HuR translocation to the cytoplasm of cancer cells in actin-independent manner

Umma Habiba[1], Takeshi Kuroshima[3], AyaYanagawa-Matsuda[1], Tetsuya Kitamura[1], AFMA Chowdhury[2], Jumond P. Jehung[2], Elora Hossain[3], Hidehiko Sano[2], Yoshimasa Kitagawa[4], Masanobu Shindoh[1], Fumihiro Higashino[1,4]

[1] Department of Oral Pathology and Biology, Hokkaido University Faculty of Dental Medicine and Graduate School of Dental Medicine, Sapporo, Japan.

[2] Department of Restorative Dentistry, Hokkaido University Faculty of Dental Medicine and Graduate School of Dental Medicine, Sapporo, Japan

[3] Department of Oral Diagnosis and Medicine, Hokkaido University Faculty of Dental Medicine and Graduate School of Dental Medicine,, Sapporo, Japan.

[4] Department of Molecular Oncology, Hokkaido University Faculty of Dental Medicine and Graduate School of Biomedical Science and Engineering, Sapporo, Japan.

ABSTRACT

Human antigen R (HuR) is a RNA-binding protein, which binds to the AU-rich element (ARE) in the 3′-untranslated region (3′ UTR) of certain mRNA and is involved in the export and stabilization of ARE-mRNA. HuR constitutively relocates to the cytoplasm in many cancer cells, however the mechanism of intracellular HuR trafficking is poorly understood. To address this question, we examined the functional role of the cytoskeleton in HuR relocalization.

We tested the effect of actin depolymerizing macrolide latrunculin A or myosin II ATPase activity inhibitor blebbistatin for HuR relocalization induced by the vasoactive hormone Angiotensin II in cancer and control normal cells. Western blot and confocal imaging data revealed that both inhibitors attenuated the cytoplasmic HuR in normal cells but no such alteration was observed in cancer cells. Concomitant with changes in intracellular HuR localization, both inhibitors markedly decreased the accumulation and half-lives of HuR target ARE-mRNAs in normal cells, whereas no change was observed in cancer cells. Furthermore, co-immunoprecipitation experiments with HuR proteins revealed clear physical interaction with ß-actin only in normal cells.

The current study is the first to verify that cancer cells can implicate a microfilament independent HuR transport. We hypothesized that when cytoskeleton structure is impaired, cancer cells can acquire an alternative HuR trafficking strategy.

Key words:

HuR, human antigen R; ARE, AU-rich element; 3′-untranslated region (3′ UTR); Latrunculin A (Lat.A); blebbistatin (blebbistat); Human Angiotensin II (Ang.II); COX-2,

cyclooxygenase-2.

INTRODUCTION

Human antigen R (HuR) is a ubiquitously expressed member of the embryonic lethal abnormal vision (ELAV) family of RNA binding proteins, most abundantly localized in the nucleus. It shuttles between the nucleus and the cytoplasm in the presence of external stimuli such as extracellular stress (1,2). HuR distribution between the nucleus and the cytoplasm is supported by its associated proteins, transportin 1 and 2 (Tran 1and 2) (3,4), pp32, APRIL, exportin and chromosome maintenance region 1 (CRM1) (5,6). However, the mechanisms underlying the cytoplasmic trafficking of HuR and its consignment mRNA are far less understood.

HuR has been shown to bind to AU-rich elements (ARE) in many mRNAs and play a role in the stabilization of these mRNAs. The cytoplasmic translocation of HuR is fundamental for the HuR mediated stabilization of ARE-mRNAs. AREs are RNA elements that enhance rapid decay of mRNA. They usually exist in the 3′-untranslated region (3′ UTR) of certain mRNAs encoding early response genes or growth-related genes, such as proto-oncogenes and growth factors. The fate of ARE-mRNA is controlled by several RNA-binding proteins. For instance, AUF1, tristetraprolin (TTP) accelerate the degradation of ARE-mRNA; whereas HuR binds to AREs to protect ARE-mRNA from rapid degradation (7,8). Under normal conditions, cytoplasmic translocation of HuR is transient; however, HuR constitutively accumulates in the cytoplasm of many cancer cells.

Cytoplasmic HuR is thought to be involved in malignant transformation of cancer cells and may contribute to the malignant phenotype of cancer (9). Increased cytoplasmic

HuR expression has been detected in cancer (10-18) and preneoplastic lesions (19,20). A deregulated HuR pathway has been suggested to have implications in cancer biology, by promoting the abnormal expression of several proteins (5). Therefore, HuR may be considered as a prognostic as well as therapeutic target for anticancer therapies. Moreover, strategies that prevent the pathological abundance of cytoplasmic HuR could be accounted for interfering the aberrant gene expression by HuR.

Previous studies have reported that intracellular mRNA transport is mediated by microfilament together with their corresponding motor protein (21-28). Moreover, Doller et al. (29,30) demonstrated that the attenuation of cytoplasmic HuR by microfilament inhibitors in Human primary mesangial cells (HMC) and Hepatocellular carcinoma (HEPG2). However, evidence regarding modulatory effect of cytoplasmic HuR in other carcinoma as well as existence of other possible mechanisms of HuR translocation to the cytoplasm of cancer cells was lacking.

