LETTERS

Anti-apoptotic function of a microRNA encoded by the HSV-1 latency-associated transcript

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MicroRNAs (miRNAs) are a class of small RNA molecules that regulate the stability or the translational efficiency of target messenger RNAs (mRNAs)[1,2]. The latency-associated transcript (*LAT*) of herpes simplex virus-1 (HSV-1) is the only viral gene expressed during latent infection in neurons[3]. *LAT* inhibits apoptosis and maintains latency by promoting the survival of infected neurons[4]. No protein product has been attributed to the *LAT* gene and the mechanism by which *LAT* protects cells from apoptosis is not yet known. Here we show that a miRNA encoded by the HSV-1 *LAT* gene confers resistance to apoptosis. Neuroblastoma cells transfected with a fragment of the *LAT* gene show reduced susceptibility to cell death. The anti-apoptotic function of *LAT* has been mapped to a region within the first exon[5,6]. We have identified and characterized a microRNA (miR-LAT) generated from the exon 1 region of the HSV-1 *LAT* gene. The *LAT* miRNA was found to accumulate in cells transiently transfected with the *LAT* gene fragment or infected with a wild-type strain of HSV-1. A mutant virus in which a 372-nucleotide fragment encompassing the mature miRNA was deleted neither protected the infected cells from apoptosis nor generated an miRNA. miR-LAT exerts its anti-apoptotic effect by downregulation of transforming growth factor (TGF)-β 1 and SMAD3 expression, both of which are functionally linked in the TGF-β pathway. Our results suggest that the miRNA encoded by the HSV-1 *LAT* gene regulates the induction of apoptosis in infected cells by modulation of TGF-β signalling and thus contributes to the persistence of HSV in a latent form in sensory neurons.

First we confirmed the anti-apoptotic function of *LAT*. As expected, a 2.9-kilobase (kb) fragment of the HSV-1 *LAT* gene (pcDNA-PstMlu) reduced susceptibility to stress-induced apoptosis in SY5Y and HeLa cells (Supplementary Fig. 1). Although it has been shown that *LAT* promotes neuronal survival after HSV infection by reducing apoptosis[4], the mechanism by which it regulates this process is still not understood. We hypothesized that the HSV-1 *LAT* gene exerts its anti-apoptotic effect by encoding a miRNA. To test this hypothesis, we attenuated the function of the RNase III enzyme Dicer by using specific short interfering RNA (siRNA) (Fig. 1a, b) and evaluated its effect on *LAT*-mediated resistance to apoptosis. Dicer-targeted siRNA abrogated the ability of a short hairpin RNA (shRNA) directed against green fluorescent protein (GFP-shRNA)[7] to block GFP expression in SY5Y cells (Supplementary Fig. 2). Dicer-targeted siRNA significantly inhibited the anti-apoptotic effect of *LAT* as compared to cells transfected with a control nonspecific siRNA (Fig. 1c).

Computational analysis using RNAMOT[8] and MFOLD[9] revealed the presence of a sequence within exon 1 of HSV-1 *LAT* containing a hairpin loop reminiscent of precursor miRNAs (Fig. 2a). Next we cloned the small RNAs from SY5Y cells transfected with pcDNA-PstMlu. Colony hybridization with the Sty-Sty region of *LAT* revealed that 7% of the clones contained miR-LAT sequences. Sequencing of positive clones confirmed the predicted sequence of miR-LAT shown in Fig. 2a. We cloned miR-LAT multiple times. No other HSV-1 miRNA was present in the sequenced clones. The sequences present in the HSV-1 *LAT* region encoding miR-LAT are conserved in 17+, F and McKrae strains of HSV-1 (Fig. 2a). Transfection of cells with a *LAT* deletion construct (ΔSty)—deletion of a 372-nucleotide fragment encompassing the predicted mature miRNA (Fig. 2 a)—showed significant reduction in protection from cisplatin-induced apoptosis (Fig. 2b).

