

## Letter to the Editor

---

### Silencing human Rb2/p130 with shRNA

To the Editor,

We designed and validated by real-time PCR, western blot and immunofluorescence, two new shRNAs directed against RB2/p130 and we set up the protocol to efficiently target this gene in a human lymphoblastoid cell line, by both transient and stable transfection. The clones, that stably express short hairpins directed against Rb2/p130, will be useful to further investigate the role of this Rb family member in lymphoid cells both in physiological and pathological conditions.

pRb2/p130 is a member of the Rb tumor suppressor family; this family is also composed of pRb/p105 and pRBL1/p107 [6,8,9]. Due to the dominant role of pRb/p105 in the family and to some overlapping functions, the roles of pRb2/p130 and RBL1/p107 were underestimated for a long time. It should be noted, however, that although they show partially overlapping functions, the three members are differentially regulated during cell cycle and interact with different E2F factors, which are the best-characterized retinoblastoma proteins targets. Through E2Fs, RBs regulate transcription and cell cycle transition [4].

In particular pRb2/p130 is abundant during the G<sub>0</sub> phase in non-proliferating cells, and its expression is altered in a large number of human cancers [1–3,7]. pRb2/p130 interacts with the repressing E2F4-5 and this interaction is responsible for the repression of cell cycle regulatory genes, such as *cdc25*, *cdc2*, E2F1-3 and RBL1/p107 [4,6,8,9]. When cells enter the cell cycle, pRb2/p130 becomes phosphorylated and this event leads to dissociation of pRb2/p130 from its E2F partner and to loss of transcriptional repression.

We chose RNAi to knockdown RB2/p130, with the purpose of fully elucidating its cellular functions in lymphoid cells. Unfortunately, no validated shRNA existed to target human Rb2/p130 to date, so we designed 10 oligos using web available software and cloned oligos in pSilencer4.1CMV vector. We show here by real-time PCR, immunofluorescence and western blot that oligo 4 and oligo 9 are effective in silencing, thus supporting their use for research purpose.

To design shRNA we used GenScript software (sequences are shown in Fig. 1a); these sequences were then adapted to pSilencer4.1CMV-Puro vector, using the siRNA converter by Ambion. After cloning shDNA 4 and 9 into pSilencer4.1CMV-Puro vector, we transiently transfected cells to screen our sequences for efficient silencing. Cells were collected 24 and 31 hours after transfection, to further proceed to RNA and protein extraction. In Fig. 1b, we show by Real-time PCR that sequences 4 and 9 showed a strong reduction in Rb2/130 mRNA.

To confirm that the downregulation at the mRNA level correlated with a corresponding protein decrease, we performed western blot analysis. In Fig. 1c, we show that pRb2/p130 levels in scrambled transfected cells were unchanged with respect to the control, whereas sequence 4 and sequence 9 are effective in silencing, showing a reduction respectively of 55% and 78% in the optimal condition.

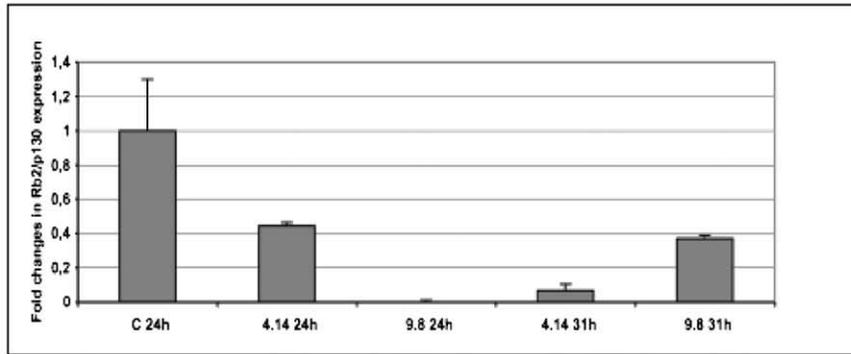
We then proceeded to create stable clones expressing shRNA against Rb2/p130. Cells nucleofected with pSilencer4.1CMV-shRb2 4, pSilencer4.1CMV-shRb2 9 or pSilencer4.1CMV-scrambled were selected using puromycin. RB2/p130 expression was then checked at the RNA and the protein levels by Real-time PCR (Fig. 1d), Western blot (Fig. 1e) and Immunofluorescence (Fig. 1f). Our results confirm transient transfection data. Sequence 4 is more efficient than sequence 9, but both are able to silence RB2/p130. Moreover, as we can detect by immunofluorescence, in sequence 9 most of pRb2/p130 signal is localized within the cytoplasm, where the protein is supposed to be inactive.