Therefore, the current study aimed to analyze human cervical (HeLa) and oral squamous cell (HSC3) carcinoma to unveil whether their intracellular trafficking mechanisms are same or different in other tumors. In an aim to decipher the underlying mechanism, we used normal cells (human foreskin fibroblast; BJ and human gingival fibroblast; HGF1) as control. To analyze the functional role of cytoskeletal element in the nucleo-cytoplasmic HuR redistribution and HuR targeted mRNA stabilization in cancer and normal cells, we directly treated the cells with the microfilaments inhibitors: Latrunculin A (Lat.A) and blebbistatin (blebbistat) after stimulation with vasoactive hormone Angiotensin II (Ang II). The involvement of Ang II in HuR reshuttling was demonstrated in a previous study (29).

A previous report (30) demonstrated an actin-myosin dependent transport of HuR in hepatocellular carcinoma (HEPG2). Based on that we assumed that intracellular HuR trafficking in cervical (HeLa) and oral squamous cell carcinoma (HSC3) will also show a similar microfilament dependent mechanism. Interestingly, the results of the current study revealed a novel microfilament-independent intracellular HuR trafficking in cervical (HeLa) and oral squamous cell carcinoma (HSC3). Based on these observations we also speculated that intracellular transport mechanism of HuR and its consignment mRNA could differ in different types of cancer.

MATERIALS AND METHODS

Cell culture

HeLa (human cervical cancer), HSC3 (human oral squamous cell carcinoma), HEPG2 (human hepato cellular carcinoma), BJ (human foreskin fibroblast) and HGF1 (human gingival fibroblast) cells were obtained from the American Type Culture Collection and were cultured at 37˚C and 5% CO2 atmosphere in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% fetal bovine serum (FBS) with antibiotics.

Reagents and antibodies

Latrunculin A was obtained from (Cayman chemical, USA). Human Angiotensin II, blebbistatin, Phalloidin-rhodamine, Protein G sepharose, Protease inhibitor Cocktail (p8340) and Actinomycin D (from Streptomyces species) were purchased from Sigma-Aldrich (USA). Antibodies against HuR (mouse monoclonal IgG), Lamin B (goat polyclonal IgG), Actin (HRP goat polyclonal IgG) were purchased from Santa Cruz Biotechnology. Antibodies against β-tubulin (AA2) were purchased from Sigma-Aldrich (St louis, MO, USA) and Myosin II were purchased from cell signaling.

Peroxidase conjugated goat anti mouse IgG (HRP conjugated) were purchased from Jackson Immuno Research Laboratory, West Grove, PA, USA. The secondary Alexa-Fluoro 488 (A 11017 goat anti mouse IgG) and Alexa-Fluoro 568 (A 11036 goat ant rabbit IgG) were purchased from Invitrogen.

Western blot analysis

Whole cell lysates were prepared using radio immunoprecipitation assay (RIPA) buffer (150 mM NaCl; 25 mM Tris-HCl, pH 7.6; 1% Nonidet P-40; 1% sodium deoxycholate; 0.1% SDS) containing protease inhibitors. Twenty micrograms of each sample were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Total protein content was confirmed by β-actin staining. In order to separate cells into cytoplasmic and nuclear fractions, cells were suspended in a fractionating buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1.5 mM MgCl2, 0.5% Nonidet P-40, protease inhibitor cocktail), followed by vigorous shaking for 5 min and centrifugation at 12,000 rpm for 30 secs. The supernatant was used as the cytoplasmic fraction. To estimate the accuracy of cell fractionation, cytoplasmic (β-tubulin) and nuclear (lamin B) proteins were detected using western blotting. Antibodies used in this study were specific to HuR, (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin, β-tubulin, Lamin B (Sigma-Aldrich, St louis, MO, USA). The secondary antibody was horse radish peroxidase-conjugated IgG (Jackson Immuno Research Laboratories, West Grove, PA, USA). Immunoreactive bands were visualized with chemiluminescence using the Supersignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, USA).

Fluorescence microscopy

Cells were grown on coverslips in 35-mmdishes at 60–80% confluence and were stimulated for 2 h with Angin II without or with microfilament inhibitor either latrunculin A (Lat.A;30 min) or blebbistatin (Blebbistat;4 hour). Immediately following treatment, cells were rinsed once with phosphate buffered saline (PBS), fixed for 20 min at RT with 4% paraformaldehyde in PBS, blocked and permeabilized in 2% BSA plus 0.1% Triton X-100 (PBS) at room temperature (RT). Subsequently, the fixed cells were incubated with anti-HuR and myosin II primary antibody for overnight at 4ºC followed by Alexa Fluor 488 and Alexa Fluor 568 secondary antibodies for 1 hour at room temperature respectively. F-actin was visualized with rhodamine-conjugated phalloidin. Cell nuclei were counterstained with DAPI before mounted on slides by using Mountant permafluor (Thermo scientific, FM 111212A). Cells were observed using an IX71 inverted microscope (Olympus). Image acquisition was performed with the Olympus Fluoview Software (FV10-ASW 4.2 viewer).

