Differential expression of microRNAs in Marek's disease virus-transformed T-lymphoma cell lines

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MicroRNAs (miRNAs) are increasingly recognized to play crucial roles in regulation of gene expression in different biological events, including many sporadic forms of cancer. However, despite the involvement of several viruses in inducing cancer, only a limited number of studies have been carried out to examine the miRNA expression signatures in virus-induced neoplasia, particularly in herpesvirus-induced tumours where virus-encoded miRNAs also contribute significantly to the miRNome of the tumour cell. Marek’s disease (MD) is a naturally occurring, rapid-onset CD4⁺ T-cell lymphoma of poultry, induced by the highly contagious Marek’s disease virus (MDV). High levels of expression of virus-encoded miRNAs and altered expression of several host-encoded miRNAs were demonstrated in the MDV-transformed lymphoblastoid cell line MSB-1. In order to identify the miRNA expression signature specific to MDV-transformed cells, we examined the global miRNA expression profiles in seven distinct MDV-transformed cell lines by microarray analysis. This study revealed that, in addition to the high levels of MDV-encoded miRNAs, these MD tumour-derived lymphoblastoid cell lines showed altered expression of several host-encoded miRNAs. Comparison of the miRNA expression profiles of these cell lines with the MDV-negative, retrovirus-transformed AVOL-1 cell line showed that miR-150 and miR-223 are downregulated irrespective of the viral aetiology, whereas downregulation of miR-155 was specific for MDV-transformed tumour cells. Thus, increased expression of MDV-encoded miRNAs with specific downregulation of miR-155 can be considered as unique expression signatures for MD tumour cells. Analysis of the functional targets of these miRNAs would contribute to the understanding of the molecular pathways of MD oncogenicity.

INTRODUCTION

MicroRNAs (miRNAs) are small (approx. 22–25 nt long), non-coding RNAs that regulate gene expression by base pairing with the RNA transcripts, targeting them for translational repression or degradation. All metazoan genomes encode miRNAs and the latest release (12.0) of miRBase (http://microrna.sanger.ac.uk) contains 8619 hairpin precursor miRNAs in various species (Griffiths-Jones et al., 2008). In the past few years, there have been huge increases in the number of studies on miRNA and cancer. Profiling of global miRNA expression levels (miRNome) has generated extensive data on miRNA expression in various forms of cancer. These studies have reiterated the important role of miRNAs in all aspects of cancer biology, including proliferation, apoptosis, invasion/metastasis and angiogenesis (Fabbri et al., 2008; Lee & Dutta, 2009). Such studies have also provided information on the developmental lineage, differentiation state and prognosis of malignant cells (Lowery et al., 2008; Schotte et al., 2008). Nearly all of these studies have been carried out on non-infectious forms of cancer. Current estimates suggest that viruses are involved in 15–20% of human cancers worldwide (Javier & Butel, 2008) and oncogenic viruses have been instrumental in delineating several molecular pathways of neoplastic transformation. Despite this, comparatively little is known on global miRNA expression profiles of virus-induced cancers (Martinez et al., 2008; Yeung et al., 2008). In many tumours, particularly those associated with oncogenic herpesviruses (Cosmopoulos et al., 2008; Cullen, 2006; Gottwein & Cullen, 2008; Pfeffer et al., 2005; Sullivan & Grundhoff, 2007), high levels of expression of virus-encoded miRNAs...
add further complexity to the miRNome of the transformed cell (Ghosh et al., 2008).

