Translin associated protein X is essential for cellular proliferation

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Abstract DNA vectors that express short hairpin RNAs (shRNAs) provide a new tool for reverse genetic analysis for selective long-term reduction of gene expression in mammalian cells. Using shRNA constructs with a cytomegalovirus promoter and an actin intron between the hairpins for stabilization, we reduce expression of an exogenously expressed gene, GFP and the endogenous protein, Translin-associated factor X (TRAX), in stably transfected Hela cell lines. The reduction of TRAX in Hela cells causes reduced cell proliferation. This decrease is specific as there is no equivalent reduction of the TRAX interacting protein, Testis brain RNA-binding protein, or any significant increase in a number of interferon-related target genes.

Keywords: RNA interference; Translin associated protein X; Cell proliferation

1. Introduction

Translin and its mouse ortholog, Testis brain RNA-binding protein (TB-RBP), are highly conserved and ubiquitously expressed DNA- and RNA-binding proteins with proposed functions in mitotic cell division [1,2], chromosomal translocations [3–5], stabilization, transport, and translational suppression of specific mRNAs [6–8]. Mice lacking TB-RBP are viable and when heterozygotes were bred, a normal Mendelian distribution was obtained [2]. However, the TB-RBP null mice were 10–30% smaller than their littermates, accumulated visceral fat, had a thin carnosus panniculus, and showed behavioral changes. The males had abnormal seminiferous tubules and reduced sperm counts, while the females were subfertile and had reduced litter sizes.

In addition to these phenotype changes in mice lacking TB-RBP, one of the TB-RBP interacting proteins, Translin associated protein X (TRAX) [9,10] was also affected, being reduced to 50% in heterozygotes and absent in the nulls. This loss of TRAX occurred post-transcriptionally as equal amounts of TRAX mRNA were detected in tissues from wild type, heterozygous, and homozygous mice [2]. Defects at the cellular level were also detected. Embryonic fibroblasts (MEFs) from TB-RBP null mice proliferated more slowly than MEFs isolated from their heterozygous or wild type littermates. Reintroduction of TB-RBP into the null MEFs remedied this defect [11]. However, upon reintroduction of TB-RBP, TRAX also began to accumulate. TB-RBP is needed to stabilize TRAX by forming hetero-oligomers because in the absence of TB-RBP, TRAX becomes ubiquitinated and degraded [11]. Thus, the concomitant loss of both TB-RBP and TRAX in TB-RBP deficient mice makes it difficult to relate specific cellular functions to TB-RBP, to TRAX, or to both proteins.

Here, we utilize an intron-based cytomegalovirus (CMV) driven short hairpin vector to selectively reduce expression of TRAX in long-term cultures without decreasing TB-RBP levels. We find that TRAX is essential to maintain normal rates of cell proliferation in cultured cells.

2. Materials and methods

2.1. Intron-based shRNAs system

A long pair of "shagging" primers containing two fragments from the GFP coding region with a 3–8 bp spacer was synthesized as follows: forward primer-cacccta cgg caa gct gac cct gaa gtt cat cta tat ata tag gct acg tcc agg agc gca cca tct tcg ctt gca ttg atc aca cgt gga tga ccc agg tga gtg gcc cgc and reverse primer-a cct acg gca agc tga ccc tga agt tca tgg ccg gct acg tcc agg agc gca cca tct tca att cac gtg tga tca ctc aaa cat gat ctg taa ggc ag (underlining indicates sequences from the GFP coding region). Genomic DNA from Hela cells or primary human fibroblasts was used as template and the β-actin intron C [12] was amplified and cloned into a pLenti6/V5-D-TOPO vector (Invitrogen, Carlsbad, CA). A similar pair of primers containing two fragments of the human TRAX coding region was designed with the following primers: forward primer-caccatt cat ctt cac ccg tga tgt tgg cct ta atc att tca gca gga act tga tgc aag cca ggt gag tgg ccc gct a and reverse primer-att cat ctt cac ccg tga tgt tgg cct ggg atc att tca gca gga act tga tgc aag ctc aaa cat gat ctg taa ggc (underlining indicates sequences from the TRAX coding region). PCR products were cloned into a lentivirus vector. The pLentivirus vectors containing the inverted repeat of GFP and TRAX were named pLenti6/GFPi and pLenti6/TRAXi, respectively.

