Virally induced changes in cellular microRNAs maintain latency of human cytomegalovirus in CD34+ progenitors

Emma Poole,† Stuart R. McGregor Dallas, †† Julia Colston, Robert Samuel V. Joseph and John Sinclair

University of Cambridge, Department of Medicine, Box 157, Level 5 Laboratories Block, Addenbrooke's Hospital, Cambridge CB2 0QQ, UK

One site of latency of human cytomegalovirus (HCMV; human herpesvirus 5) is known to be CD34+ haematopoietic progenitor cells, and it is likely that carriage of latent virus has profound effects on cellular gene expression in order to optimize latency and reactivation. As microRNAs (miRNAs) play important roles in regulating stem-cell gene expression, this study asked whether latent carriage of HCMV led to changes in cellular miRNA expression. A comprehensive miRNA screen showed the differential regulation of a number of cellular miRNAs during HCMV latency in CD34+ progenitor cells. One of these, hsa-miR-92a, was robustly decreased in three independent miRNA screens. Latency-induced change in hsa-miR-92a results in an increase in expression of GATA-2 and subsequent increased expression of cellular IL-10, which aids the maintenance of latent viral genomes in CD34+ cells, probably resulting from their increased survival.

INTRODUCTION

Human cytomegalovirus (HCMV; human herpesvirus 5), like all herpesviruses, has two phases in its life cycle: lytic and latent. The lytic phase comprises transcription of viral immediate-early (IE), early and late genes, which results in the production of infectious virions. In contrast, during latent infection, which can be established in CD34+ haematopoietic stem cells (Goodrum et al., 2002; Hahn et al., 1998; Reeves et al., 2005b), the viral genome is carried in the absence of infectious virus production. During natural latency, a much more restricted transcription profile occurs in which only a limited number of viral genes are expressed – the so-called latency-associated transcripts (Bego et al., 2005; Goodrum et al., 2007; Kondo & Mocarski, 1995).

In a normal healthy individual, primary HCMV infection routinely results in a subclinical infection, which is rapidly ameliorated by the host immune response (Rook, 1988). However, due to the ability of HCMV to express multiple immune avoidance genes, as well as its ability to undergo latent infection, the virus is never cleared from the infected individual, who can routinely undergo sporadic reactivation events that are tightly controlled by the host immune system. Reactivation from latency can occur in naturally latently infected CD34+ progenitor cells upon their differentiation to immature dendritic cells (DCs) and subsequent maturation to mature DCs (Reeves et al., 2005b; Sinclair, 2008). The control of HCMV latency and reactivation is not fully understood, but it is known that the restricted viral transcription programme that occurs during latency is dependent on repression of viral IE gene expression (Reeves et al., 2005b) – CD34+ progenitor cells do not support robust viral IE gene expression. Similarly, roles for the limited number of viral genes that are expressed during latency are only just being elucidated, but it is likely that these will be involved in the establishment, maintenance and reactivation of latent virus and they are likely to modify the latently infected cell to ensure efficient carriage and reactivation of virus in vivo.

It is becoming increasingly clear that cellular microRNAs (miRNAs) play important roles in the regulation of development, apoptosis and differentiation of haematopoietic stem cells (Garzon & Croce, 2008; Jovanovic & Hengartner, 2006) – all cellular functions that could impinge upon the carriage of latent virus. miRNAs are RNA molecules of ~21 nt and have been shown to exert their effects by mediating translational silencing, which is achieved by recruitment of the Dicer and Argonaute proteins, leading to a reduction in protein levels of the target gene by either RNA degradation or translational suppression (Meister et al., 2004; Tomari & Zamore, 2005). Consequently, cellular miRNAs are viewed as an important additional mechanism to control cellular gene expression. This mechanism of regulating gene expression is also

†These authors contributed equally to this work.
††Present address: Princeton University Department of Molecular Biology, Lewis Thomas Laboratory, Princeton, NJ 08544, USA.