A synthetic RNA oligonucleotide corresponding to pre-miR-LAT was processed *in vitro* by a recombinant Dicer enzyme to yield a ~20-nucleotide product (Fig. 2c). Next we performed a northern blot analysis to evaluate the expression of miR-LAT in *LAT*-expressing cells. Only one of the strands of the double-stranded hairpin precursor is energetically favoured to enter the RISC complex and accumulate in the cell as the mature miRNA[10]. Therefore we used two probes: the 5′ probe comprising of the 5′ arm of the hairpin, and the 3′ probe comprising of the 3′ arm of the hairpin. The 3′ probe revealed a ~60-nucleotide and a ~20-nucleotide band (Fig. 2d), corresponding to pre-miRNA and mature miRNA, respectively. The 5′ probe did not show any mature miRNA (Fig. 2d); however, a ~60-nucleotide pre-miRNA band was detected by the 5′ probe. This indicates that

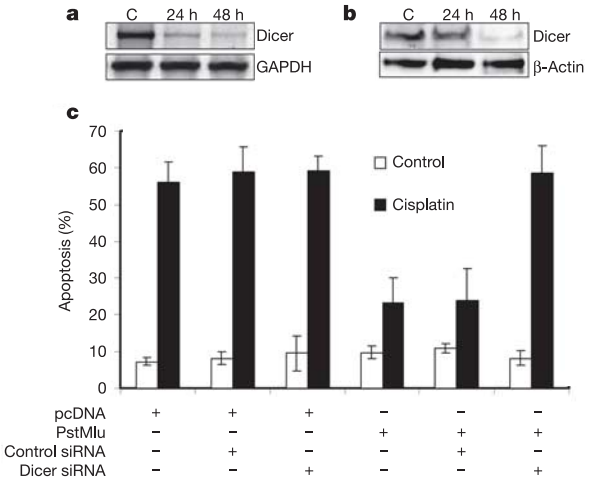

Figure 1 | *LAT* inhibits apoptosis by a Dicer-dependent mechanism. **a**, RT–PCR analysis of the transcript levels of Dicer and GAPDH after transfection of SY5Y cells with siRNA targeting Dicer. **b**, Western blot analysis for Dicer and β-actin using whole-cell lysates of SY5Y cells transfected with siRNA targeting Dicer. **c**, SY5Y cells were transfected with the indicated plasmids, treated with cisplatin and the percentage of apoptosis was determined. Data represent the mean of three independent experiments ±s.d.

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only the 5′ arm of the pre-miRNA hairpin constitutes the mature miR-LAT. Cells transfected with the plasmid pcDNA-ΔSty, a deletion mutant of *LAT*, were not protected from apoptosis (Fig. 2b) and northern blot analysis did not detect any pre-miRNA or mature miRNA (data not shown). Next we determined the generation of miR-LAT in virus-infected cells. For this purpose we used the wild-type virus (17+), mutant virus (ΔSty) with a 372-nucleotide deletion in exon 1 (position +76 to +447) of the *LAT* gene, and a ΔSty rescued virus (StyR). The mutation did not affect the ability of the virus to establish infection in tissue culture or the expression of other viral genes (data not shown). As shown in Fig. 2e, ~20-nucleotide mature miR-LAT was detected in cells infected with wild-type and StyR virus. No mature miR-LAT was found in cells infected with the mutant virus (Fig. 2e). Furthermore, the ΔSty virus was less efficient in protecting cells from stress-induced apoptosis as compared to the wild-type and StyR virus (Fig. 3a, b; see also Supplementary Fig. 3).