2.2. Lentivirus infection

293FT cells (Invitrogen, Carlsbad, CA) were cultured in complete culture medium (DMEM containing 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin) and Geneticin (500 μg/ml). The day before transfection, 5×10^6 293FT cells were plated onto 100 mm plates with 10 ml of culture medium. On the day of transfection, the culture medium was replaced with 5 ml of growth medium containing serum without antibiotics. ViraPower Packaging Mix (Invitrogen, Carlsbad, CA) (9 μg) and 3 μg of pLenti6/GFPi or pLenti6/TRAXi were mixed with 1.5 ml Opti-MEM I medium without serum. At the same time, 36 μl of Lipofectamine 2000 (Gibco BRL, Rockville, MD) was added to the Opti-MEM I medium. The DNA and Lipofectamine 2000 were gently mixed, incubated for 20 min at room temperature and added to the DNA-Lipofectamine 2000 complexes dropwise. After gentle

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mixing, the cells were incubated overnight at 37 °C in a CO_2 incubator. The next day, the medium was replaced with complete culture medium and the virus-containing supernatants were harvested 72 h post-transfection. The cell debris was removed by centrifugation at 3000 rpm for 15 min at 4 °C and the viral supernatants were pipetted into cryovials in 1 ml aliquots and stored at −80 °C. U2, H1299, HFF and Hela cells (ATCC, Manassas, VA), propagated with complete culture medium (DMEM) except U2, were cultured in McMoy's 5A medium (Gibco BRL, Rockville, MD) and plated at 5×10^5 cells per 100 mm plate. The next day, the virus-containing supernatants were diluted 3-fold and added to the cells with polybrene (6 μg/ml) (Sigma, St. Louis, MO). The following day, the medium was replaced with complete culture medium. Seventy two hours after transfection, the medium was replaced with fresh medium containing 50 μg/ml of blasticidin. This was repeated every 3–4 days to produce the stably transfected cells expressing GFP or TRAX shRNAs. The colonies were monitored for 3 weeks for pLenti6/GFPi and 6 weeks for pLenti6/TRAXi transduction and picked with clone cylinders (Sigma, St. Louis, MO).