The results of an NCode miRNA screen showing changes in cellular miRNAs in CD34+ cells following HCMV latent infection are available with the online version of this paper.
employed by a number of viruses that encode their own miRNAs to fine tune viral gene expression: human immunodeficiency virus type I (HIV-1), herpes simplex virus, Simian virus 40, Marek’s disease virus, Epstein–Barr virus, murine cytomegalovirus and human herpesvirus 8 (Bennasser et al., 2006; Burnside & Morgan, 2007; Dölken et al., 2009; Fannin Rider et al., 2008; Grey & Nelson, 2008; Grey et al., 2008; Niu et al., 2009; Omoto et al., 2004; Pfeffer, 2007; Pfeffer et al., 2004, 2005; Samols et al., 2005; Sullivan et al., 2005, 2006; Swaminathan 2008; Tang et al., 2008, 2009). Many of these viruses are known to encode multiple miRNAs. For example, there are 13 in silico predicted miRNAs for HCMV, which are scattered throughout the genome and expressed at various times during productive infection. The function of many of these miRNAs is still being elucidated, but the HCMV-encoded miR-UL112-1 miRNA has been ascribed a role in the regulation of viral IE gene expression (Grey & Nelson, 2008; Murphy et al., 2003), as well as the inhibition of killing by natural killer cells as a result of decreasing the levels of the major histocompatibility complex class I chain-related molecule B protein (Stern-Ginossar et al., 2008).

In addition to encoding their own miRNAs, viruses are known to modulate the levels and functions of cellular miRNAs and vice versa. For instance, the adenoviral VA1 non-coding RNA prevents the nuclear export of miRNA precursors (Lu & Cullen, 2004), whereas cellular miRNA-122 targets the 5’ end of hepatitis C virus and enhances virus replication (Jopling, 2008). Similarly, HIV-1 down-regulates many cellular miRNAs, including the polycistrionic cluster miR17-92 (Triboulet et al., 2007), which has been implicated in a number of roles including cellular differentiation and proliferation.

Although HCMV has also been shown to differentially regulate a limited number of cellular miRNAs during lytic infection (Stern-Ginossar et al., 2009; Wang et al., 2008), to date there have been no reports on changes in cellular miRNAs associated with HCMV latent infection. On the basis that changes in cellular miRNAs could have profound effects on the expression of cellular genes, which, in turn, could impact on latent carriage of virus, we analysed the ability of HCMV to affect cellular miRNAs during latency. We have shown here that a small number of cellular miRNAs are robustly altered during latent carriage of HCMV and describe how these changes could manipulate the cellular micro-environment to regulate both cellular and viral gene expression to the benefit of viral latency.

RESULTS

Specific cellular miRNAs are differentially regulated during experimental latency in CD34+ progenitor cells

One major problem associated with any analysis of global changes in HCMV-infected cells of the myeloid lineage is the lower frequency at which HCMV appears to infect these cells in vitro unless extremely high m.o.i. are used (Hertel et al., 2003). Analysis of the number of CD34+ cells carrying the HCMV genome after experimental latent infection with clinical isolates of HCMV at an m.o.i. of 5 (based on fibroblast infection) have shown that approximately 60–70% of cells carry the HCMV genome by in situ hybridization assays (S. R. McGregor Dallas & J. Sinclair, unpublished observations). Nevertheless, we reasoned that this level of experimental latent infection should still result in global changes in total cellular miRNAs that would be discernible. We therefore analysed the changes in cellular miRNAs associated with experimentally latent HCMV infection of CD34+ progenitor cells using an Invitrogen NCode system Sanger miRBase 10.0 cellular miRNA screen (Lakshmipathy et al., 2007). CD34+ progenitor cells latently infected with HCMV were harvested at 10 days post-infection (p.i.) and analysed for cellular miRNA content. Routinely, such latently infected cells showed no viral IE gene expression (Fig. 1a), consistent with our previous analysis detailing these experimentally infected cells as a robust model of latency (Reeves & Sinclair 2010; Reeves et al., 2005a). In contrast, expression of UL138 RNA, a transcript already established to be expressed during natural latent infection (Goodrum et al., 2007), was clearly detectable (Fig. 1a). Only a few changes in the levels of cellular miRNAs were observed during latency with high statistical significance (see Methods), and a representative total screen is proved in Supplementary Table S1 (available in JGV Online). The array results passed all standard blank, positive and negative quality controls and were subject to additional filters to ensure identification of the most significantly and robustly altered cellular miRNAs associated with latency. Table 1 shows the cellular miRNAs that showed a greater than twofold change during latency. All of these were downregulated but, more importantly, were observed in a totally independent second miRNA screen using different cellular RNA preparations from latently infected cells analysed some 3 months apart. Of those with the highest confidence limits and highest fold decrease were hsa-miR-let-7a, hsa-miR-let-7b, hsa-miR-297 and hsa-miR-92a, which have been shown to have numerous effects on cellular functions, many associated with neoplasia. It should be noted that the two- to threefold changes in cellular miRNAs resulting from our experimental latent infection would probably be much more profound in populations of CD34+ cells with higher levels of latent infection, which at present we have found impossible to achieve. Nevertheless, the levels of change of individual cellular miRNAs we observed during latency (two- to threefold) were similar to those reported for many of the changes in levels of cellular miRNAs at early times of productive infection with HCMV (Wang et al., 2008). Each of the changes in levels of cellular miRNAs we observed are in the process of being validated and their possible effects are being assessed. However, our attention was initially drawn to hsa-miR-92a, which has also been implicated in the regulation of haematopoiesis and immune functions (Bonauer & Dimmel, 2009), which
might be of importance for a virus that is carried latently in cells of the myeloid lineage.