Our results show clearly that sh4 and sh9 are effective in silencing RB2/p130, both in transient and stable transfection assays.

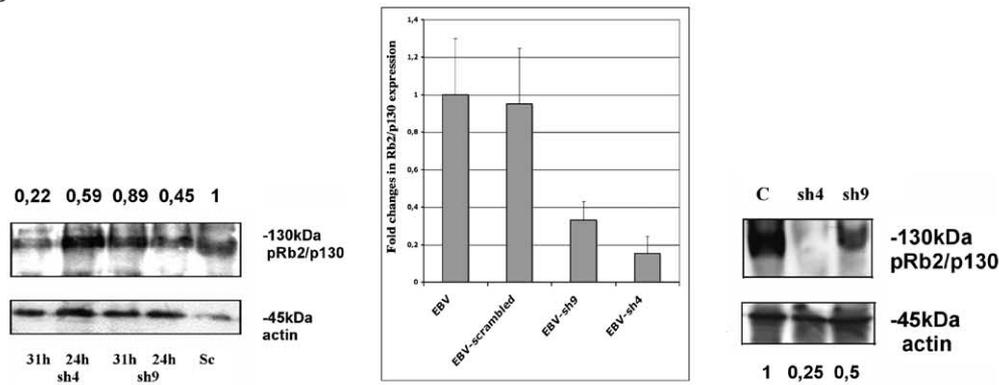
Moreover, despite of the rapid transgene expression obtained by nucleofection, our analysis demonstrates that silencing is still efficient 31 hours after transfection. We also found that the two shRNAs showed different silencing dynamics during this time; in fact sh9 is more efficient 31 hours after transfection while sh4 shows higher efficiency at 24 hours.

Name	Top strand	Bottom strand
shRb2 4	5'-GATCCTCAGCTGTAGTCCTCATAATTCA AGAGATTATGAGGACTACAGCTGACCA-3'	5'AGCTTGGTCAGCTGTAGTCCTCATA ATCTCTTGAATTATGAGGACTACAGC TGAG-3'
shRb2 9	5'-GATCCCGAATACAGTTGTCTCAAATTCA AGAGATTTGAGACAACTGTATTTCGGCA-3'	5'AGCTTGCCGAATACAGTTGTCTCAA ATCTCTTGAATTTGAGACAACTGTATT CG G-3'