Marek’s disease virus (MDV) is a highly contagious, oncogenic alphaherpesvirus of the genus Marevirinae; it is associated with Marek’s disease (MD), a naturally occurring, rapid-onset T-cell lymphoma of chicken (Calnek, 1986). The MDV genome encodes several miRNAs that map to the MDV-encoded oncogene Meq and the LAT (latency-associated transcript) regions of the virus (Burnside et al., 2006; Burnside & Morgan, 2007). Although the target genes regulated by MDV-encoded miRNAs are yet to be discovered, high levels of expression in MDV-transformed cell lines and tumours suggest that they play important roles in oncogenesis (Morgan et al., 2008; Xu et al., 2008). From a small RNA library generated from MDV-transformed lymphoblastoid cell line MSB-1 (Akiyama & Kato, 1974), we have previously demonstrated high levels of expression of MDV-encoded miRNAs (Yao et al., 2007, 2008). Elevated levels of expression of some of these miRNAs were also confirmed by real-time quantitative PCR in these cells (Xu et al., 2008). Several host miRNAs that are associated directly with oncogenicity, such as miR-17-92, miR-21 and let-7i, were also present at a high frequency in the MSB-1 library, suggesting a role for these miRNAs in neoplastic transformation of these cells (Yao et al., 2008). Although analysis of miRNAs by cloning (Yao et al., 2008) or high-throughput sequencing (Burnside et al., 2008) is used to identify upregulated miRNAs in tumours or transformed cells, such studies do not provide differential expression profiles of miRNAs in different cell types. Comparisons of miRNA expression profiles between neoplastically transformed and normal cells using miRNA microarrays have enabled the identification of specific miRNA expression signatures in different types of cancer cell, some of which have been shown to be useful indicators of cell type, stage of differentiation and even prognosis of the cancer (Calin & Croce, 2006; Rosenfeld et al., 2008). Only a limited number of studies comparing the global miRNA expression profiles of virally transformed tumour cells have been carried out, particularly in tumour cells transformed by oncogenic herpesviruses that themselves encode multiple miRNAs (Sullivan & Grundhoff, 2007). Here we describe the results of the comparison of the miRNA expression profile of seven different MDV-transformed T-lymphoblastoid cell lines with that of normal chicken splenocyte or CD4+ T-cell populations (Petherbridge et al., 2004). Reticuloendotheliosis virus T (REV-T strain)-transformed CD4+ T-cell line AVOL-1 (Yao et al., 2008) and avian leukosis virus (ALV) HPRS F42 strain-transformed B-cell line HP45 (Nazerian, 1987) were included in the experiments as MDV-negative transformed cell lines. Cell lines were grown at 38.5 °C in 5% CO2 in RPMI 1640 medium containing 10% fetal calf serum, 10% tryptose phosphate broth and 1% sodium pyruvate.

**Chicken splenocytes, CD4+ T cells and magnetic cell sorting.** Single-cell suspensions of lymphocytes were prepared from spleen tissues of uninfected birds by using Histopaque-1083 (Sigma-Aldrich) density-gradient centrifugation. CD4+ T cells were isolated by magnetic cell sorting using mouse anti-chicken CD4 antibodies (Chan et al., 1988) and goat anti-mouse IgG microbeads (Miltenyi Biotec). After each antibody treatment, cells were washed three times with PBS containing 0.5% bovine serum albumin. At each wash, the cell suspension was centrifuged at 450 g for 10 min. Positively stained cells were sorted through an AutoMACS Pro Separator (Miltenyi Biotec). Purity of the sorted cells was confirmed to be >99% by flow cytometry after labelling with monoclonal anti-goat/sheep IgG-fluorescein isothiocyanate (Sigma) antibody (data not shown).

**Microarray analysis of miRNA expression.** Preparation of probes and hybridization to the arrays were carried out by using methods described previously (Lawrie et al., 2007, 2008). Briefly, 500 ng purified miRNA from lymphoblastoid cell lines, normal splenocytes or CD4+ T-cell populations was labelled with either Cy3 or Cy5 dye using an Array 900microRNA RT kit from Genisphere and hybridized to the miRNA microarray described previously (Lawrie et al., 2008). The array contains miRNA probe sets (designed from miRBase v. 9.2) spotted in quadruplicate non-adjacently (the sequences of the probes are available at http://www.microRNAworld.com). Where the homologous sequences of miRNAs in different species are identical, miRNA sequences from only one species was spotted on the array. In addition, probes for the mature MDV-1-encoded miRNAs miR-M2-3p, miR-M2-5p, miR-M3-3p, miR-M3-5p, miR-M4-3p, miR-M4-5p, miR-M5-3p, miR-M5-1p and miR-M12-3p (Yao et al., 2008) were also included on the array. A model design with splenocytes and/or CD4+ T cells as reference was used and the expression values are depicted as log ratios of test and reference samples. Image analysis was done by using BlueFuse software (BlueGnome).

