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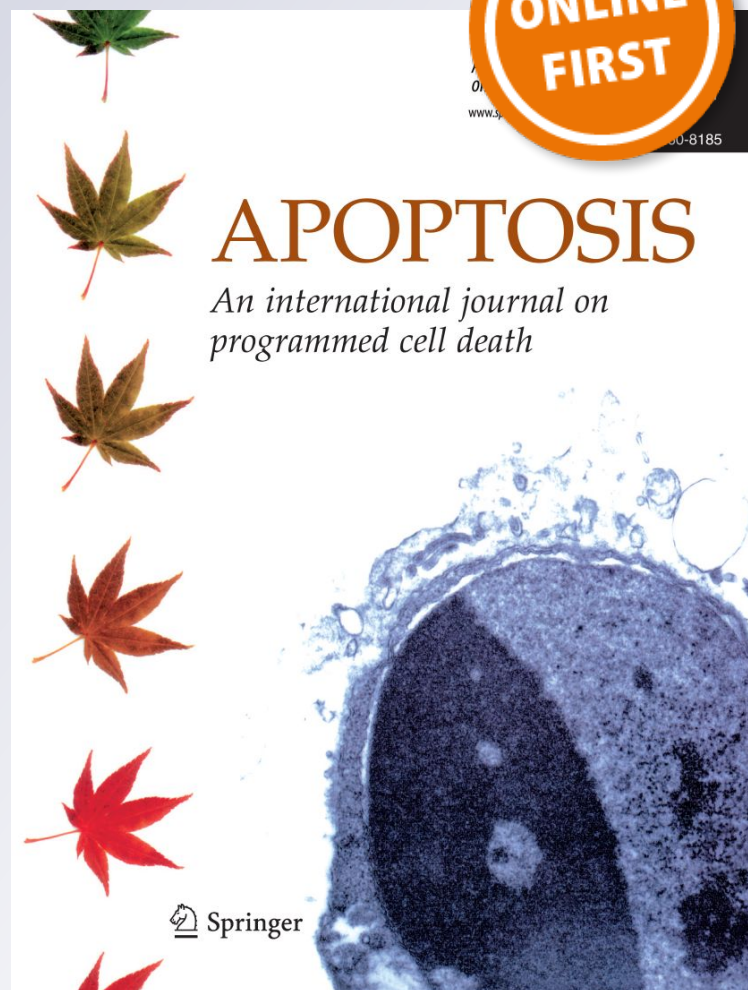
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# Baculovirus-based gene silencing of Humanin for the treatment of pituitary tumors

María Florencia Gottardo<sup>1,2</sup> · Matías L. Pidre<sup>3</sup> · Camila Zuccato<sup>2</sup> · Antonela S. Asad<sup>2</sup> · Mercedes Imsen<sup>2</sup> · Gabriela Jaita<sup>1,2</sup> · Marianela Candolfi<sup>2</sup> · Víctor Romanowski<sup>3</sup> · Adriana Seilicovich<sup>1,2</sup> 

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## Abstract

Pituitary tumors are the most common primary intracranial neoplasms. Humanin (HN) and Rattin (HNr), a rat homolog of HN, are short peptides with a cytoprotective action. In the present study, we aimed to evaluate whether endogenous HNr plays an antiapoptotic role in pituitary tumor cells. Thus, we used RNA interference based on short-hairpin RNA (shRNA) targeted to HNr (shHNr). A plasmid including the coding sequences for shHNr and dTomato fluorescent reporter gene was developed (pUC-shHNr). Transfection of somatolactotrope GH3 cells with pUC-shHNr increased apoptosis, suggesting that endogenous HNr plays a cytoprotective role in pituitary tumor cells. In order to evaluate the effect of blockade of endogenous HNr expression in vivo, we constructed a recombinant baculovirus (BV) encoding shHNr (BV-shHNr). In vitro, BV-shRNA was capable of transducing more than 80% of GH3 cells and decreased HNr mRNA. Also, BV-shHNr increased apoptosis in transduced GH3 cells. Intratumor injection of BV-shHNr to nude mice bearing s.c. GH3 tumors increased the number of apoptotic cells, delayed tumor growth and enhanced survival rate, suggesting that endogenous HNr may be involved in pituitary tumor progression. These preclinical data suggests that the silencing of HN expression could have a therapeutic impact on the treatment of pituitary tumors.

**Keywords** Humanin · Rattin · Baculovirus · ShRNA · Pituitary tumor · Apoptosis

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María Florencia Gottardo and Matías L. Pidre contributed equally to this work.

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✉ Adriana Seilicovich  
adyseili@fmed.uba.ar

<sup>1</sup> Departamento de Biología Celular e Histología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

<sup>2</sup> Instituto de Investigaciones Biomédicas (INBIOMED), Facultad de Medicina, CONICET- Universidad de Buenos Aires, Paraguay 2155, piso 10, C1121ABG Buenos Aires, Argentina

<sup>3</sup> Instituto de Biotecnología y Biología Molecular (IBBM), Universidad Nacional de La Plata - CONICET, La Plata, Argentina

## Introduction

Pituitary tumors account for about 8% of primary intracranial neoplasms [1, 2]. Prolactin-secreting adenomas are the most frequent type among pituitary tumors. Although prolactinomas are benign, they can also be locally aggressive, and compress adjacent structures, resulting in neurological dysfunction [3]. These tumors can be effectively treated with dopaminergic agonists, but about 20% of patients develop resistance to this treatment [4]. Somatotropinomas comprise about 20% of pituitary tumors [5]. Although their primary treatment is transsphenoidal surgery, the probability of surgical success does not exceed 40% in the case of macroadenomas. Thus a high percentage of patients with this disease require alternative forms of treatment [1]. Since our previous results indicate that the antiapoptotic peptide Humanin protects pituitary cells from proapoptotic stimuli [6, 7], we evaluated whether this molecular target could hold therapeutic value in these tumors.

