Apoptosis (2018) 23:143–151
https://doi.org/10.1007/s10495-018-1444-0

ORIGINAL PAPER

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

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

Published online: 19 January 2018
© Springer Science+Business Media, LLC, part of Springer Nature 2018

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

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 macroadenomes. 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

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

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10495-018-1444-0) contains supplementary material, which is available to authorized users.

✉ Adriana Seilicovich
adyseili@fmed.uba.ar

[1] Departamento de Biología Celular e Histología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

[2] Instituto de Investigaciones Biomédicas (INBIOMED), Facultad de Medicina, CONICET- Universidad de Buenos Aires, Paraguay 2155, piso 10, C1121ABG Buenos Aires, Argentina

[3] Instituto de Biotecnología y Biología Molecular (IBBM), Universidad Nacional de La Plata - CONICET, La Plata, Argentina

[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 somatolactotrope tumor cells overexpress HNr when compared to normal anterior pituitary cells, and that exogenous HN protects them from TNF-α-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: **gaccctatggagcttcaatttagagaacttaaattgaagctccatagggtc**) 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 *Eco*RV-*Not*I 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 co-transfected in insect cell line *Trichoplusia ni* BTI-TN-5B1-4 (High FiveTM 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 µl/ml MEM amino acids, 2 mM glutamine, 100 µg/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

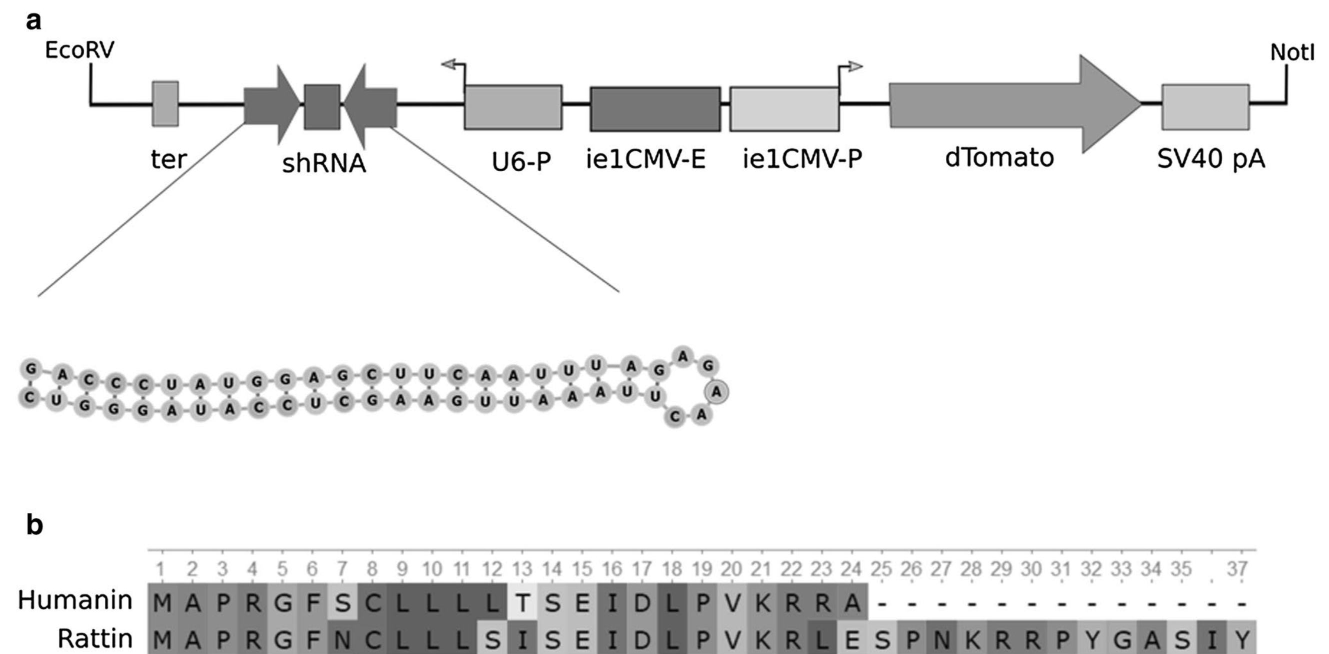

Fig. 1 **a** Scheme of the silencing cassette. HNr specific shRNA is encoded under the control of the U6 promoter and the reporter gene dTomato is encoded under CMV *IE* promoter. Amplification of the shRNA sequence is depicted below. The control construct contains only the CMV (IE)-dTomato sequence (not shown). **b** Sequence homology of Humanin and Rattin [32]

