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Activation of gga-miR-155 by reticuloendotheliosis virus T strain and its contribution to transformation

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Running title: v-rel induces gga-miR-155 through the NF-κB pathway
Abstract

The v-rel oncoprotein encoded by reticuloendotheliosis virus T strain (Rev-T) is a member of the rel/NF-κB family of transcription factors capable of transformation of primary chicken spleen and bone marrow cells. Rapid transformation of avian haematopoietic cells by v-rel occurs through a process of deregulation of multiple protein-encoding genes through its direct effect on their promoters. More recently, upregulation of oncogenic miR-155 and its precursor pre-miR-155 were demonstrated in Rev-T-infected chicken embryo fibroblast cultures as well as Rev-T-induced B-cell lymphomas. Through electrophoresis mobility shift assay and reporter analysis on gga-miR-155 promoter, we show that the v-rel-induced miR-155 overexpression occurs by the direct binding to one of the putative NF-κB binding sites. Using v-rel-induced transformation model on chicken embryonic splenocyte cultures, we could demonstrate dynamic increase in miR-155 levels during the transformation. Transcriptome profiles of lymphoid cells transformed by v-rel showed upregulation of miR-155 accompanied by downregulation of a number of putative miR-155 targets such as Pu.1 and CEBPβ. We also show that v-rel can rescue the suppression of miR-155 expression observed in Marek’s disease virus-transformed cell lines, where its functional viral homolog MDV-miR-M4 is overexpressed. Demonstration of gene expression changes affecting major molecular pathways including organismal injury and cancer in avian macrophages transfected with synthetic mature miR-155 underline its potential direct role in transformation. Our study suggests that v-rel-induced transformation involves complex set of events mediated by the direct activation of NF-κB targets together with the inhibitory effects on miRNA targets.

Keywords: v-rel, NF-κB, miR-155, transformation
Introduction

The rel/NF-κB family of transcription factors (1, 2) play a key role in the control of cell proliferation and apoptosis, two functions critical in cancer. The involvement of rel/NF-κB in malignancy is best demonstrated by the acute oncogenicity of their viral derivative, v-rel, first identified in reticuloendotheliosis virus T (Rev-T) strain (3, 4). Rev-T is an acutely transforming variant of REV, the aetiological agent of reticuloendotheliosis in birds, carrying the viral oncogene v-rel, a variant of the turkey cellular proto-oncogene c-rel (5-7). Because of the rapidity and efficiency of transformation of the cells, the v-rel provides a valuable model for studying the role of rel/NF-κB family in neoplastic transformation and cancer. The v-rel-mediated transformation occurs predominantly through the modulation of transcription of rel/NF-κB targets (8-10), the examples of which include AP-1 (11, 12), IRF-4 (13), SH3BGRL (14), TGFβ/Smad (15) and telomerase reverse transcriptase (TERT) subunit (16). More recently, repression of BLNK and BCAP proteins (17) and a novel interaction of CAPERα and the transactivating domain of v-rel (18) were shown to be important for lymphocyte transformation by the v-rel oncoprotein.

Several studies have also implicated microRNAs (miRNAs) as key mediators of a number of cell regulatory processes including the induction of cancer (19-21). Among the numerous miRNAs expressed in hematopoietic cells, miR-155 was shown to have the most wide ranging effects on the biology of lymphocytes (22-25). It is processed from a primary transcript, known as ‘Bic’ (B-cell integration cluster), whose upstream region was originally found to be a frequent site of integration of the avian leukosis virus in lymphomas (26). A number of recent miRNA profiling studies have shown elevated levels of miR-155 in a wide array of cancers including lymphomas (27-30).

In a recent study on chicken embryo fibroblast (CEF) cultures infected with reticuloendotheliosis virus (Rev) HA1101 strain, differential expression of a number of genes...
leading to changes in several signalling pathways were reported (31). We and others have shown upregulation of miR-155 in Rev-T-transformed cell lines and CEF (32, 33). For further analysis of the global changes in miRNA profiles induced by v-rel, we used an in vitro model of v-rel-induced transformation of embryonic splenocytes to demonstrate the sequential upregulation of miR-155 during the transformation process. Our studies confirm that v-rel-mediated upregulation of gga-miR-155 occurs through the direct binding to at least one of the putative NF-κB sites on the Bic/miR-155 promoter. Analysis of the gene expression changes in the v-rel-transformed cells further demonstrated downregulation of a number of known miR-155 targets potentially affecting a number of important biological pathways. Demonstration of the targeting of a number of cancer-related genes in chicken macrophages overexpressing miR-155 demonstrated the importance of this miRNA as a major regulator of v-rel-induced transformation.