Humanin (HN) is a 24-amino-acid peptide originally discovered in surviving neurons of Alzheimer's disease brain

[8, 9]. Rattin (HNr), a homolog of HN in rat, exhibits a cytoprotective action in several cell types such as neurons, lymphocytes and testicular germ cells [10–15]. Previously, we showed that rat GH3 somatotrophic tumor cells overexpress HNr when compared to normal anterior pituitary cells, and that exogenous HN protects them from TNF- $\alpha$ -induced apoptosis [6, 7]. The aim of the present study was to evaluate the effect of endogenous HNr blockade on the apoptotic response of pituitary tumor cells. We used RNA interference based on short-hairpin RNA (shRNA), to suppress gene expression [16–18]. Transfection of GH3 cells with a plasmid encoding shRNA specific for HNr downregulated its expression and sensitized them to proapoptotic stimuli in vitro. In order to evaluate the effect of HNr inhibition in vivo, a baculovirus (BV) derived from AcMNPV encoding shRNA to silence HNr was constructed and characterized. BVs are insect pathogens unable to replicate in mammalian hosts. Nevertheless, they can efficiently transduce mammalian cells [19]. Lack of toxicity and replication, as well as the absence of pre-existing immunity in mammalian hosts make BVs very attractive vectors for gene therapy applications [20, 21]. Our results indicate that endogenous HNr plays an antiapoptotic role in pituitary tumor cells and suggest that this peptide may be involved in pituitary tumor progression. BV-mediated silencing of HNr may have therapeutic value for the treatment of tumors that overexpress HN.

## Materials and methods

All chemicals and reagents were obtained from Sigma Aldrich. (St. Louis, MO) except for phenol red-free Dulbecco's modified Eagle's medium and supplements (D-MEM; GIBCO, Invitrogen, Carlsbad, CA), fetal bovine serum (Natocor, Buenos Aires, Argentina), all terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) reagents (Roche Molecular Biochemicals, Mannheim, Germany), anti-rabbit IgG and anti-rabbit fluorescein-conjugated secondary antibody (Vector Laboratories Inc., Burlingame, CA), plasmids and primers (GenScript Inc, Piscataway, NJ), and the materials indicated below.

## Animals

Adult female athymic N:NIH Swiss (*nude*) mice (6–8 weeks old) were kept under controlled conditions of light (12:12 h light–dark cycles) and temperature (20–25 °C). Mice were fed standard laboratory chow and water ad libitum and kept in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal procedures were previously approved by the Ethics Committee of the School of Medicine, University of Buenos Aires.

## Plasmid constructs for HN-specific shRNA

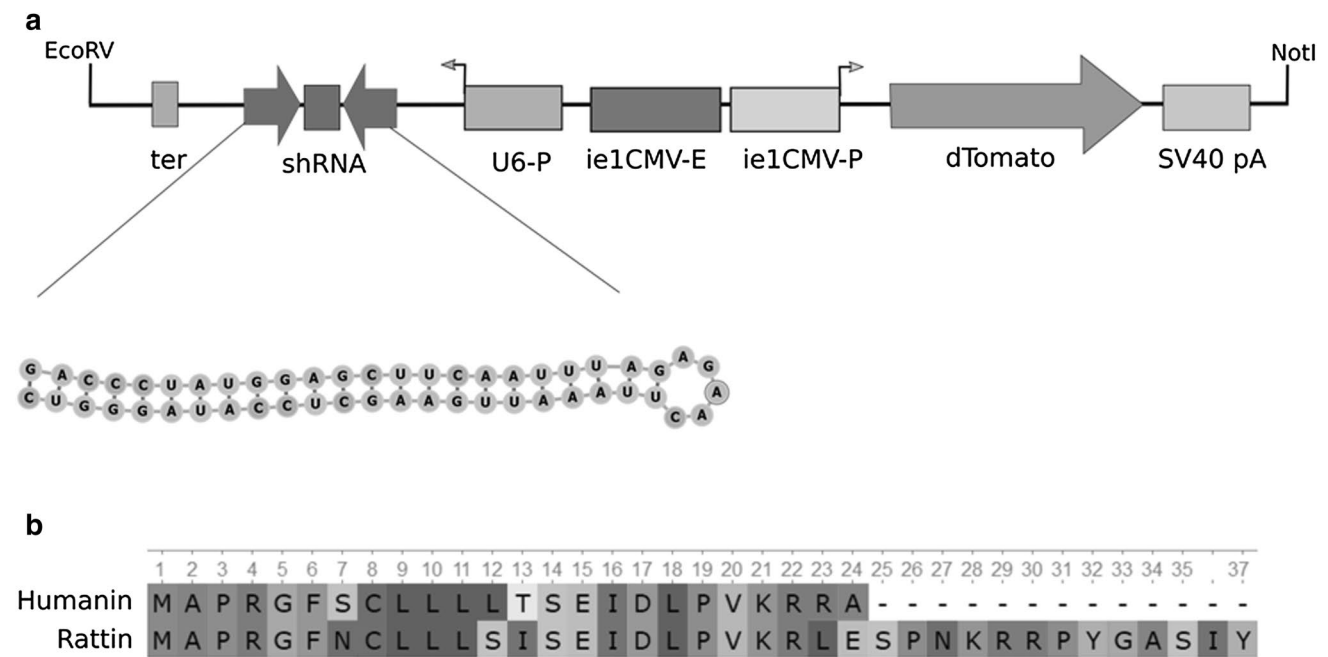
The 21-nucleotide-long shRNA-coding dsDNA comprising the HNr RNA sequence (shRNA-HNr: **gaccctatggagcttcaatttagagaacttaattgaagctccatagggtc**) was synthesized fused to the U6 promoter and cloned in a bicistronic pUC57 vector (pUC-shHNr). In order to detect successfully transfected cells the construct included the coding sequence for dTomato fluorescent reporter gene under the control of the cytomegalovirus (CMV) major immediate-early (IE) promoter (Fig. 1). This construct is referred to as shRNA cassette in the following sections. A similar pUC57-derived construct containing only the CMV (IE)-dTomato sequence was constructed for control experiments (pUC-Control).