To determine the role of miR-LAT in providing resistance to apoptosis we used 2′-*O*-methyl antisense oligonucleotides specific for miR-LAT. As shown in Fig. 3c, d, co-transfection of the 2′-*O*-methyl antisense oligonucleotide with the *LAT* fragment (pcDNA-PstMlu) significantly attenuated its anti-apoptotic effect, whereas a control 2′-*O*-methyl oligonucleotide had no such effect. Both antisense and control 2′-*O*-methyl oligonucleotides did not show any effect on apoptosis when co-transfected with control (pcDNA) plasmid (Fig. 3c, d; see also Supplementary Fig. 4). To elucidate further the anti-apoptotic function of miR-LAT we transfected cells with a synthetic double-stranded RNA oligonucleotide corresponding to mature miR-LAT. This synthetic miR-LAT oligonucleotide caused significant reduction in apoptosis as compared to control miRNA (Fig. 3e, f; see also Supplementary Fig. 5).

To gain an insight into the mechanism by which miR-LAT regulates apoptosis, we sought to identify targets of miR-LAT. Computational analysis using miRanda[11] identified transforming growth factor-β1 (TGF-β) and SMAD3 as targets of miR-LAT, both of which are functionally related in TGF-β signalling. TGF-β is a potent inhibitor of cell growth and an inducer of apoptosis[12]. SMAD2 and SMAD3 are phosphorylated by the activated TGF-β receptor, form complexes with SMAD4, and together accumulate in the nucleus to regulate transcription of target genes that have important roles in diverse cellular processes[13]. TGF-β-induced cell death is associated with changes in the expression, localization and activation of both pro- and anti-apoptotic members of the Bcl2 family, as well as activation of caspases[14]. The 3′ untranslated region (UTR) of TGF-β and SMAD3 contain sequences with partial homology to miR-LAT (Fig. 4a). The miR-LAT response element in the 3′UTR of TGF-β is evolutionarily conserved (Fig. 4a). Given that miRNAs function in RNA silencing pathways either by targeting mRNAs for degradation or by repressing translation, we determined the effect of HSV-1 *LAT* on mRNA and protein levels of TGF-β and SMAD3. We observed a substantial decrease in the mRNA levels of TGF-β and SMAD3 in cells transfected with pcDNA-PstMlu or infected with