RNA isolation and quantitative real time-PCR (RT-PCR)

Total RNA was extracted using the TRI reagent (Sigma-Aldrich, St louis, MO, USA) and the RNA was subjected to reverse transcription using Rever Tra Ace qPCR RT master mix with genomic DNA remover (Toyobo, Osaka, Japan). Quantitative real-time RT-PCR was performed in the DNA Engine Opticon2 (MJ Research, Watertown, MA, USA), and cDNA was amplified using the following primers:

GAPDH, 5′-ATCCTGGGCTACACTGAGCA-3′, 5′-TGCTGTAGCCAAATTCGTTG-3′,

COX-2,

5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′,5′-AGATCATCTCTGCCTGAGTAT

CTT-3′ and Cyclin A,5′-ATTAGTTTACCTGGACCCAG-3′,

5′-CACAAAGCTACTTCTGCTCT

-3′. GAPDH was used for normalization.

The C(T) values of mRNA levels were normalized to the C(T) values of GAPDH mRNA within the same sample. To evaluate the half-life of total COX-2 mRNA, cells were treated with 5μg/ml actinomycin D for 30, 60, 120 min. Total cellular RNA of each cell was used for quantitative real time RT-PCR.

Protein interaction Assay

To search proteins that interact with HuR in cancer and normal cells, Hela, HSC3 and BJ were lysed in TENT lysis buffer [25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS]. For immunoprecipitation, protein G sepharose beads was equilibrated with lysis buffer. After preclearing the lysates, proteins were immunoprecipitated with anti HuR and mouse normal IgG and incubated for 4 hours at 4ºC under gentle rotation. Afterwards,50ul beads were added with 500ug protein and incubated for 4 hours at 4ºC under rotary agitation. After centrifugation for 5 min at 3000g, the supernatant was discarded followed by washing for several times with lysis buffer. The Ag-Ab complex was eluded by boiling the samples in loading buffer for 5 min. Finally, equal volumes of proteins were subjected to SDS-PAGE and analyzed by immunoblotting.

Statistical analysis

Statistical analysis was performed by using one-way ANOVA. Post-hoc multiple comparisons were done by Tukey’s test at 5% level of significance. All statistical

analysis was done by using SPSS 22.0 for Windows (SPSS, Chicago, IL, USA). * indicates $p < 0.05$).

RESULTS

Effects of Actin- myosin cytoskeleton interfering drugs on cancer and normal fibroblast cells and actin cytoskeletal organization

Cancer and normal fibroblasts cells were grown to confluence and stimulated 2 hours with Angiotensin II followed by in the absence or presence of either Latrunculin A or blebbistatin for 30 minutes and 4 hours respectively. Sufficient concentration of each drugs was chosen to reduce cytoplasmic HuR accumulation without inducing any significant cytotoxic or apoptotic cell response according to Doller et al. (30); 0.1uM for latrunculin A and 5 uM for blebbistatin. Treatment with Angiotensin II and Angiotentin II+Blebbistatin resulted in cell-cell separation and a stellate appearance in some cells compared with control cells. Cells treated with Angiotensin II+Latrunculin A showed the greatest degree of change in cell morphology, with rounding up of cells, cell detachment and cell-cell separation **(Fig.1)**.

These treatments also induced alterations in staining patterns for F actin and myosin **(Fig.3)**. Actin and myosin stress fiber were observed throughout the control cells, whereas a significant decrease was observed in the treated cells. Angiotensin II, Angiotensin II+ Blebbistatin displayed some stress fiber at the peripheral region of the cell body but weak (Angiotensin II) to undetectable fibers (Angiotensin II + Blebbistatin) in the center of the cell body whereas cells treated with Angiotensin II+ Latrunculin A showed complete loss of stress fiber. In addition, the strong shrinkage was observed in many cells and nuclei after administration of Latrunculin A resulting from the disruption of F actin filament. Similar results were recorded from three

independent experiments.

Under these conditions, though the morphology of drug treated cells changed and detached from their surface, reculturing of the detached cells in the fresh medium led to recovery of normal cell shape over a period of 24 hours (data not shown).

Modulatory effect of nucleo-cytoplasmic HuR shuttling by microfilament inhibitors

To evaluate the possible effect of microfilament inhibitors on the cytoplasmic HuR level, we examined the subcellular localization of HuR by western blot analysis after the treatment of indicated period mentioned above. Interestingly, cytoplasmic HuR levels were differentially modulated in control normal cells and cancer cells. Both Latrunculin A and blebbistatin showed inhibitory effect on cytoplasmic HuR levels in normal cells **(Fig.2, A and B)** but none of these inhibitors showed any inhibitory effect on nucleocytoplasmic HuR shuttling **(Fig.2, C and D)** in cancer cells. However, irrespective of cell types used (both cancer and normal cells), the total and nuclear content of HuR remained unchanged **(Fig.2; middle panel and lower panel respectively)**. From these observation, it is tempting to speculate that both microfilament inhibitors largely interfere with the nucleo-cytoplasmic HuR distribution without affecting HuR expression.