2.3. Western blotting and flow cytometry

U2, H1299, HFF and Hela cell derived lines expressing shRNAs of GFP and TRAX were propagated as above and maintained with blasticidin (30 μg/ml). HA tagged TB-RBP was amplified using a pair of primers containing the HA TB-RBP-coding sequence in the forward primer (5′ primer, gccgctagcgccgccaccatgggatcctacccttacgacgttcctgattacgctagcctcgaattc-atgtctgtgagcgagatcttc; 3′ primer, gcctctagactatttttcaccacaagc) and cloned into the pCI-Neo expression vector (Promega, Madison, WI). The cell lines expressing GFP shRNAs were transfected with the pEGFP-C1 vector (Clontech, Palo Alto, CA) and analyzed 72 h after transfection. Whole cell lysates were prepared in RIPA buffer [(1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS, containing freshly added 1/10 volume of protease inhibitor cocktail) (Sigma, St. Louis, MO) in PBS], incubated on ice for 20 min, and centrifuged at 12 000×*g* for 10 min at 4 °C. The protein concentration in the supernatant was quantitated with a BCA Protein Assay Kit (Pierce, Rockford, IL). Aliquots (20 μg) were mixed with 2SDS loading buffer containing 0.35 M 2-mercaptoethanol, subjected to 10% SDS-PAGE and then transferred onto PVDF membranes (Millipore, Bedford, MA). Primary antibodies for mouse anti-GFP (Roche, Indianapolis, IN), mouse anti-HA (Roche Diagnostics, Germany), mouse anti-α-tubulin (Sigma, St. Louis, MO), affinity purified anti-TB-RBP [13] and mouse anti-TRAX (Pharmagen, San Diego, CA) were used to detect each of the proteins. Horseradish peroxidase-conjugated anti-mouse IgG antibody, protein A and the Enhanced Chemiluminescent (ECL) Detection Kit (Amersham Pharmacia, Piscataway, NJ) were used to visualize each protein. Hela cell lines expressing shRNAs were trypsinized 72 h after transfection. Cells were resuspended in complete DMEM, centrifuged at 3000×*g* for 5 min, and washed twice with ice cold PBS. Samples were sorted using an EPICS XL (Beckman-Coulter, Miami, FL). The Hela cell lines expressing TRAX shRNAs were propagated and maintained in blasticidin (30 μg/ml). When the cells reached confluency, whole cell lysates were harvested and analyzed by Western blotting as described above. TRAX expression was measured by densitometry using ImageQuant software and relative expression levels were normalized to TB-RBP and the tubulin loading control. Hela cell lines expressing TRAX and GFP shRNAs and mock infected Hela cells were plated at 5×10^5 in 100 mm dishes in duplicate. Cell numbers were counted every 24 h for 5 consecutive days. At 72 h, cells were harvested and sorted by FACs for cell cycle analysis. Briefly, the cells were resuspended in 1 ml of −20 °C methanol and incubated for a minimum of 30 min on ice. The cells were then centrifuged at 3000 g at 4 °C for 5 min, the supernatants aspirated, and 400–500 μl of propidium iodide staining solution (0.15 mM propidium iodide, 0.1% Triton X-100 and 1 mg/ml RNase (Sigma, St. Louis, MO)) was added.

2.4. Quantitative real-time RT-PCR

Total Hela cell RNA was isolated with the Rnase Easy kit (Qiagen, Valencia, CA). RNA was incubated with deoxyribonuclease I (Invitrogen,Carlsbad, CA) for 15 min at room temperature and the DNase I was inactivated by addition of 1 μl of 25 mM EDTA to the reaction mixture and heated for 10 min at 65 °C. The RNA samples were transcribed into cDNAs using SUPERSCRIPT RT (Invitrogen, Carlsbad, CA). The primers for real-time RT-PCR were designed using Primer Express 1.5 software. The following primers were synthesized. OAS1, forward primer: agg tgg taa agg gtg gct cc, reverse primer: aca acc agg tca gcg tca gat; MX1, forward primer: acctgtgcagccagtatgagg, reverse primer: agcccgcagggagtcaat; ISG20, forward primer: cctgcacaagagcatccaga, reverse primer: cgttgcccteg catcttc; IFI44, forward primer: tggcagtgacaactcgtttga, reverse primer: ccgcttccctccaaaa; IFIT1, forward primer: gccacaaaaaatcacaagcca, reverse primer: ccattgtctggatttaagcgg; TRAX, forward primer: ccg tga tgt tgg cct tta aat c, reverse primer: cct atg gag gag aaa aat tgt cct t; TB-RBP, forward primer: agc gtg act gct gga gac tac tc, reverse primer: gaa aac cgg aat cca gct ca; and GAPDH, forward primer: aac gac ccc ttc att gac ctc, reverse primer: ttg aat ttg ccg tga gtg ga. Real-time RT-PCR was performed with the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA) using SYBR green. Relative gene expression was quantitated by the comparative CT method as described in the user's manual. In three Hela cell lines, samples were examined in duplicate and the relative amounts of TRAX, TB-RBP, MX1, ISG20, IFI44, IFIT1 and OAS1 mRNAs were normalized to Hela GAPDH mRNA.

3. Results and discussion

3.1. Intron-based shRNA construct

Many RNA interference methodologies are available to selectively reduce gene expression in cells and animals [14–19].

