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Hu/elav RNA-binding protein HuR regulates parathyroid hormone related peptide expression in human lung adenocarcinoma cells

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Summary. In 54 stage I and II human lung adenocarcinomas, HuR and PTHrP levels were positively correlated and the PTHrP-HuR status of the tumor was an independent prognostic marker of the clinical outcomes of patients. The possibility that HuR could upregulate PTHrP expression in lung adenocarcinoma was investigated by immunohistochemical, Western blot and RT-PCR analyses in HCC44 and DV90 human lung adenocarcinoma cell lines. In both cell lines, knockdown of HuR by specific siRNAs reduced PTHrP mRNAs and both cellular and secreted protein. Moreover, it inhibited cell growth and induced cell apoptosis, as revealed by the increase of caspase-3 activity. These effects were partially rescued by the addition of exogenous PTHrP (1-34). Analysis by actinomycin D assay revealed that in both cell lines HuR silencing produced a decrease of PTHrP mRNA half-life by about 70%. These findings add PTHrP to the list of lung cancer-associated genes, whose mRNA is stabilized by HuR.

Key words: Human lung adenocarcinoma, HuR expression, HuR knockdown, PTHrP expression, PTHrP mRNA stability, human lung adenocarcinoma cell lines

Introduction

The family of Hu/elav RNA-binding proteins participate in the post-transcriptional regulation of mRNAs bearing U- and AU-rich sequences and comprises the neuronal proteins HuB, HuC and HuD, and the ubiquitously expressed protein HuR (Hu antigen R or ELAVL1) (Antic and Keene, 1997).

Several studies focusing on breast, colon, ovarian and lung cancer showed a significant correlation between HuR expression and advanced stages of malignancy, supporting a role for HuR in cancer development in these tissues (Blaxall et al., 2000; López de Silanes et al., 2003; Denkert et al., 2004, 2006; Heinonen et al., 2005; Wang et al., 2011). Broad evidence indicated that HuR expression plays crucial roles in mRNA stabilization/translation of numerous growth, motility and angiogenic factors involved in malignant transformation (López de Silanes et al., 2005; Abdelmohsen and Gorospe, 2010].

The identification of HuR as a shuttling protein rationalizes the possibility that HuR may have different subcellular locations during different functional and/or developmental stages (Fan and Steitz, 1998). Recently, it has been reported that cytoplasmic, but not nuclear, expression of HuR behaved as a prognostic marker of a poor clinical outcome in non-small cell lung carcinomas (NSCLC) (Wang et al., 2011; Lauriola et al., 2012).

Parathyroid hormone related peptide (PTHrP) regulates cell growth and differentiation and is expressed during fetal and adult life (Wysolmerski and Stewart, 1998). PTHrP expression could be associated with both positive and/or negative effects on tumor progression and clinical outcome, suggesting that PTHrP can induce tissue-specific responses (Nishihara et al., 2007).

Previously, we observed that in the same series of patients with lung adenocarcinomas, the expressions of both cytoplasmic HuR (Lauriola et al., 2012) and PTHrP (Monego et al., 2010) were associated with a worse clinical outcome. The finding that in human renal cell

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carcinoma, PTHrP, a tumor survival factor, is upregulated by HuR through the increase of PTHrP mRNA stability (Danilin et al., 2009), led us to hypothesize that HuR can also regulate the expression of PTHrP in lung adenocarcinoma.

In the present study, we found that HuR is involved in the regulation of PTHrP expression in two human lung adenocarcinoma cell lines. Moreover, in stage I and II lung adenocarcinomas HuR and PTHrP were co-expressed and PTHrP-HuR status of the tumor behaved as an independent prognostic marker of the clinical outcome of patients.

Materials and Methods

Patients

The clinico-pathological characteristics of this patient series have been previously reported (Monego et al., 2010). Briefly, 54 patients (39 males and 15 females) with completely resected pathological stage I and II lung adenocarcinoma, with age range 18 to 75 years were enrolled in this study, after approval by the Institutional Review Board of the Università Cattolica del S. Cuore. The tumors were classified as lung adenocarcinomas of mixed histologic type (20 acinar predominant, 14 papillary predominant and 20 solid predominant with mucin production) according to the more recent classification criteria based on the predominant growth pattern of adenocarcinoma (Travis et al., 2011). With a median follow-up of 36 months (range 3-194), metastases occurred in 19 of 54 (35.2%) cases. At the end of the study, 12 of 54 (22.2%) patients died of cancer.

Cell lines

The HCC44 and DV90 human lung adenocarcinoma cell lines (DSMZ, Braunschweig, Germany) were grown in RPMI medium containing 2 mM glutamine and antibiotics (100 U/ml penicillin and 100 μg/ ml streptomycin), supplemented with 10% foetal bovine serum, at 37°C in a humidified atmosphere containing 5% CO_2. Cell lines were serially sub-cultured with trypsin-EDTA treatment and seeded twice a week at a density of $3x10^5$ cells/ml.

Small interfering RNA (siRNA) transfection for knockdown of HuR.

Two different HuR-specific siRNAs duplex oligonucleotides were purchased from Qiagen (Milan, Italy). The transfection was performed as indicated by the manufacturer. HCC44 and DV90 cells were seeded at a density of $5x10^4$ cells/well in 24-well culture plates for cell growth assays, $3x10^6$/plate in 100 mm plates for Western blot analysis and $3x10^6$/well in six-well plates for apoptosis detection and for the experiments of PTHrP mRNA stability. Cells were transfected with 20 nM HuR-specific siRNAs or control siRNA or fluorescently labeled siRNA as transfection efficiency control, using the HiPerfect Transfection reagent (Qiagen). The target sequences were 5'-AAGTAGCAGGACACAGCTTGG-3' (Hs-ELAVL1_1 siRNA) and 5'-ACCAGTTTCAATGGTCATAAA-3' (Hs-ELAVL1_11 siRNA). The two siRNAs used achieved the same extent of reduction in HuR expression, thus Hs-ELAVL1_1 siRNA was used in the reported experiments. The effect of siRNA knockdown on cell viability was determined by trypan blue exclusion method.

Cell growth evaluation

After plating, HCC44 and DV90 cells were transfected and cultured for the specified time periods. When indicated, 100 nM PTHrP 1-34 (Bachem, Weil am Rhein, Germany) was added to cell cultures. Quadruplicate hemocytometer counts of triplicate cultures were performed. Only viable cells were comprised in the count of growing cells.

Cell proliferation assay

After plating, cells ($5x10^3$/well) were transfected and cultured for different time periods (0-72 h). Then 10 μM BrdU was added to each well and plate was incubated overnight at 37°C. The amount of BrdU incorporated into newly synthesized DNA at each time point was monitored by an ELISA-based approach following the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany).

Apoptosis detection

Apoptosis was measured by acridine orange-ethidium bromide method (Gorman et al., 1996) and analyzed by fluorescence microscopy. The activity of caspase-3 was assessed fluorimetrically as previously reported (Serini et al., 2008).

