR4A1 and NR4A3 would therefore be hypothesised to increase the sensitivity of EBV blasts to apoptosis, and this will be investigated using lentiviral expression vectors.

As lentiviral infection of resting B cells and CD40L blasts is currently very difficult with the common vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped lentiviruses which we have used to date [729-731], we have obtained a recently described measles glycoprotein pseudotyped lentivirus packaging system which has been reported to enable the successful transduction of resting B cells and CD40L generated B cell blasts [732]. This will facilitate an investigation into the requirement for TSG down-regulation during B cell transformation. B cells could be transduced prior to EBV infection, allowing the transformation efficiency of TSG expressing (GFP positive) B cells to be compared with non-transduced B cells. Furthermore, this would enable the TSG expression to be knocked-down in CD40L blasts, following transduction with a siRNA containing lentivirus; allowing the function of the candidate TSGs to be comprehensively investigated in B cells.

In summary, we have shown that EBV modulates cellular gene expression during B cell transformation. We have identified a subset of miRNAs and genes which are differentially expressed between EBV and CD40L blasts, which may represent genes and miRNAs which are specific, and therefore important, to B cell transformation. These include miRNAs which are specifically down-regulated in EBV positive BL cell lines relative to EBV loss clones, and 10 miRNAs which are up-regulated during transformation and which are all predictd to negatively regulate Bim expression. In addition, we identified three candidate TSGs which are

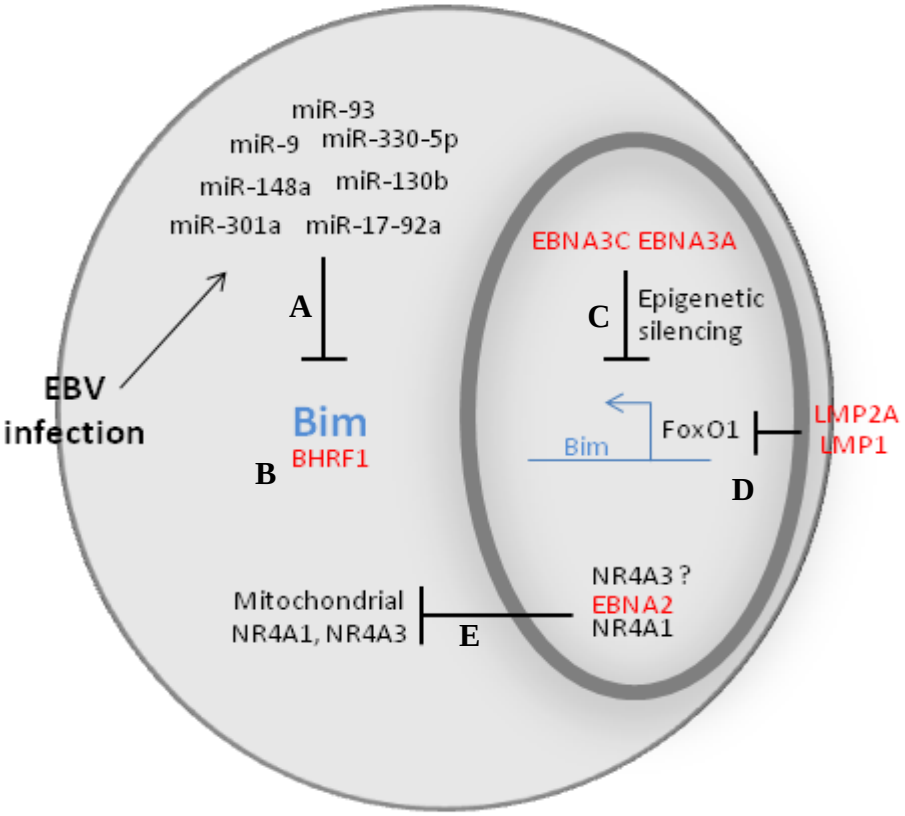

Figure 6.1: EBV modulation of the pro-apoptotic Bim and NR4A proteins during B cell transformation. A cartoon representing the various methods employed by EBV to modulate the expression and function of Bim, NR4A1 and NR4A3 following EBV infection of B cells. Bim is an antagonist of the pro-survival protein Bcl2 whereas NR4A1 and NR4A3 have been shown to convert Bcl2 from a pro-apoptotic to an anti-apoptotic protein, thus, representing two possible strategies employed by EBV to enhance cell survival during B cell transformation. **(A)** Our own data has shown that EBV infection of B cells results in the up-regulation of 10 miRNAs which are predicted to post-transcriptionally repress Bim expression, providing another possible mechanism by which Bim expression is controlled by EBV during transformation. **(B)** It has also been reported that the viral Bcl2 homolog, BHRF1, can bind to and sequester Bim protein, inhibiting the apoptotic function of Bim [733]. **(C)** EBNA3A and EBNA3C have been shown to inhibit the transcription of Bim [699] and this down-regulation may be partially mediated via increased histone methylation at the Bim promoter during transformation, which in late passage LCLs is replaced by CpG island methylation of the Bim promoter [734]. **(D)** LMP1 and LMP2A have been shown to repress the forkhead box class O1 (FoxO1) transcription factor which is known to activate the transcription of Bim [735, 736], providing another mechanism by which EBV may down-regulate the expression of Bim during transformation. **(E)** We have demonstrated that the pro-apoptotic proteins NR4A1/3 are both down-regualted following EBV infection of B cells. The pro-apoptotic function of NR4A1/3 in T cell requires their nuclear export to the mitochondria where they bind to Bcl2 and convert it to a pro-apoptotic protein. Thus, NR4A1/3 may also function in the induction of apoptosis in B cells. EBNA2 has been reported to bind to NR4A1 preventing its export to the mitochondria, providing an additional meacnism by which EBV may negatively regulate the expression and function of these two pro-apoptotic receptors during transformation.

down-regulated following EBV infection. In addition, we have also demonstrated that virus binding alone induces a significant number of gene expression changes in resting B cells, the majority of which are maintained in LCLs, suggesting a possible mechanism where EBV stimulates some of the gene expression changes necessary for transformation prior to entering the cell.

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