## Generation of recombinant baculoviruses

In order to improve delivery efficiency of the shRNA-expressing DNA construct for in vivo experiments, a recombinant AcMNPV baculovirus was produced (BV-shHNr) containing the complete cassette described in the previous section. Briefly, the shRNA cassette was cloned into the *EcoRV-NotI* digested pBacPAK9 transfer vector (Clontech, Mountainview, CA). Flanking AcMNPV sequences allow homologous recombination with viral DNA in insect cells to transfer the expression cassette to the polyhedrin locus of the viral DNA. To this end the recombinant pBacPAK was cotransfected in insect cell line *Trichoplusia ni* BTI-TN-5B1-4 (High Five<sup>TM</sup> cells; Thermo Fisher Scientific, Waltham, MA) with bApGOZA DNA [22]. Cells were maintained in Grace's medium (Thermo Fisher Scientific) supplemented with 10% of fetal bovine serum at 27 °C in T-flasks until signs of infection became apparent (Supplementary Fig. 1). The same strategy was used to generate the control baculovirus expressing dTomato without the shRNA sequence (BV-Control). Recombinant dTomato expression was verified by epifluorescence microscopy (Nikon Eclipse Ti-S). BVs were titrated on Sf9 cell monolayers as plaque forming units (PFU); these titers were coincident with infectious *foci* as determined in a reporter cell line expressing GFP under the control of polyhedrin (*polh*) promoter [23].

## Cell culture

GH3 cell monolayers were grown in flasks containing DMEM supplemented with 10  $\mu$ l/ml MEM amino acids, 2 mM glutamine, 100  $\mu$ g/ml streptomycin, 10% fetal bovine serum. GH3 cells were harvested using 0.025% trypsin–EDTA in PBS. Cell viability assessed by trypan blue exclusion was over 90%. For in vitro experiments, GH3 cells were seeded on cover slips placed in 24-well tissue culture



**Fig. 1 a** Scheme of the silencing cassette. HNr specific shRNA is encoded under the control of the U6 promoter and the reporter gene dTomato is encoded under CMV *IE* promoter. Amplification of the

shRNA sequence is depicted below. The control construct contains only the CMV (*IE*)-dTomato sequence (not shown). **b** Sequence homology of Humanin and Rattin [32]

plates ( $1 \times 10^5$  cells/ml/well) for TUNEL assay or in 24-well tissue culture plates ( $2 \times 10^5$  cells ml/well) for immunocytochemical assay or ( $1 \times 10^6$  cells ml/well) for Western blot.

### Gene transfection and transduction

GH3 were transfected with 1  $\mu$ g of pUC-shHNr or pUC-Control plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and incubated for 16 h. In some experiments, cells were incubated with TNF- $\alpha$  (50 ng/ml) for a further 24 h.

To transduce GH3 cells with recombinant baculoviruses, cells were incubated for 1 h with BV-shHNr (20 BV/cell and 50 BV/cell) in DMEM and, after addition of supplemented medium for 16, 48 or 96 h, cells were fixed with 4% paraformaldehyde (PFA) for immunocytochemical detection of HNr and assessment of apoptosis by TUNEL or processed for protein extraction.

### Experimental model of pituitary adenoma

Eight-week-old *nude* mice were injected subcutaneously (s.c.) into the right flank with  $3 \times 10^6$  GH3 cells. Tumor size was determined every 2 days with caliper and tumor volume was estimated according to the formula:  $[\text{width}^2 \times \text{length}]/2$  ( $\text{mm}^3$ ). When the tumor volume reached approximately 200  $\text{mm}^3$ , animals were injected intratumorally with  $10^8$  PFUs of BV-Control or BV-shHNr (40  $\mu$ l/

mouse). Tumor growth was evaluated for 15 days and survival was determined until the tumor reached 2000  $\text{mm}^3$ . Mice were monitored daily and when the first signs of distress appeared, they were euthanized by cervical dislocation. A group of mice were euthanized 15 days after baculovirus injection and tumors were removed within minutes. Tumors were fixed in 4% PFA in PBS and embedded in paraffin. Sections (4  $\mu$ m) were deparaffinized in xylene, rehydrated in graded ethanol and processed for determination of apoptosis by TUNEL in tissue slices.

### Microscopic detection of DNA fragmentation by TUNEL

Deparaffinized and re-hydrated tumor sections or fixed GH3 cells were irradiated in a microwave oven (370 W for 5 min) in 10 mM sodium citrate buffer, pH 6 and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 5 min at 4  $^\circ$ C. Non-specific labeling was prevented by incubating the preparations with blocking solution (5% blocking reagent; Roche Molecular Biochemicals) for 30 min at room temperature. DNA strand breaks were labelled with digoxigenin-deoxyuridine triphosphate (DIG-dUTP) using terminal deoxynucleotidyl transferase (0.18 U/ $\mu$ l) according to the manufacturer's protocol. After incubation with 10% donkey serum in PBS for 90 min, tissue sections or cells were incubated for 1 h with fluorescein conjugated anti-digoxigenin antibody (1:10) to detect addition of DIG-dUTPs to

3'-OH ends of fragmented DNA. The slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) containing 4,6-diamidino-2-phenylindolehydrochloride (DAPI) for DNA staining and visualized in a fluorescent light microscope (Axiophot; Carl Zeiss, Jena, Germany). Data of apoptotic cells in tissue sections were expressed as the number of TUNEL-positive cells/field. The percentage of cultured apoptotic cells was calculated as [(TUNEL+)/total cells]  $\times$  100.

### Expression of HNr by immunofluorescence

The presence of HNr in GH3 cells was evaluated by indirect immunofluorescent staining. Cells were incubated for 1 h with anti-HNr antibody (Sigma, 1:100), washed and incubated for 1 h with anti-rabbit IgG-FITC (Vector Laboratories, 1:50). Finally, slides were mounted with mounting medium for fluorescence (Vectashield) containing DAPI. Control slides were incubated with the corresponding normal serum or IgG subtype instead of primary antibody. Cells were visualized in a fluorescence light microscope (Axiophot).