**Results**

**Upregulation of miR-155 in Rev-T transformed cell lines.** During the analysis of the global changes in miRNA expression in chicken lymphocyte lines transformed by avian oncogenic viruses, we observed that miR-155 is overexpressed in v-rel-transformed chicken lymphocytes, compared to the normal spleen cells and MDV-transformed cell lines (32). For confirmation of the higher expression of miR-155 in v-rel-transformed cells, we examined Rev-T-transformed cell lines AVOL-1, AVOL-2, AVOL-3 and RIR-Rev-T cells by Northern blot analysis. An ALV transformed B-cell line HP45 was used as positive control where miR-155 is upregulated due to insertional activation and normal spleen cells which doesn’t express detectable levels of miR-155 was used as negative control. High levels of miR-155 transcripts were readily observed in all Rev-T transformed cell lines (Fig 1).
v-rel binds to the NF-κB sites in the Bic/miR-155 promoter. Having demonstrated the upregulation of miR-155 in Rev-T transformed cells, we examined the potential mechanisms of miR-155 overexpression by v-rel. Analysis of the chicken Bic/miR-155 promoter sequence for potential transcription factor binding sites using the program TFSEARCH (34) identified a number of transcription factor binding sites, including two putative NF-κB sites (NF-κB1 & NF-κB2) located at positions -581 and -66 respectively (relative to the transcription start site).

In order to establish that v-rel binds directly to the putative NF-κB sites in the Bic/miR-155 promoter, electrophoresis mobility shift assay was carried out using recombinant GST-v-rel fusion protein. Briefly, purified GST-v-rel protein was incubated with dsDNA oligonucleotides probe spanning the two putative NF-κB sites. The intense shifted bands were observed with incubation of GST-v-rel and wild type labelled probes for both sites (lane 2, Fig 2A). The bands are competed by an excess of cold competitor (lane 3, Fig 2A), but not the same amount of a mutant competitor that is not bound by v-rel protein (lane 4, Fig 2A).

NF-κB site 2 in Bic/miR-155 promoter is required for miR-155 activation. Having demonstrated the direct binding of v-rel to the NF-κB sites, we next examined the possible contribution of these elements in mediating Bic regulation. To this end, we carried out reporter assays to examine the ability of v-rel to drive the expression of renilla luciferase reporter gene using constructs containing the wild type or the mutant chicken Bic/miR-155 promoter. For this, the chicken Bic/miR-155 promoter region extending from -1829 to +3 nucleotides from transcription start site (+1) was cloned upstream renilla luciferase gene of psiCHECK™-2 vector (Promega) to replace the SV40 promoter generating the reporter construct pBic-WT. Mutagenesis of the two NF-κB sites was carried out by overlapping PCR generating pBic-M1, pBic-M2 and pBic-M1M2 constructs, where the NF-κB1, NF-κB2 or both sites respectively, were mutated (Fig 2B). For the reporter assay, each of the reporter and pcDNA3-v-rel constructs were co-transfected into DF-1 cells and the luciferase
expression was assayed 48 hours later using the Dual-Glo Luciferase Assay System (Promega) following manufacturer’s instructions. As shown in Fig 2C, mutation of the first NF-κB site (pBic-M1) did not show obvious changes in the luciferase levels compared to the wild type promoter (pBic-WT) construct. In contrast, mutation of the second NF-κB site (pBic-M2) decreased the promoter activity by 63% compared to that of the pBic-WT, suggesting that the v-rel-mediated transactivation occurs mainly through this NF-κB site. The promoter activity of double mutant pBic-M1M2 construct was similar to that of pBic-M2 further confirming that the second NF-κB site in the Bic/miR-155 promoter is important for the v-rel-mediated upregulation of miR-155.