Western blot analysis

SDS-PAGE, Western blotting, and densitometric analysis were performed by standard protocols (Serini et al., 2008). The membrane was blocked overnight at 4°C in 5% dried milk (w/v) in PBS plus 0.05% Tween 20 and then incubated with specific mouse monoclonal antibodies to HuR (clone 3A2, catalog # sc-5261, 1:1000, Santa Cruz Laboratories, Santa Cruz, CA, USA) and PTHrP (clone 212-10.7, 1:1000, Calbiochem, Germany). As a loading control, the blots were reprobed with anti-α-actinin antibody (clone B-12, catalog # sc-166524, 1:1000, Santa Cruz Laboratories). Following incubation with secondary anti-mouse antibody (1:10000, Amersham, Pharmacia Biotech Italia, Milan, Italy), the immunocomplexes were visualized using the enhanced chemiluminescence detection system

(Amersham) and quantitated by densitometric analysis.

Evaluation of PTHrP secretion

The amount of PTHrP 1-34 secreted by HCC44 and DV90 cells in the culture medium was evaluated by a commercially available enzyme immunoassay kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA) following the manufacturer's instructions. The minimum detectable levels of PTHrP were 0.01 ng/ml. Cells were counted to normalize PTHrP 1-34 concentrations in the conditioned medium among culture wells.

RNA extraction and one-step real-time PCR for PTHrP and HuR

Cell sample was digested for 10 min at 55°C in 200 μl of TENS 1x (10 mMTris pH 7.4, 10 mM EDTA, 100 mM NaCl, 1% SDS) with 100 mg/ml proteinase K and RNA was then extracted by RNAsi mini kit (Qiagen, Milan, Italy), following the manufacturer's protocol. We assessed the quantity and quality of the RNA spectrophometrically (E260, E260/E280 ratio, spectrum 220-320 nm; Biochrom, Cambridge, UK) and by separation on an Agilent 2100 Bioanalyzer (Palo Alto, CA, USA). RNA was treated with RQ1 RNase-Free DNase (Promega, Milan, Italy). Levels of PTHrP and Hur mRNA were assessed by real time PCR using iscript one-step RT-PCR kit with Sybr Green (Bio-rad, Hercules, CA). 100 ng of RNA was added to a 1x RT-PCR mix containing specific buffer and SYBR, each of forward and reverse primers (300 mM) and 1X iScript reverse transcriptase to make up a final volume of 25 μl. Cycling conditions were: 10 min at 50°C for cDNA synthesis followed by 5 min at 95°C for reverse inactivation, 40 cycles of 95°C for 10s, 60°C for 30s, and 80 cycles of 55 + 0.5°C per cycle for melting curve analysis in a CFX96 Real-Time PCR detection system (Bio-Rad). Primers used for PTHrP common region were: forward 5'-GTC TCA GCC GCC GCC TCA A-3' and reverse 5'-GGA AGA ATC GTC GCC GTA AA-3'; Hur primers were: forward 5'-GAC ATC GGG AGA ACG AAT TT-3' and reverse 5'-TGC TGA ACA GGC TTC GTA-3'; GAPDH primers were: forward 5'-GAA GGT GAA GGT CGG AGT C-3' and reverse 5'-GAA GAT GGT GAT GGG ATT TC-3'. Each assay was performed in triplicate and data were processed by CFX96 Real-Time optical system software (Bio-Rad). To determine relative changes in mRNA expression, the average obtained for PTHrP and Hur were normalized to the average amount of GAPDH using the 2-DCq method. Specificity of RT-PCR product was assessed by direct sequencing, using the same primers employed for PCR amplification.

Evaluation of PTHrP mRNA stability

PTHrP mRNA stability was evaluated in both HCC44 and DV90 cells, transfected with HuR siRNA or control siRNA for 72 hrs, by adding actinomycin D (Act D, 5 μg/ml) to block transcription for an additional 240 min. At the indicated time points cells were harvested and the remaining PTHrP mRNA was analyzed by RT-PCR as described above.

Immunohistochemistry

HuR and PTHrP immunohistochemical staining of consecutive tumor sections from the same paraffin block and quantitative evaluation of PTHrP and HuR levels, expressed as integrated density (ID) units, were performed as previously reported (Ranelletti et al., 2001; Monego et al., 2010; Lauriola et al., 2012). Immunohistochemical double staining procedure for HuR and PTHrP proteins was performed as previously reported (Gessi et al., 2005), utilizing rabbit polyclonal antibodies against HuR (1:100; Santa Cruz Biotechnology, Inc., CA) and PTHrP 1-34 (1:250; D.B.A., Milano, Italy) and the Vector M.O.M immunodetection kit (Vector Laboratories). The HuR and PTHrP proteins were detected by ABC peroxidase kit (Vector Laboratories), utilizing as substrates 3-amino-9-ethylcarbazole for the first antigen and diaminobenzidine for the second one. After treatment, HCC44 and DV90 cells were fixed with 4% paraformaldehyde for 7 min, then permeabilized with 0.2% BSA and 0.1% Triton X-100 in PBS and labeled with anti-HuR mouse monoclonal (clone 3A2, sc:5261, 1:250, Santa Cruz Biotechnology) or anti-PTHrP 1-34 rabbit polyclonal (T-4513, 1:300, Peninsula Laboratories, San Carlos, CA) antibodies. Binding was visualized using a biotinylated secondary antibody and the strepdavidin-biotin peroxidase complex (UltraTek HRP kit, UCS Diagnostics, Morlupo, Italy) developed with diaminobenzidine. Negative controls were performed using rabbit or mouse irrelevant matched immunoglobulins or by omitting primary antibodies.

Statistical analysis

The significance of difference between mean values was analyzed using Kruskal-Wallis test. Two-way frequency tables were evaluated by Fisher exact test. All P values were two-sided. Spearman's r was used to analyze the correlation between PTHrP ID and nuclear or cytoplasmic HuR ID. The evaluation of the PTHrP mRNA half-life was performed by best fitting of the experimental curves with non-linear one phase exponential decay, utilizing GraphPad software Inc. (La Jolla, CA). For survival analysis, PTHrP and cytoplasmic Hur cut off values were chosen according to the results of ROC curve analyses. Tumors with cytoplasmic HuR IDs >13.5 and PTHrP IDs >8.0 (29 out of 54) were classified as positive. All medians and life tables were computed using the product- limit estimate by Kaplan and Meier, and the curves were examined by the log-rank test. Statistical analyses were carried out by JMP 10 software (SAS Institute Inc.; Cary, NC).