a



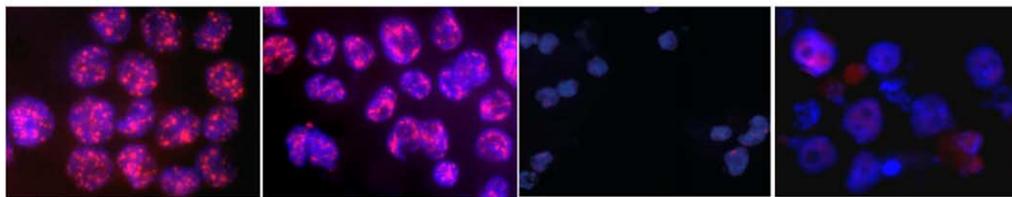
b



c

d

e



EBV-b

EBV-b-scrambled

EBV-b-sh4

EBV-b-sh9

f

Fig. 1. a. shDNA sequences. b. pRb2/p130 expression changes during transient transfection: RB2/p130 mRNA changes in clones transiently transfected with 2 different shDNAs directed against RB2/p130. c. pRb2/p130 levels after transient transfection: pRb2/p130 protein levels in control scrambled sequence (SC) or cells transfected with sh4 or sh9 respectively 24 and 31 hours after transfection. Levels of pRb2/p130 in scrambled transfected cells did not change between 24 and 31 hours (data not shown). Densitometry was shown on the top of the panel. d. Rb2/p130 expression during stable short hairpin transfections: RB2/p130 mRNA changes in clones transfected with sh4 and sh9 compared with EBV-b untransfected control (C). e. pRb2/p130 levels during permanent expression: pRb2/p130 protein levels in control scrambled sequence (SC) or cells that stably express sh4 or sh9. Densitometry was shown at the bottom of the panel. f. Visualization of pRb2/p130 by immunofluorescence: DAPI staining (blue) shows nuclei. Alexafluor568 staining (red) shows pRb2/p130 localization. pRb2/p130 decreases in sh4 and sh9 expressing cells was evident. Scrambled-expressing cells did not show any significant change in pRb2/p130 expression or localization.

Finally, we created two clones that stably express short hairpins directed against Rb2/p130, which will be useful to further investigate the role of this Rb family member in lymphoid cells both in physiological and pathological conditions. Many issues remain to be clarified, especially about the role of pRb2/p130 in lymphomagenesis; it has been found to be mutated in 16/19 cases of endemic Burkitt's lymphoma and 6/13 cases of the sporadic type [1,2]. For these reasons we are interested, by means of these stable clones and microarray technology, in extensively studying global modulation in gene expression and in promoter activation, following RB2/p130 knockdown.

## Methods

### *Design of shRNA and plasmid construction*

To design shRNA, the Genescript software ([www.genscript.com](http://www.genscript.com)) was used. To adapt oligos to pSilencer4.1CMV (Ambion, TX), siRNA converter by Ambion was used. Oligo sequences showed no significant homology with other Rb family members. RNAi positive control, provided by Ambion, consists in the introduction in the cell of a short hairpin directed against GAPDH mRNA (Data not shown). Oligos were annealed in DNA Annealing solution and ligated in pSilencer4.1CMV (Ambion, TX), according to manufacturer's instructions. Scrambled sequences and positive controls were already cloned into pSilencer4.1CMV, and were provided by Ambion with the empty vector.

The plasmids were sequenced using BigDye Terminator V1.1 and AbiPrism310 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany) according to manufacturer's instructions.

### *Cell lines and transfection*

EBV-b, a human B lymphoblastoid cell line immortalized by EBV, was kindly provided by Prof. Lanzavecchia (IRB, Bellinzona, Swiss). Cells were splitted a day before nucleofection.  $5 \times 10^6$  cells were transfected with 8  $\mu$ g of each plasmid, using Nucleofector II and solution V with program A-23 (Amaxa, Cologne, Germany). Transfection efficiency and cell death were checked by FACS analysis. Stable clones were obtained by selection with 0.5  $\mu$ g/ml puromycin, starting selection 6 hours after transfection.

### *Real-time PCR*

Cells were harvested 24 h or 31 h after transfection and RNA extracted using Versagene Kit (Gentra, Celbio, Italy). DNase treated RNA (500 ng) was retrotranscribed using M-MLV (Ambion, TX) and random hexamers according to manufacturer's instructions. Equal volumes of cDNA for each sample were then subjected to real-time PCR using DNA engine Opticon 2 (MJ research, Biorad, CA) and SYBR GREEN I (Finnzyme, Espoo, Finland).

Primers used for real-time PCR were designed between two adjacent exons of the gene of interest, using the Autoprime software available at [www.autoprime.de](http://www.autoprime.de). Hypoxanthine-guanine phosphorybosyl transferase 1 (HPRT) was used as a reference gene and relative quantification was performed by the use of the comparative  $\Delta\Delta C_T$  method [5].

### *Western blot*

Cells were lysed in EBC buffer (50 mM tris pH 8.0, 150 mM NaCl, 1% NP40) and the amount of proteins evaluated by Bradford assay (Biorad, CA, USA); 50  $\mu$ g of cell extract were then subjected to western blot analysis using anti-pRb2/p130 (Transduction, BD, Italy) or anti-actin (Sigma-Aldrich, Milano, Italy) respectively, at the concentration of 1 : 400 and 1 : 500. Relative quantification was performed using Quantity-One software (Biorad, CA, USA) applying the global background subtracting method.