**Statistical analysis of microarray data.** Data were normalized within each microarray by subtracting the mean log2 ratio from each measurement. Quantile normalization was then used to standardize the data across arrays, and a linear model was fitted to each miRNA using Limma (Smyth, 2005). The resultant P-values were adjusted for multiple testing by using the Benjamini–Hochberg correction of the false-discovery rate (Benjamini & Hochberg, 1995). The P-values and mean expression were calculated for each cell type. Those miRNAs showing consistent differential expression across all cell types were subjected to a second analysis where all samples were treated as ‘virus-infected’, regardless of cell type. A similar analysis in Limma was performed, resulting in P-values calculated for expression across all cell types. The data were sorted according to the log2 ratio and heat maps were produced by using R (http://www.R-project.org).

**Inducible expression of mature miRNAs.** Constructs for inducible expression of miRNAs were generated in the pRTS-1-SVP-Tom(−) vector, constructed from the pRTS-1 plasmid (Bornkamm et al., 2005) by replacing the hygromycin B-resistance gene with a puromycin-resistance gene. The inducible bidirectional promoter in this construct expresses monomeric red fluorescent protein td-tomato (Shaner et al., 2004) and the miRNA of interest simultaneously in a tightly regulated, doxycycline (Dox)-inducible system. The inserted sequence of each miRNA consists of the stem-loop structure and 100–200 bp of upstream and downstream flanking genome sequences, a feature
designed to ensure that the expressed miRNAs are processed as naturally as possible. The approximately 500 bp fragment of each miRNA was obtained by RT-PCR using RNA extracted from chicken embryo fibroblasts by using the following primers: gga-miR-155 (5’- AGATCTCTGATGTCGTACCTTTATGAC-3’ and 5’-CTCGAGGGC- GATTGCGCTTTAACTTAG-3’); gga-miR-223 (5’-AGATCTGCAGA- CGTCTGTCTTGCTTC-3’ and 5’-CTCGAGGAGAATCTTACGACC- AGCAC-3’). As the sequence of the chicken orthologue was not available, we used hsa-miR-150 for generating the expression constructs of miR-150, using RNA prepared from HEK293T cells with primers 5’- AGATCTCTTGGCTCTTGGAT-3’ and 5’-CTCGAGGAGACCTTTACGACA- AGCAC-3’). More recently, the mature gga-miR-150 sequence was shown to be identical to the hsa-miR-150 sequence except for a single nucleotide substitution (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL6541). Agarose gel-purified PCR fragments were cloned into the BglII and XhoI sites of the pRTS-1-SVP-Tom(–) vector and confirmed by sequence analysis. For the expression of cloned miRNAs, HEK293T cells were transfected with the pRTS-1-miRNA constructs by using Lipofectamine 2000 (Invitrogen) and stable cells were selected with puromycin (1 μg ml−1). Respective miRNAs induced with the addition of Dox (1 μg ml−1) were detected by Northern blotting analysis.

Luciferase reporter assays. In order to demonstrate that the Dox-induced miRNAs in stably transfected HEK293T cells are functional, we carried out reporter assays on one of the stable cell lines expressing gga-miR-155 by analysing its effect on the previously characterized target Pu.1. For this, we generated a reporter construct in which a 110 bp fragment of the chicken Pu.1 3′ untranslated region (UTR) transcript (GenBank accession no. NM_205023) that contained the predicted miR-155-response element (MRE) is inserted downstream of Renilla luciferase in the psiCHECK-2 vector (Promega) to generate the Pu.1-3′UTR-wt construct. A mutant construct (Pu.1-3′UTR-mt) with mutations in the MRE sequences was also constructed. The details of the oligonucleotides used for generating the chicken Pu.1-3′UTR reporter constructs are shown elsewhere (Zhao et al., 2009). HEK293T cells stably expressing gga-miR-223 were used as a control. Transfection into HEK293T cells for luciferase reporter assays was carried out in 96-well plates with Lipofectamine 2000 (Invitrogen). Firefly and Renilla luciferase activities were measured consecutively with the Dual-Luciferase Reporter Assay system (Promega), using a Luc-Pert II luminoimeter (Anthos Labtec). In all cases, the constitutively expressed firefly luciferase activity in the psiCHECK-2 vector served as a normalization control for transfection efficiency.