### RNA isolation and real time PCR (qRT-PCR)

GH3 cells were transduced with BV-Control or BV-shHNr and incubated for 48 h. Cells were collected as described above and RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol. One  $\mu$ g of total RNA was reverse transcribed using SuperScript II Reverse Transcriptase according to the manufacturer's protocol (Invitrogen). After incubation for 5 min at 65 °C with 1  $\mu$ l primers (1  $\mu$ g/ $\mu$ l) and 1  $\mu$ l of dNTP Mix (10 mM), RNA samples were incubated for 2 min at 42 °C with a mix containing 0.1 mM DTT and RNaseOUT™ (Invitrogen). After addition of 1  $\mu$ l SuperScript II RT (Invitrogen), samples were incubated for 50 min at 42 °C and the reaction was heat-inactivated (70 °C for 15 min). Finally, samples were treated with RNase H (Invitrogen).

For real time PCR, a pair of forward and reverse primers mapping to HNr and cyclophilin was used as a reference mRNA (Table 1). Cyclophilin was already used for gene expression quantification in anterior pituitary [24, 25] and was chosen as reference because its expression does not vary

in the pituitary during the estrous cycle. All primers were obtained from GenBiotech (Buenos Aires, Argentina). Real time PCR was performed using an StepOne™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA). For each reaction, 25  $\mu$ l of solution containing 5  $\mu$ l cDNA, 9  $\mu$ M forward and reverse primers and 10  $\mu$ l Power SyberGreen PCR Master Mix (Applied Biosystems) were used. All reactions were performed in duplicate. Negative controls included amplification of RNA (without reverse transcription) and water. Amplification was initiated by a 2 min pre-incubation at 50 °C, followed by 40 cycles at 95 °C for 15 s, 60 °C for 1 min, terminating at 95 °C for the last 15 s (melting). PCR product specificity was verified by a melting curve analysis. Linearity of real-time RT-PCR signaling was determined with wide-range serial dilutions of reference cDNA and linear correlations were found between the amount of cDNA and the Ct. Gene expression was normalized to the endogenous reference gene cyclophilin by the  $\Delta\Delta$ Ct method [26] using Step-One Software (Applied Biosystems), and expressed as fold-changes relative to the control group. We validated this method in our experiments by confirming that amplification efficiencies of target gene and housekeeping gene were similar, following the procedure published by Livak and Schmittgen [26]. The products of PCR were visualized in an agarose gel to confirm correct fragment size.

### Western blot

GH3 cells were transduced with BV-Control or BV-shHNr and incubated for 48 h. Total proteins were extracted in lysis buffer containing 250 mM NaCl, 5 mM MgCl<sub>2</sub>, 50 mM NaF, 1 mM dithiothreitol (DTT), 1% Igepal, 0.02% sodium azide, 0.1% sodium dodecyl sulphate (SDS) in 50 mM Tris-HCl pH 7.4 and protease inhibitor cocktail (1:100). Following centrifugation, the supernatant was used for immunoblot assay. Protein concentration of each sample was determined by Bradford assay (BioRad Laboratories, Hercules, CA). Sixty micrograms of proteins were size-fractionated in 12% SDS-polyacrylamide gel, then electrotransferred to polyvinyl difluoride (PVDF) membranes. Blots were blocked for 90 min in 5% non-fat dry milk-TBS-0.1% Tween 20 and incubated overnight with anti-Bax (1:50, BD Biosciences, San Jose, CA) or anti-Bcl-2 (1:30, Santa Cruz Biotechnology), and anti- $\beta$ -actin (1:500, Santa Cruz Biotechnology) in the same buffer at 4 °C. This was followed by 1 h incubation with the corresponding anti-rabbit secondary antibody (1:1000, Millipore, Temecula, CA). Immunoreactivity was detected by enhanced chemiluminescence (Productos Biológicos, Buenos Aires, Argentina). Chemiluminescence was detected by chemiluminescence imaging system (G Box Chemi HR16, Syngene, Cambridge, U.K.) and bands were quantified using Gene Tools software (Syngene). Intensity data from Bax, and Bcl-2 in total proteins were normalized

**Table 1** Oligonucleotides used as primers in real time PCR

Rattin (HNr) forward	5'-GAGGGTTCAACTGTCTTACTTTCA-3'
Rattin (HNr) reverse	5'-GTGAAGAGGCTGGAATCTCCC-3'
Cyclophilin forward	5'-CTGGTGGCAAGTCCATCTAC-3'
Cyclophilin reverse	5'-CCCGCAAGTCAAAGAAATTA-3'

with respect to the corresponding  $\beta$ -actin blot and expressed as relative increments compared to respective controls.

### Statistical analysis

Data were graphed and analyzed using GraphPad Prism version 5.00 software (GraphPad Software). Real Time-PCR data were analyzed by Student's *t* test. The number of apoptotic cells evaluated by TUNEL in slides from three independent experiments was expressed as percentage of TUNEL positive cells  $\pm$  95% confidence limits (CL) of the total number of cells counted in each specific condition and analyzed by  $\chi^2$  test. The mean of TUNEL-positive cells per field from 10 to 24 fields of 3 tumor sections from each mouse was considered an individual value and data were analyzed by Student's *t* test. Western blot data were evaluated by Student's *t* test. Tumor growth data were analyzed by multiple regression analysis and Kaplan–Meyer survival curves were evaluated by log-rank test. Differences between groups were considered significant when  $p < 0.05$ . All the experiments were performed at least twice.