Consequently, we validated this change by qRT-PCR specific for hsa-miR-92a (Fig. 1b), which confirmed that this cellular miRNA was indeed downregulated during experimental HCMV latency of CD34+ progenitor cells (this change represents ΔΔCt values of 12 in mock-infected cells compared with 32 in CD34+ cells that had been latently infected with HCMV). Furthermore, UV treatment...
Table 1. Cellular miRNAs are regulated during HCMV latency

CD34+ cells were mock infected or latently infected with HCMV and harvested in Trizol at 10 days p.i. An NCode miRNA screen was carried out from two independent experiments and analysed in triplicate for each screen. The table shows those miRNAs with a greater than twofold change and their P values from one of the screens.

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Fold change during latency compared with mock</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-let-7a</td>
<td>-2.5</td>
<td>0.0050</td>
</tr>
<tr>
<td>hsa-miR-let-7b</td>
<td>-3.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>hsa-miR-206</td>
<td>-2.0</td>
<td>0.0200</td>
</tr>
<tr>
<td>hsa-miR-296 3p</td>
<td>-2.6</td>
<td>0.0070</td>
</tr>
<tr>
<td>hsa-miR-297</td>
<td>-2.9</td>
<td>0.0010</td>
</tr>
<tr>
<td>hsa-miR-32</td>
<td>-2.0</td>
<td>0.0700</td>
</tr>
<tr>
<td>hsa-miR-608</td>
<td>-2.4</td>
<td>0.0080</td>
</tr>
<tr>
<td>hsa-miR-92a</td>
<td>-2.3</td>
<td>0.0030</td>
</tr>
</tbody>
</table>

of the inoculum in CD34+ cells eliminated the latency-associated reduction in hsa-miR-92a levels when compared with latently infected cells (Fig. 1c), suggesting that the observed decrease in hsa-miR-92a was specifically due to latent HCMV infection and not, for instance, the effects of virus binding. Additionally, incubation of CD34+ cells for 3 days with supernatant from latently infected CD34+ cells, following 10 days of latent infection, showed only slightly decreased levels of hsa-miR-92a (72.3%) compared with those incubated with supernatants from uninfected cells (Fig. 1d). This indicated that, whilst there may be a minor bystander effect on uninfected CD34+ cells in the latently infected population, the predominant factor inducing the decrease in hsa-miR-92a is latent virus infection.

hsa-miR-92a functionally targets the cellular transcription factor GATA-2 in CD34+ myeloid cells

A number of predicted targets of hsa-miR-92a have already been defined using miRNA target algorithms (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) and, as with most miRNAs, these are substantial in number. However, one of these targets, identified in two different prediction algorithms (Targetscan, http://www.targetscan.org; EMBL-EBL MicroCosm http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) is the cellular transcription factor GATA-2.

This cellular transcription factor is known to be important in the proliferation, lineage commitment and survival of haematopoietic progenitor cells (Pan et al., 2000), cellular functions that could all be of importance for carriage of HCMV in this cell type.

If latent infection does result in a reduction in hsa-miR-92a, and if hsa-miR-92a does authentically target GATA-2 in CD34+ cells, then latent infection of CD34+ cells with HCMV should lead to an increase in GATA-2 levels. Fig. 2(a) clearly shows that latent infection of CD34+ progenitor cells increased protein levels of GATA-2 approximately threefold when analysed by Western blot analysis and densitometry. This increase in GATA-2 protein during latent infection was also reflected in the levels of GATA-2 RNA when analysed by RT-PCR and qRT-PCR (Fig. 2(b–d))

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and is consistent with recent analyses showing that miRNAs can also have profound effects on RNA stability (Guo et al., 2010). However, although hsa-miR-92a levels do change during latent HCMV infection and this is accompanied by changes in levels of GATA-2, it was still important to show formally that changes in hsa-miR-92a could, in themselves, cause such increases in GATA-2 expression.