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

Gene transfection and transduction

GH3 were transfected with 1 µg 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-α (50 ng/ml) for a futher 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 3×10^6 GH3 cells. Tumor size was determined every 2 days with caliper and tumor volume was estimated according to the formula: [width^2 × length]/2 (mm^3). When the tumor volume reached approximately 200 mm^3, animals were injected intratumorally with 10^8 PFUs of BV-Control or BV-shHNr (40 µl/mouse). Tumor growth was evaluated for 15 days and survival was determined until the tumor reached 2000 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 µm) 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 °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/µl) 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-digoxygenin 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-phenylindoledihydrochloride (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] × 100.

Expression of HNr by inmunofluorescence

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 µg 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 µl primers (1 µg/µl) and 1 µl 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 µl 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 µl of solution containing 5 µl cDNA, 9 µM forward and reverse primers and 10 µl 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 ΔΔCt 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.

Table 1 Oligonucleotides used as primers in real time PCR

Rattin (HNr) forward	5′-GAGGGTTCAACTGTCTCTTACTTTCA-3′
Rattin (HNr) reverse	5′-GTGAAGAGGCTGGAATCTCCC-3′
Cyclophilin forward	5′-CTGGTGGCAAGTCCATCTAC-3′
Cyclophilin reverse	5′-CCCGCAAGTCAAAGAAATTA-3′

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 $MgCl_2$, 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-β-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 Bio-Lógicos, 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

with respect to the corresponding β-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 ± 95% confidence limits (CL) of the total number of cells counted in each specific condition and analyzed by χ^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-α increased apoptosis of both normal and tumor pituitary cells [27, 28]. We also showed that HN per se did not modify basal apoptosis of pituitary cells but inhibited the apoptotic effect of TNF-α 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-α. To this end, GH3 cells were transfected with pUC-Control or pUC-shHNr plasmids, incubated for 24 h, and after addition of TNF-α, incubated for an additional period of 24 h (Fig. 2b). At 48 h after transfection, TNF-α 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-α-induced apoptosis, suggesting that endogenous HNr also plays a cytoprotective role against an external apoptotic stimulus.

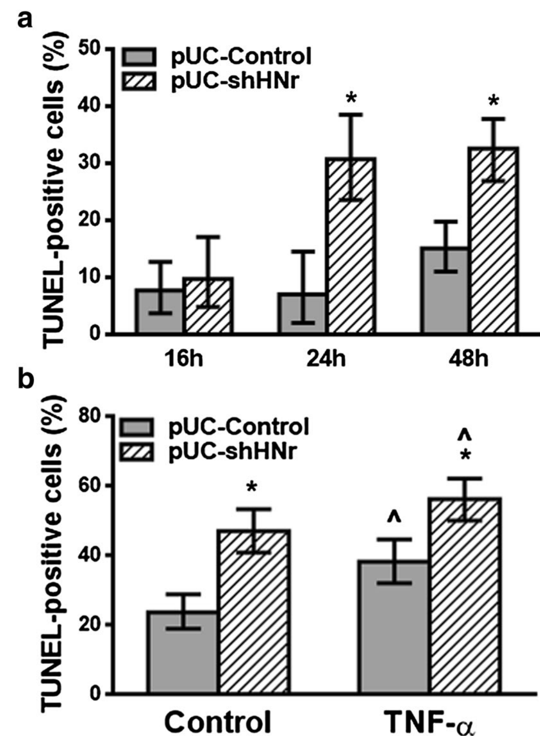

Fig. 2 Inhibition of endogenous HNr reduced apoptosis of GH3 cells. **a** GH3 cells were transfected with 1 µg of plasmid DNA control (pUC-Control) or plasmid with interfering RNA for HNr (pUC-shHNr) for 16, 24, and 48 h. Apoptosis was assessed by the TUNEL method in transfected (dTomato-positive) cells. Each point represents the percentage ± CL of TUNEL-positive GH3 cells (n ≥ 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 µg of pUC-Control or pUC-shHNr DNA for 24 h and then incubated without (Control) or with TNF-α (50 ng/ml) for further 24 h. Apoptosis was assessed by the TUNEL method in transfected (dTomato-positive) cells. Each column represents the percentage ± CL of TUNEL-positive GH3 cells (n ≥ 700 cells/group). Data from at least two independent experiments were analyzed by χ2. *$p < 0.05$ versus respective pUC-Control, ^$p < 0.05$ versus respective control without TNF-α

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-shHN 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-shHN 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-HNr did not modify Bcl-2 expression (Fig. 4d), it significantly increased the Bax/Bcl-2 ratio in GH3 cells (Fig. 4e).