Results

HuR and PTHrP expressions in adenocarcinomas

PTHrP was expressed in the cytoplasm (Fig. 1A), while HuR immunostaining was localized both in the nucleus and cytoplasm of tumor cells (Fig. 1B). Tumors expressing high levels of PTHrP also showed high amounts of cytoplasmic and nuclear HuR, while tumors negative for PTHrP showed low or no HuR expression. Fig. 1A,B show consecutive sections from the same tumor expressing high levels of both PTHrP and HuR, while Fig. 1C,D show consecutive sections from the same tumor expressing low levels of both proteins. Double immunohistochemical staining revealed that PTHrP and HuR were co-expressed in the same tumor cells (Fig. 1E-G). In a series of 54 lung adenocarcinomas the level of PTHrP ID was directly correlated with cytoplasmic, but not nuclear (not shown), HuR levels. At the cut off points chosen, 29 (53.7%) tumors were positive for both PTHrP and cytoplasmic HuR, while 25 (46.3%) tumors were negative for both proteins (Fig. 1H; Table I). PTHrP and cytoplasmic HuR (PTHrP-HuR) status of tumors was not associated with age, sex, smoking and tumor diameter, while a significant association was found with lymph-node involvement at presentation, with none of the PTHrP-HuR negative tumors associated with lymph-node involvement at presentation. Moreover, 16 out of 19 (84.2%) patients with a PTHrP-HuR positive tumor developed metastases (Table 1).

The Kaplan-Meier analyses of survival curves revealed that at 5-year follow-up, the estimated proportions of metastasis-free and overall surviving patients with PTHrP-HuR positive tumors were significantly lower as compared to those with PTHrP-HuR negative tumors (Table 2).

As revealed by the multivariate analysis, PTHrP-HuR positivity retained an independent negative prognostic significance relative to the risk of metastasis, while tumor diameter > 3 cm, lymph-node involvement at presentation and positive PTHrP-HuR status retained an independent negative prognostic significance relative to the risk of death (Table 3).

HuR knockdown decreases PTHrP expression in lung adenocarcinoma cell lines

Western blot analyses clearly showed that HuR gene

Table 1. Distribution of PTHrP and cytoplasmic HuR status according to clinicopathological characteristics in stage I-II lung adenocarcinomas.

Parameter	Number of cases	PTHrP-HuR negative[1]	PTHrP-HuR positive[2]	p[3]
All cases	54	25 (46.3%)	29 (53.7%)	
Age (years)				
<60	32	14 (43.8%)	18 (56.2%)2	
>60	22	11 (50.0%)	11 (50.0%)	0.783
Sex				
Male	39	20 (51.3%)	19 (48.7%)	
Female	15	5 (33.3%)	10 (66.7%)	0.36
Smoking				
No	14	4 (28.6%)	10 (71.4%)	
Yes	40	21 (52.5%)	19 (47.5%)	0.21
Tumor diameter (cm)				
<3	23	15 (65.2%)	8 (34.8%)	
>3	31	10 (32.2%)	21 (67.8%)	0.027
Lymph-node involvement at presentation				
No	43	25 (58.1%)	18 (41.9%)	
Yes	11	0	11 (100%)	0.0004
Metastasis				
No	35	22 (62.9%)	13 (37.1%)	
Yes	19	3 (15.8%)	16 (84.2%)	0.0014

[1]: Percent of PTHrP-HuR negative; [2]: Percent of PTHrP-HuR positive; [3]: Fisher exact test

Table 2. Five-year follow-up according to PTHrP and cytoplasmic HuR status of lung adenocarcinomas.

PTHrP-HuR status	N (%)	Metastasis-free surviving	Overall surviving
Negative	25 (46. 3)	91.3% (95% C.I. : 71.2-97.7)	95.6% (95% C.I. : 34.1-77.0)
Positive	29 (53.7)	43.6% (95% C.I. : 24.3-65.3)	51.6% (95% C.I. : 29.4-73.3)
Log-Rank test		p = 0.0001	p =0.0007

Table 3. Multivariate analysis of prognostic variables in stage I-II lung adenocarcinoma patients.

	Metastasis-free Survival			Overall Survival		
Covariates	RR[1]	(CI 95%)[2]	p	RR	(CI 95%)	p
Age (years)						
< 66	1			1		
> 66	0.68	(0.2-1.9)	0.40	1.37	(0.2-8.6)	0.72
Sex						
Male	1			1		
Female	2.14	(0.6-7.1)	0.22	3.10	(1.3-34.2)	0.08
Smoking						
No	1			1		
Yes	1.86	(0.5-8.0)	0.37	3.35	(0.6-33.1)	0.19
Tumor diameter (cm)						
< 3	1			1		
> 3	1.72	(0.5-5.1)	0.35	40.88	(3.5-718.5)	0.0012
Lymph-node involvement at presentation						
No	1			1		
Yes	2.31	(0.8-7.4)	0.14	18.58	(3.4-169.6)	0.0004
PTHrP-HuR status						
Negative	1			1		
Positive	9.06	(1.9-56.1)	0.006	15.69	(1.5-434.8)	0.02

[1]: Unadjusted relative risk. [2]: 95% confidente intervals.