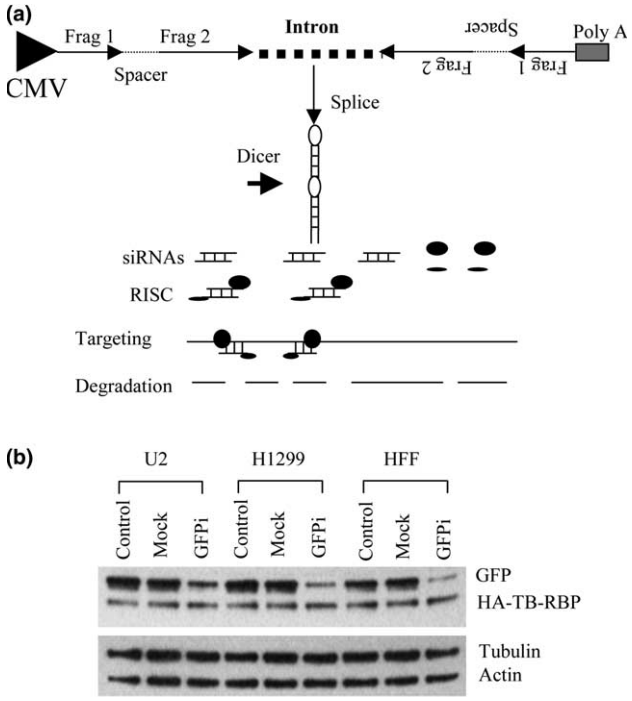

Fig. 1. Selective reduction of gene expression in human cell lines by an intron-based shRNA vector. (a) Schematic diagram showing the formation of intron-based shRNA vectors. Fragments of 28 and 29 bp from the coding region of GFP with a small spacer and their inverted repeats linked by human β-actin intron C were cloned into a mammalian expression vector driven by the CMV promoter. The intron-junction sequence is predicted to be spliced during pre-mRNA processing to produce a loopless hairpin structure similar to miRNA precursors. The shRNAs are further processed by Dicer to siRNAs that guide cleavage of the cognate mRNA. (b) Selective reduction of an exogenously expressed mRNA, GFP in three human cell lines, U2, H1299 and HFF, following infection with a lentivirus construct expressing GFP shRNAs. Antibiotic resistant pools of cells were selected and transfected with pEGFP and control HA tagged TB-RBP. Seventy two hours after transfection, whole cell lysates were prepared and aliquots (25 μg) were analyzed by Western blotting. GFP expression was measured in the control parental line, in a mock transfection, and following transfection with the GFP shRNAs cassette.

To determine whether TB-RBP or TRAX are essential to maintain normal rates of cellular proliferation, we have utilized a novel intron-based shRNA vector driven by a CMV promoter. Two short fragments from the GFP coding region and its inverted repeat linked to a human β-actin intron C [12] were cloned into a lentivirus mammalian expression vector driven by a CMV promoter. The intron contributes to the stability of the two short perfect inverted-repeat sequences, which are not a prerequisite for the specificity of gene silencing (Fig. 1(a)). The intron-based construct elicits total silencing in plants when spliced during pre-mRNA processing to produce a loopless hairpin structure [20]. The fold back shRNA helix similar to the precursors of miRNAs [21,22] is readily processed by Dicer and assembled into an RNA-induced silencing complex (RISC) to guide sequence specific cleavage of mRNA (Fig. 1(a)).