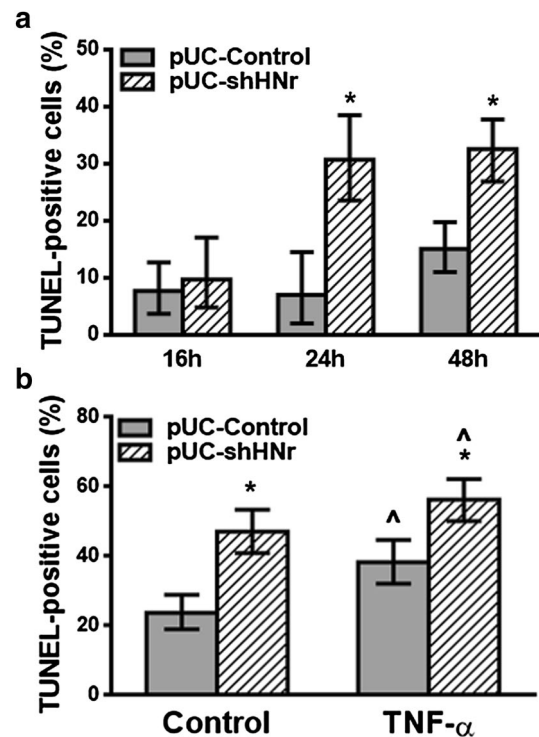
## Results

### Silencing of HNr in pituitary tumor cells in vitro

In order to evaluate the role of endogenous HNr in the apoptotic response of pituitary tumor cells, we used a plasmid (pUC-shHNr) encoding both a shRNA specific for HNr and dTomato, a red fluorescent reporter gene (Fig. 1). Transfection efficiency of plasmid pUC-shHNr in GH3 cells was about 30% as assessed by dTomato expression at 48 h post-transfection (Supplementary Fig. 2a and b).

To evaluate the effect of HNr silencing, we assessed the percentage of GH3 cells showing apoptosis at different times post-transfection with pUC-shHNr and control (pUC-Control) plasmids. The percentage of apoptotic cells was determined by TUNEL assay. At 16 h post-transfection no differences were detected in the percentage of apoptosis in GH3 cells transfected (dTomato-positive) with either pUC-shHNr or pUC-Control plasmid (Fig. 2a). However, at 24 and 48 h post-transfection, the percentage of TUNEL-positive cells was higher in pUC-shHNr transfected cells than in those transfected with pUC-Control plasmid (Fig. 2a), indicating that endogenous HNr exerts an antiapoptotic action in pituitary tumor cells. In contrast, in dTomato-negative cells, no differences were detected between the percentage of apoptotic GH3 cells incubated with pUC-shHNr with respect to that incubated with pUC-Control (data not shown).

We previously reported that TNF- $\alpha$  increased apoptosis of both normal and tumor pituitary cells [27, 28]. We



**Fig. 2** Inhibition of endogenous HNr reduced apoptosis of GH3 cells. **a** GH3 cells were transfected with 1  $\mu$ g of plasmid DNA control (pUC-Control) or plasmid with interfering RNA for HNr (pUC-shHNr) for 16, 24, and 48 h. Apoptosis was assessed by the TUNEL method in transfected (dTomato-positive) cells. Each point represents the percentage  $\pm$  CL of TUNEL-positive GH3 cells ( $n \geq 1500$  cells/group). Data from at least two independent experiments were analyzed by ANOVA. \* $p < 0.05$  versus pUC-Control. **b** GH3 cells were transfected with 1  $\mu$ g of pUC-Control or pUC-shHNr DNA for 24 h and then incubated without (Control) or with TNF- $\alpha$  (50 ng/ml) for further 24 h. Apoptosis was assessed by the TUNEL method in transfected (dTomato-positive) cells. Each column represents the percentage  $\pm$  CL of TUNEL-positive GH3 cells ( $n \geq 700$  cells/group). Data from at least two independent experiments were analyzed by  $\chi^2$ . \* $p < 0.05$  versus respective pUC-Control, <sup>^</sup> $p < 0.05$  versus respective control without TNF- $\alpha$

also showed that HN per se did not modify basal apoptosis of pituitary cells but inhibited the apoptotic effect of TNF- $\alpha$  on them [6, 7]. Therefore, we also determined the effect of silencing of HNr on the sensitivity of pituitary tumor cells to a proapoptotic stimulus, such as TNF- $\alpha$ . To this end, GH3 cells were transfected with pUC-Control or pUC-shHNr plasmids, incubated for 24 h, and after addition of TNF- $\alpha$ , incubated for an additional period of 24 h (Fig. 2b). At 48 h after transfection, TNF- $\alpha$  increased apoptosis in pUC-Control- or pUC-shHNr-transfected (dTomato-positive) cells. As shown in Fig. 2b, pUC-shHNr increased basal as well as TNF- $\alpha$ -induced apoptosis, suggesting that endogenous HNr also plays a cytoprotective role against an external apoptotic stimulus.

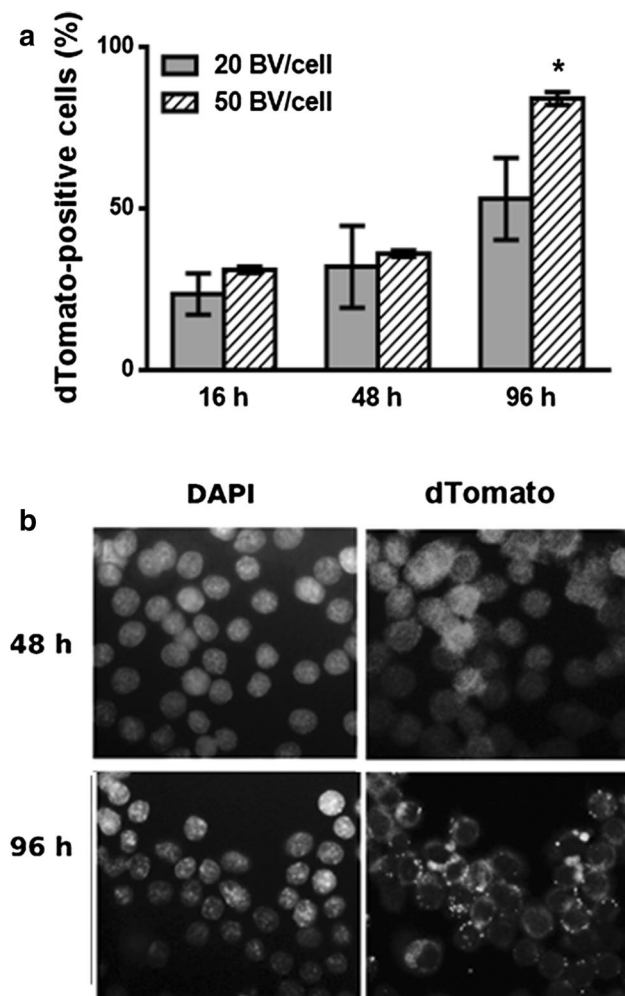
## Generation of viral vectors to block HNr in pituitary tumors in vivo