v-rel relieves the inhibition of miR-155 expression in MSB-1 cells. We have previously shown that miR-155 is consistently downregulated in MDV-transformed tumours and cell lines (32). Although the mechanisms for this downregulation are not known, this could be due to the complementation of miR-155 functions by the high levels of the viral homolog MDV-miR-M4 expressed in these cells. We wanted to examine whether the downregulation of miR-155 in MDV transformed cell lines can be rescued by expressing v-rel in these cells. RCAS(A)-v-rel-GFP virus stocks were used for transduction of v-rel into MSB-1 and 265L, where the GFP marker allowed sorting of the infected cells. Analysis of the sorted cells by Western blotting showed expression of v-rel-GFP in both infected MSB-1 and 265L but not in uninfected cells (Fig 3A). Expression of v-rel increased the level of miR-155 expression by approximately 700-fold in MSB-1 cells and by about 900-fold in 265L cells which is much higher than the miR-155 level in untransformed CD4+ cells (Fig 3B), demonstrating that ectopic expression of v-rel can induce expression of miR-155 in avian lymphoid cells.

Induction of miR-155 is accompanied by downregulation potential targets. For further analysis of the dynamic global changes in miRNA profiles during v-rel-induced transformation, we examined the changes in RCAS(A)-v-rel-infected chicken embryonic
splenocytes undergoing transformation. Induction of v-rel in these cells resulted in rapid transformation resulting in the appearance of continuously proliferating cell lines usually in 8-10 days. The dynamic changes of miR-155 expression during the transformation process of splenocytes measured by qRT-PCR are shown in Fig 4A. Quite clearly, miR-155 is significantly upregulated during the time-course of v-rel transformation, with levels showing increases of 5 fold (day 1), 6 fold (day 4), 50 fold at day 7, 150 fold at day 9 and nearly 1500 fold at day 14, as compared with the level at day 0.

In order to assess the simultaneous changes in gene expression during transformation, we carried out the transcriptome analysis using the chicken Affymetrix platform on the RNA samples extracted from these cells. To focus on miRNA-induced repression of gene expression, we used the Bioconductor package Limma (35) to extract 1242 genes that showed significant downregulation at day 14 compared to day 0. Table 1 shows the top 20 statistically enriched predicted miRNA targets in this list. Of the 1242 downregulated genes, 73 are predicted targets of gga-miR-155 (Fig 4B) making it the top hit of the most enriched miRNA targets. Analysis also showed that the enrichment of the targets of other miRNAs such as gga-miR-9*, gga-miR-217, gga-miR-19a and gga-miR-23b were also significant.

These data highlighted the importance of miR-155 and other miRNAs in v-rel induced transformation. MiR-155 is a well-studied oncogene of hematopoietic cells. Considering the complexity of targets analysis in v-rel induced transformation system as lots of miRNAs and mRNAs are affected by v-rel, we overexpressed miR-155 in chicken macrophages derived from line 0 chicken by transfection of miR-155 mimics into bone-marrow derived macrophages. ‘Allstars’ negative control (Qiagen) was used as control in an attempt to get a cleaner result on miR-155 targets. The RNA extracted from transfected cells were analysed by deep sequencing. The significant down regulated genes with miR-155 target sites in 3’UTR were subject to the pathway analysis using Ingenuity Pathway Analysis tool. As
shown in Fig 5, several potential miR-155 targets are involved in a number of diseases and cellular processes. The number of cancer-related genes targeted by miR-155 ranks the second implicating the importance of miR-155 as a regulator in disease pathogenesis, particularly in tumorigenesis.

Discussion

The Rev-T avian retrovirus encodes the v-rel oncoprotein, which is a member of the Rel/NF-κB transcription factor family. Although Rel/NF-κB transcription factors have been associated with oncogenesis in mammals, v-rel is the only member of this family that is oncogenic in animal systems. Due to its pervasive role in oncogenesis, there is great interest in NF-κB signalling, and v-rel provides a valuable model for studying NF-κB signalling in lymphoid cell cancers because of its ability to transform chicken lymphoid cells (12, 15). In this study, we demonstrate that v-rel can readily induce transformation of lymphocyte populations, and the establishment of CD4+ T-cell (AVOL-1) and B-cell (AVOL-2) lineages suggested that v-rel-induced transformation function is not restricted to specific lineages.