Preparation of RNA for Northern blotting. Preparation of RNA for Northern blotting analysis from splenocytes, CD4+ and CD8+ populations of T lymphocytes, MDV- and retrovirus-transformed cell lines, and HEK293T cells stably selected for recombinant pRTS-1 vectors expressing miRNAs was carried out by using standard methods as described previously (Yao et al., 2007, 2008). Antisense oligonucleotides to the miRNAs gga-miR-155, gga-miR-223 and hsa-miR-150 were used as probes.

RESULTS

In order to determine the miRNA expression signature in MDV-induced tumours, we compared the global gene expression profiles of seven MDV-transformed cell lines by microarray analysis using miRNA probe sets designed from miRBase v. 9.2. The tests validating the sensitivity, specificity and reproducibility of these arrays have been described previously (Lawrie et al., 2008). Ratios of miRNA expression levels in transformed cell lines normalized to the reference samples of normal chicken splenocytes or CD4+ cells were used for the analysis. These studies showed that the grouping of miRNAs in the seven MDV-1-transformed cell lines was distinct from that in the MDV-1-negative lymphocyte cell line AVOL-1 when splenocytes were used as a reference (Fig. 1a). When purified CD4+ T cells were used as a reference, the expression profiles of miRNAs in four of these cell lines (Fig. 1b) were largely in agreement with those obtained by using reference splenocytes (detailed data on the log, fold changes and the P-values for each of the cell lines are provided in Supplementary Tables S1 and S2, available in JGV Online). The miRNA profiles of the cell lines in both of these analyses were generally consistent, although individual cell lines did show differences (Supplementary Figs S1 and S2).

MDV-1-encoded miRNAs are upregulated in transformed cell lines

The inclusion of mature probe sequences of nine MDV-1-encoded miRNA sequences (Yao et al., 2008) alongside the host miRNA probe sets in the miRNA microarray enabled assessment of the levels of expression of virus- and host-encoded miRNAs in these cell lines. Compared with the MDV-negative REV-T-transformed cell line AVOL-1, all of the MDV-transformed cell lines showed upregulation of MDV-1-encoded miRNAs (Fig. 1a), although the expression levels of individual miRNAs were not uniform in these cells.

Changes in host miRNA profiles in MD tumour cell lines

Examination of the global miRNA expression profiles of the seven MDV-1-transformed cell lines revealed changes in several host miRNAs (Fig. 1). Major differences in the host miRNA expression profiles could also be observed between MDV-1-transformed cell lines and the MDV-negative cell line AVOL-1, demonstrating the differences in the molecular oncogenic pathways between these cell lines. Microarray readouts from our studies did demonstrate differences between cell lines with regard to the expression of individual miRNAs. Although such differences could be important for individual cell lines, our main interest was to look for miRNA profiles that are conserved in all MDV cell lines, as this could give insights into the fundamental molecular pathways of miRNA-mediated gene regulation in MDV transformation. Our results showed that several host-encoded miRNAs, such as miR-155, miR-223, miR-150, miR-451, miR-26a and miR-126, were downregulated in all MDV-transformed cell lines relative to the levels in normal splenocytes or CD4+ T cells (Fig. 1). As miR-223 and miR-150 were also downregulated in retrovirus-transformed AVOL-1 cells, the reduced expression of these two miRNAs is thought to be a broader feature of transformed T cells, irrespective of the viral aetiology. However, this was not the case with miR-155, the levels of which were consistently reduced in all of the seven
MDV-1-transformed lymphoblastoid cell lines whilst the expression levels in the AVOL-1 cell line were very high (Fig. 1a), demonstrating that the downregulation of miR-155 is a feature unique to MDV transformation of T cells.