Consequently, we tested whether changes in cellular hsa-miR-92a in isolation could directly regulate GATA-2 expression in CD34\(^+\) cells. Unfortunately, it is well established that primary CD34\(^+\) cells are notoriously difficult to transfect. However, a number of CD34\(^+\) cell lines are available that can be transfected more easily. The CD34\(^+\) myelomonocytic cell line KG-1 has many characteristics of CD34\(^+\) progenitors: in particular, KG-1 cells can be differentiated along the myeloid lineage using standard conditions, and mature DCs can be derived from them (Teobald et al., 2008). These cells are, therefore, a biologically relevant cell type with respect to HCMV latent infection (Sinclair, 2008).

Firstly, we tested whether KG-1 cells could be transfected with miRNAs or miRNA antagonists to efficiently alter steady-state levels of hsa-miR-92a. Fig. 3(a) shows that KG-1 cells transfected with an inhibitor of hsa-miR-92a (anti-hsa-miR-92a) showed a good reduction of endogenous hsa-miR-92a as detected by qRT-PCR. The decreased level of hsa-miR-92a in cells electroporated with the inhibitor may be due to degradation of the hsa-miR-92a (Krützfeldt et al., 2005), or may be due to duplex formation preventing the qRT-PCR primers from recognizing the target efficiently. Regardless, the results are consistent with the belief that anti-hsa-miR-92a specifically targets hsa-miR-92a in these cells. Similarly, KG-1 cells that had been transfected with mature hsa-miR-92a resulted in increased levels of hsa-miR-92a (Fig. 3a).

We next confirmed a direct effect of hsa-miR-92a on GATA-2 expression using assays employing luciferase expression vectors engineered to contain the predicted hsa-miR-92a seed region, which we identified in the 3′-untranslated region of GATA-2. Fig. 3(b) shows that the presence of the GATA-2 seed region, predicted to be responsive to hsa-miR-92a, caused a decrease in luciferase expression in the presence of an hsa-miR-92a antagonist, presumably due to the ability of the antagonist to reduce endogenous levels of hsa-miR-92a. These low but reproducible effects of hsa-miR-92a on luciferase vectors containing the target GATA-2 sequence are consistent with levels of repression observed in similar assays for other miRNA targets, both viral and cellular (Grey et al., 2008; Wang et al., 2008). In contrast, no such effects were observed using parental luciferase vectors. Perhaps, more importantly, we also observed that delivery of an antagonist of hsa-miR-92a to KG-1 cells resulted in an increase in the levels of endogenous cellular GATA-2 protein, which was clearly detected by Western blot analysis and densitometry (Fig. 3c). These data argue that hsa-miR-92a directly targets GATA-2 in CD34\(^+\) cells.

Cellular interleukin-10 (cIL-10) expression is regulated by hsa-miR-92a via GATA-2

The increase in GATA-2 accompanying latent infection of CD34\(^+\) cells should result in changes in the level of expression of cellular genes normally regulated by GATA-2. Interestingly, in a separate analysis of the latency-induced changes in the secretome of CD34\(^+\) progenitor cells that we
carried out recently (unpublished data), we noted that latently infected CD34\(^+\) cells secreted increased levels of cellular IL-10 (Fig. 4a). This increase in cIL-10 was not observed following infection with UV-inactivated virus (Fig. 4a). Recently, Shin et al. (2003) identified a GATA-2-binding site in the cIL-10 promoter, suggesting that the cIL-10 promoter is regulated by GATA-2.

To test whether GATA-2 directly, or by modification of its levels by hsa-miR-92a, could, in isolation, regulate cIL-10 in CD34\(^+\) cells, supernatants from KG-1 cells that had been electroporated with either GATA-2 or anti-hsa-miR-92a were analysed for levels of cIL-10 by ELISA (Fig. 4b). Levels of secreted cIL-10 in KG-1 cell supernatants were significantly increased following transfection with GATA-2 or anti-hsa-miR-92a (Fig. 4b). To confirm that this increase in cIL-10 resulting from treatment of cells with anti-hsa-miR-92a was mediated through the resulting increase in GATA-2, as we would predict, we also treated KG-1 cells with anti-hsa-miR-92a in the presence of small interfering RNAs (siRNAs) to GATA-2 (Fig. 4c, d). Clearly, the anti-hsa-miR-92a-mediated increase in cIL-10 was dependent on GATA-2, as removal of GATA-2 by specific siRNA, which worked efficiently as shown in (c), prevented anti-hsa-miR-92a from upregulating cIL-10 (Fig. 4d). This argued that changes in the levels of hsa-miR-92a, which lead to regulation of cIL-10, do so in a GATA-2-dependent manner.