One of the major disadvantages of using plasmids expressing a specific shRNA is the low transfection efficiency in vivo. In order to evaluate the effect of the blockade of endogenous HNr expression in animal models of pituitary adenomas, we generated a recombinant baculovirus (BV-shHNr) carrying the shRNA-dTomato cassette. After obtaining the recombinant baculoviruses (BV, Supplementary Fig. 1), GH3 cells were incubated with 20 or 50 BV/cell of BV-shHNr for 16, 48 or 96 h. The presence of dTomato-positive cells was detected at all time-points evaluated (Fig. 3a and b). BVs were capable of transducing more than 80% of GH3 cells as assessed by expression of the reporter gene after 96 h post-transduction. In addition, BV-shHNr decreased HNr mRNA

in GH3 cells compared to BV-Control-transduced cells as assessed by qRT-PCR at 48 h post-transduction (Fig. 4a). To evaluate the effect of HNr silencing with BV-shHNr on the apoptotic response of GH3 cells, we determined the percentage of TUNEL-positive cells at 48 h post-transduction with BV-Control or BV-shHNr. BV-shHNr increased apoptosis in dTomato-positive GH3 cells (Fig. 4b), suggesting that vector-mediated inhibition of endogenous HNr expression was effective enough to trigger apoptosis in these cells. We also explored the effect of BV-shHNr on the expression of antiapoptotic protein Bcl-2 and proapoptotic protein Bax in GH3 cells. As determined by Western blot, BV-shHNr up-regulated the expression of Bax (Fig. 4c). Although BV-shHNr did not modify Bcl-2 expression (Fig. 4d), it significantly increased the Bax/Bcl-2 ratio in GH3 cells (Fig. 4e).

## Effect of BV-shHNr on tumor growth and survival of mice inoculated with GH3 cells

To evaluate the effect of blockade of endogenous HNr expression in vivo, nude female mice were inoculated s.c. with GH3 cells (Fig. 5a). When the tumor volume reached 200 mm<sup>3</sup>, the animals were injected intratumorally with BV-Control or BV-shHNr. Tumor size was measured for 15 days starting one day post-inoculation and survival was monitored until the tumor reached 2000 mm<sup>3</sup>. In a group of animals, tumors were dissected and processed to assess apoptosis by the TUNEL method. We found that BV-shHNr treatment delayed tumor growth (Fig. 5c), suggesting that inhibition of endogenous HNr expression exerts an antitumoral effect. Also, the survival rate of mice treated with BV-shHNr was significantly higher than that of controls (Fig. 5d). These observations were in agreement with an increase in the number of TUNEL-positive cells/field detected in the tumors treated with BV-shHNr when compared to BV-Control (Fig. 5b).



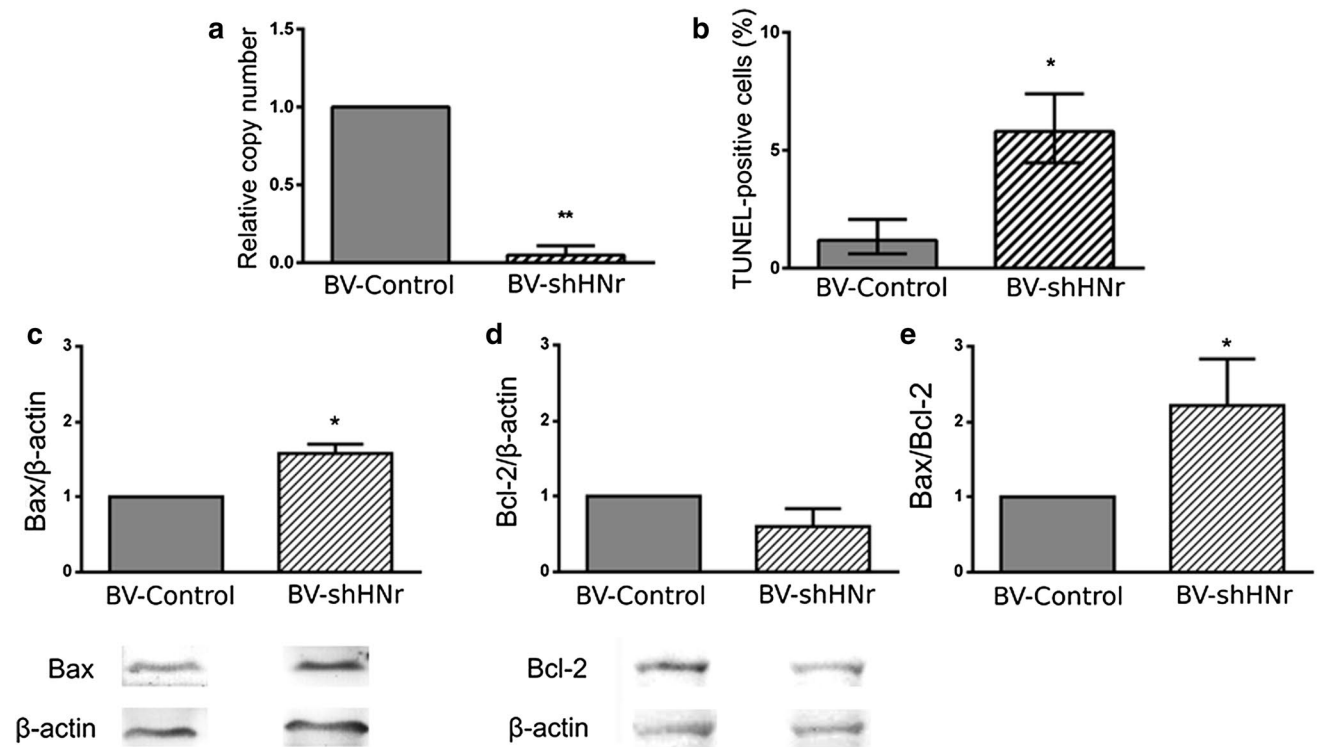
**Fig. 3** Baculovirus transduction with BV-shHNr in GH3 cells. **a** Baculovirus transduction with BV-shHNr in GH3 cells incubated for 16, 48 or 96 h, using 20 and 50 BV/cell. **b** Representative images of transfected cells incubated with 50 BV/cell of BV-shHNr. Upper panels: DAPI; lower panels: dTomato reporter gene

## Discussion

We have previously reported that HNr expression in rat somatotrope tumor cells was higher than in normal pituitary cells from female rats [7], suggesting that overexpression of HNr could be involved in the growth of pituitary tumors. We also reported that HN inhibited TNF- $\alpha$ -induced apoptosis in GH3 cells, suggesting that this peptide has antiapoptotic activity in pituitary tumor cells [6]. In agreement with our results, it was reported that HN was overexpressed in gastric cancer cells, where this peptide exerted an antiapoptotic action, suggesting that HN overexpression could be an important molecular event in gastric tumorigenesis [29].

