Figure 1. **Induction of MCF in calves.** **(a)** Body temperature recorded daily after intravenous inoculation of two groups of four calves with mock-infected BT cells (mock, solid symbols) or cells infected with the AlHV-1 WT C500 strain (AlHV-1, open symbols). Calves were euthanized at 14 (AlHV-1) and 16 (mock) days pi. **(b)** Inguinal and mediastinal LN mass at time of death. **(c)** Percentages of CD4$^+$ and CD8$^+$ cells in the gated CD3$^+$ T cell population in inguinal LN at time of euthanasia. **(d)** Multicolour flow cytometry analysis of CD4$^+$ and CD8$^+$ T cells in PBMC before and after propagation in culture with rhIL-2 (10ng/ml). Gate was placed on CD3$^+$ T cells. LCLs propagated from all calves showed >90% CD3$^+$ T cells. Error bars in (b) and (c) indicate SE.

Table 1. Distribution of the mapped reads against the cellular and viral databases

| Organism	Target	Percentage of reads
Bos taurus	microRNA	28.8%
	genome	22.5%
	rRNA	19.1%
	tRNA	6.9%
	mRNA	5.3%
	ncRNA	2.6%
	snoRNA	1.7%
	MtRNA	0.9%
	snRNA	0.8%
	Mt_tRNA	0.2%
	Repeats	<0.1%
	Ψgene	<0.1%
	Retrotransposed	<0.001%
	teloRNA	<0.0001%
	scRNA	<0.00001%
AlHV-1	LUR	1.5%
	prDNA	<0.001%
Ambiguously mapped reads		3.8%
Unmapped reads		5.8%

Table 2. AlHV-1 small RNA sequences, genomic locations, and distribution in the library[a]

AlHV-1 miRNA	Cloned sequence (major form)	Strand	Size (nt)	No of reads	Genomic location	Walz et al., 2010[b]