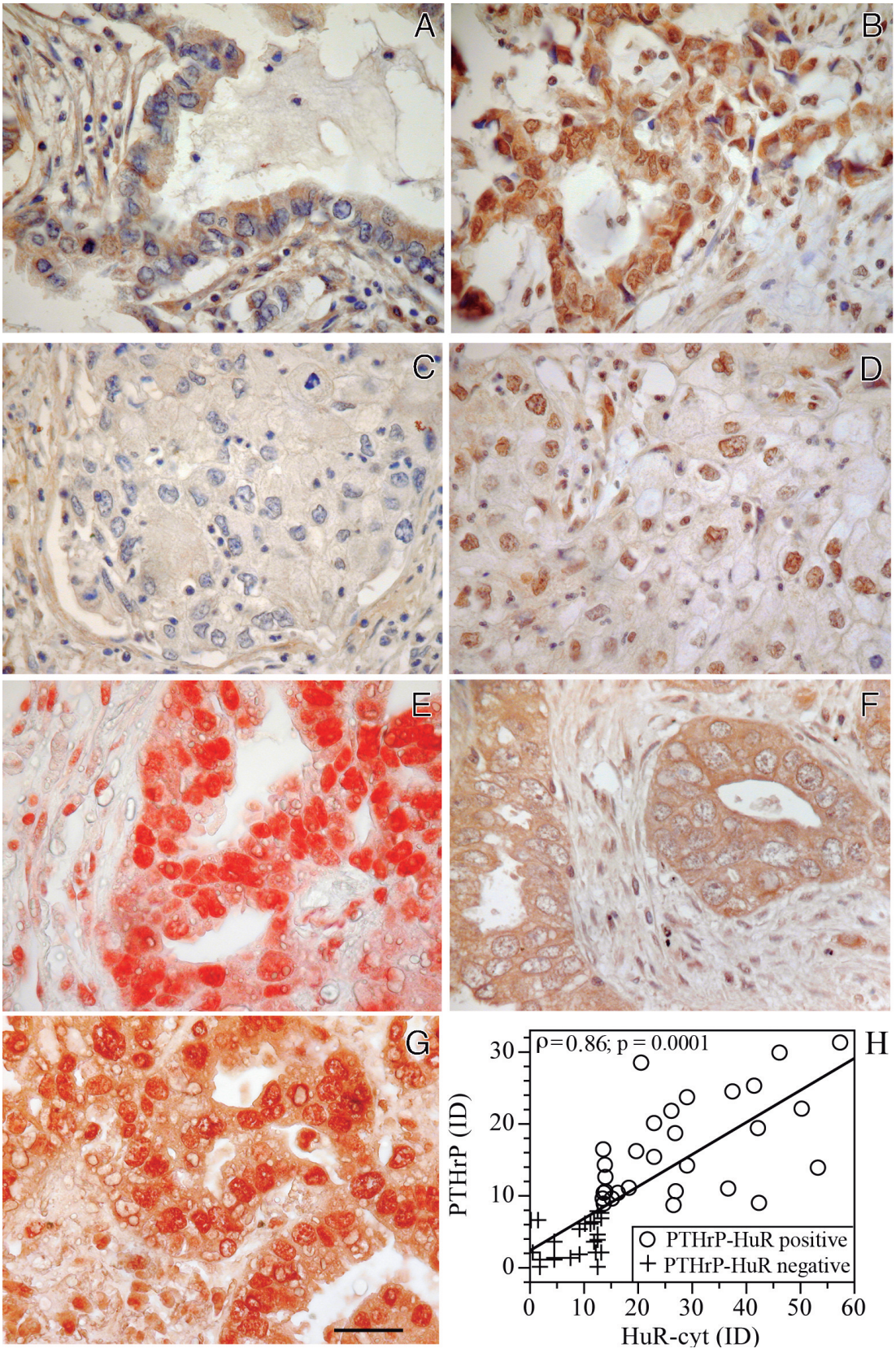

Fig. 1. Immunohistochemical staining with a-PTHrP (1-34) **(A, C, F)** and a-HuR **(B, D, E)** antibodies. **A** and **B** show consecutive sections from the same tumor expressing high levels of both PTHrP and HuR, while **C** and **D** show consecutive sections from the same tumor expressing low levels of both proteins. Double immunostaining of adenocarcinoma **(G)** with a-PTHrP (1-34) (**F**, brown) and a-HuR (**E**, red) antibodies. Correlation between PTHrP and cytoplasmic HuR levels and distribution of double positive and negative cases according to PTHrP and cytoplasmic Hur cut off values **(H)**. Scale bar: 50 μm.

silencing greatly reduced the amount of both HuR and PTHrP in DV90 and HCC44 cells after 72 hrs and 96 hrs of culture (Fig. 2A-F). These results were also confirmed by immunocytochemical tests revealing that HuR knockdown decreased the expression of both HuR and PTHrP proteins in both cell lines (Fig. 3). Moreover, HuR knockdown also reduced, in a time-dependent manner, the amount of PTHrP 1-34 secreted in the medium by HCC44 and DV90 cells after 48 and 72 hours of culture (Fig. 4A,B).

HuR knockdown decreases PTHrP mRNA expression and stability in adenocarcinoma cells

The transient transfection with specific HuR siRNA decreased HuR mRNA expression in DV90 and HCC44 cells by up to 75% at 48 hrs and 72 hrs, respectively. As revealed by RT-PCR quantitations, DV90 constitutively contained about one log less amount of PTHrP mRNA than HCC44 cells (not shown). After HuR gene silencing, the expression of PTHrP was significantly