Taken together, these data suggested that GATA-2 and hsa-miR-92a can indeed mediate regulation of cIL-10; whilst GATA-2 upregulates cIL-10 in CD34\(^+\) cells, hsa-miR-92a downregulates cIL-10 expression.

cIL-10 upregulation during latency leads to increased carriage of the viral genome in CD34\(^+\) progenitor cells

It is well established that CD34\(^+\) progenitor cell survival can be enhanced following treatment with recombinant cIL-10 via induction of Bcl-2 expression (Weber-Nordt et al., 1996). We reasoned that latent infection with HCMV may well result in signals to the cell that might be interpreted as pro-death, as has been established for HCMV lytic gene expression (Baillie et al., 2003), and, unless counteracted, could result in loss of the reservoir of latently infected CD34\(^+\) progenitor cells. Consequently, we tested directly whether increased cIL-10 expression was important for carriage of the viral genome. We addressed this using a cIL-10 neutralization assay (Fig. 5a). CD34\(^+\) progenitor cells were infected with HCMV and, after the establishment of latency (3 days), were incubated in the presence of an isotype control antibody or a neutralizing cIL-10 antibody (Fig. 5a). Following 10 days of latency, the HCMV genome could readily be detected in cells that had been treated with the isotype control antibody (Fig. 5a, top panel, lane 6), but genome carriage was much reduced when a cIL-10 neutralization antibody was used (Fig. 5a, top panel, lane 8). As a loading control, GAPDH PCRs were also carried out (Fig. 5a, lower panel). We did note a minor decrease in the relative level of GAPDH present in latently infected cells in the presence of the antibody to cIL-10, which we believe is consistent with increased cell death.
and does not compromise the analysis. Consistent with the view that cIL-10 does increase CD34<sup>+</sup> progenitor cell survival (Weber-Nordt et al., 1996), CD34<sup>+</sup> progenitor cells latently infected with HCMV, which will result in increased levels of cIL-10, did show increased Bcl-2 expression by Western blot and densitometric analysis as expected (Fig. 5b).

To test whether the cIL-10 induced by latent virus infection did actually result in increased resistance to apoptosis, a cIL-10 neutralization assay was carried out in the presence of virus infection in CD34<sup>+</sup> progenitor cells. Fig. 5(c) shows that apoptosis, as detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, was increased in virus-infected cells where cIL-10 had been neutralized by antibody. Furthermore, latently infected cells were protected from apoptosis induced by Fas-L (Fig. 5e). As expected, in the absence of virus infection or apoptosis inducer, little cell death was observed (Fig. 5e, first column), but following incubation with Fas-L, apoptosis was induced, resulting in cell death (Fig. 5e, second column). Removal of IL-10 made little difference, in the absence of virus infection, to the induction of cell death by Fas-L (Fig. 5e, third column), but supplementing the cells with the apoptosis inhibitor Z-VAD-FMK prevented Fas-L-induced apoptosis (Fig. 5e, fourth column). Apoptosis was minimal in the presence of virus in the absence of Fas-L (Fig. 5e, fifth column); however, the addition of Fas-L did not induce apoptosis to the levels of mock-infected cells during virus infection (Fig. 5e, compare columns 2 and 6). Furthermore, protection from apoptosis appeared to be, at least to some extent, cIL-10 dependent, as neutralization of cIL-10 with an anti-cIL-10 antibody rescued the ability of Fas-L to induce apoptosis (Fig. 5e, compare columns 3 and 7).