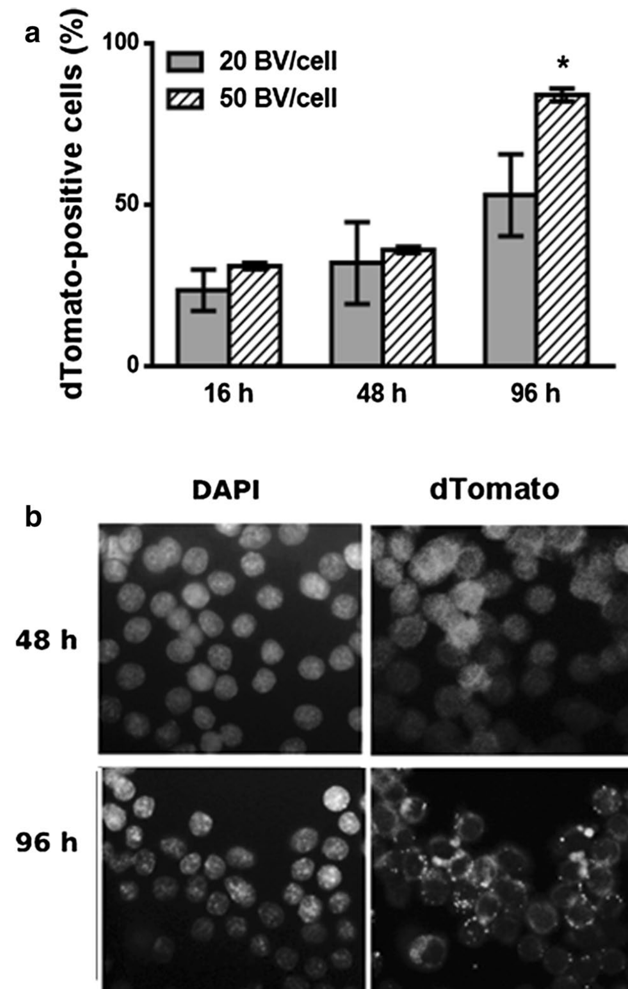

Fig. 3 Baculovirus transduction with BV-shHNr in GH3 cells. **a** Baculovirus transduction with BV-shHNr in GH3 cells incubated for 16, 48 or 96 h, using 20 and 50 BV/cell. **b** Representative images of transfected cells incubated with 50 BV/cell of BV-shHNr. Upper panels: DAPI; lower panels: dTomato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

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^3, 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^3. 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).

Discussion

We have previously reported that HNr expression in rat somatolactotrope 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-α-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