Cluster 1						
miR-4-5p	UCUGCUGCGCGGCGCUUCUUU(CU)	-	21	1,878	122-142	pre-miR-1
miR-4-3p	GAGGAGCCUCGCACGGCAGAGA(A)		22	5,273	84-105	
miR-3-5p	CAGAGACCGCACGGGUGUUUCU	-	22	407	486-507	pre-miR-3
miR-3-3p	UCACACCCAGGCGGUCUCUGCA(A)		22	7,779	433-460	
miR-2-5p	UUAAGAAAUAUAGGCUCAAGGU(U)	-	22	3,994	996-1017	/
miR-2-3p	UCUUGUGCCUAGAUUUCUUAUA		22	4,929	963-984	
miR-1-5p	UACAAGGGCUAAGCAUGAGCU(GU)	-	21	4,086	1126-1146	/
Cluster 2						
miR-32-5p	UGCGGGGUUGUGGGAAGCAGACG	-	23	5,470	26392-26414	pre-miR-7
miR-32-3p	UCUGCUUCGCCAUCUCCGUAC		21	375	26358-26378	
miR-31-5p	AAAGUGCACCCCUGGUUGUUUG(UG)	-	22	11,043	26500-26522	pre-miR-8
miR-31-3p	UAAAACCUCGGGUGCACUUAU		21	7,139	26464-26484	
miR-30-5p	UGUUGGCACCUGGGUUAUUACAU	-	23	196	26610-26632	pre-miR-9
miR-30-3p	UGUAAUAGCACUGUGUGCUAC		21	26	26-572-26592	
miR-29-5p	UGGAAAAGCUAUGUUUCAAAUACG	-	24	1,033	26760-26783	pre-miR-10
miR-29-3p	UAUUUGAAAAUAGCUUUUCCG		21	206	26722-26742	
miR-28-5p	GUAUAGCAUGAGCAUAACUGUU	-	22	6,528	26980-27001	pre-miR-11
miR-28-3p	ACAGUUUUGACCAUGCUAGCC		21	24	26946-26966	
miR-27-5p	UAGGCUAGUCUGGACAAAACACU	-	23	4,784	27096-27711	pre-miR-12
miR-27-3p	UGUUUUGUACAGGCUACCUAGC		22	1,592	27054-27075	
miR-26-5p	UAGCUUGUGUGUUGUGUCUGU	-	21	892	27234-27254	pre-miR-13
miR-25-5p	UAAGAGCUCUUGGCGAAGGCU(CU)	-	21	2,403	27334-27354	pre-miR-14
miR-25-3p	AUCUUUGCCAAGUACUCUGGU	-	21	1,196	27298-27318	