As all of the MDV-1-transformed cell lines used in this study have a CD4+ T-cell phenotype, we also wondered whether it is appropriate to use whole splenocyte populations as the reference sample in the analysis. In order to rule out the possibility that the altered expression profiles of miRNAs in the MDV-transformed cell lines are not due to the use of splenocytes as the reference, we also repeated the analysis of miRNA expression in four of the above MDV-transformed cell lines with purified CD4+ T cells as the reference. Overall, the results were largely consistent with the values obtained by using normal splenocytes as the reference (Fig. 1b). However, the use of purified CD4+ cells as the reference resulted in the demonstration of increased expression of several more host miRNAs, such as miR-146b, miR-454, miR-7b, miR-34a, miR-18a, miR-133a, miR-29a and gga-1et-7d (Fig. 1b). As the splenocyte reference data represent the miRNA expression of multiple

**Fig. 1.** Heat maps showing clustering of differentially expressed miRNAs (adjusted $P<0.05$) in MDV-transformed cell lines. (a) miRNAs identified in the seven MDV-transformed cell lines (names of cell lines are shown below each lane) in comparison to those in the MDV-1-negative REV-transformed AVOL-1 cell line. The data shown are normalized by using uninfected splenocytes as the reference. (b) Heat map showing differentially expressed miRNAs in four of the above MDV-transformed cell lines (shown below each lane), normalized against expression in normal CD4+ T cells as the reference. A colour key indicating low (green) to high (red) values and the $P$-values is also shown.
lymphocyte populations, it was not surprising to see changes in the miRNA expression patterns when purified CD4⁺ T cells were used as the reference. Data on the log₂ fold changes and P-values for each cell line using splenocytes or purified CD4⁺ T cells as the reference can be seen in Supplementary Tables S1 and S2, respectively.

**Analysis of miRNA expression by Northern blotting**

For further validation of the microarray data demonstrating the reduced expression of miR-155, miR-223 and miR-150 in MDV-transformed tumour cell lines, we carried out Northern blotting analysis comparing the levels of these miRNAs in four MDV-transformed cell lines with those in normal lymphocyte populations and retrovirus-transformed cell lines AVOL-1 and HP45. Northern blotting analysis confirmed the observations of reduced expression of these three miRNAs in the microarray readouts of MDV-transformed cells. The levels of gga-miR-155 signal detected were very low in the normal splenocyte and CD4⁺/CD4⁻ T-cell populations (Fig. 2a). These low levels were reduced further in all MDV-transformed cell lines included in the assay. In contrast, stronger gga-miR-155 signals were evident in avian retrovirus-transformed cell lines HP45 and AVOL-1. The levels of gga-miR-223 were lower in normal CD4⁺ T cells than in whole spleen-cell or CD4⁻ T-cell populations. However, no hybridization signals for gga-miR-223 expression were evident in any of the cell lines transformed by either MDV-1 or avian retroviruses, suggesting that the downregulation of gga-miR-223 is a broader feature of lymphocyte transformation, irrespective of the viral aetiology. The expression of miR-150 also appeared to be restricted to the untransformed cells, as there was no evidence of its expression in transformed cells. Although miR-150 and miR-223 expression appeared to be similar in this respect, the levels of miR-150 were much higher in CD4⁺ T cells (Fig. 2a). We also evaluated the Dox-inducible expression of miR-155, miR-223 and miR-150 from the pRTS-1 vector (Bornkamm et al., 2005). Northern blotting analysis of HEK293T cells expressing the pRTS-1–miRNA constructs showed high levels of expression of each of the three mature miRNAs, regulated tightly in a Dox-inducible manner (Fig. 2b).

For functional evaluation of the efficacy of this inducible expression system for identifying potential miRNA targets, we examined the ability of the pRTS-1–miR-155 expression vector to silence the reporter construct containing the wild-type or the mutant MRE region of the 3' UTR of Pu.1, a validated target of miR-155 (Zhao et al., 2009). This assay showed that the relative Renilla luciferase levels of reporter constructs with wild-type MRE sequences were reduced specifically by nearly 60 % compared with the mutant MRE construct (Fig. 3a). This reduction in luciferase levels was dependent on the induction of miR-155 in these cells by Dox treatment. The specificity of the reporter assay was demonstrated further by the absence of reduction in luciferase levels in cells expressing gga-miR-223 (Fig. 3a). The tightly regulated nature of the Dox-inducible expression system used here was demonstrated by the non-leaky expression of the td-tomato marker gene in the untreated (Dox⁻) cells (Fig. 3b).