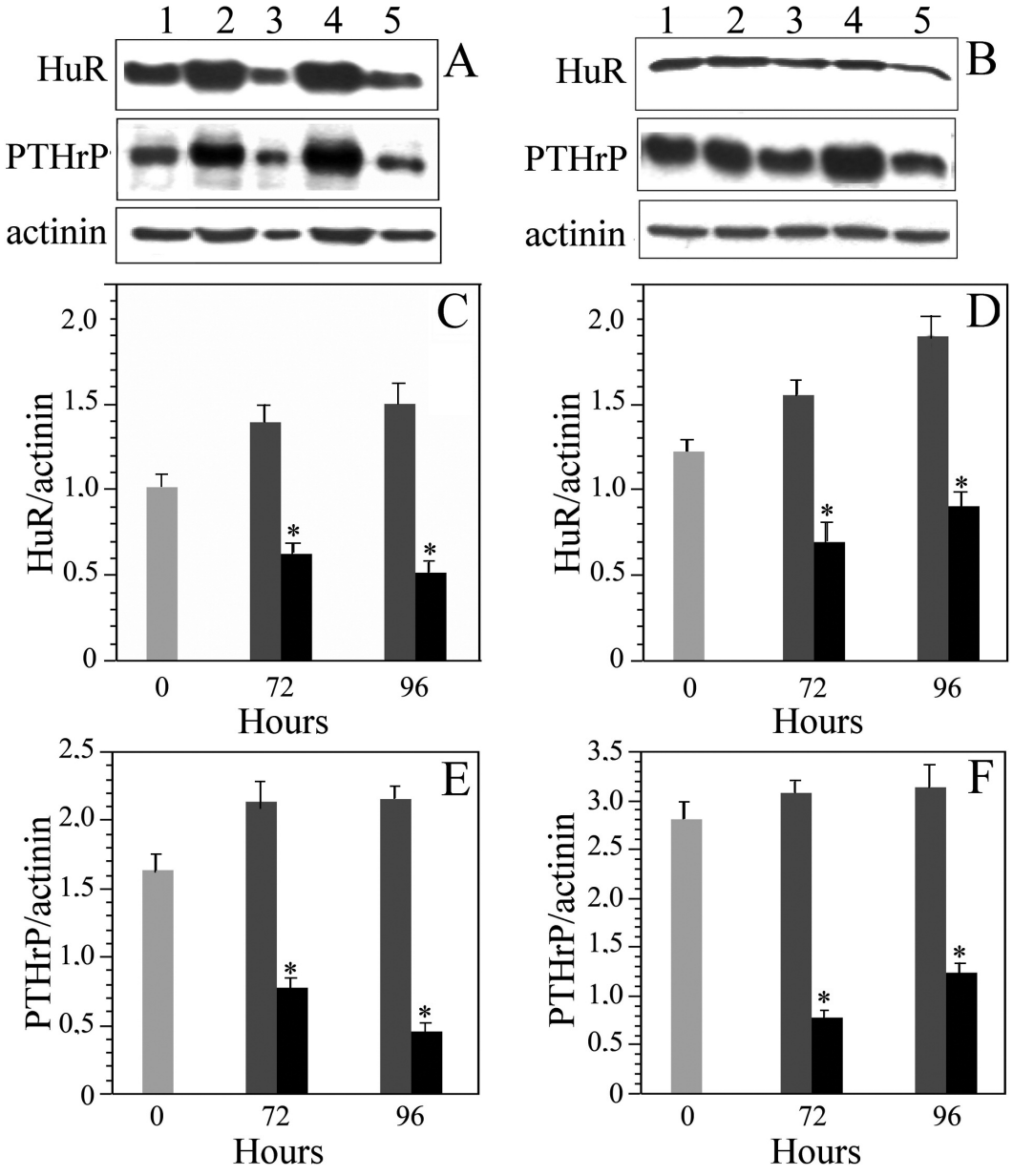

Fig. 2. HuR knockdown decreases the expression of PTHrP in HCC44 **(A, C, E)** and DV90 **(B, D, F)** lung adenocarcinoma cell lines. Immunoblots **(A, B)** for HuR, PTHrP and corresponding α-actinin (1 = t 0; 2 = control siRNA, 72 hrs; 3 = HuR siRNA, 72 hrs; 4 = control siRNA, 96 hrs; 5 = HuR siRNA, 96 hrs) and relative densitometric readings **(C-F)** (gray bars: control siRNA; black bars: specific HuR siRNA). Shown are representative immunoblots of at least three independent experiments. *p< 0.001 relative to control siRNA.

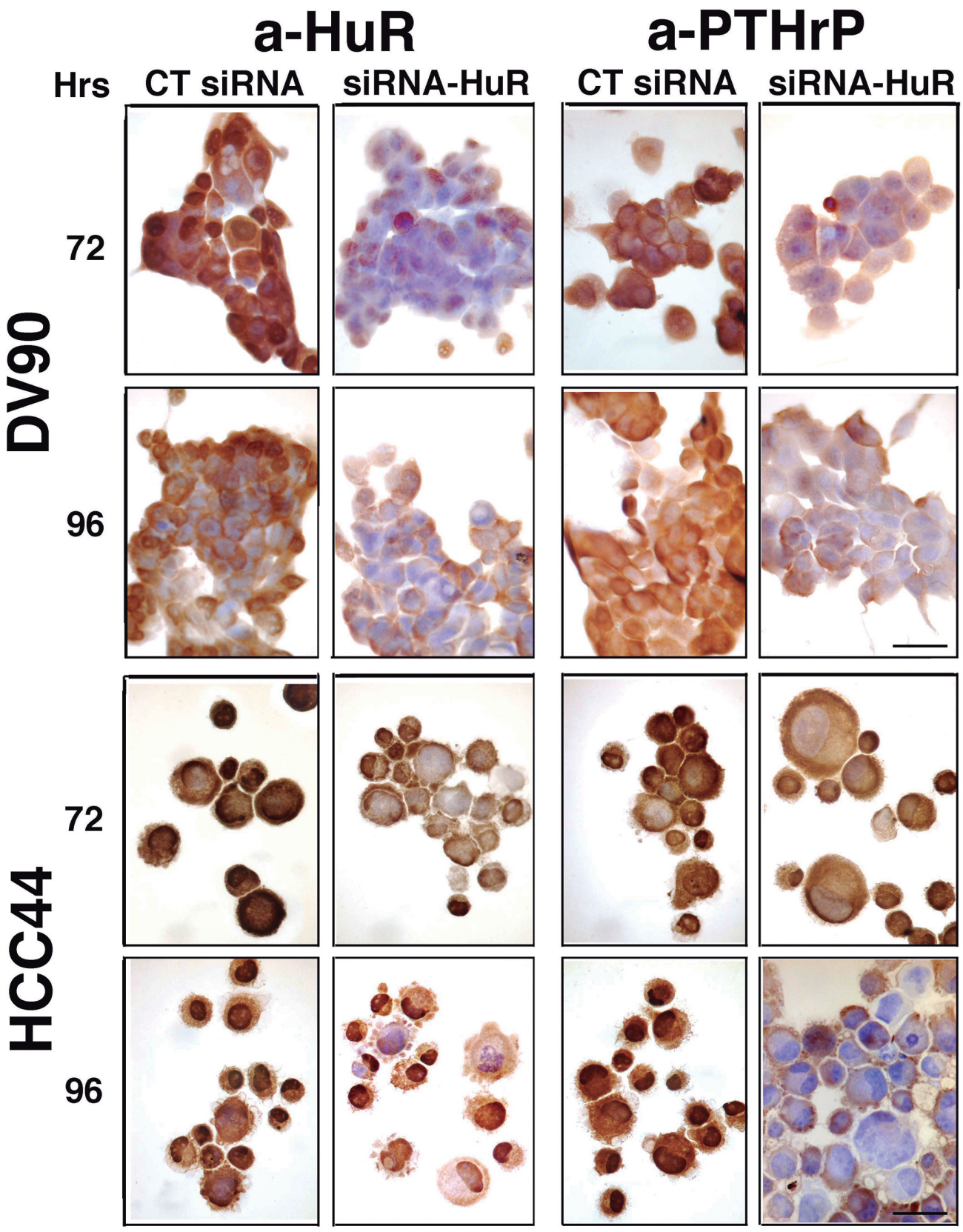

Fig. 3. HuR knockdown decreases PTHrP expression in DV90 and HCC44 lung adenocarcinoma cell lines. Cells, transfected with HuR specific siRNA (siRNA-HuR) or control siRNA (CT siRNA), were harvested at the indicated time, cytocentrifuged and immunostained with anti-HuR or anti PTHrP 1-34 antibodies. Scale bars: 25 μm.

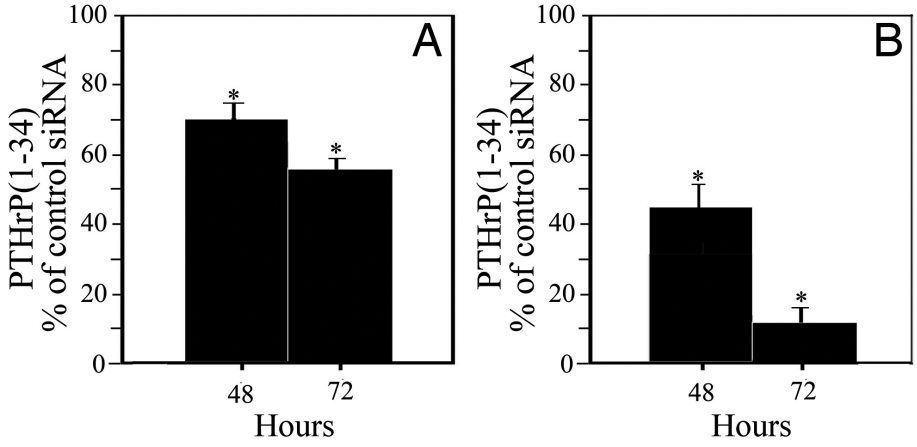

Fig. 4. Enzyme immunoassay of the amount of PTHrP secreted in the culture medium by HCC44 **(A)** and DV90 **(B)** cells transfected for 48 and 72 hours with control siRNA or specific HuR siRNA. After normalizing for cell number (pg/10^6 cells), the results were expressed as a percentage of PTHrP (1-34) secreted by specific HuR siRNA treated cells relative to that produced by control siRNA transfected cells. Results represent mean ± SE of triplicate experiments. PTHrP concentrations (pg/ 106 cells) in the control siRNA transfected cell medium, at 48 hours and 72 hours, rspectively, were: 4.5±0.6 SE and 23.2±3.1 SE for HCC44 and 1.2±0.07 and 6.4±0.8 for DV90. * p<0.001 72 hrs versus 48 hrs.