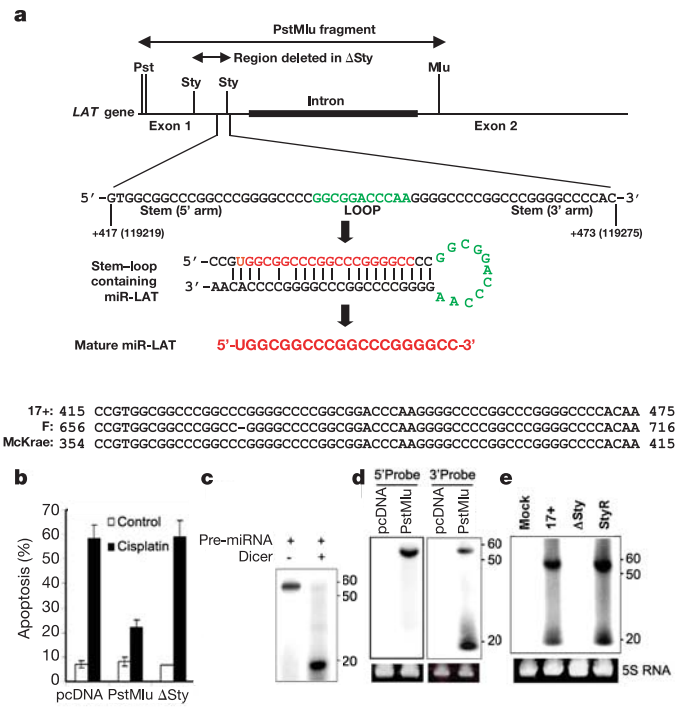

Figure 2 | The *LAT* gene of HSV-1 codes for a miRNA. **a**, Schematic representation of the HSV-1 *LAT* gene. Sequence of mature miRNA is shown in red. Bottom panel: sequence conservation of miR-LAT-encoding region in 17+, F and McKrae strains of HSV. **b**, SY5Y cells were treated with cisplatin 24 h after transfection and the percentage of apoptosis was determined. Data represent the mean of three independent experiments ±s.d. **c**, Pre-miR-LAT is processed *in vitro* by recombinant Dicer. **d**, Northern blot analysis of the *LAT* miRNA in SY5Y cells transfected with pcDNA or pcDNA-PstMlu. **e**, Northern blot detection of LAT miRNA in cells infected with the indicated viruses. An ethidium-bromide-stained picture of 5S RNA is shown as a loading control. The migration of a DNA oligonucleotide ladder (bp) is indicated.

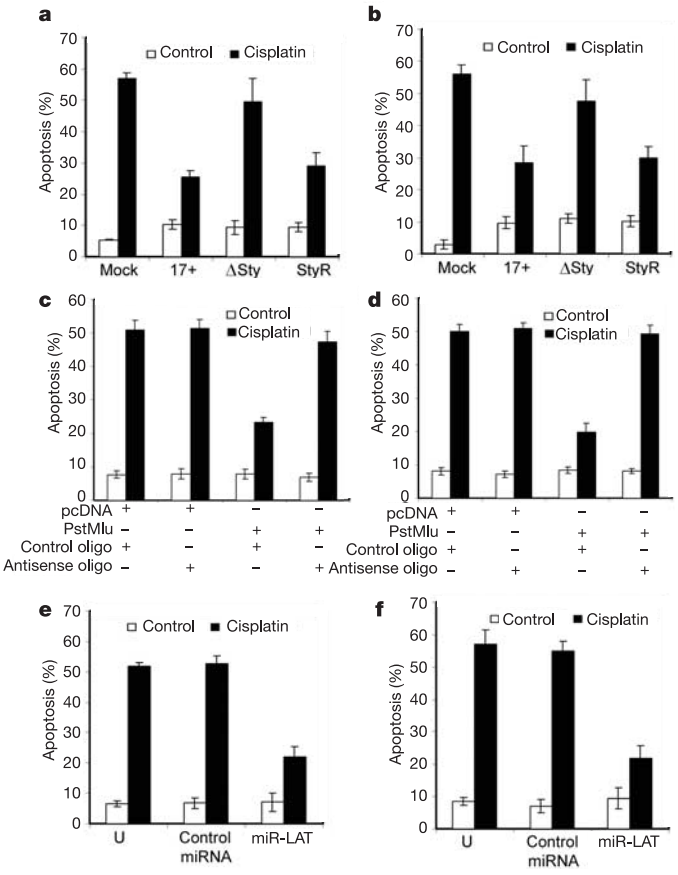

Figure 3 | The *LAT* region of HSV-1 17+ protects cells from apoptosis. **a**, **b**, SY5Y (**a**) and HeLa (**b**) cells were infected as indicated and treated with cisplatin 16 h after infection. Data represent the mean of three independent experiments ±s.d. **c**, **d**, SY5Y (**c**) and HeLa (**d**) cells were transfected as indicated, treated with cisplatin 24 h after transfection, and the percentage of apoptosis was determined. Data represent the mean of three independent experiments ±s.d. **e**, **f**, SY5Y (**e**) and HeLa (**f**) cells were treated with cisplatin 24 h after transfection with the indicated miRNA, and the percentage of apoptosis was determined. Data represent the mean of three independent experiments ±s.d. U, untransfected.