miR-24-5p	CACGAUAGUUUAGAAAAAAUCUGU	-	24	216	27476-27499	pre-miR-15
miR-24-3p	UGAUUUUUACUAUGCUAUCAGA		22	801	27445-27466	
miR-23-5p	AGAUAGUUUGGGGGAGGCCUUU	-	22	654	27676-27697	pre-miR-16
miR-23-3p	UGGCUCAGCCAAACUAUCACG		21	941	27642-27662	
miR-22-5p	UGAUGAUCCUAAUGGAGGACU(UU)	-	21	3,724	27861-27881	pre-miR-17
miR-22-3p	AGGUGCUACAUUAAGAUCAUGA		22	1,445	27826-27847	
miR-21-5p	UGUCAGUUACAGCAGGAAUUGUU	-	23	1,792	27984-28006	pre-miR-18
miR-21-3p	CAUUCCGGGCUUUAACUGACAAGC		24	14,227	27947-27970	
miR-20-5p	GAUAGAUUGGGUUAGGAUCUGU	-	22	24,011	28122-28143	pre-miR-19
miR-20-3p	AGAUCCUUGCUCAAUCUGUCUU		22	2,320	28086-28107	
miR-19-5p	UGAUAGCUUAAGGUGCUCUCUG	-	22	60	28245-28266	pre-miR-20
miR-18-5p	CAGUAGUUGGAAGUACCCUAUU	-	22	666	28465-28486	pre-miR-21
miR-18-3p	AAUAAGGUGCAGCCGACUCUUA		22	253	28425-28446	
miR-17-5p	UAGUGGUCAGGCGAGUCUUAUU	-	22	964	28672-28693	pre-miR-22
miR-17-3p	UACGCACGCGUCUGACUACUCC		21	826	28634-28654	
miR-16-5p	UAAUGGUUGCCUGUGCUAUAUU(U)	-	22	750	28875-28897	pre-miR-23
miR-16-3p	AUUGGCGCAGUUAACUAUUGC		21	411	28838-28858	
miR-15-5p	GAGCACCAUCAGUAGUUAUGUGU	-	23	972	29072-29094	pre-miR-24
miR-15-3p	AAUAACUGCUGACAGUGCAAGU		22	7,609	29035-29056	
miR-14-5p	AUCCAGUACACAAGCUUCCCCCGU	-	24	2,814	29218-29241	pre-miR-25
miR-14-3p	CGGGUGCGAGUGUACUGGCAU		21	6,575	29178-29198	
miR-13-5p	CAAGUACACUACGUAUCACGCGGU	-	24	493	29341-29364	pre-miR-26