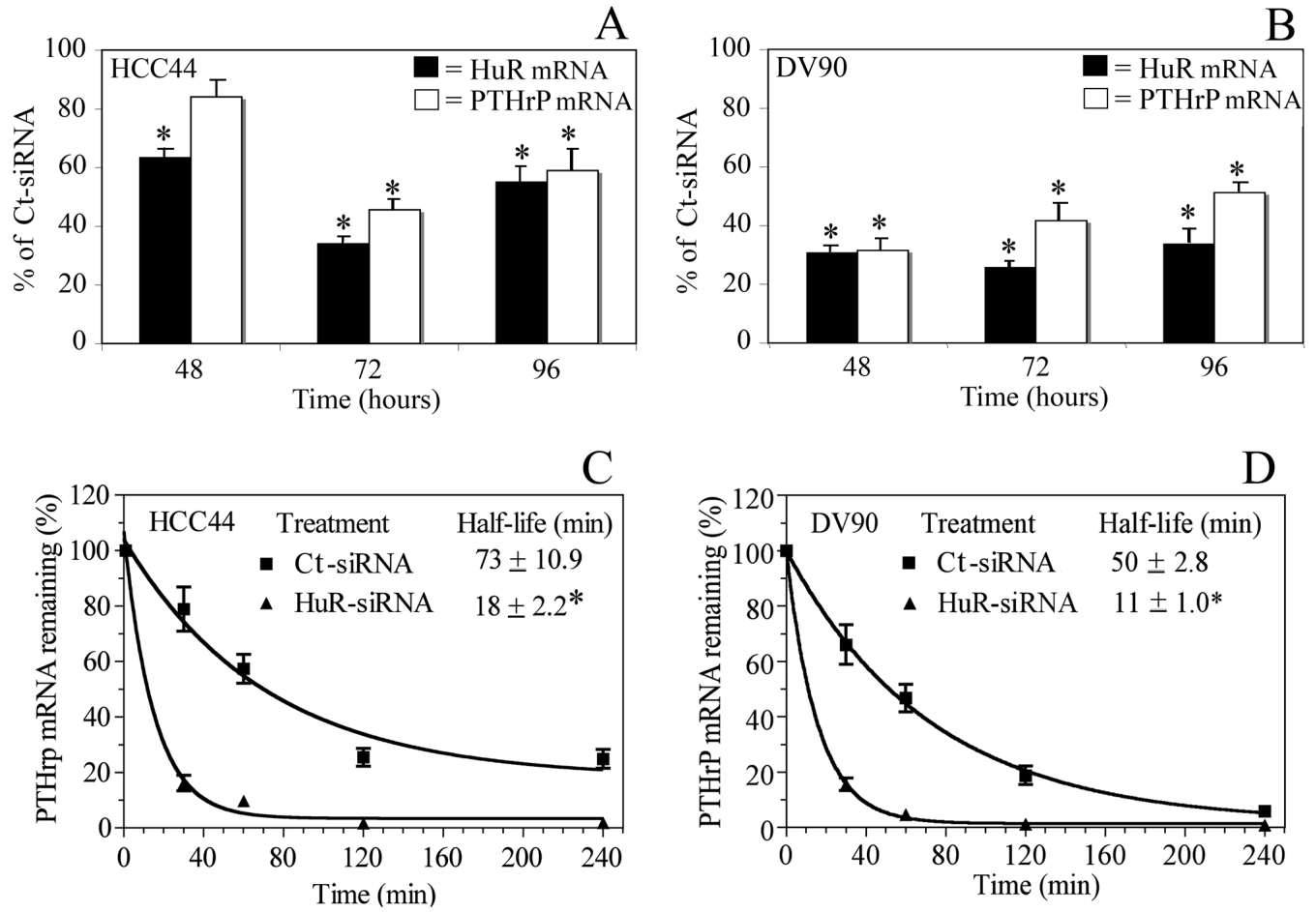

Fig. 5. Effects of transfection with HuR siRNA, for the indicated time, on HuR and PTHrP mRNAs in HCC44 **(A)** and DV90 **(B)** cells. Results are expressed as a percentage of mRNA in HuR siRNA transfected cells relative to control siRNA transfected ones and represent mean ± SE of triplicate experiments. (* p< 0.001). Stability of PTHrP mRNA in HCC44 **(C)** and DV90 **(D)** cells transfected with HuR siRNA and control siRNA for 72 hours and then exposed to actinomycin D for the indicated times. Results are expressed as a percentage of PTHrP mRNA remaining relative to the amount at time 0 and represent mean ± SE of triplicate determinations. (* p< 0.001). Shown is one of two similar experiments.