wild-type 17+ virus (Fig. 4b), which was accompanied by a concomitant decrease in endogenous SMAD3 (Fig. 4b) and TGF-β protein (Supplementary Fig. 6). We observed a significant decrease in the activity of the *Renilla* luciferase reporter gene fused to the full-length 3′UTRs of TGF-β or SMAD3 in *LAT*-expressing SY5Y cells, which was accompanied by a decrease in *Renilla* luciferase mRNA (Fig. 4c). There was no effect on the activity and mRNA levels of a *Renilla* luciferase reporter gene lacking the miR-LAT response elements (Fig. 4c). Furthermore, *LAT* expression had no effect on mRNA levels of firefly luciferase, which was used as a transfection control (Fig. 4c). *LAT* expression had no effect on the activity of a *Renilla* luciferase reporter construct having the 3′UTR of TGF-β or SMAD3 in the presence of a 2′-*O*-methyl antisense oligonucleotide specific for miR-LAT (Fig. 4d). To confirm that repression of TGF-β and SMAD3 by miR-LAT is via the predicted miR-LAT-binding site in their 3′ UTRs (Fig. 4a), we cloned double-stranded oligonucleotides comprising the wild-type target site or a mutant target site that disrupts miR-LAT binding at the 3′ end of the *Renilla* luciferase gene (Supplementary Fig. 7). These clones contained a single copy of either the wild-type or the mutated target site. The wild-type construct (TGF-β and SMAD3) showed up to a 90% reduction in luciferase activity, which was accompanied by a decrease in *Renilla* luciferase mRNA levels (Fig. 4e). The mutant construct did not significantly decrease luciferase activity or mRNA levels of a *Renilla* luciferase reporter gene (Fig. 4e). Thus, the proposed site (Fig. 4a) is the major miR-LAT-binding site in these target genes.

Next we inhibited the expression of TGF-β and SMAD3 by using siRNAs that specifically target TGF-β and SMAD3 (Fig. 4f). Down-regulation of TGF-β and SMAD3 independently of miR-LAT also confers resistance to apoptosis (Fig. 4f), whereas a control scrambled siRNA did not show any such effect (Fig. 4f). This suggests that inhibition of the TGF-β pathway by miR-LAT is sufficient for its anti-apoptotic effect.

Furthermore, we analysed the effect of miR-LAT on TGF-β/SMAD-dependent transcription using SBE4-Luc, a TGF-β-responsive reporter gene construct that contains four copies of the SMAD-binding element (SBE)[15]. As shown in Fig. 4g, pcDNA-PstMlu inhibited TGF-β-mediated induction of the SBE4-Luc construct. The *LAT* deletion construct (ΔSty) had no effect on induction of the SBE4-Luc reporter gene as compared to pcDNA (Fig. 4g). *LAT* expression had no effect on TGF-β-mediated induction of the SBE4-Luc reporter gene in the presence of 2′-*O*-methyl antisense oligonucleotides specific for miR-LAT (Fig. 4h). To elucidate further the role of miR-LAT in TGF-β signalling, SY5Y cells were co-transfected with a synthetic double-stranded RNA oligonucleotide corresponding to mature miR-LAT along with the SBE4-Luc reporter gene. As shown in Fig. 4i, the synthetic miR-LAT oligonucleotide caused a significant reduction in TGF-β-mediated induction of SBE4-Luc reporter gene activity as compared to a control (scrambled) double-stranded RNA oligonucleotide.

Taken together, our results show that the HSV-1 *LAT* gene (position +415 to +475) codes for a miRNA that can inhibit apoptosis. The *LAT* gene maintains latent infections by protecting infected neurons from undergoing apoptosis[4]. Furthermore, it has been shown that an unrelated anti-apoptotic gene can substitute for the HSV-1 *LAT* gene during reactivation of the virus in mice[16,17]. Our results suggest that the anti-apoptotic miRNA miR-LAT, encoded by the *LAT* region of HSV-1, has an important role in the survival of latently infected neurons and thus contributes to the persistence of