miR-13-3p	UGCAGUGUUGCUUAGUGUGCUUAG		24	235	29302-29325	
miR-12-5p	UAGUUACAUUGACUGGUAAUA	-	21	4,535	29481-29501	pre-miR-27
miR-12-3p	GUUACCAGUUUUUGUACCAG		20	14	29448-29467	
miR-11-5p	GCGGUACUGGGGUUGUUAAAGA	-	22	1,022	29663-29684	pre-miR-28
miR-11-3p	GUUAACAUCCCCAAUAUCGGU(U)		21	14,878	29481-29501	
miR-10-5p	ACGGUGCAGUAACUGAUAUAGA	-	22	139	29790-29811	pre-miR-30
miR-10-3p	UAUAACGGAACACCUGCAUCGGCU		24	17,758	29751-29773	
miR-9-5p	UGAAGCAGGCUAUCUCUCACCUG	-	23	708	29909-29930	pre-miR-31
miR-9-3p	CAAGUGCAAGUGGCCUGCCUCAA		22	45	29870-29892	
miR-8-5p	AAUCAGACGGCUUGUGCAUAAGCU	-	24	3,183	30068-30091	pre-miR-32
miR-8-3p	UUAUGCACGGCUACCUGGUAG		21	2,876	30030-30050	
miR-7-5p	AGACAGCAGUGAGCGGGUGCG	-	22	2,295	30205-30226	pre-miR-33
miR-7-3p	CAAUCCACUCCCUGUCUGUCACU		23	5,923	30167-30189	
miR-6-3p	UAAUCUGCAAACUUCUGUCCCCU	-	23	10,632	30291-30312	pre-miR-34
miR-5-5p	AAUCGAGAAAGGCACUCCUCACCGG	-	25	8,866	30472-30496	pre-miR-35
miR-5-3p	UGUGGGUCUAGUGUCCUUCCGAGA		24	5,657	30431-30454	
Isolated :						
miR-33-5p	UGAGCAACACUCCCUGCCCCAG	+	22	64	32375-32395	pre-miR-36
"miR"-34-5p	AUCCCGGGGCCGAGGG	+	16	210	38132-38147	/
pr-DNA:						
"miR"-35-5p	CCCCGCCCCCGCUCU	+	16	48	82-97	/
miR-36-5p	UCCCCCCGCGGGGGCCCGGG	+	20	1,926	252-271	/

[a] The predominantly cloned sequences are indicated. 5p, 5' arm of the hairpin precursor; 3p, 3' arm of the hairpin precursor. Positions are given relative to the published genomic sequence of the C500 strain of AlHV-1. Small RNAs of = 16bp were not considered as miRNA and displayed as "miR".