In addition to the changes in protein-coding genes, many changes in the miRNA profiles also occur in v-rel transformed cells, and one of the miRNAs expressed at significantly higher levels in v-rel-derived tumor cell lines such as KBMC and CM758 is gga-miR-155 (33). Higher expression of miR-155 is reported in a number of haematopoietic malignancies (36-40). The precursor of miR-155, termed c-Bic, was first observed to co-operate with myc in chicken B-cell lymphomas induced by avian leukosis proviral integrations (26, 41). Southern blot hybridization of genomic DNA from AVOL-1 and AVOL-2 cells showed no evidence of genomic rearrangements in Bic loci (data not shown) discounting insertional activation of miR-155 in these cell lines. It is known that miR-155 can also be induced by a variety of immune cell stimuli such as TLR ligands, TNF-α, IFN-β and other antigens (41-45).
conserved AP-1 element in the human Bic/miR-155 promoter was shown to be essential for some of these functions (46). Transcriptional regulation of miR-155 by TGF-β/Smad4 pathway using the Smad response elements in the human miR-155 promoter has also been reported (47). Epstein–Barr virus (EBV) latent membrane protein-1 (LMP1) is a potent inducer of miR-155 and the NF-κB sites in the Bic/miR-155 promoter have been shown to be pivotal for this function (48, 49).

Both Northern blotting and microarray data showed that miR-155 is significantly increased in v-rel-transformed T and B lymphocytes compared to the normal spleen cells. These observations are similar to the findings reported previously (33). Despite the consistent demonstration of transformation of B and T-lymphocytes by v-rel, the precise mechanisms have not been demonstrated. As an NF-κB homolog (8), the most likely mechanism of miR-155 upregulation would be through the direct activation of the miR-155 promoter through the NF-κB binding sites. EMSA showed that v-rel binds directly to both NF-κB binding sites. To assess the ability of v-rel to activate transcription from miR-155 promoter, we performed reporter assays using the miR-155 promoter and its derivative lacking each of the NF-κB binding sites. Our results demonstrated that indeed v-rel controls miR-155 through one of the NF-κB binding sites in the Bic/miR-155 promoter.

A number of previous studies have demonstrated robust expression of Bic in EBV-infected cells (50, 51). It has been shown later that EBV-encoded latent membrane protein-1 (LMP-1), a functional homologue of the tumor necrosis factor receptor family, upregulates the expression of miR-155 mainly by activating the NF-κB pathway (48). The data here is the first evidence showing miR-155 being regulated by an NF-κB transcription factor, the v-rel oncogene encoded by Rev-T in avian systems. It has been shown previously that v-rel exerts downstream effects through the transcription factor AP-1 (12, 46). AP-1 sites are present in
chicken Bic/miR-155 promoter sequences and the contribution of AP-1 in regulation of miR-155 expression in v-rel-transformed lymphocytes remains to be determined.

Interestingly, while miR-155 was upregulated in Rev-T transformed cell lines, it was consistently downregulated in MDV-transformed lymphocytes (52). Although miR-155 functions are probably rescued by the high level expression of the MDV1-miR-M4 homolog in these cells (53), the precise molecular mechanisms of downregulation of miR-155 in MDV-transformed cells are not clear. RCAS-mediated transduction of v-rel did rescue the expression of miR-155 in two of the MDV transformed cell line MSB-1 and 265L. The increased level of miR-155 expression after introduction of v-rel into these cells indicated that the upregulation of miR-155 is a direct effect. It is interesting to know that common occurrence of MDV with REV in chickens could lead a part or entire genome of REV integrating into MDV genome (54, 55). Although a number of field MDV isolates with REV insertion have been characterized, the precise molecular mechanisms for the altered pathogenic properties and the increased virulence are still not clear (55, 56).