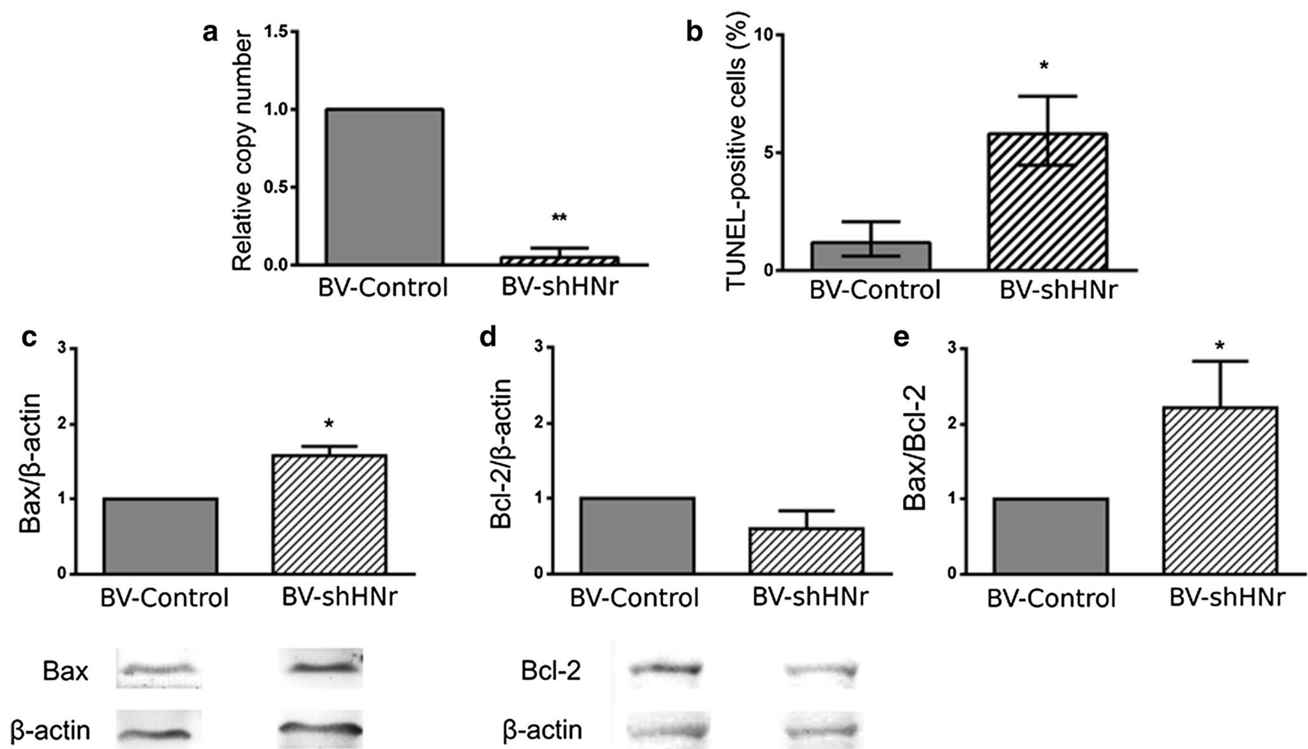

Fig. 4 Effect of BV-shHNr transduction in GH3 tumor cells in vitro. GH3 cells were incubated with BV-Control or BV-shHNr for 48 h. **a** Expression of HNr mRNA. Each column represents the mean ± SE of the concentration of HNr RNA relative to its internal control of cyclophilin. (n = 3), **p < 0.01 Student's *t* test. **b** Apoptosis was evaluated by the TUNEL method. Each column represents the percentage ± CL of TUNEL-positive GH3 cells (n ≥ 1000 cells/group). *p < 0.05. χ^2. Expression of Bax (**c**) and Bcl-2 (**d**) was determined by Western blot. Each column represents the mean ± SE of the relative increment respect to BV-Control. Lower panels show representative blots. β-actin was assessed as loading control. Raw data from 3 independent experiments were normalized by the corresponding β-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-κB 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