Finally, the ability of the apoptosis inhibitor to rescue apoptosis induction in virus-infected cells in the absence of cIL-10 was analysed. To do this, the cIL-10 neutralization assay was carried out in the presence of the apoptosis inhibitor Z-VAD-FMK. The levels of apoptosis observed in the absence of cIL-10 were reduced when cells were supplemented with Z-VAD-FMK (Fig. 5d). Although cIL-10 clearly had a significant anti-apoptotic role, which would probably aid viral carriage in HCMV latently infected cells, this protection from apoptosis was not 100% complete. This may indicate that virus-induced protection from apoptosis may also involve other additional factors. Therefore, from these experiments, we concluded that the latency-induced reduction of hsa-miR-92a leads to
an increase in levels of GATA-2, which in turn promotes cell survival and carriage of the latent viral genome by the induction of cIL-10.

**DISCUSSION**

Given the limited viral gene expression that occurs during HCMV latency, it is likely that the virus will use multiple methods to modulate and fine tune cellular gene expression in order to optimize carriage and reactivation of latent virus. In this report, we showed that, during HCMV latency in CD34+ progenitor cells, the levels of a small number of cellular miRNAs were reproducibly differentially regulated: these changes were observed in three independent screens of different batches of latently infected CD34+ progenitor cells.

One of these cellular miRNAs was hsa-miR-92a and, interestingly, one of the predicted targets of this miRNA is GATA-2. Transfection of KG-1 cells with hsa-miR-92a or an inhibitor of hsa-miR-92a led to decreases and increases, respectively, of endogenous cellular GATA-2, validating this cellular gene as a target for hsa-miR-92a in CD34+ cells. Similarly, luciferase expression vectors engineered to contain the predicted hsa-miR-92a seed region, which we have identified in the 3′-untranslated region of GATA-2, were also inhibited by hsa-miR-92a, confirming that this miRNA does indeed specifically target GATA-2.

Consistent with the latency-associated regulation of hsa-miR-92a also affecting GATA-2 expression, we observed a reproducible increase in GATA-2 levels in latently infected CD34+ progenitor cells. Our prediction that latent infection would, therefore, modify the expression of GATA-2-regulated genes was supported by the observation that cIL-10, a cytokine known to be activated by GATA-2 (Shin et al., 2003; see also Fig. 4) was upregulated during latent infection of progenitor CD34+ cells and that inhibition of hsa-miR-92a in CD34+ cell lines led to an increase in cIL-10 expression.

Taken together, these data suggest that latent infection of CD34+ cells with HCMV results in changes in levels of GATA-2, at least in part, through the latency-associated inhibition of hsa-miR-92a expression, which in turn leads to changes in cIL-10 expression through this increase in GATA-2 levels. Pleasingly, this is consistent with data we have derived from a comprehensive screen of the secretome of latently infected CD34+ progenitor cells, which also showed increases in secreted cIL-10 among a number of changes in cellular chemokines and cytokines during latency (E. Poole, G. Mason, S. R. McGregor Dallas, M. Wills & J. Sinclair, unpublished data).

Our belief that this secretion of cIL-10 by latently infected cells helps to maintain carriage of the latent genome in the cell population was supported by the observation that antibody depletion of cIL-10 during experimental latency resulted in loss of the viral genome in the latent population, probably by decreasing the survival of the latently infected CD34+ cell population. Consistent with previous observations that cIL-10 is known to cause the upregulation of Bcl-2 (Weber-Nordt et al., 1996), latent infection of CD34+ cells with HCMV also resulted in increased levels of Bcl-2 expression. It should be stated that, whilst we favour the interpretation that loss of viral genomes in latently infected cells in the absence of cIL-10 results directly from the death of latently infected cells, formally we cannot rule out that the lack of cIL-10 simply reduces the copy number of viral genomes during latency. At present, we are unable to distinguish between these, as we are unable to analyse latency on a cell-by-cell basis.

Interestingly, cIL-10 is also capable of inhibiting synthesis of pro-inflammatory cytokines and suppressing antigen presentation by multiple mechanisms (Jonuleit et al., 2003; Sanjabi et al., 2009). It is of note that HCMV also encodes a cIL-10 homologue. The HCMV IL-10 transcript is differentially spliced to generate an IL-10 isoform only expressed during lytic infection (cmvIL-10), as well as an isoform expressed during both lytic and latent infection (LaCmvlIL-10) (Jenkins et al., 2004). Little is known of the function of LaCmvlIL-10 – it has some, although not all, of the functions of cIL-10 and does not appear to signal through the cellular IL-10 receptor (Spencer et al., 2008). However, as the anti-IL-10 antibody used here was specific for cIL-10, the effects we observed in our analyses can only have resulted from inhibition of cIL-10 function. Consequently, it would appear that functions encoded by LaCmvlIL-10 are not independently able to take the place of latently induced cIL-10, at least with respect to apoptosis protection.