To verify the results from western blot analysis, confocal microscopy was applied to visualize actin-myosin cytoskeleton changes by microfilament inhibitors and nucleo-cytoplasmic redistribution of HuR. Confocal microscopy shows staining of HuR

exclusively in the nuclei of untreated cells and cytoplasmic extension of HuR staining was observed in cells that were treated with Ang II for 2 h **(Fig.3)**. In contrast to the result of western blot analysis, the nuclear HuR signal was diminished to some extent. These phenomenon was observed independent of cell types used in this experiment. Short incubation of cells with Latrunculin A that causes disruption of F-actin filament results in impairment of Ang II induced increase in cytoplasmic HuR in control fibroblast cells (BJ, HGF1) **(Fig.3; A, B)** but such impairment was not observed in cancer cells (HeLa, HSC3) **(Fig.3; C, D)**. Similar to Latrunculin A, a reduction in the cytoplasmic HuR abundance by Ang II was achieved by blebbistatin in control fibroblast cells as expected but showed no effect on cancer cells **(Fig.3; A-D)**. These findings indicate that HuR relocates to the cytoplasm of cancer cells, such as HeLa and HSC3 cells, in actin fiber-independent export system.

Down regulation of HuR triggered mRNA expression by microfilament inhibitors

Then the modulatory effect of cytoskeletal inhibitors on HuR triggered mRNA expression and stabilization were performed with HeLa and BJ cells. To address whether the microfilament inhibitors would functionally relevant with a reduced mRNA expression of the HuR target mRNAs, COX-2 and cyclin A, we administered Ang II followed by in the absence or presence of microfilament inhibitor Latrunculin A and blebbistatin. Our data showed that Ang II induced increase level of COX-2 and Cyclin A mRNA **(Fig.4; A and B)**. The levels of the tested mRNA were significantly reduced if cells were treated with microfilament inhibitors for the indicated period in control human fibroblast cells. In contrast, none of the inhibitors affected the levels of these mRNAs in cancer cells. These results indicate that in HeLa cells, cytoskeletal inhibitors

don’t have any inhibitory effects on HuR-triggered mRNA expression.

HuR target mRNA coding for COX-2 stabilization in microfilament treated cells

Upon confirming the expression changes of the HuR target mRNA, we examined the half-lives of COX-2 mRNA to evaluate its stabilization. To elucidate the half-lives of the mRNA, transcription was stopped by addition of actinomycin D before cells were further treated with Angin II or together with Latrunculin A and blebbistatin. The time dependent decay of mRNA by quantitative real time RT-PCR revealed that in control human fibroblast cells (BJ), the half-life of COX-2 mRNA was reduced if cells were treated with microfilament inhibitors **(Fig.5; A)**. In contrast, in cervical carcinoma (HeLa) the COX-2 mRNA was not affected by any of the treated inhibitors **(Fig.5; B)**. These data indicate that concomitant with their suppressive effect on HuR target mRNA, both microfilaments especially blebbistatin showed strong suppressive effect on COX-2 mRNA stability in control fibroblast cells. Whereas, in a clear contrast, in HeLa cells, the half-life of COX-2 mRNA was not affected by either of the mentioned inhibitors. These findings unveiled a cytoskeletal independent HuR targeted mRNA expression and stabilization in HeLa cell.

Constitutive interaction of HuR protein with Beta actin in normal and cancer cells

To search whether HuR would constitutively bind to actin in cancer and normal cells, we performed immunoprecipitation assay with cell lysates from cancer (HeLa, HSC3) and normal cell (BJ). As postulated, normal (BJ) cells showed a clear physical interaction of HuR with actin. Importantly this association was not observed in cell lysates which were derived from cancer cells (HeLa and HSC3). Collectively, these data suggest that HuR exported to the cytoplasm of cancer cells without actin fiber pathway

(Fig.6).

DISCUSSION

The results of this study are different from previous results that intracellular transport of HuR follows a microfilament-dependent mechanism in all types of cancer cells (). Therefore, we propose a novel microfilament independent HuR transport in oral and cervical cancer when cytoskeletal structure is impaired.

We employed cytoskeleton inhibitors to test the potential role of actin-myosin based cytoskeleton in the constitutive HuR shuttling, HuR dependent mRNA expression and stabilization in cancer and normal cell. Latrunculin A is the marine derived macrolide which reversibly binds the cytoskeleton actin monomers, forming complex with G-actin and disrupt its polymerization (31). Some studies reported that Latrunculin A inhibits microfilament mediated cell process without affecting the organization of the microtubule dependent cytoskeleton and showed potent antitumorigenic properties (32,33). Blebbistatin is a highly selective inhibitor of non-muscle myosin II ATPase, that interfere the myosin II mediated cell motility, migration and adhesion. Additionally, blebbistatin can interfere with the cellular invasiveness of pancreatic adenocarcinoma (34,35).

Although the association of intracellular mRNA transport with microfilament and their associated motor proteins was reported previously (21-28), the actual nature and significance of the interaction is not yet understood in detail. In previous studies it was shown that both actin and myosin inhibitors exert as potent anti-tumorigenic properties including inhibition of cell invasiveness as demonstrated for pancreatic adenocarcinoma cells (34). Concomitantly, both compounds strongly reduced the high abundance of cytoplasmic HuR in human hepatocellular carcinoma (HEPG2) (30). Contrary to these

findings, our study unveiled the high constitutive cytoplasmic HuR abundance appeared insensitive to both actin and myosin inhibitors in human cervical cancer (HeLa) and human oral squamous cell carcinoma (HSC3) **(Fig.2 and Fig. 3)** suggesting cytoskeleton independent HuR shuttling in HeLa and HSC3 carcinoma. Our immunocytochemistry results revealed that Angiotensin II treatment increases cytoplasmic HuR abundance in both normal and cancer cells with diminished nuclear HuR signal to some extent **(Fig 3)**. After treatment with microfilament inhibitors, normal cells (BJ, HGF1) showed a decrease in cytoplasmic HuR that is not reconcile with an increased HuR import back to the nucleus **(Fig 3; A and B)**. This data suggests that microfilament disruption impair nuclear HuR export which furthermore implies a cytoskeleton dependent communication between the nucleus and the cytoplasm. It should be mentioned that angiotensin II induced cytoplasmic HuR abundance and reduced nuclear HuR level could not be visualized clearly in western blot analysis because the relative changes of the HuR protein in nucleus and cytoplasmic compartment is too low to be detected.