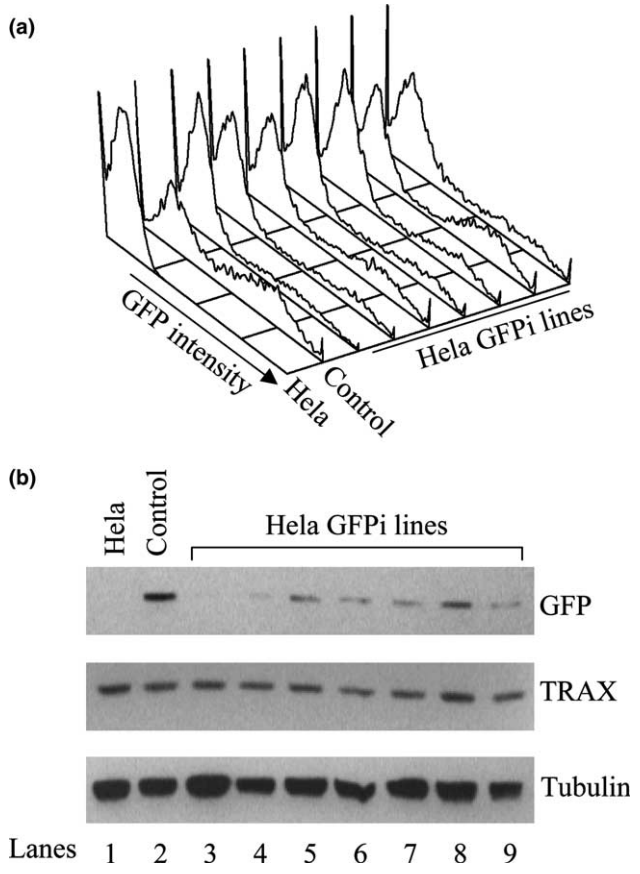

Fig. 2. Exogenously expressed GFP is reduced in stably transduced Hela cell lines expressing two fragments of GFP shRNAs. (a) Analysis of GFP expression by FACs analysis. Stably selected Hela cell lines were transfected with the pEGFP vector and 72 h later cells were harvested and analyzed by FACs. A negative control, untransfected Hela cells (Hela), was sorted as GFP-negative cells (rear peak indicates GFP-negative Hela cells and the forward peak indicates GFP-positive cells); control transfected Hela cells expressing GFP transduced with lentivirus without GFP shRNAs; and seven different stably selected Hela cell lines expressing GFP shRNAs (showing reduced GFP expression in forward peaks) are illustrated. (b) Western blot showing expression level of GFP in cell lysates (25 μg) from stably transduced Hela cell lines. Lane 1, Hela cell control; lane 2, GFP transfection control; lanes 3–9, seven different stably selected Hela lines. Upper panel, GFP expression; middle panel, endogenous TRAX; lower panel, tubulin loading control.

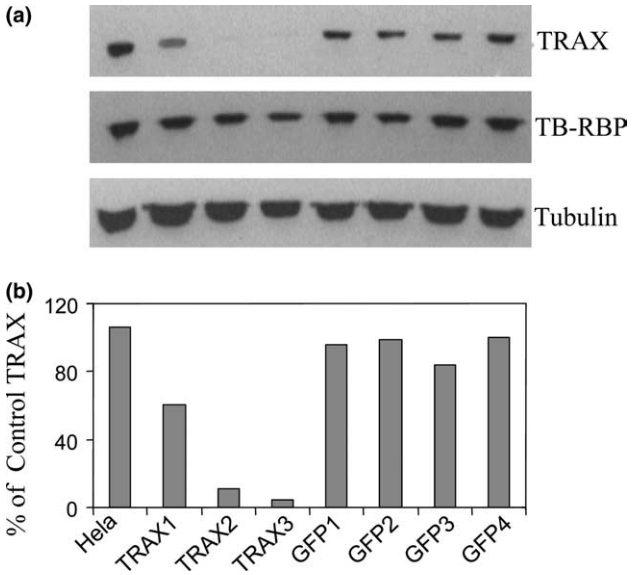

Fig. 3. Endogenous TRAX is selectively reduced in Hela cell lines expressing two TRAX shRNAs. Hela cells were infected with lentivirus containing two different fragments of 27 bp from the TRAX coding region with a small spacer and its inverted repeat linked by a human β-actin intron C. Three clones expressing TRAX shRNAs and four clones expressing GFP shRNAs were analyzed by Western blotting. (a) Upper panel, TRAX; middle panel, TB-RBP; lower panel, tubulin. (b) TRAX expression was quantitated by densitometry using ImageQuant software and its percentage of expression was normalized to that of control Hela cells.