A number of targets of miR-155 have been identified previously. C-Maf (43), AID (57, 58), Pu.1 (59), SOCS1 (60), interleukin-1 (61) and IKKε (49, 62) have been implicated in mediating functions of miR-155 in the immune system. Ets-1 and Meis1 mediate megakaryopoiesis (63). SHIP1 and C/EBP have been implicated in myeloproliferative disorders (64, 65), Peli1 controls the generation and function of T follicular helper cells through promoting the degradation of the NF-κB family transcription factor c-Rel (66), tumor protein p53 inducible nuclear protein 1 (Tp53INP1) is involved in pancreatic cancer (67) and SOCS1 in promoting γ-chain cytokine signalling to ensure effector and memory CD8+ T cell differentiation (68). Additionally, miR-155 targets JARID2, a cell cycle regulator and part of a histone methyltransferase complex, to promote cell survival (33). From microarray data on RNA of v-rel transformed cells, 73 out of 1242 significantly downregulated genes are
potential targets of miR-155. Not only was miR-155 the most statistically enriched target within the list of significantly down-regulated genes, but members of the miR-17-92 cluster are also implicated, a cluster which is known to be involved in cancer (69-72), this further emphasized the role of oncogenic miRNAs in transformation. The oncogenic effects of miR-155 are mediated through its target mRNAs. The known miR-155 targets Pu.1, CEBPβ are present in the down regulated genes from microarray analysis in v-rel transformed cells. Together with the evidence that the potential miR-155 targets in macrophages involved in cancer are standing out of other diseases and functions related targets, demonstrating the important role of miR-155 in v-rel induced transformation. Although the precise roles and molecular pathways of miR-155 in v-rel induced transformation are not fully known, its repressive function on transcriptional factors such as Pu.1 and CEBPβ can have wide-ranging effects on the cellular milieu and the global gene expression profiles seen for lymphocytes. Further studies will be required to ascertain the involvement of Pu.1, CEBPβ and/or other miR-155 regulated transcription factors in the regulation of miR-155-inhibited genes. Similarly, the repression of some of the other target genes is also likely to contribute to the induction of hematopoietic cell malignancy. Although upregulation of miR-155 appears to add complexity to regulation of gene expression in v-rel–induced malignant transformation, the downstream network of miR-155 targets or the importance of those target genes in v-rel induced transformation could be an interesting area to explore.

**Materials and methods**

**Transformed cell lines**
Rev-T-transformed cell lines AVOL-1 (CD4$^+$ T-cell line) (32), AVOL-2 (B-cell origin), AVOL-3, RIR-RevT (a transformed cell line derived from outbred Rhode Island Red chickens) and avian leukosis virus (ALV) HPRS F42 strain-transformed B-cell line HP45 (73) were used. MDV cell lines MSB-1 (74) and 265L (32) were used to study the effects of induction of v-rel. All the cell lines were grown at 38.5 °C in 5% CO$_2$ in RPMI 1640 medium containing 10% fetal calf serum, 2% chicken serum, 10% tryptose phosphate broth, 0.1% 2-mercaptoethanol and 1% sodium pyruvate. CEF-derived cell line DF-1 was grown using methods described (75).

**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).

**Plasmid constructs**

The construct pcDNA3.1-v-rel was used for reporter assay. For electrophoresis mobility shift assay, recombinant v-rel fused in-frame with GST in pGEX2T (GE Healthcare) vector was used. RCAS(A) retroviral vector (Replication Competent ALV LTR with a Splice acceptor) (76) with v-rel cloned into the ClaI site was used for *in vitro* transformation of embryonic splenocytes. The orientation of the insert was verified by restriction enzyme digestion and
sequencing. RCAS (A)-EGFP-v-rel construct with the N-terminal enhanced green fluorescent protein (EGFP) tag was used for the expression of v-rel in MSB-1 and 265L cells.

**Cloning and mutagenesis of Bic/miR-155 promoter**

The chicken Bic/miR-155 promoter region extending from -1829 to +3 nucleotides from transcription start site (+1) was amplified by PCR from the genomic DNA prepared from CEF. The isolated fragments were digested with Bg/II and NheI and cloned into Bg/II and NheI cut psiCHECK™-2 vector (Promega) to replace the SV40 promoter driving the renilla luciferase gene to generate the pBic-WT reporter construct. Mutagenesis of the two NF-κB sites on the pBic promoter was carried out by overlapping PCR using primers 5’-CCACATATTTCCTGGCTGGAGCACATAAATTTTCTGAG-3’ and 5’-CTCAGAAAAATTATGTCTCGAGCCAGCAAGGAAATATGTGG-3’ for NF-κB site 1, 5’-GAAAAGGAAAGCAGGCTCGAGACTCAAGACGGTTAG-3’ and 5’-CTAACCGTCTTGAGTCTCGAGCCTGCTTTCCTTTTC-3’ for NF-κB site 2. The mutant constructs were used to replace the corresponding fragment in the pBic-WT vector to generate pBic-M1, pBic-M2 and pBic-M1M2 constructs, where the 1st, 2nd and both NF-κB sites respectively, were replaced. In each case, the XhoI restriction site introduced during the replacement of the NF-κB motifs allowed the screening of the constructs by XhoI digestion. The sequences of the promoter region of all the constructs were confirmed by sequence analysis.