In spite of the marked antiapoptotic action of HN in many cell types [30–32], reports on the role of HN in





**Fig. 4** Effect of BV-shHNR transduction in GH3 tumor cells in vitro. GH3 cells were incubated with BV-Control or BV-shHNR for 48 h. **a** Expression of HNR mRNA. Each column represents the mean  $\pm$  SE of the concentration of HNR RNA relative to its internal control of cyclophilin. (n=3), \*\*p<0.01 Student's *t* test. **b** Apoptosis was evaluated by the TUNEL method. Each column represents the percentage  $\pm$  CL of TUNEL-positive GH3 cells (n $\geq$ 1000 cells/group).

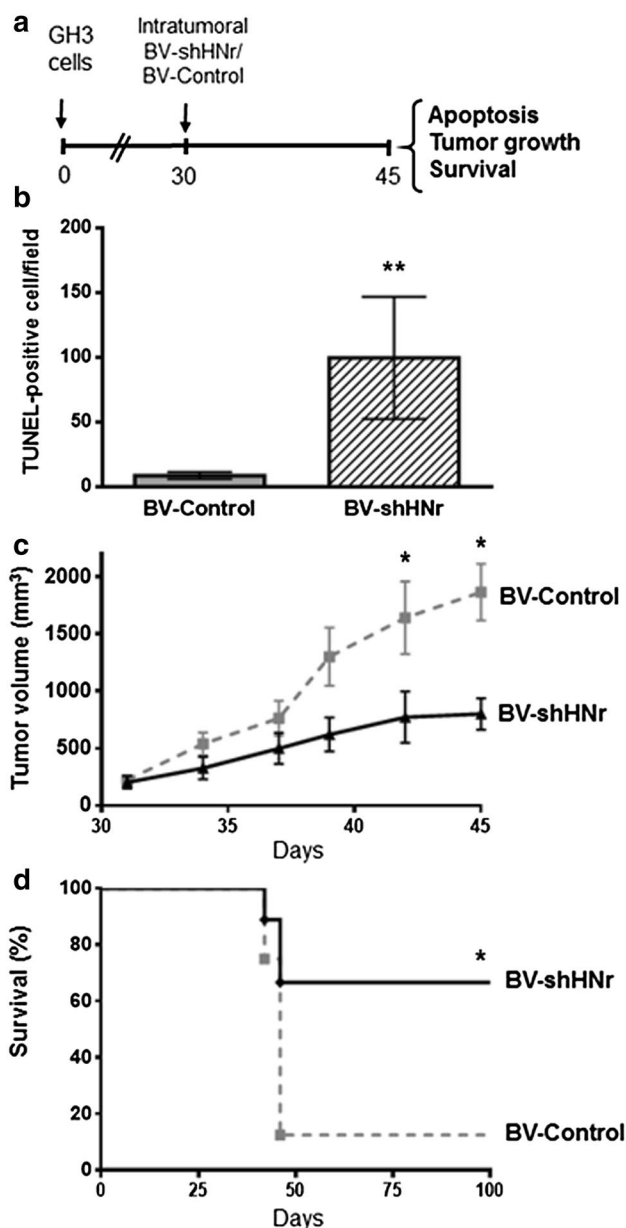
\*p<0.05,  $\chi^2$ . Expression of Bax (**c**) and Bcl-2 (**d**) was determined by Western blot. Each column represents the mean  $\pm$  SE of the relative increment respect to BV-Control. Lower panels show representative blots.  $\beta$ -actin was assessed as loading control. Raw data from 3 independent experiments were normalized by the corresponding  $\beta$ -actin value. \*p<0.05 Student's *t* test. **e** Bax/Bcl-2 ratio. \*p<0.05 Student's *t* test

cancer development are surprisingly scarce. Eriksson et al. [33] demonstrated that a synthetic HN analog prevented the chemotherapy-induced impairment of linear bone growth and apoptosis of growth plate cartilage, whereas it did not interfere with the anticancer effect of chemotherapy in human tumor xenografts as well as in several human tumor cell lines. It was also shown that a HN agonist protected male germ cells and leucocytes from chemotherapy-induced apoptosis [13, 34]. Based on these reports, it was suggested that endogenous and exogenous HN may protect from the toxic effects of chemotherapy although its tumor inhibiting action requires further investigation [35]. It is very likely that HN could exert different effects depending on the cell type and physiopathological context. In fact, we show here that inhibition of endogenous HNR with a specific shRNA decreased HNR expression while increasing apoptosis of GH3 cells, clearly suggesting that endogenous HNR plays an antiapoptotic role in pituitary tumor cells. We have previously reported that the antiapoptotic effect of exogenous HN involves multiple pathways, including STAT3 and NF- $\kappa$ B pathway activation and inhibition of Bax translocation to mitochondria [7]. Our new findings indicate that endogenous

HN also modulates the Bcl-2 pathway. BV-HNR up-regulated Bax and increased the Bax/Bcl-2 ratio, an index of proapoptotic phenotype. Our in vivo results show that silencing of HNR expression with BV-shRNA reduced the progression of xenografted pituitary tumors, thus decreasing tumor growth and enhancing survival. These findings suggest that HNR plays a role in pituitary tumor development and that inhibition of HNR expression could be effective as an additional treatment of pituitary tumors.