### *Immunofluorescence*

Cells were smeared on positively charged slides and fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature. Permeabilization was obtained by washing cells with PBS, 0.2% Triton X-100, 1% BSA. Saturation was performed for 1 hour at room temperature in goat serum (Zymed laboratories, Milano, Italy). All of the antibodies were diluted in goat serum. Primary antibody incubation was carried out at room temperature for 1 hour, using anti-Rb2/p130 (Neomarkers, Lab Vision Corporation, USA), at a concentration of 1 : 75. Secondary goat anti-mouse antibody, conjugated with Alexafluor568 (Molecular Probes, Invitrogen, Italy), was diluted 1 : 100 and incubated at room temperature for 45 minutes. The slides were examined on an Axiovert 200 microscope (Carl Zeiss, Germany) and processed with proprietary software.

Eleonora Leucci<sup>a</sup>, Anna Onnis<sup>a</sup>, Giulia De Falco<sup>a,b</sup>,  
Anna Luzzi<sup>a</sup>, Giovanna Cerino<sup>a</sup>,  
Antonio Giordano<sup>a,b</sup>, Lorenzo Leoncini<sup>a,\*</sup>

<sup>a</sup> Department of Human Pathology and Oncology,  
University of Siena, Siena, Italy

<sup>b</sup> Sbarro Institute for Cancer Research  
& Molecular Medicine,  
Biolife Science Building Suite 333,  
Temple University Philadelphia, PA 19122, USA

\*Corresponding author: Prof. Lorenzo Leoncini, MD,  
Department of Human Pathology and Oncology,  
University of Siena, Policlinico "Le Scotte",  
via delle Scotte, 6, 53100 Siena, Italy  
Tel.: +39 0577 233 237; Fax: +39 0577 233 235;  
E-mail: leoncinil@unisi.it

## References

- [1] C. Cinti, P.P. Claudio, C.M. Howard, L.M. Neri, Y. Fu, L. Leoncini, G.M. Tosi, N.M. Maraldi and A. Giordano, Genetic alterations disrupting the nuclear localization of the retinoblastoma-related gene RB2/p130 in human tumor cell lines and primary tumors, *Cancer Res.* **60** (2000), 383–389.
- [2] C. Cinti, L. Leoncini, A. Nyongo, F. Ferrari, S. Lazzi, C. Bellan, R. Vatti, A. Zamparelli, G. Cevenini, G.M. Tosi, P.P. Claudio, N.M. Maraldi, P. Tosi and A. Giordano, Genetic alterations of the retinoblastoma-related gene RB2/p130 identify different pathogenetic mechanisms in and among Burkitt's lymphoma subtypes, *Am. J. Pathol.* **156** (2000), 751–760.
- [3] P.P. Claudio, C.M. Howard, C. Pacilio, C. Cinti, G. Romano, C. Minimo, N.M. Maraldi, J.D. Minna, L. Gelbert, L. Leoncini, G.M. Tosi, P. Micheli, M. Caputi, G.G. Giordano and A. Giordano, Mutations in the retinoblastoma-related gene RB2/p130 in lung tumors and suppression of tumor growth in vivo by retrovirus-mediated gene transfer, *Cancer Res.* **60** (2000), 372–382.
- [4] N. Dyson, The regulation of E2F by pRB-family proteins, *Genes Dev.* **12** (1998), 2245–2262.
- [5] K.J. Livak and T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, *Methods* **4** (2001), 402–408.
- [6] J.R. Nevins, E2F: a link between the Rb tumor suppressor protein and viral oncoproteins, *Science* **258** (1992), 424–429.
- [7] M.G. Paggi, A. Baldi, F. Bonetto and A. Giordano, Retinoblastoma protein family in cell cycle and cancer: a review, *J Cell Biochem.* **62** (1996), 418–430.
- [8] P. Stiegler and A. Giordano, The family of retinoblastoma protein, *Crit. Rev. Eukariot. Gene Expr.*, **11** (2001), 59–76.
- [9] J.Y. Wang, E.S. Knudsen and P.J. Welch, The retinoblastoma tumor suppressor protein, *Adv. Cancer Res.* **64** (1994), 25–85.