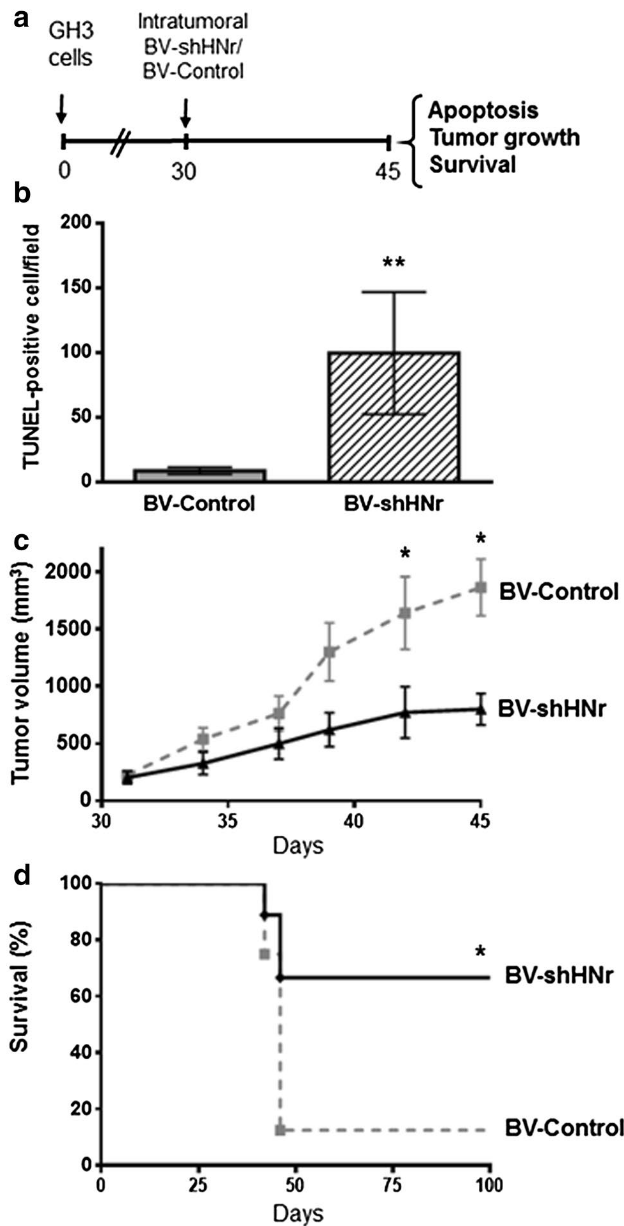

Fig. 5 Effect of baculovirus-mediated HNr gene silencing on apoptosis in xenografted pituitary tumor and tumor progression. **a** *Nude* mice were inoculated s.c. with GH3 cells in the flank. When tumor volume reached approximately 200 mm^3, mice were injected intratumorally with control baculovirus (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 ± 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.

Acknowledgements This work was supported by the Consejo Nacional de Investigaciones Científicas y Tecnológicas, National Research Council (PIP 11420110100353 to M.C.; PIP 11220120100261 to A.S, doctoral fellowships to MFG, MP, ASA), Agencia Nacional de Promoción Científica y Tecnológica (PICT 2013-0310, PICT-2015-3309 to M.C.; PICT 2014-0334 to A.S.; PICT 2014-1827 to V.R., doctoral fellowship to CZ), the University of Buenos Aires (20020130100020 to A.S.) and the National University of La Plata (X703 to V.R.).

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

1. Melmed S (2009) Acromegaly pathogenesis and treatment. J Clin Invest 119:3189–202
2. Melmed S (2003) Mechanisms for pituitary tumorigenesis: the plastic pituitary. J Clin Invest 112:1603–1618
3. Gillam MP, Molitch ME, Lombardi G, Colao A (2006) Advances in the treatment of prolactinomas. Endocr Rev 27:485–534
4. Benveniste RJ, King WA, Walsh J, Lee JS, Delman BN, Post KD (2005) Repeated transsphenoidal surgery to treat recurrent or residual pituitary adenoma. J Neurosurg 102:1004–1012
5. Osamura RY, Kajiya H, Takei M et al (2008) Pathology of the human pituitary adenomas. Histochem Cell Biol 130:495–507
6. Gottardo MF, Jaita G, Magri ML et al (2014) Antiapoptotic factor humanin is expressed in normal and tumoral pituitary cells and protects them from TNF-alpha-induced apoptosis. PLoS ONE 9:e111548
7. Gottardo MF, Moreno Ayala M, Ferraris J et al (2017) Humanin inhibits apoptosis in pituitary tumor cells through several signaling pathways including NF-kappaB activation. J Cell Commun Signal 11:329–340
8. Hashimoto Y, Niikura T, Ito Y et al (2001) Detailed characterization of neuroprotection by a rescue factor humanin against various Alzheimer's disease-relevant insults. J Neurosci 21:9235–9245
9. Hashimoto Y, Niikura T, Tajima H et al (2001) A rescue factor abolishing neuronal cell death by a wide spectrum of familial Alzheimer's disease genes and Abeta. Proc Natl Acad Sci USA 98:6336–6341
10. Niikura T, Tajima H, Kita Y (2006) Neuronal cell death in Alzheimer's disease and a neuroprotective factor, humanin. Curr Neuropharmacol 4:139–147
11. Zapala B, Kaczynski L, Kiec-Wilk B et al (2010) Humanins, the neuroprotective and cytoprotective peptides with antiapoptotic and anti-inflammatory properties. Pharmacol Rep 62: 767–777