In summary, the recombinant baculovirus encoding an interfering RNA for HNR efficiently transduced GH3 cells and increased apoptosis of GH3 cells. Our results could help to design therapies using HN as a potential target for the alternative treatment of tumors using BV-derived vectors for gene therapy.

Several viral vectors for gene therapy of cancer and other pathologies are currently used in experimental phase [8, 21, 36]. Regardless of differences in gene delivery efficiencies, pre-existing immunity is one of the most important obstacles that vectors must overcome. Baculoviruses, although highly immunogenic, do not exhibit this disadvantage since human anti-baculovirus-specific pre-existing immunity



**Fig. 5** Effect of baculovirus-mediated HNr gene silencing on apoptosis in xenografted pituitary tumor and tumor progression. **a** *Nude* mice were inoculated s.c. with GH3 cells in the flank. When tumor volume reached approximately 200 mm<sup>3</sup>, mice were injected intratumorally with control baculovirus (BV-control) or Baculovirus encoding shHNr (BV-shHNr). **b** Tissue sections from tumors were analyzed for detection of apoptosis by TUNEL. Each column represents the mean  $\pm$  SE of TUNEL-positive cells per field. \*\* $p < 0.01$  versus BV-Control. Student's *t* test ( $n = 3$  animals per group). **c** Tumor growth was evaluated for 15 days. \* $p < 0.05$  versus BV-Control. Multiple regression analysis ( $n = 8$  animals per group). **d** Kaplan Meyer survival curves. \* $p < 0.05$  versus BV-Control. Log rank test ( $n = 6$  animals per group)

has not been detected [21, 37, 38]. In addition, baculovirus DNA has not been found to integrate in the host cell genome. These advantages over other viral gene therapy vectors make baculoviruses a promising tool for future clinical trials. In

conclusion, bearing in mind the previously documented evidence on antiapoptotic activity of HN and the effects of silencing HNr expression in GH3 cells in vitro and in vivo shown in the present study, we envisage the therapeutic potential for baculovirus-vectored silencing of HN as an alternative treatment of pituitary tumors.

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### Compliance with ethical standards

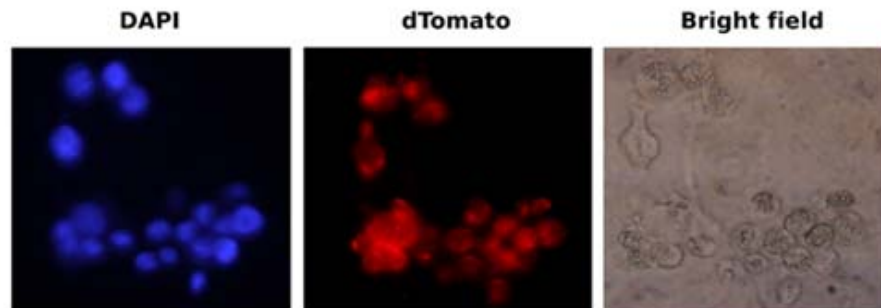
**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the Ethics Committee of the School of Medicine, University of Buenos Aires.

### References

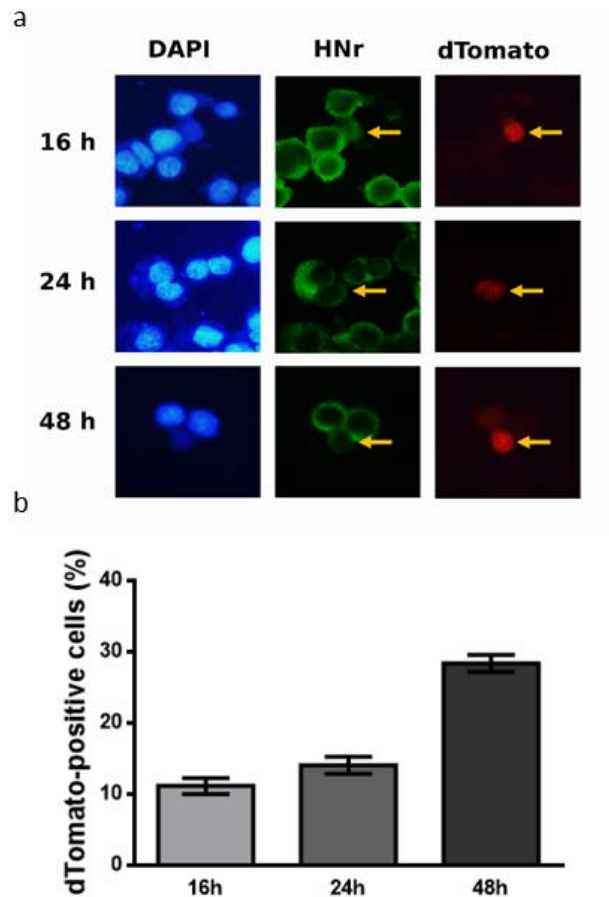
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**Supplementary Fig. 1** Generation of recombinant baculovirus for delivery of shRNAs in mammalian cells. Representative images of Sf9 insect cells co-transfected with bAcGoza bacmid and the plasmid pBacPAK9-shHNR; observation made at 96 h post-transfection. Left panel: DAPI; medium panel: cells with recombinant baculovirus expressing dTomato protein; right panel: cells infected with recombinant baculovirus BV-shHNR observed under phase contrast microscopy (TIF 124 KB)



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**Supplementary Fig. 2** Transfection efficiency of the plasmid with HNr interfering RNA. GH3 cells were transfected with 1  $\mu$ g of plasmid DNA pUC-shHNr, incubated for 16, 24 or 48 h and processed for the detection of HNr by immunofluorescence. (a) Representative microphotographs of transfected cells. Arrows indicate transfected cells showing dTomato and HNr expression. Nuclei were counterstained with DAPI. Left panels: DAPI; medium panels: HNr; right panels: dTomato. (b) Percentage of transfected (dTomato-positive) cells. Each column represents the percentage  $\pm$  CL of dTomato-positive GH3 cells ( $n \geq 1000$  cells/group) (TIF 143 KB)