We are aware that changes in miRNAs generally act to fine tune the regulation of gene expression. The effects of hsa-miR-92a during latent infection were modest but totally reproducible, and were consistent with the levels of effects on gene expression observed in other studies of both cellular and viral miRNAs (Grey et al., 2008; Wang et al., 2008). Consequently, we think it possible that these effects of HCMV latency-induced changes in cellular miRNAs may augment changes in the expression of cellular genes that may also be the targets of other latency-associated cellular functions. It would not surprise us, therefore, if cIL-10 expression is targeted by a miRNA-independent mechanism (such as a specific latency-associated viral gene) and that concomitant latency-induced regulation of expression of hsa-miR-92a augments this regulation. Experiments to address this are in progress. Regardless, our data point to a role for cellular miRNAs as a mechanism by which cellular gene expression is regulated during latent HCMV infection.

In conclusion, our data show that specific changes in cellular miRNAs accompany latent carriage of HCMV. Downregulation of one of these miRNAs, hsa-miR-92a, had significant effects on cIL-10 secretion via regulation of the cellular transcription factor GATA-2, and expression of
cIL-10 helped maintain the latent carriage of HCMV in CD34+ progenitor cells by pro-life signalling.

The changes we observed in other cellular miRNAs during HCMV latency are also likely to be important for targeting of cellular gene expression, but not knowing the specific cellular targets of these miRNAs makes it difficult to predict, at this time, how they may impinge on cellular gene expression. Nonetheless, we believe it likely that such changes in multiple cellular miRNAs could result in global changes in cellular gene expression, which could lead to a coordination of regulation of cellular gene expression that ultimately leads to an environment that optimizes establishment, maintenance or reactivation of latent virus in vivo.

**METHODS**

**Cells and viruses.** HFFF cells were maintained in Eagle’s minimum essential medium, supplemented with 10% FCS. The myelomonocytic CD34+ cell line KG-1 was maintained in RPMI 1640 containing 10% FCS, as described previously (Teobald et al., 2008). Frozen bone marrow-derived CD34+ cells were obtained from Lonza and resuscitated and maintained in X-Vivo 15 medium (Lonza), as described by the suppliers, plus 2.5 mM l-glutamine (Gibco).

Low-passage-number clinical isolates of HCMV (strain TB40e or Merlin) were used for all virus infections (Griffin et al., 2005).

**Virus infections.** Productive infection of HFFF cells with HCMV was carried out as described previously (Baillie et al., 2003; Murphy et al., 2003). Experimental latent infection of CD34+ cells with HCMV has been described elsewhere (Reeves et al., 2005a). Briefly, for experimental latent infection of CD34+ cells, the cells were infected with HCMV at an m.o.i. of 5 in X-vivo 15 medium and maintained for 10 days before harvesting for subsequent analyses.

Virus infection levels were between 60 and 70% as determined by fluorescent in situ hybridization (Everett & Murray, 2005) to the HCMV genome (S. R. McGregor Dallas & J. Sinclair, unpublished observations).

**Isolation of DNA and RNA.** RNA was isolated from cells using Trizol (Invitrogen) as described by the manufacturer. DNA was isolated as described previously (Roback et al., 2001).

**miRNA screen.** Each RNA (350 ng), isolated as described above, was analysed using an Invitrogen NCode Human miRNA Microarray containing probes for the complete Sanger mirBASE 10.0 (http://orf.invitrogen.com/ncode/). The experiment was performed using a loop design (Kerr et al., 2000). Following hybridization of the labelled RNA, the arrays were washed following the standard protocol recommended in the NCode Rapid miRNA Labelling System Manual and the arrays were scanned using a GenePix 4000B microarray scanner (Molecular Devices) for Alexa Fluor 3 (excitation 556 nm, emission 573 nm) and Alexa Fluor 5 (excitation 650 nm, emission 665 nm). The scanned images were then annotated and analysed using GenePix software. The annotated data were normalized using a Latin squares algorithm by the NCode Profiler data analysis software (http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/RNAi-Epiphenetics-and-Gene-Regulation/miRNA-Profiling--.html). The array data were checked to ensure that the median backgrounds were below 75 relative fluorescence units and to make sure that the geometric mean ratios of the NCode Multi-Species miRNA Microarray controls were in the range of 0.8–1.2 for each array. The pairwise median fold-change P value was obtained. The values for each individual miRNA probe sequence were determined in triplicate, and miRNAs with values of P<0.05 for two out of three replicates on the array were considered statistically significant.