Although a complete understanding of the underlying mechanisms awaits further experiments, it is unlikely that a single pathway or mechanism exists for regulating intracellular HuR trafficking in all tumors. Eberhardt W et al (36) reported the possibility that the molecular transport system utilized by HuR and its consignment mRNA does not only rely on the cancer cell type but in addition may depend on the stimulus or signaling cascade which triggers in HuR shuttling. This speculation was further corroborated by the fact that in agreement with the previous report (30) both microfilament inhibitors showed attenuation of cytoplasmic HuR abundance in HEPG2

cells. **(Supplementary Fig.1; A and B)**. Moreover, concordant with the previous study (29) which reported microfilament dependent HuR transport in Human primary mesangial cells (HMC), we also observed the inhibitory effect on cytoplasmic HuR abundance in control human fibroblast cells (BJ, HGF1) **(Fig; 2 and Fig. 3)**.

Next we focused our attention on HuR because this mRNA binding protein plays a role in the regulation of mRNA stability. Administration of microfilament inhibitors Latrunculin A and blebbistatin significantly reduced the expression of HuR target mRNA COX-2 and Cyclin A, which contain HuR binding sites (ARE) within their 3′ untranslated region (37,38), with no such impairment was observed in cancer cells (HeLa and HSC3) **(Fig 4)**. Moreover, the half life of the COX -2 mRNA was not altered in cancer cells by any of the inhibitors **(Fig.5)**. In line with these observations coimmunoprecipitation experiments confirmed a clear physical interaction of HuR with actin in normal cell (BJ)**(Fig.6)**. Importantly this association was not observed in cell lysates which were derived from cancer cells (HeLa and HSC3). Collectively, these data suggest that HuR exported to the cytoplasm of cancer cells without actin fiber pathway.

To the best of our knowledge, this is the first report to demonstrate that nucleo-cytoplasmic shuttling of HuR can occur without actin in oral and cervical cancer. Our observations point to the possible differential molecular transport system of HuR in individual cancer types. We hypothesized that molecular transport system of HuR is different in individual cancer and cancer cells acquire an alternative HuR trafficking strategy when actin-myosin cytoskeleton is disrupted. However further studies are needed to elucidate specific cancer types. In addition, discovery of cancer types that

shows microfilament involvement in HuR trafficking is needed for an evaluation of therapeutic potential of microfilaments inhibitors.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

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Figure Legends

Fig.1 Expression of morphological changes after application of microfilament inhibitor. BJ (A) and HeLa (B) Cells were treated with Ang II (2 h) followed by in the absence or presence of either latrunculin A (Lat.A) or blebbistatin (Blebbistat), which were treated for 30 min (Lat. A) or 4 h (Blebbistat) respectively. Treatment with Angiotensin II and Angiotentin II+Blebbistatin resulted in cell-cell separation and a stellate appearance in some cells compared with control cells. Cells treated with Angiotensin II+Latrunculin A showed the greatest degree of change in cell morphology

characterized by remarkable shrinkage of actin stress fibers, rounding up of cells, cell detachment and cell-cell separation (marked with arrow). (A, B, magnification x4; inset magnification x10).

Fig.2 Cytoplasmic HuR abundance in different human fibroblast cells is modulated by microfilament inhibitors but cancer cells show microfilament independent exportation. Human fibroblasts (A) BJ (human foreskin fibroblast; left panel) (B) HGF1 (human gingival fibroblast; left panel), and cancer cells (C) HeLa (human cervical cancer; right panel) (D) HSC3 (human oral squamous cell carcinoma; right panel) were stimulated for 2 h with Angin II without or with either latrunculin A (Lat.A; 30 min) or blebbistatin (Blebbistat 4 hour) as indicated. Equal amount (20µg) of total protein and cytoplasmic and nuclear fractions were subjected to SDS-PAGE and immunoblotted with anti-HuR, anti-ß actin, anti-ß tubulin and anti-lamin B respectively. The level of HuR protein was monitored in total cell lysates (TP) and cytoplasmic (CP) and nuclear extracts (NP). The data shown are from a single experiment representative for three independent experiment giving similar results.

Fig.3 Actin-myosin cytoskeleton disruption impairs nucleo-cytoplasmic HuR shuttling in control fibroblast cells but in cancer cells this shuttling is independent of the actin-myosin cytoskeleton disruption. Confocal microscopy was performed to visualize changes in the actin–myosin cytoskeleton by microfilament inhibitors and nucleo-cytoplasmic redistribution of HuR. (A) BJ (human foreskin fibroblast) (B) HGF1 (human gingival fibroblast) (C) HeLa (human cervical cancer) (D) HSC3 (human oral squamous cell carcinoma) cells were stimulated for 2 h with Angin II without or with either latrunculin A (Lat.A;30 min) or blebbistatin (Blebbistat;4 hour) as indicated.