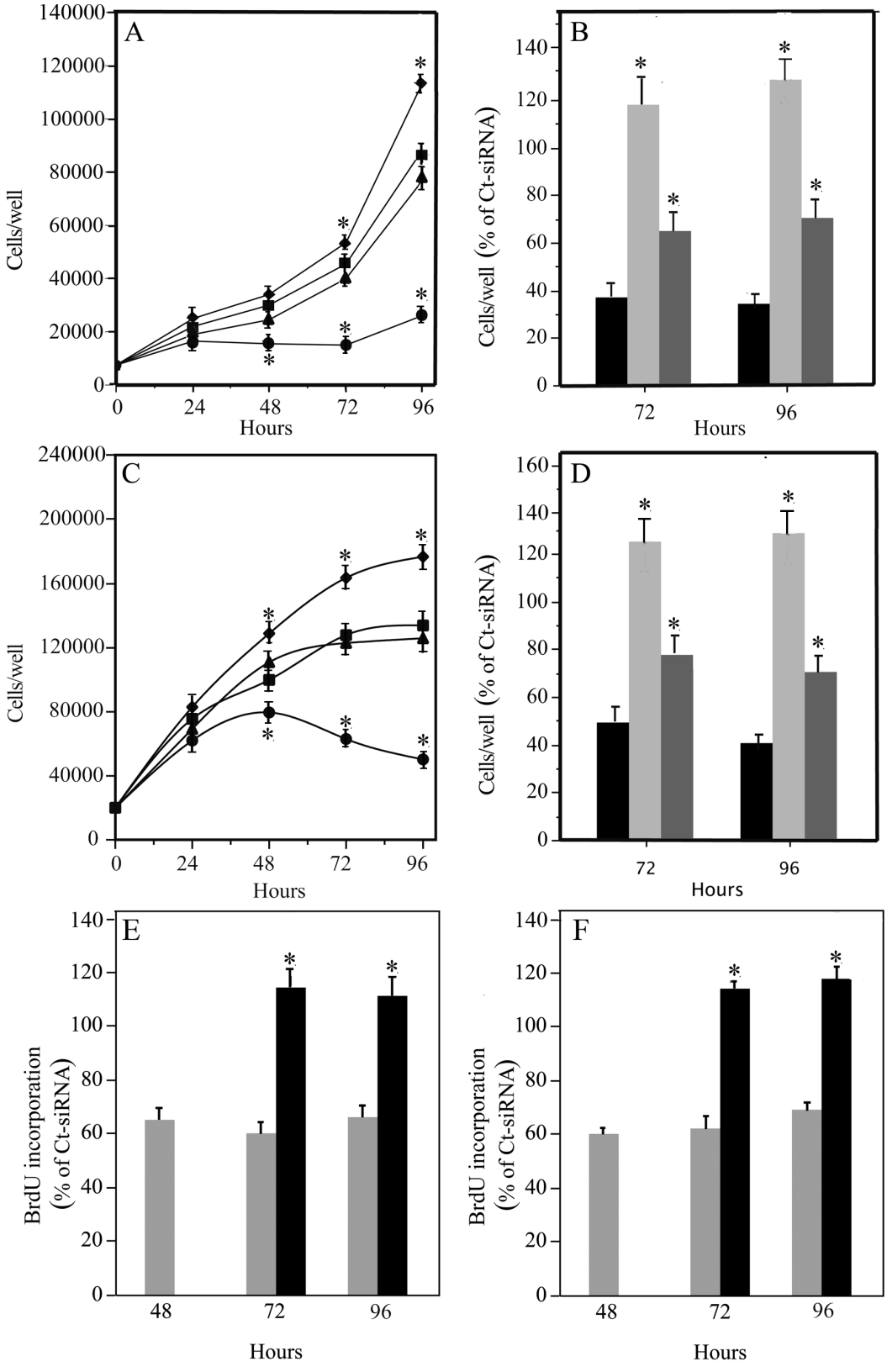

Fig. 6. Time course of the growth effects of HuR siRNA and exogenously added PTHrP (1-34) on DV90 **(A)** and HCC44 **(C)** lung adenocarcinoma cell lines. Cells were seeded in 24-well culture plates at a density of 5 x 10^4 cells/well and, after plating, cells were transfected with HuR specific siRNA (circles) or control siRNA (triangles) or treated with siRNA diluent alone (squares) or with 100 nM PTHrP (1-34) (diamonds). Cells were harvested at the indicated time and their number assessed by quadruplicate hemocytometer counts. Results represent mean + SE of triplicate experiments. *p<0.001 relative to siRNA diluent alone. Reversibility by PTHrP (1-34) of the antiproliferative effect of HuR specific siRNA transfection in DV90 **(B)** and HCC44 **(D)** cells. Cells transfected with HuR specific siRNA (black bars); cells transfected with HuR specific siRNA and treated with 100 nM PTHrP (1-34) at 48 hr of culture (dark gray bars); cells cultured in the presence of siRNA diluent and treated with 100 nM PTHrP (1-34) at 48 hr of culture (light gray bars). Cells were harvested at the indicated time and their number assessed by quadruplicate hemocytometer counts. Results are expressed as percentage of cells tranfected with control siRNA and represent mean ± SE of triplicate experiments. *p<0.001 relative to HuR specific siRNA. Reversibility by PTHrP (1-34) of the inhibitory effect of HuR specific siRNA transfection on BrdU incorporation in HCC44 **(E)** and DV90 **(F)** cell lines. Cells transfected with HuR specific siRNA (light gray bars); cells transfected with HuR specific siRNA and treated with 100 nM PTHrP (1-34) at 48 hr of culture (black bars). Results are expressed as percentage of OD of cells tranfected with control siRNA and represent mean + SE of triplicate experiments. *p<0.001 relative to HuR specific siRNA.

decreased in DV90 and HCC44 cells up to 60% at 48 and 72 hrs, respectively (Fig. 5A,B).

Actinomycin D assay revealed that in both cell lines HuR knockdown significantly reduced the PTHrP mRNA stability, as estimated by the reduction of PTHrP mRNA half-life (Fig. 5C,D).

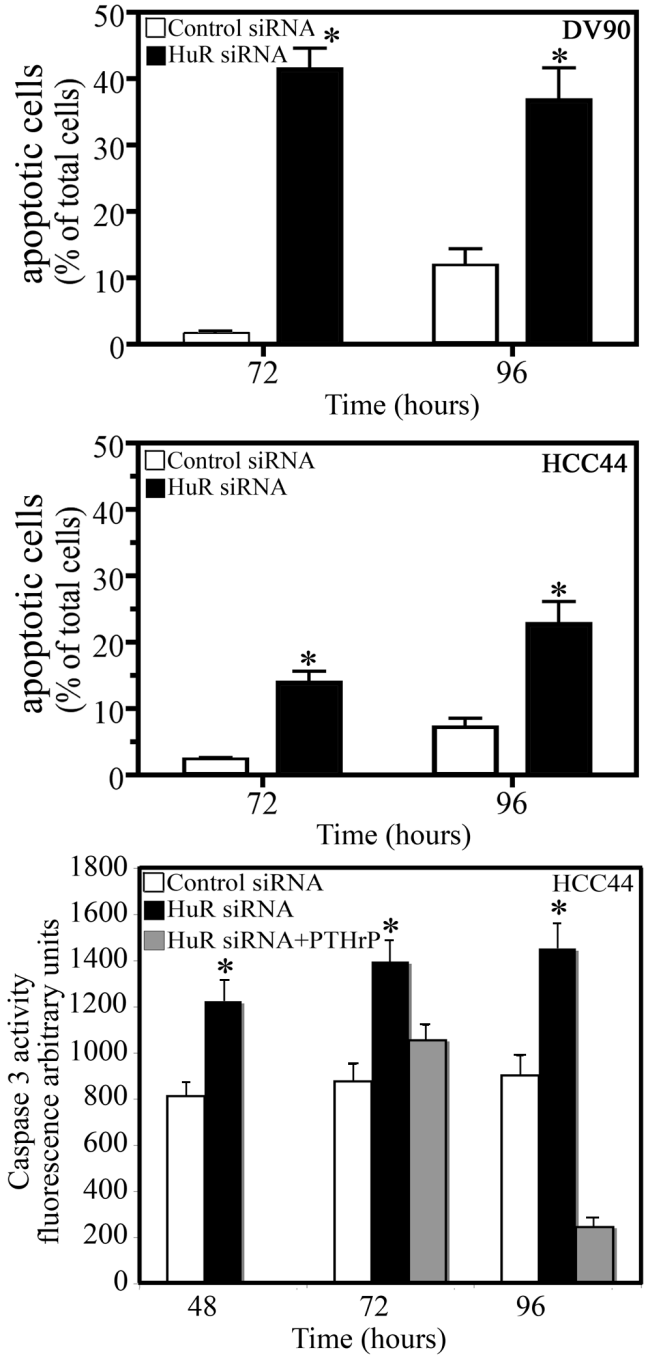

Fig. 7. HuR knockdown increases the percentage of apoptosic DV90 and HCC44 lung adenocarcinoma cells as revealed by acridine orange-ethidium bromide method. HuR knockdown increases Caspase 3 activity in HCC44 cells while the exogenous addition of PTHrP 1-34 reverts the effect of HuR knockdown in a time-dependent fashion. Results are expressed as mean ± SE of triplicate experiments. *p< 0.01, HuR siRNA versus control siRNA and HuR siRNA+PTHrP.