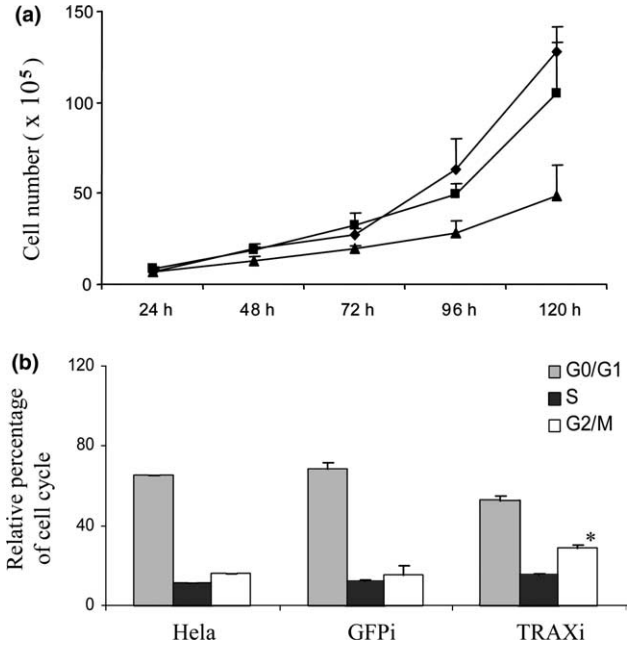

Fig. 4. Reduction of TRAX in Hela cell lines causes a slower growth rate and an abnormal cell cycle. (a) Cells (5×10^5) from three Hela cell lines expressing TRAX shRNAs, three cell lines expressing GFP shRNAs and one Hela cell control were plated onto 100 mm dishes. Cells were trypsinized and counted every 24 h for five consecutive days. Hela cell controls (diamonds), GFP expressing cell lines (squares) and TRAX expressing cell lines (triangles). (b) Cells were propagated as above, harvested at 72 h, stained with PI and analyzed by FACs. The G2/M phase in Hela cell lines expressing TRAX shRNAs is significantly increased. Asterisk indicates significant differences between Hela cell controls and Hela cell lines expressing GFP siRNAs ($P < 0.01$).

Table 1
Real-time RT-PCR analysis of gene expression in Hela cell lines expressing shRNAs

Clone types	TRAX	TB-RBP	OAS1	MX1	ISG20	IFI44	IFIT1
Hela	1 (0.95–1.04)	1 (0.98–1.01)	1 (0.99–1.00)	1 (0.67–1.48)	1 (0.98–1.01)	1 (0.98–1.01)	1 (0.75–1.33)
Hela/GFPi	1.91 (1.74–2.09)	0.92 (0.88–0.95)	2.97 (2.74–3.22)	1.91 (1.76–2.06)	1.59 (1.45–1.73)	0.97 (0.79–1.19)	0.49 (0.46–0.53)
Hela/TRAXi	0.007 (0.002–0.01)	1.73 (1.45–2.06)	0.67(0.56–0.80)	0.24 (0.21–0.28)	2.09(1.79–2.45)	0.13 (0.10–0.16)	0.02 (0.02–0.02)

Real-time PCR was performed with the ABI PRISM 7700 sequence detection system using the SYBR green PCR master mix. Relative gene expression was analyzed by the comparative CT method as described in the user's manual. Samples were assayed in duplicate. The relative amounts of TRAX, TB-RBP, OAS1, MX1, ISG20, IFI44 and IFIT1 mRNAs were compared to Hela GAPDH mRNA levels.

3.2. The CMV-shRNA construct can reduce exogenous GFP expression in both transiently and stably transduced cells

To determine if we can specifically reduce the expression of a gene with our shRNA constructs, we have infected three human derived cell lines, U2, H1299 and HFF, with a lentivirus containing a GFP shRNA expression cassette and we have selected for pools of antibiotic resistant cells. Seventy two hours after transfection with GFP and tagged TB-RBP vectors, whole cell lysates were prepared and analyzed by Western blotting (Fig. 1(b)). The blot reveals a selective reduction of GFP, while the HA tagged TB-RBP and two endogenous proteins, β-actin and α-tubulin, remain unchanged in these three human lines (Fig. 1(b)).