After fixation and permeabilization, cells were stained with an anti-HuR antibody and subsequently with the Alexa-Fluoro 488 coupled (green) secondary antibody. F-actin was visualized with rhodamine conjugated phalloidin (A- D upper panel). Cells were also treated as mentioned above and immunostained for myosin IIA (green), HuR (red) using either Alexa-Fluoro 488 secondary antibodies (A -D lower panel). In all cases, cell nuclei were visualized by staining with DAPI (40,60-DAPI). Bars,20μm. Data shown are from a single experiment representative of three repeats giving similar results.

Fig.4 Microfilament inhibitors down-regulated the expression of HuR target mRNA in normal cells but remain unaffected in cancer cells. (A) BJ and (B) HeLa cells were stimulated for 2 h with Angin II with or without Latrunculin A (Lat.A;30 min) or blebbistatin (Blebbistat;4 hour). The content of the HuR target mRNAs coding for COX-2, Cyclin A is assessed by quantitative real-time PCR experiments using GAPDH mRNA as a normalization control. Data are shown as the mean ± standard deviation of three independent experiments (one-way ANOVA and Tukey's post-hoc test; * indicates $p < 0.05$).

Fig.5 HuR target mRNAs coding for COX-2 stabilization in microfilament inhibitors treated cells. The stability of COX-2 mRNAs is reduced by microfilament inhibitors in normal cell (A) but remains unaffected in cancer cell (B). Transcription of BJ (human foreskin fibroblast; left panel) and HeLa cells (human cervical cancer; right panel) were blocked by the addition of actinomycin D (5 μg/ml) before cells are further treated with Angin II, or together with different microfilament as indicated. After the indicated time periods cells are harvested and extracted for total cellular RNA. mRNA

levels are quantified by quantitative real time RT-PCR experiments using GAPDH as a normalization control. Graphs depict the percentage of remaining COX-2 mRNA levels in relation to GAPDH mRNA and compared with the levels of normalized mRNA species measured immediately after the addition of actinomycin D and which are set as 100%. The half-lives of the mRNAs (min) are indicated as t½. The plot shows the mean of three independent experiments.

Fig.6 Protein interaction with HuR in cancer and normal cells. To search protein that interact with HuR in cancer (HeLa and HSC3) and normal cell (BJ), cells were lysed with TENT lysis buffer. Protein G sepharose beads was equilibrated with lysis buffer. After preclearing the lysates, proteins were immunoprecipitate with anti HuR and mouse normal IgG and incubate for 4 hours at 4ºC. 50ul beads were added with 500ug protein and incubate for 4 hours at 4ºC followed by centrifugation for 5 min at 3000g. Supernatant was discarded and the Ag-Ab complex was eluded by boiling the samples in loading buffer for 5 min. Finally, equal volumes of proteins were subjected to SDS-PAGE and analyzed by immunoblotting.

Supplementary Fig.1. Actin-myosin cytoskeleton disruption impairs nucleo-cytoplasmic HuR shuttling in HEPG2 (human hepato cellular carcinoma) cell. Confocal microscopy was performed to visualize changes in the actin-myosin cytoskeleton by microfilament inhibitors and nucleo-cytoplasmic redistribution of HuR. HEPG2 cell was stimulated for 2 h with Angin II without or with either Latrunculin A (Lat.A;30 min) or blebbistatin (Blebbistat;4 hour) as indicated. After fixation and permeabilization, cells were stained with an anti-HuR antibody and subsequently with

the Alexa-Fluoro 488 coupled (green) secondary antibody. F-actin was visualized with rhodamine conjugated phalloidin (Upper panel). Cells were also treated as mentioned above and immunostained for myosin IIA (green), HuR (red) using either Alexa-Fluoro 488 secondary antibodies (Lower panel). In all cases, cell nuclei were visualized by staining with DAPI (40,60-DAPI). Bars,20μm. Data shown are from a single experiment representative of three repeats giving similar results.