[b] Correspondence with predicted pre-miRNAs in Walz *et al.*, 2010
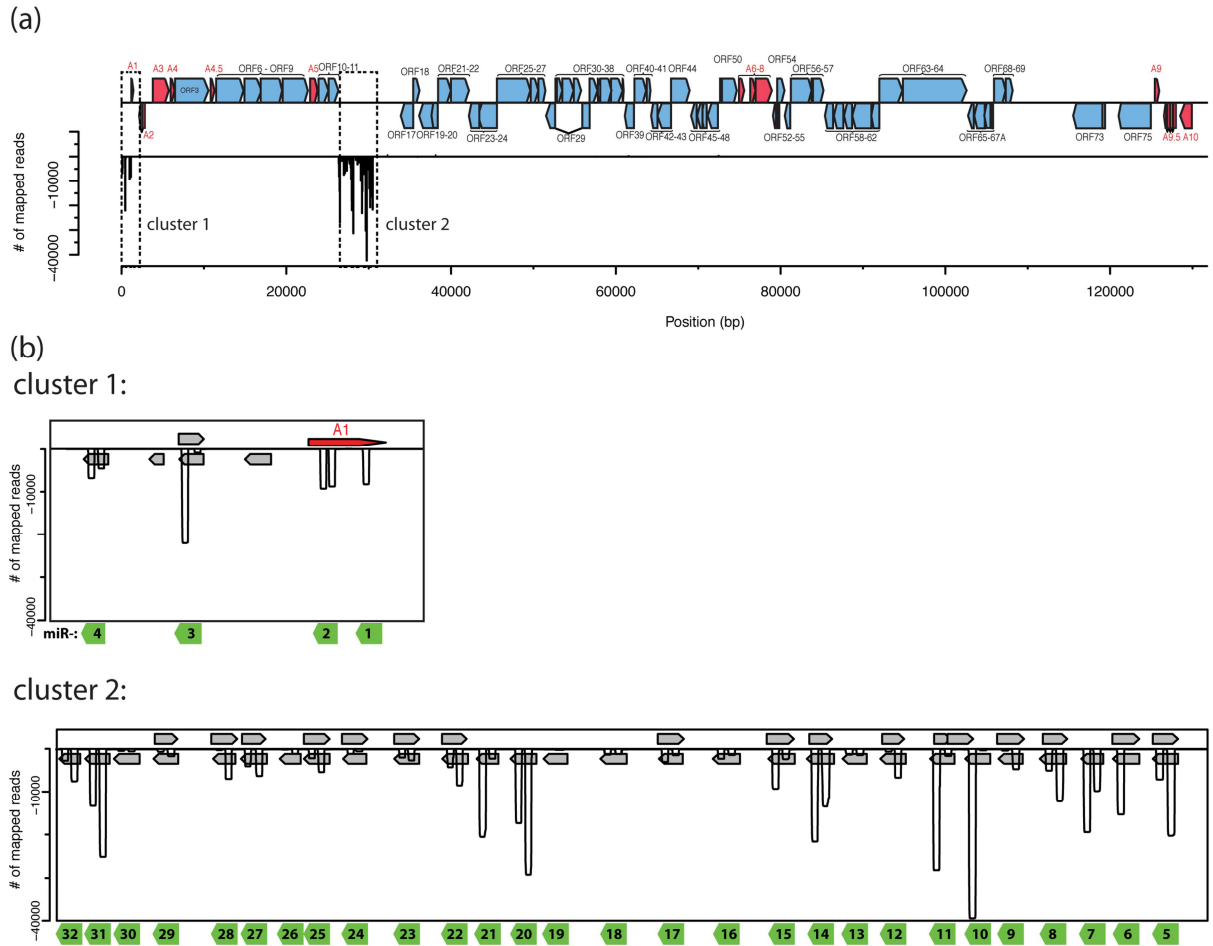
Figure 2. Genomic distribution of AlHV-1 small RNA sequences. (a) Small RNA cloning from total RNA of LCL propagated from a calf infected by the AlHV-1 C500 WT strain and developing MCF (LCL718). Predicted ORF are represented by the blue boxes and AlHV-1 specific ORFs are depicted in red. Small RNA reads are mapped on the genome relative to their position and abundance. **(b)** Inset zooms of identified small RNA cluster 1 and 2. Small RNA reads are mapped on the reverse strand of the genome relative to their position (from 5'- to 3'-end) and abundance. The grey boxes represent previously predicted pre-miRNA (Walz *et al.*, 2010). Negative values represent reads mapping on the reverse strand of the genome.
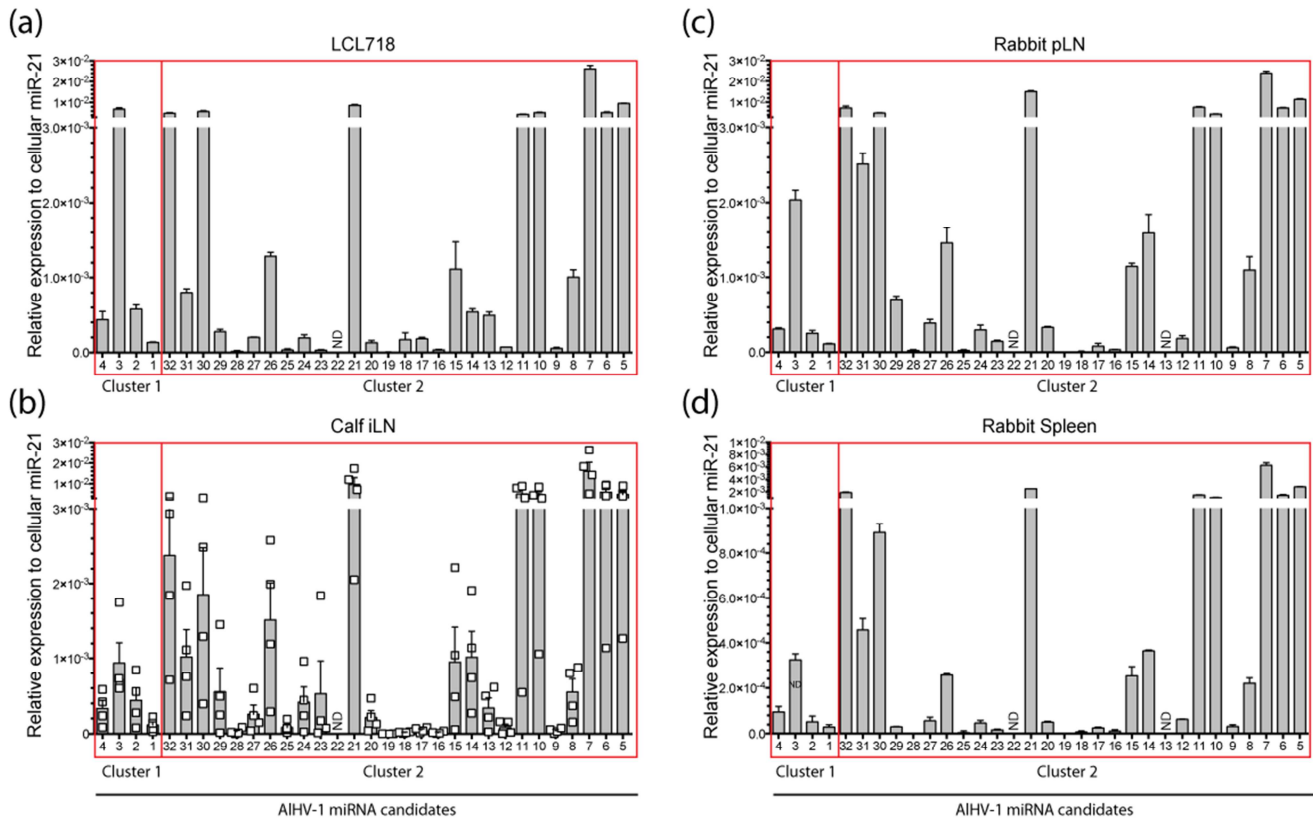
Figure 3. Detection of AlHV-1 miRNA candidates by quantitative RT-PCR. Total RNA (500 ng) was obtained from: **(a)** LCL718, **(b)** iLN of MCF-developing calves infected with the AlHV-1 WT C500 parental strain (n=4), open square symbols represent measurement for individual calves, **(c)** pLN cells of a MCF-developing rabbit infected with the AlHV-1 WT C500 BAC-excised strain, and **(d)** splenocytes of a MCF-developing rabbit infected with the AlHV-1 WT C500 BAC-excised strain. RNA was subjected to cDNA synthesis and miRNA-specific real-time PCR using a previously established protocol described in the Methods (Balcells *et al.*, 2011). Mock-infected calves (n=4) were used as controls for PCR specificity. Expression of viral miRNAs relative to host miR-21 are shown as $2^{-\Delta Ct}$ values.

```
                               U    A U–
miR-291b-3p:     5’– AAAGUGCA CC U   UUGUUUGU –3’
AlHV-miR-31-5p:  5’– AAAGUGCA CC U   UUGUUUGU –3’
                               C    C GG
```

Figure 4. **Sequence comparison of AlHV-1 miR-31-5p and miR-291b-3p.** Seed sequences are shown in bold.
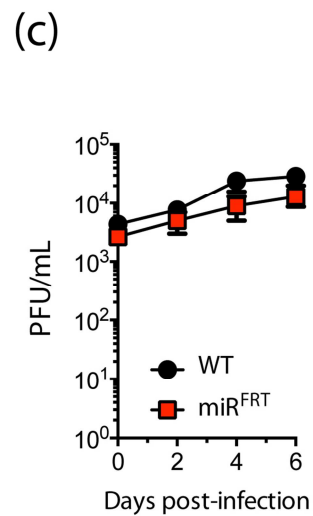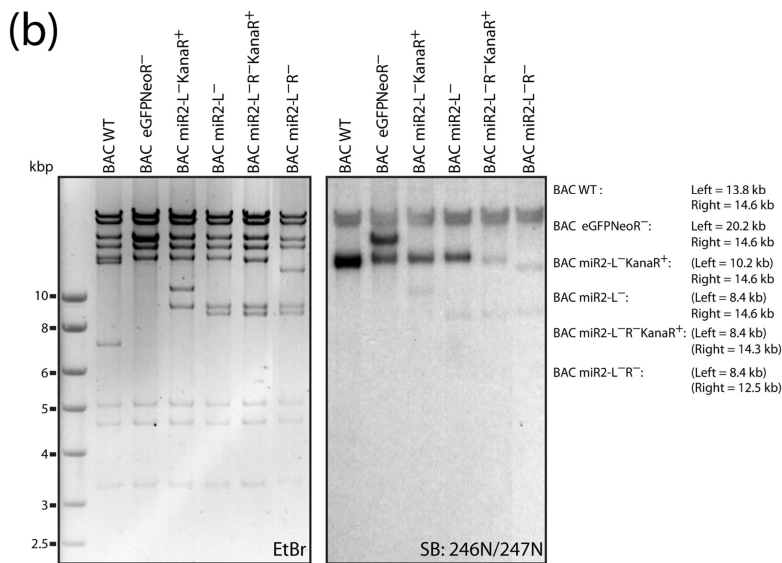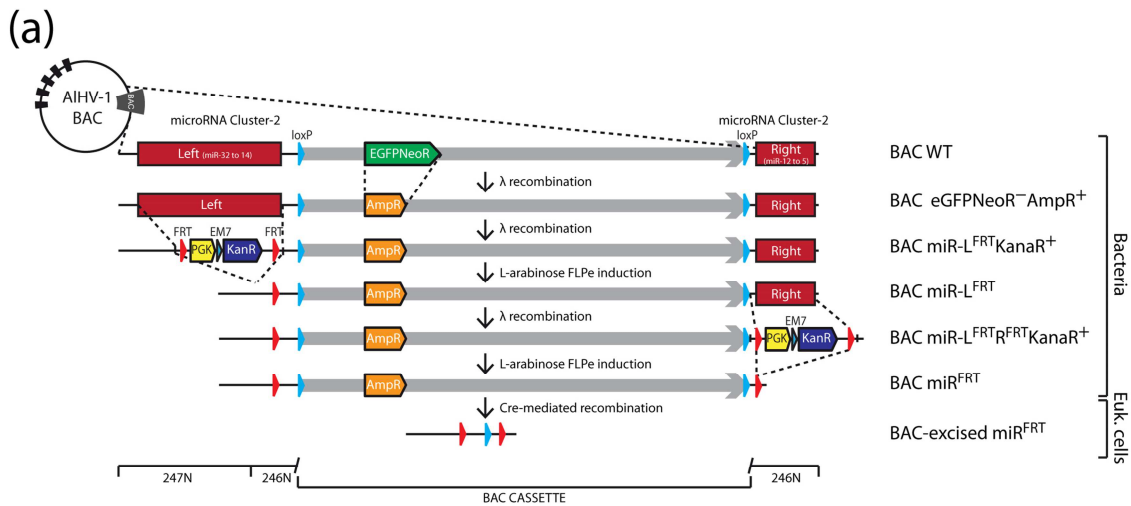
Figure 5. Deletion of cluster 2 miRNAs. (a) Recombineering methodology used to delete cluster 2 miRNAs flanking the BAC cassette and generation of the miR^{FRT} strain. **(b)** Southern blotting analysis of produced BAC plasmid DNA after *Eco*RI restriction and ethidium bromide staining (EtBr). The probes corresponding to region 246N and 247N were used (Ensser *et al.*, 1997). **(c)** Multi-step growth curves of WT and miR^{FRT} strains in BT fibroblasts. The data presented are means ± SD of results from measurements in triplicate.