**DISCUSSION**

Global changes in miRNA expression profiles using microarray analysis are used increasingly to identify specific miRNA expression signatures associated with several human malignancies (Calin & Croce, 2006, 2007; Lawrie et al., 2008). Most of these studies have been carried out on tumour tissues or cell lines derived from various sporadic forms of cancer (Ozen et al., 2008; Ruike et al., 2008). These studies have highlighted the direct oncogenic potential of the cluster of miRNAs such as miR-21, miR-155 and miR-17-92, providing valuable insights into the
molecular pathways of oncogenesis (Wiemer, 2007). Oncogenic viruses account for a large proportion of neoplasms in man and animals (Javier & Butel, 2008).

Although the induction of many of these tumours has until recently been attributed mainly to virus-encoded oncoproteins, an increasing amount of data indicates that miRNAs, encoded either by the host or by viruses themselves in the case of oncogenic herpesviruses (Cullen, 2006, 2009; Pfeffer et al., 2004, 2005; Sullivan & Grundhoff, 2007), play significant roles in oncogenesis.

We and others have documented recently that the highly oncogenic MDV-1 encodes several novel miRNAs (Burnside & Morgan, 2007; Burnside et al., 2008; Morgan et al., 2008; Yao et al., 2008). High levels of expression of these miRNAs in lymphomas and transformed cell lines have been demonstrated by direct cloning, Northern blotting and quantitative RT-PCR (Burnside et al., 2006; Xu et al., 2008; Yao et al., 2008). Although these studies have been valuable in identifying miRNAs that are expressed at high levels in these cells, they do not always provide comprehensive miRNA expression profiles, particularly of those miRNAs that are downregulated in the transformed cells. With a view to examining the global expression of miRNAs in MDV-transformed cells, we carried out miRNA expression profiling of seven independent MDV-transformed tumour cell lines by using miRNA microarray analysis. Each of these cell lines was derived from an independent MD lymphoma. As demonstrated previously with MSB-1 cells (Yao et al., 2007, 2008), MDV-1-encoded miRNAs were indeed the most abundant miRNAs in all of the cell lines. High-level expression of virus-encoded miRNAs appears to be a feature common to virus-transformed cell lines, as cells transformed by other oncogenic herpesviruses, such as Kaposi’s sarcoma-associated herpesvirus (KSHV) and Epstein–Barr virus, also showed high levels of expression of virus-encoded miRNAs (Cai et al., 2005; Lawrie et al., 2008; Pratt et al., 2009). Higher copy numbers of viral genomes and active transcription of miRNA genes may account for the higher expression of virus-encoded miRNAs in the virus-transformed cell lines, although one cannot rule out the possibility of differential processing of virus-encoded miRNAs in these cells. The functions and putative targets of most of the MDV-encoded miRNAs remain unknown. However, we have shown recently that MDV-miR-M4, one of the most abundantly expressed virus-encoded miRNAs in all of the cell lines, is a functional orthologue of miR-155 with the potential to target important lymphocyte-specific transcription factors such as Pu.1 (Zhao et al., 2009). More efforts in the future to identify the potential targets of other MDV-encoded miRNAs (Morgan et al., 2008) will unravel more molecular pathways of oncogenesis.

Microarray data analysis of the changes in the global expression profiles of host-encoded miRNAs in MDV-transformed cell lines could be grouped into (i) those that are restricted only to some of the MDV-transformed cell lines and (ii) those that appear to be conserved across all of the cell lines. The changes in miRNA expression in individual cell lines, such as the increased expression of miR-221/miR-222 in MSB-1 cells (Lambeth et al., 2009),

![Fig. 3. Functional analysis of inducible miR-155 by using a reporter assay on the Pu.1 3’ UTR. (a) Reporter assays in HEK293T cells expressing gga-miR-155 and gga-miR-223 from the Dox-inducible promoter in the pRTS-1 vector. Relative Renilla luciferase levels (RL) of Dox+ and Dox- HEK293T cells transfected with luciferase reporter constructs containing the wild-type (Pu.1-wt) or mutant (Pu.1-mt) MRE-containing region of the 3’ UTR of the chicken Pu.1 transcript (Zhao et al., 2009) are shown. (b) Fluorescence microscopic image of td-tomato expression in Dox+ (top left) and Dox- (top right) HEK293T cells stably expressing pRTS-1–gga-miR-155. Bright-field images of the two cell populations are also shown (bottom left and right).](image-url)
are likely to be important in the regulation of respective target proteins in individual cell lines. However, in this paper, we focus on the global changes in miRNA expression common to all MDV-transformed cell lines.