12. Niikura T, Hashimoto Y, Tajima H et al (2003) A tripartite motif protein TRIM11 binds and destabilizes Humanin, a neuroprotective peptide against Alzheimer's disease-relevant insults. Eur J Neurosci 17:1150–1158
13. Jia Y, Ohanyan A, Lue YH et al (2015) The effects of humanin and its analogues on male germ cell apoptosis induced by chemotherapeutic drugs. Apoptosis 20:551–561
14. Lue Y, Swerdloff R, Liu Q et al (2010) Opposing roles of insulin-like growth factor binding protein 3 and humanin in the regulation of testicular germ cell apoptosis. Endocrinology 151:350–357
15. Gong Z, Tas E, Muzumdar R (2014) Humanin and age-related diseases: a new link? Front Endocrinol 5:210
16. Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev 16:948–958
17. Lambeth LS, Smith CA (2013) Short hairpin RNA-mediated gene silencing. Methods Mol Biol 942:205–232
18. Snove O Jr, Rossi JJ (2006) Expressing short hairpin RNAs in vivo. Nat Methods 3:689–695
19. Pidre ML, Ferrelli ML, Haase S, Romanowski V (2013) Baculovirus display: a novel tool for vaccination. In: Romanowski V (ed) Biochemistry genetics and molecular biology. Intech, Rijeka, pp 137–164
20. Hu YC (2008) Baculoviral vectors for gene delivery: a review. Curr Gene Ther 8:54–65
21. Kwang TW, Zeng X, Wang S (2016) Manufacturing of AcMNPV baculovirus vectors to enable gene therapy trials. Mol Ther Methods Clin Dev 3:15050
22. Je YH, Chang JH, Choi JY et al (2001) A defective viral genome maintained in Escherichia coli for the generation of baculovirus expression vectors. Biotech Lett 23:575–582
23. Haase S, McCarthy CB, Ferrelli ML, Pidre ML, Sciocco-Cap A, Romanowski V (2015) Development of a recombination system for the generation of occlusion positive genetically modified *Anticarsia gemmatalis* multiple nucleopolyhedrovirus. Viruses 7:1599–1612
24. Ferraris J, Boutillon F, Bernadet M, Seilicovich A, Goffin V, Pisera D (2012) Prolactin antagonism in mouse anterior pituitary: effects on cell turnover and prolactin receptor expression. J Physiol Endocrinol Metab 302:E356–E364
25. Ferraris J, Zárate S, Jaita G, Boutillon F, Bernadet M, Auffret J, Seilicovich A, Binart N, Goffin V, Pisera D (2014) Prolactin induces apoptosis of lactotropes in female rodents. PLoS ONE 9:e97383
26. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408
27. Candolfi M, Jaita G, Zaldivar V, Zárate S, Ferrari L, Pisera D, Castro MG, Seilicovich A (2005) Progesterone antagonizes the permissive action of estradiol on tumor necrosis factor-alpha-induced apoptosis of anterior pituitary cells. Endocrinology 146(2):736–743
28. Candolfi M, Jaita G, Pisera D, Ferrari L, Barcia C, Lui JC, Castro MG, Seilicovich A (2006). Adenoviral vectors encoding tumor necrosis factor-alpha and FasL induce apoptosis of normal and tumoral anterior pituitary cells. J Endocrinol 189(3):681–690
29. Mottaghi-Dastjerdi N, Soltany-Rezaee-Rad M, Sepehrizadeh Z, Roshandel G, Ebrahimifard F, Setayesh N (2014) Genome expression analysis by suppression subtractive hybridization identified overexpression of Humanin, a target gene in gastric cancer chemoresistance. Daru 22:14
30. Matsuoka M, Hashimoto Y (2010) Humanin and the receptors for humanin. Mol Neurobiol 41:22–28
31. Yen K, Lee C, Mehta H, Cohen P (2013) The emerging role of the mitochondrial-derived peptide humanin in stress resistance. J Mol Endocrinol 50:R11–R19
32. Niikura T, Chiba T, Aiso S, Matsuoka M, Nishimoto I (2004) Humanin: after the discovery. Mol Neurobiol 30:327–340
33. Eriksson E, Wickstrom M, Perup LS et al (2014) Protective role of humanin on bortezomib-induced bone growth impairment in anticancer treatment. J Natl Cancer Inst https://doi.org/10.1093/jnci/djt459
34. Surampudi P, Chang I, Lue Y et al (2015) Humanin protects against chemotherapy-induced stage-specific male germ cell apoptosis in rats. Andrology 3:582–589
35. Cohen P (2014) New role for the mitochondrial peptide humanin: protective agent against chemotherapy-induced side effects. J Natl Cancer Inst 106:dju006
36. Felberbaum RS (2015) The baculovirus expression vector system: a commercial manufacturing platform for viral vaccines and gene therapy vectors. Biotechnol J 10:702–714
37. Makkonen KE, Airenne K, Yla-Herttulala S (2015) Baculovirus-mediated gene delivery and RNAi applications. Viruses 7:2099–2125
38. Luo WY, Shih YS, Lo WH et al (2011) Baculovirus vectors for antiangiogenesis-based cancer gene therapy. Cancer Gene Ther 18:637–645