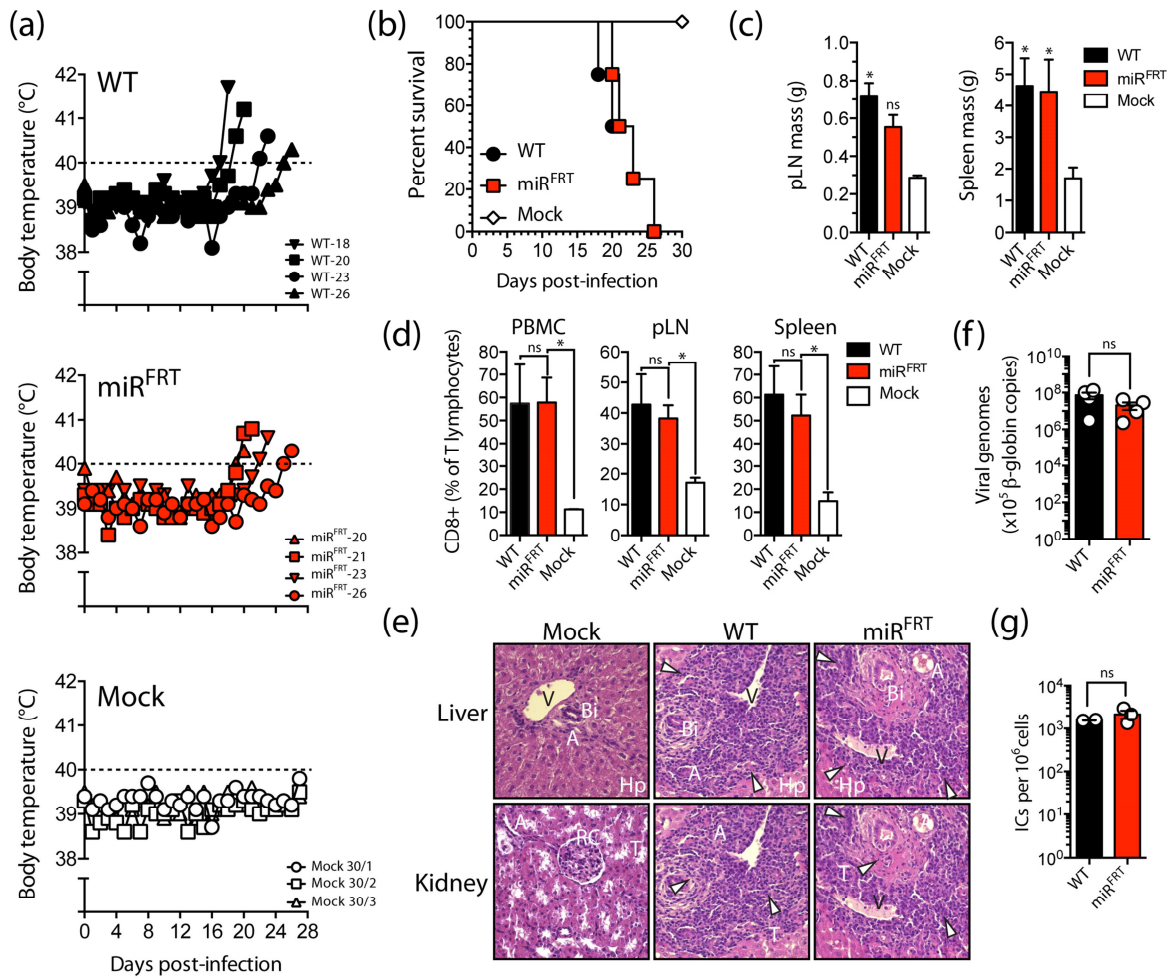
Figure 6. AlHV-1 cluster 2 miRNAs are not involved in MCF pathogenesis. (a) Body temperature of 3 groups of rabbits infected intravenously with mock-infected BT cells (mock, n=3), or BT cells infected with the WT eGFPNeoR⁻Amp^{R+} (WT, n=4) or miR^{FRT} (n=4) virus strains. **(b)** Cumulative incidence of survival. **(c)** Spleen and popliteal LN mass at time of euthanasia. Bars represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 (one-way ANOVA with Bonferroni's post test). **(d)** Percentages of CD8⁺ cells in the gated T cell population of PBMC, pLN and spleen analysed by flow cytometry at time of euthanasia. **(e)** Histopathological characterization of MCF lesions observed in liver and kidney of one rabbit representative of each group. White arrowheads indicate typical infiltrations of lymphoblastoid cells. A: arterioles; Bi: small bile ducts; Hp: hepatocytes; RC: renal corpuscules; T: uriniferous tubules; V: veins. **(f)** qPCR of viral genome copies in pLN at time of euthanasia of rabbits infected with the recombinant strains. Real-time PCR quantification was normalized on 10^5 copies of beta-globin cellular genomic sequence. Data are plotted as means ± SEM of triplicate measurements for each sample (n= 4). Unpaired Student *t* test. **(g)** Infectious center assay per million cells of pLN. Data are plotted as means ± SE of triplicate measurements for each sample (n= 4). Unpaired Student *t* test.