HuR knockdown decreases proliferation and increases apoptosis of lung adenocarcinoma cells

HuR knockdown induced a time-dependent decrease of DV90 (Fig. 6A) and HCC44 (Fig. 6C) cell number, while no significant effects were produced by transfection with control siRNA. The addition of 100 nM PTHrP 1-34 to the culture medium enhanced the cell number, particularly at 72 and 96 hrs of culture. The growth inhibitory effect of HuR knockdown in DV90 (Fig. 6B) and HCC44 (Fig. 6D) cells was partially prevented by the addition of 100 nM PTHrP 1-34 after 48 hours of culture. This effect of exogenously added PTHrP was confirmed by BrdU incorporation studies (Fig. 6E,F).

HuR knockdown induced a significant increase in the percentage of HCC44 and DV90 apoptotic cells after 72 and 96 hours of culture, as revealed by acridine orange-ethidium bromide staining (Fig. 7). Moreover, caspase 3 activity significantly increased after 48, 72 and 96 hours of culture in HuR siRNA transfected cells. After 48 hours of culture, the addition of 100 nM PTHrP 1-34 to cells transfected with HuR siRNA reduced, in a time-dependent manner, the effect of HuR knockdown on caspase 3 activity in HCC44 cells (Fig. 7).

Discussion

Previous studies found PTHrP to be expressed in the majority of NSCLC (Burton and Knight, 1992; Hidaka et al., 1998; Hastings et al., 2001) and we found that expression of N-terminal PTHrP (1-34) behaved as a poor prognostic marker in early stage lung adenocarcinoma (Monego et al., 2010). However, the literature reports are contradictory regarding the role of PTHrP in the biology of lung cancer cells (Burtis et al., 1990; Hiraki et al., 2002; Hastings et al., 2003, 2006; Sellers et al., 2004; Lorch et al., 2011; Montgrain et al., 2011). These apparent discrepancies could be due to the different effects, depending on cell types and paracrine-autocrine and/or intracrine signaling, exerted by NH_2-terminal (PTHrP 1-34 or 1-36), midmolecule (PTHrP 38-94 or 38-102), and COOH-terminal (PTHrP 109-141) regions of PTHrP peptides (Luparello et al., 1995, 2001; Massfelder et al., 1997; Hastings et al., 2002; Tovar Sepulveda and Falzon, 2002; Tovar Sepulveda et al., 2002).

Despite the long recognized role of HuR as a regulator of the expression of proliferative genes, its potential involvement in cancer has not been directly investigated. Studies examining HuR expression in lung tumors revealed that the abundance of HuR protein was significantly greater in malignant tumors than in benign tumors (Blaxall et al., 2000; Wang et al., 2011; Lauriola et al., 2012).

In the present study we report, for the first time, that the expression of cytoplasmic but not nuclear HuR are positively correlated with N-terminal PTHrP (1-34) in human lung adenocarcinomas and that HuR is involved in the regulation of total PTHrP mRNA content and stability in adenocarcinoma cell lines. These findings are consistent with the observations that HuR is predominantly localized in the nucleus of most unstimulated cells, but upon cell stimulation, it can translocate to the cytoplasm where it binds target mRNAs and prevents their decay (Abdelmohsen and Gorospe, 2010).

Our findings in lung adenocarcinoma cell lines are consistent with the observations reported by Danilin et al. that in human renal cell carcinoma HuR knockdown decreased the stability of the 141-PTHrP mRNA isoform, as well as total expression levels of VEGF and TGF-ß mRNAs (Danilin et al., 2009). In the present study, we used primers for PTHrP mRNA common region as all three PTHrP mRNA isoforms were detected in primary lung cancer (Sellers et al., 2004). Consequently, we did not study the effect of HuR silencing on the stability of individual PTHrP mRNA isoform.

The PTHrP-HuR status of the adenocarcinomas behaved as an independent prognostic marker relative to both metastasis-free and overall survival of the patients. In fact, at 5-year follow-up, the estimated proportions of metastasis-free and overall surviving patients were lowest for patients with lung adenocarcinoma expressing high levels of both cytoplasmic HuR and PTHrP. This finding is consistent with our observation on the HuR capacity to increase the levels of both cellular and secreted PTHrP (1-34) in human lung adenocarcinoma cell lines. The amount of secreted PTHrP (1-34) may be important in the control of PTHrP signaling through PTH1R in early stage lung adenocarcinoma progression (Monego et al., 2010) and in the onset of humoral hypercalcemia of malignancy (Burtis et al., 1990; Lorch et al., 2011).

HuR is increasingly recognized to regulate the expression of many proteins that enhance proliferation, inhibit apoptosis, increase angiogenesis, reduce immune recognition, and facilitate invasion and metastasis (Abdelmohsen and Gorospe, 2010).

Our findings, in the adenocarcinoma cell lines, showed that exogenous-added PTHrP (1-34) stimulated proliferative activity and partially reverted the anti-proliferative and pro-apoptotic effects of HuR knockdown. Considering the capacity of HuR to regulate PTHrP mRNA content and stability, these findings strongly suggest that the effects of HuR silencing are, at least in part, a consequence of the reduction in PTHrP (1-34) level. In this respect, our results are consistent with those by Hastings and colleagues who recently observed that selected lead-thiourea compounds, although through a different mechanism, decreased both cell content of PTHrP (1-34) protein and the rate of cell proliferation in BEN and H727 lung cancer cell lines. Interestingly, these compounds did not exert anti-proliferative effects in lines that showed no inhibition of PTHrP (1-34) protein (Hastings et al., 2010).

Molecular aspects of lung cancer, and particularly adenocarcinoma, have been deeply investigated, providing a rationale for molecular targeted therapy. The present results add PTHrP to the list of numerous growth, motility and angiogenetic factors, including VEGF, EGF and TGF-ß, all shown to be important in lung cancer tumorigenesis, in which HuR plays a crucial role in mRNA stabilization and/or translation.

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