**RT-PCR and quantitative RT-PCR (qRT-PCR).** Reverse transcription of RNA was carried out from total RNA using a Promega reverse transcription kit and amplified using a 2x PCR Red Mix (Bioline) using parameters that have been described previously (Goodrum et al., 2007).

qRT-PCR analysis of endogenous hsa-miR-92a from CD34+ cells following HCMV infection, as described above, was carried out as follows. Isolated total RNA (100 ng) was polyadenylated using an NCode miRNA First-Strand cDNA Synthesis kit, version 3 (Invitrogen), for first-strand cDNA synthesis. Three identical qPCRs were prepared with the miRNA primer for hsa-miR-92a (5'-TATTGACACTTGCCGCCGTG-3') and primer sets to detect the housekeeping genes 60S ribosomal protein L4 (RPL4) and β2-microglobulin (B2M) using an NCode SYBR GreenER miRNA qRT-PCR kit (MIRQER-100; Invitrogen) and an ABI 7900HT qPCR machine. Mean threshold cycle (Ct) values ± SD for all sample sets were calculated. Levels of hsa-miR-92a were compared with levels of RNA of housekeeping genes and used to calculate the relative levels of expression. Alternatively, to test levels of hsa-miR-92a following transfection with miRNA molecules, a TaqMan microRNA Cells-to-CT kit (Ambion) was used, as described by the manufacturer, using an Applied Biosystems 7500 Fast machine. Primers for amplification of hsa-miR-92a (GenBank accession no. NR_029508) as well as the housekeeping miRNA hsa-miR-16 (GenBank accession no. NR_029486) were purchased from Ambion. Ct values ± SD were calculated for all sample sets and used to calculate the relative levels of expression.

**miRNA/siRNA and plasmid delivery by electroporation, followed by RT-PCR and Western blot analysis.** miRNA molecules (see below and as described in the text) were transfected using the Amaxa electroporation system using a KG-1 Amaxa kit with the recommended protocol and settings (Lonza).

CD34+ progenitor cells or KG-1 cells were lysed directly in Laemmli sample buffer for analysis by SDS-PAGE and Western blotting. Primary antibodies for GATA-2 and Bcl-2 were obtained from Cell Signalling. RNA was isolated from KG-1 cells that had been infected with HCMV using Trizol (Invitrogen) followed by DNase and RT-PCR as described above. GATA-2 and GAPDH RNAs were amplified using primers that have been described previously (Pan et al., 2000; Poole et al., 2006). Primary actin antibody was obtained from Abcam. Films were scanned with Canoscan-8000F and densitometry was carried out using Image J freeware (http://rsweb.nih.gov/ij/download.html).

cIL-10 ELISA. Supernatants were harvested from control or latently infected CD34+ cells as described above, concentrated fivefold using a Centricron 3 kDa concentrator (Amicon) and assayed for cIL-10 content (NCBI protein no. P22301) by ELISA (R&D Systems), as described by the manufacturer. Alternatively, supernatants were analysed using a Chemokine and Cytokine Array C Series 1000 (RayBiotech), following the manufacturer’s guidelines.

**miRNAs/siRNA and electroporation.** The following miRNAs were obtained from Ambion: negative control 1, hsa-miR-92a and the hsa-miR-92a miRNA inhibitor. GATA-2 siRNA was obtained from Qiagen (Hs_GATA2_5 FlexiTube siRNA; GenBank accession nos NM_001145661, NM_001145662 and NM_032638).

All plasmids and miRNAs were electroporated into KG-1 cells using the Amaxa system (Lonza). After 24 h, cell lysates were harvested for a luciferase assay, as described previously (Poole et al., 2006, 2008).
TUNEL staining and apoptosis induction. CD34+ progenitor cells were mock infected or infected with HCMV in the presence or absence of neutralizing antibody to cIL-10. They were then prepicted with the apoptosis inhibitor Z-VAD-FMK (Sigma) at the recommended concentration for 6 h before induction with anti-Fas-L (human, activating) mAb (clone CH11; Millipore). Cells were then left overnight before fixing in 70% ethanol and TUNEL staining using an In situ Cell Death Detection kit, TMR red (Roche).

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REFERENCES