The miRNA profile of the seven MD tumour cell lines showed changes in the expression of several miRNAs. These included the downregulation of miR-150, miR-223 and miR-155, confirmed by Northern blotting analyses (Figs 1 and 2a). Of these, miR-150 and miR-223 were also downregulated in AVOL-1 cells, demonstrating it to be a broader feature of lymphocyte transformation, regardless of the viral aetiology. Reduced expression of miR-150 has also been reported in human lymphoid malignancies such as diffuse large B cell lymphoma (Garzon & Croce, 2008; Landgraf et al., 2007; Lawrie et al., 2008), indicating its conserved function across different species. Increasing evidence suggest that miR-150 functions through the transcription factor c-myb (Garcia & Frampton, 2008; Lin et al., 2008; Lu et al., 2008; Xiao et al., 2007; Zhou et al., 2007), and the dysregulation of c-myb and its targets could be important in T-cell transformation. In the case of miR-223, although all of the regulatory mechanisms are not yet understood fully, recent studies have indicated a clear role for miR-223 in haematopoiesis, as well as in malignancies (Baek et al., 2008; Garzon & Croce, 2008; Johnnidis et al., 2008; Merkerova et al., 2008). Whilst identification of the potential targets is important to understand fully the molecular pathways, involvement of miR-223 appears to be logical in MDV-induced lymphocyte transformation.

The downregulation of miR-155 observed by microarray analysis was unique to MDV-transformed cell lines, as it was upregulated in the MDV-negative AVOL-1 cell line (Fig. 1). Although the levels of miR-155 in the normal lymphocyte populations were low by Northern blotting analysis, it was clear that MDV-transformed cell lines showed a distinct reduction in hybridization signals, especially when compared with the retrovirus-transformed lymphocyte cell lines HP45 and AVOL-1 (Fig. 2a). Several recent studies have highlighted the potential multiple roles of miR-155 in functions ranging from innate immune responses to oncogenicity (Garzon & Croce, 2008). The molecular mechanisms that drive down the expression of miR-155 in MDV-transformed cell lines are not known. However, some of its functions on targets such as Pu.1 could be rescued by MDV-miR-M4, a highly expressed MDV-1-encoded functional orthologue of miR-155 (Zhao et al., 2009). Although the regulatory expression dynamics of miR-155 and MDV-miR-M4 are not understood fully, the existence of autoregulatory mechanisms of miR-155 expression mediated through a common set of targets cannot be ruled out. It is interesting that, in KSHV-infected primary effusion lymphoma cell lines, miR-155 was also found to be downregulated in favour of the KSHV-encoded miR-K12-11 homologue (Skalsky et al., 2007).

The data from this study have enabled us to characterize the miRNome of MDV-transformed tumours. Although this has provided valuable insights into the expression profiles of miRNAs in these cell lines, the major challenge will be in the identification of the putative targets of the differentially expressed miRNAs in these cells. Although bioinformatic predictions of miRNA targets are valuable, the development of systems for functional characterization of miRNA targets is important to understand the pathways of oncogenesis. The tightly regulated, Dox-inducible miRNA expression system of the differentially expressed miRNAs that we developed in HEK293T cells will be valuable in identifying the putative functional targets of these miRNAs. Demonstration of the expression of mature miRNAs in a Dox-dependent manner clearly showed the proper processing of these miRNAs in this system. For functional validation of the system, we analysed the putative targeting of miR-155 on one of the validated target proteins, Pu.1 (Zhao et al., 2009). The tightly regulated expression of miR-155 and the specific silencing of the relative luciferase levels with reporter assays with wild-type 3’ UTR reporter constructs (Fig. 3) provide a platform for functional analysis of the putative targets of differentially expressed miRNAs.

In summary, the data presented here demonstrate that miRNA expression profiling using microarrays is a powerful approach for analysing the relative levels of several miRNAs simultaneously. This study, the first of its kind in MDV-transformed cell lines, demonstrates that, in addition to the overexpression of several MDV-encoded miRNAs, downregulation of some of the host-encoded miRNAs is also a hallmark of MDV transformation. Determination of the miRNA profile is a first step towards identification of the regulatory networks of gene expression in these cell types.

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