To extend this approach to stably transfected lines of cells, we infected Hela cells with the lentivirus construct and obtained seven stable Hela cell clones which express GFP shRNAs. Seventy two hours after the GFPi Hela cell lines were transfected with the pEGFP vector, the cells were sorted by FACs (Fig. 2(a)). GFP was reduced in the seven Hela GFPi lines (forward peaks). The reduction of GFP in the transfected Hela GFPi lines was confirmed by Western blotting (Fig. 2(b), lanes 3–9). The blot reveals a variable but substantial reduction of exogenously expressed GFP, while the endogenous proteins, TRAX and tubulin, were unchanged.

3.3. The CMV-shRNA vector selectively suppresses TRAX expression leading to reduced cell proliferation

Previous studies with mice lacking TB-RBP [2] and MEFs (MEFs) derived from TB-RBP null mice revealed that the loss of TB-RBP caused an equivalent loss of its partner protein, TRAX, and the MEFs from null mice showed a slower growth rate [11]. Having established that our intron-based vector works with the exogenous GFP gene, we targeted TRAX in Hela cells with a similar vector expressing TRAX shRNAs to determine whether TRAX deficiency affected cell proliferation. Western blotting revealed that three stable Hela cell TRAXi lines (TRAX 1, 2 and 3) express reduced amounts of TRAX compared to control Hela and Hela GFP (GFP 1–4) cell lines (Fig. 3). The intron-based shRNAs vector selectively reduced TRAX, while TB-RBP remained unchanged (Fig. 3). Most importantly, the Hela cells expressing TRAX shRNAs proliferate more slowly (Fig. 4(a)) with a build up of cells in the G2/M phase (Fig. 4(b)). Of 15 lines of Hela cells expressing TRAX shRNAs, 14 out of 15 had a slower growth rate (data not shown). A similar slower growth rate of NIH 3T3 cells was observed when TRAX levels were transiently reduced with TRAX siRNAs [11]. To confirm that the reduction of TRAX expression was due to the specific loss of TRAX mRNAs, we measured TRAX mRNA levels by real-time RT-PCR. TRAX mRNA was reduced to about 1% of the level of control Hela cells and the Hela cell lines expressing GFP shRNAs (Table 1), establishing that the intron-based vectors act through siRNAs as reported for other shRNA vectors [14–19].

3.4. The intron-based vector does not induce an interferon response

To rule out the possibility that the intron-based vectors used in this study were triggering a generalized interferon response, we analyzed by real-time RT-PCR a panel of interferon target genes, including 2′5′-oligoadenylate synthetase (OAS1), interferon-inducible protein p78 (MX1), interferon stimulated gene 20 (ISG20), interferon-induced protein 44 (IFI44) and interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) known to be induced by H1 or U6 driven shRNA vectors [23]. Compared to control Hela cells, the mRNA levels of the interferon markers in Hela cell lines expressing GFP or TRAX shRNAs only fluctuated slightly (Table 1). The decreases of MX1, IF144 and IFIT1 mRNAs in cell lines expressing TRAX shRNAs are likely due to the slower growth rate of the cells [11]. The non-induction of the interferon mRNAs indicates that the reduction in TRAX was not part of a global interferon response in the infected cells. We propose that the miRNA-like precursors produced by intron-based vectors can "knock down" gene expression for a wide range of genes in a variety of mammalian cells with specificity. This approach, using shRNA vectors containing a CMV promoter and inverted repeat sequences that are stabilized by an intron, could also be useful in generating loss-of-function phenotypes in transgenic animals. The success of this strategy indicates that gene silencing can be successfully obtained in mammalian cells using an expression cassette transcribing a short hairpin without the use of an RNA polymerase III promoter.

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