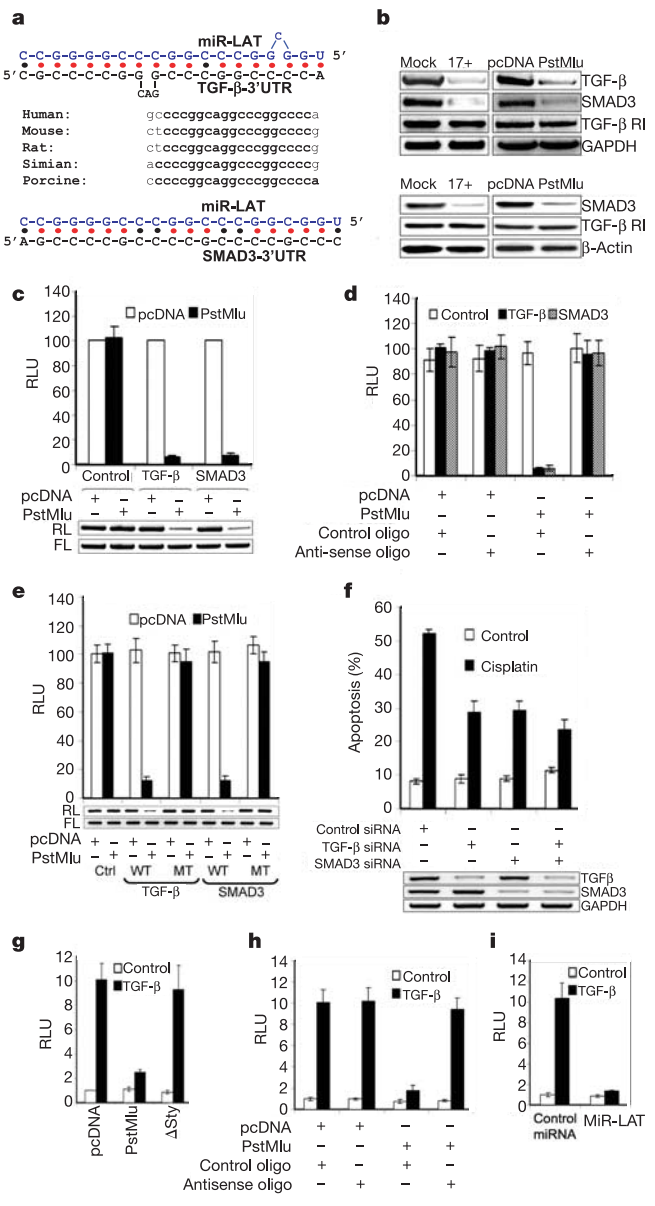

Figure 4 | Modulation of TGF-β signalling by miR-LAT. a, The predicted duplex of miR-LAT and its target site in the 3′UTR of TGF-β (top panel) or the 3′UTR of SMAD3 (bottom panel); canonical base pairs are marked with red circles; mismatches are shown as black circles. The sequence alignments in the middle panel show nucleotide conservation of the miR-LAT-binding site within the 3′UTR of TGF-β. **b**, Top panel: RT–PCR analysis of the transcript levels of TGF-β, SMAD3, TGF-β receptor I and GAPDH. Bottom panel: western blot analysis for SMAD3, TGF-β receptor I and β-actin. **c**, Top panel: SY5Y cells were co-transfected with *Renilla* luciferase constructs containing the full-length 3′UTRs of the indicated genes along with pSVβ-gal (β-gal), pcDNA or PstMlu. Data represent the mean of normalized *Renilla* luciferase/β-gal activities of four independent experiments ±s.d. RLU, relative luciferase units. Lower panel: transcript levels of *Renilla* luciferase and firefly luciferase (as a normalization control) mRNAs as determined by RT–PCR. **d**, SY5Y cells were transfected with the indicated UTR constructs. Data represent the mean of normalized *Renilla* luciferase/β-gal activities of four independent experiments ±s.d. **e**, Top panel: SY5Y cells were transfected with *Renilla* luciferase constructs containing the indicated wild-type or mutated target sites along with pcDNA or PstMlu. Data represent the mean of normalized *Renilla* luciferase/β-gal activities of four independent experiments ±s.d. Bottom panel: transcript levels of *Renilla* luciferase and firefly luciferase (as a normalization control) as determined by RT–PCR. **f**, Top panel: SY5Y cells were treated with cisplatin, 24 h after transfection, and the percentage of apoptosis was determined. Data represent the mean of four independent experiments ±s.d. Bottom panel: RT–PCR analysis of the transcript levels of TGF-β, SMAD3 and GAPDH. **g–i**, SY5Y cells were transfected as indicated along with the SBE4-Luc reporter gene. Cells were treated with or without recombinant human TGF-β and analysed for luciferase activity. Data represent the mean of four independent experiments ±s.d.