**Dual Luciferase reporter assay**

Transfection of DF-1 cells was carried out with Lipofectamine 2000 (Invitrogen) as per manufacturer’s protocols. Approximately $3 \times 10^4$ DF-1 cells were seeded in each well of a 96-well plate. Each of the reporter and pcDNA3-v-rel constructs were co-transfected into DF-1 cells and the luciferase expression was assayed 48 hours later using the Dual-Glo Luciferase Assay System (Promega) following manufacturer’s instructions. The relative expression of
renilla luciferase was determined with the normalised levels of firefly luciferase. For each sample, values from four replicates representative of at least two independent experiments were used in the analysis.

**Electrophoresis mobility shift assay (EMSAs)**

Recombinant full length v-rel from pGEX2t-v-rel plasmid in BL21 (DE3) induced with 0.5 mM isopropyl-b-D-thiogalactopyranoside (IPTG) for 3h was purified by Glutathione Sepharose 4 Fast Flow (GE Healthcare) according to the manufacturer’s instructions. EMSAs were performed using gel shift assay system (Promega) according to the manufacturer’s instructions. Double-stranded synthetic oligonucleotides were radiolabeled using $[^{32}P]$ ATP (Amersham) and T4 polynucleotide kinase. For each binding reaction, 3µg of purified protein was incubated with 0.25µg/µl poly[dI-dC] containing 50,000cpm of radiolabelled probes and a 50-fold molar excess of unlabelled competitor oligonucleotide when indicated. DNA-binding reactions were carried out for 30 min at room temperature. Competition experiments were performed by pre-incubation with protein in binding buffer for 10 min, after which labelled probe was added for a further 20-min incubation at room temperature. The DNA-protein complexes were resolved on 6% DNA Retardation Gel (Invitrogen) and detected by autoradiography.

**Immunoblotting and Northern blotting**

For Western blotting, cells were lysed in protein gel sample buffer (8M urea, 2% SDS, 10 mM Tris/HCl pH 6.8, 0.05% bromophenol blue) and separated on a NuPAGE 4–12% Bis Tris gel (Invitrogen) and transferred onto nitrocellulose membranes using an iBlot gel transfer system (Invitrogen). Western blotting was performed with c-rel and v-rel-specific HY87 mouse monoclonal antibody (77), followed by anti-mouse IgG–peroxidase conjugate (Sigma-Aldrich). Membranes were developed with an ECL Western blotting analysis system (Amersham). For Northern blot analysis, total RNA was extracted from cultured cells with
miRNeasy Mini Kit (Qiagen), and 20 µg total RNA resolved using a 15% polyacrylamide-1×Tris-borate-EDTA-8 M urea gel was blotted to a GeneScreen Plus membrane (Perkin-Elmer). DNA oligonucleotides with sequences complementary to candidate miRNAs, end-labelled with [γ-32P]ATP (Amersham) using T4 polynucleotide kinase (New England Biolabs), were used as high-specific-activity probes. Hybridization, washing and autoradiography were carried out as previously described (78).

**RCAS virus infection**

Virus stocks generated from DF-1 cells transfected with RCAS(A)-v-rel and RCAS(A)-v-rel-EGFP constructs approximately 5 days after transfection, when nearly 100% cells were EGFP positive in the case of the latter construct. For *in vitro* transformation assay, one ml (~10^6 TCID_{50}) of RCAS(A)-v-rel virus was used to infect 5×10^6 of embryonic splenocytes, and harvested at day 0, 1, 4, 7, 9 and 14 days post infection for mRNA microarray analysis and miR-155 quantitation. EGFP-expressing RCAS(A)-v-rel-EGFP-infected MSB-1 and 265L cells were also sorted and examined for v-rel and miR-155 expression.

**Stem-loop qRT-PCR for miR-155**

The expression levels of miR-155 were analysed using the TaqMan MicroRNA Assay System (Applied Biosystems) using 10 ng of total RNA as a template for reverse transcription. Each reverse transcription reaction was performed twice independently, and each reaction was tested by PCR in triplicates. All values were normalized to the expression of the endogenous let-7a, and levels calculated as fold-expression change relative to those from uninfected 265L cells.

**Microarray Analysis**

Triplicate RNA samples for each of the six time-points (0, 1, 3, 4, 7 and 14 dpi) were analysed using the Affymetrix GeneChip Chicken Genome Array. Expression values were calculated using the Robust Multi-Array Average (RMA) function within the Affy
bioconductor package (79). Affymetrix probes were linked to Ensembl genes using Ensembl (v70) and genes linked to microRNA predicted targets data from the MicroCosm targets database (80).

For the naïve prediction of miRNAs involved in the activation of genes from the mRNA expression data, the following analysis was performed: down-regulated probes at 14 DPI compared to 0 DPI were determined using Limma (35), with a FDR<=0.01 (81) and log fold change <= -1 (two-fold down-regulated). Statistical enrichment of miRNA targets within the down-regulated gene list was calculated using the CORNA package (82). Fisher’s exact test was used to calculate p-values for statistical enrichment, and adjusted for multiple testing (81). Heatmaps were drawn in R using the Pearson correlation coefficient as a similarity measure (83).

In order to analyse the behaviour of predicted gga-miR-155 targets, expression data from Affymetrix probes representing genes predicted to be targets of gga-miR-155 were extracted and analysed as a set.

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**Conflict of interest**

The authors declare no conflict of interest.
Ethics statement

No animals were used for the work presented in this manuscript.

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Table 1 Top 20 enriched miRNA targets in the list of 1242 downregulated genes

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$^1$Predicted: The total number of genes predicted to be targets of the microRNA in the population; $^2$Expected: The number we would expect to see in our sample by random chance based on our sample size; $^3$Observed: The number we actually observed; $^4$FDR: The Benjamini and Hochberg adjusted p-value from a two-tailed Fisher's exact test. **indicates FDR <= 0.05
**Figure legends**

**Figure 1. Northern blotting analysis for determining miR-155 expression.** Twenty micrograms of total RNA extracted from the indicated cells was separated on a 15% denaturing polyacrylamide gel, blotted and hybridized with end-labelled antisense oligonucleotide probes to gga-miR-155. Size markers to indicate the positions of the pre-miRNA and the mature miRNA are shown. The cellular U6 small nuclear RNA served as the loading control.

![Northern blotting analysis](image)

**Figure 2. Activation of miR-155 by v-rel occurs through the NF-κB pathway.** (A) Electrophoresis mobility shift assay using purified v-rel on the two putative NF-κB binding sites NF-κB1 (−581) and NF-κB2 (−66) on the chicken Bic/miR-155 promoter. WT = 50-fold molar cold wild-type competitor, mu = 50-fold molar cold mutant competitor. (B) Schematic diagram of luciferase reporter constructs carrying the wild type (WT) and mutant (M1, M2...
and M1M2) chicken Bic/miR-155 promoter. **(C)** Relative levels of luciferase in DF-1 cells co-transfected with pcDNA3-v-rel and the reporter constructs. Error bars represent the data from 4 replicates.

**Figure 3. Upregulation of miR-155 in MDV-transformed cell lines by v-rel.** (A) Cell lysates from MSB-1 and 265L infected with RCAS(A)-v-rel-GFP were analysed by Western blot using HY87 antibody for v-rel expression. Uninfected MSB-1 and 265L were included as negative control and AVOL-1 cells were included as positive control. (B) Expression levels of miR-155 in RCAS(A)-v-rel-GFP infected and uninfected MSB-1 and 265L. RCAS(A)-GFP infected cells were also included as a control.
Figure 4. Upregulation of miR-155 during v-rel transformation is associated with downregulation of targets. (A) Expression levels of miR-155 in RCAS(A)-v-rel transformed embryonic splenocytes on RNA samples harvested on day 0, 1, 4, 7, 9 and 14 days post infection. (B) Heatmap of 73 down-regulated genes predicted to be targets of gga-miR-155. Affymetrix probes were analysed using Limma, comparing d14 to d0 and those with an FDR<=0.01 and fold-change <= -1 (two-fold) selected. The list was further filtered for those genes predicted to be targeted by gga-miR-155. Heatmap was drawn in R using the Pearson correlation coefficient as a distance measure.
Figure 5. The potential miR-155 targets are involved in a number of diseases and functions. Top 20 functions (sorted by p-value) of the miR-155 targets identified in primary avian macrophages transfected with miR-155 mimics. The grey bars indicate the number of potential target genes for each disease or function.