infection. Recently it has been shown that mammalian cells can use RNA interference (RNAi) mechanisms to restrict viral propagation[18,19]. Some viruses have, in turn, evolved mechanisms to counter these RNA-mediated anti-viral responses of the host cell[18,19]. Our results suggest that miR-LAT inhibits apoptosis by modulation of TGF-β signalling. Thus, HSV uses the RNAi pathway to regulate host cell apoptosis to ensure survival of infected neurons and maintenance of latent infection. Such a mechanism would circumvent the need for the expression of a viral protein during the latent infection and thus help the virus to evade immune detection. Our results highlight a novel role for the RNAi pathway in host–virus interactions.

METHODS

See Supplementary Methods for information regarding detailed protocols.

Cells and virus. SY5Y cells were maintained in RPMI medium with 10% fetal calf serum and antibiotics. HeLa cells were grown in DMEM medium supplemented with 5% fetal calf serum and antibiotics. Plasmids having the PstMlu fragment of *LAT* and its deletion mutant ΔSty have been described previously[5]. The wild-type strain of HSV-1 (17+) and the mutant virus (ΔSty) have been described earlier[5].

Apoptosis assay. At 24 h after transfection cells were treated with cisplatin (10 μM) or etoposide (1–100 μM), followed by staining with Annexin V FITC (BD Biosciences). Cells showing FITC staining, loss of cell volume, loss of refractility and membrane blebbing were scored as apoptotic. At least 500 cells were counted in each well.

RNA isolation, RT–PCR, northern blotting and western analysis. RNA was isolated using Trizol reagent (Invitrogen). RNA was reverse transcribed using Superscript II RT enzyme (Invitrogen). Northern blot analysis of 50–60 μg total RNA was done as described previously[21] (see also http://web.wi.mit.edu/bartel/pub/protocols/miRNA_Nrthrns_Protocol.pdf). Western blot analysis was performed on whole-cell lysates using Dicer polyclonal antibody[20] and β-actin antibody (Amersham). SMAD3 and TGF-β receptor I antibodies were from Abcam.

Reporter assays. Cells were co-transfected with pcDNA or PstMlu (1 μg) and the respective reporter constructs (500 ng) along with pSV-βgal (100 ng). For induction of TGF-β signalling, 24 h after transfection the cells were treated with 10 ng ml^{-1} recombinant TGF-β1 protein (R&D systems). Reporter activity was measured using appropriate assay kits (Promega).

miRNA cloning. Small RNA molecules were cloned from PstMlu-transfected cells according to the protocols described previously[22].

Computational methods. RNAMOT[8] was used to search for RNA hairpin. The detected duplexes were further analysed by MFOLD[9]. Targets of miR-LAT were identified using the software miRanda[11]. Statistical analysis was performed using JMPIN 4 software. One-way analysis of variance (ANOVA) was performed at an alpha level of 0.01–0.05 to compare all the variants in a data set.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions A.G. conceived the project and carried out all experiments described. J.J.G. provided technical assistance. P.S. and A.G.H. developed and applied computational algorithm for miRNA detection and target prediction. N.W.F. directed and supervised the experimental work and interpretation of data. The manuscript was prepared by A.G. and N.W.F.

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