Fig 1

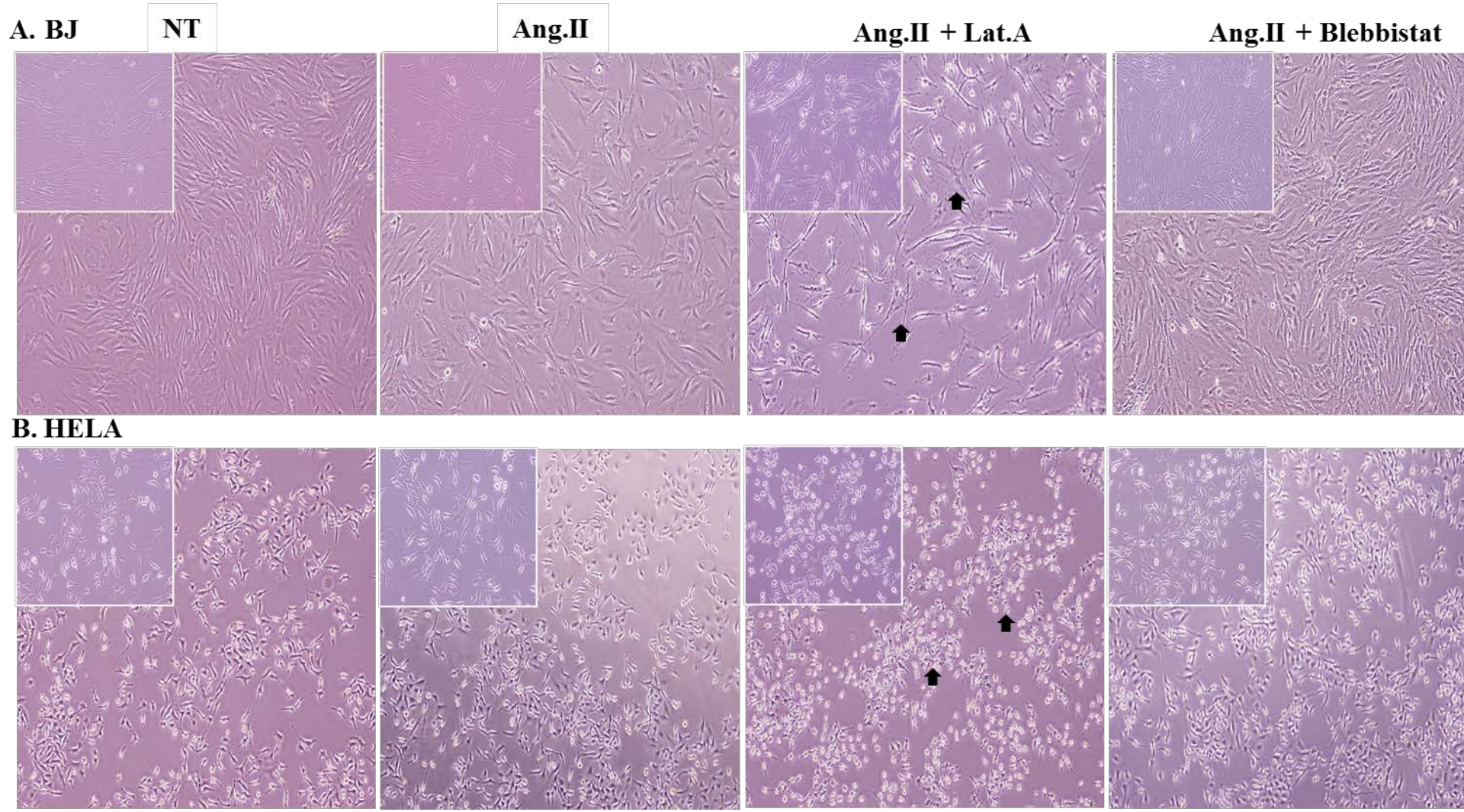


Fig 2

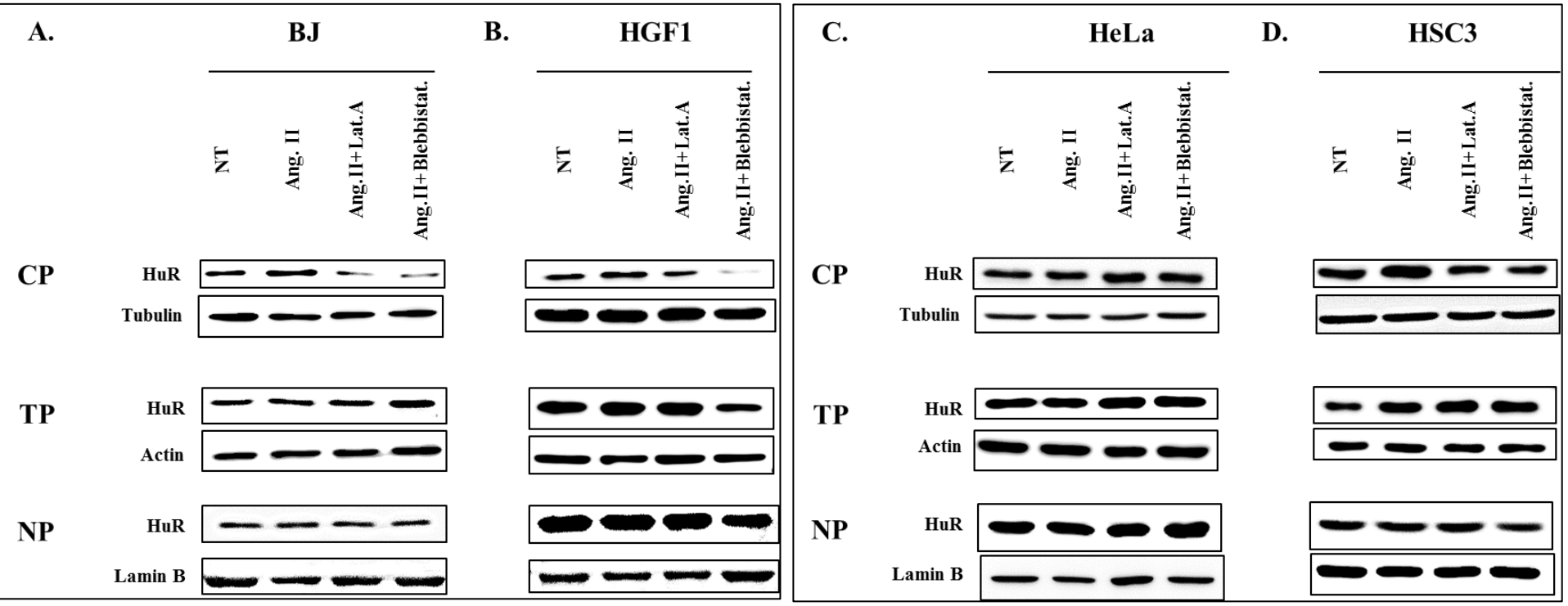

A.
BJ
B.
HGF1
C.
HeLa
D.
HSC3
NT
Ang. II
Ang.II+Lat.A
Ang.II+Blebbistat.
CP
TP
NP
HuR
Tubulin
Actin
Lamin B

Fig 3

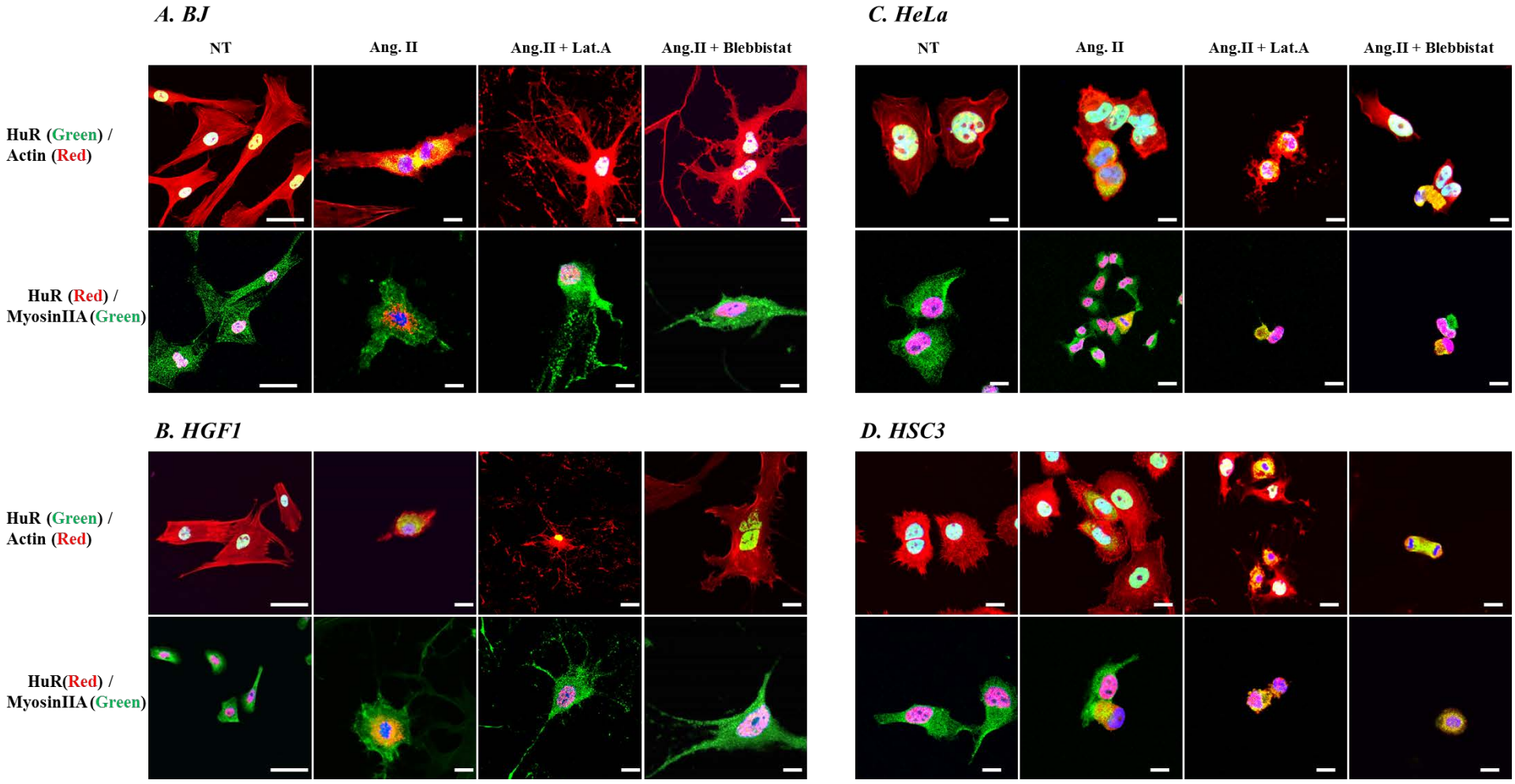

A. BJ
NT
Ang. II
Ang.II + Lat.A
Ang.II + Blebbistat
HuR (Green) / Actin (Red)
HuR (Red) / MyosinIIA (Green)
C. HeLa
NT
Ang. II
Ang.II + Lat.A
Ang.II + Blebbistat
B. HGF1
HuR (Green) / Actin (Red)
HuR(Red) / MyosinIIA (Green)
D. HSC3

Fig 4

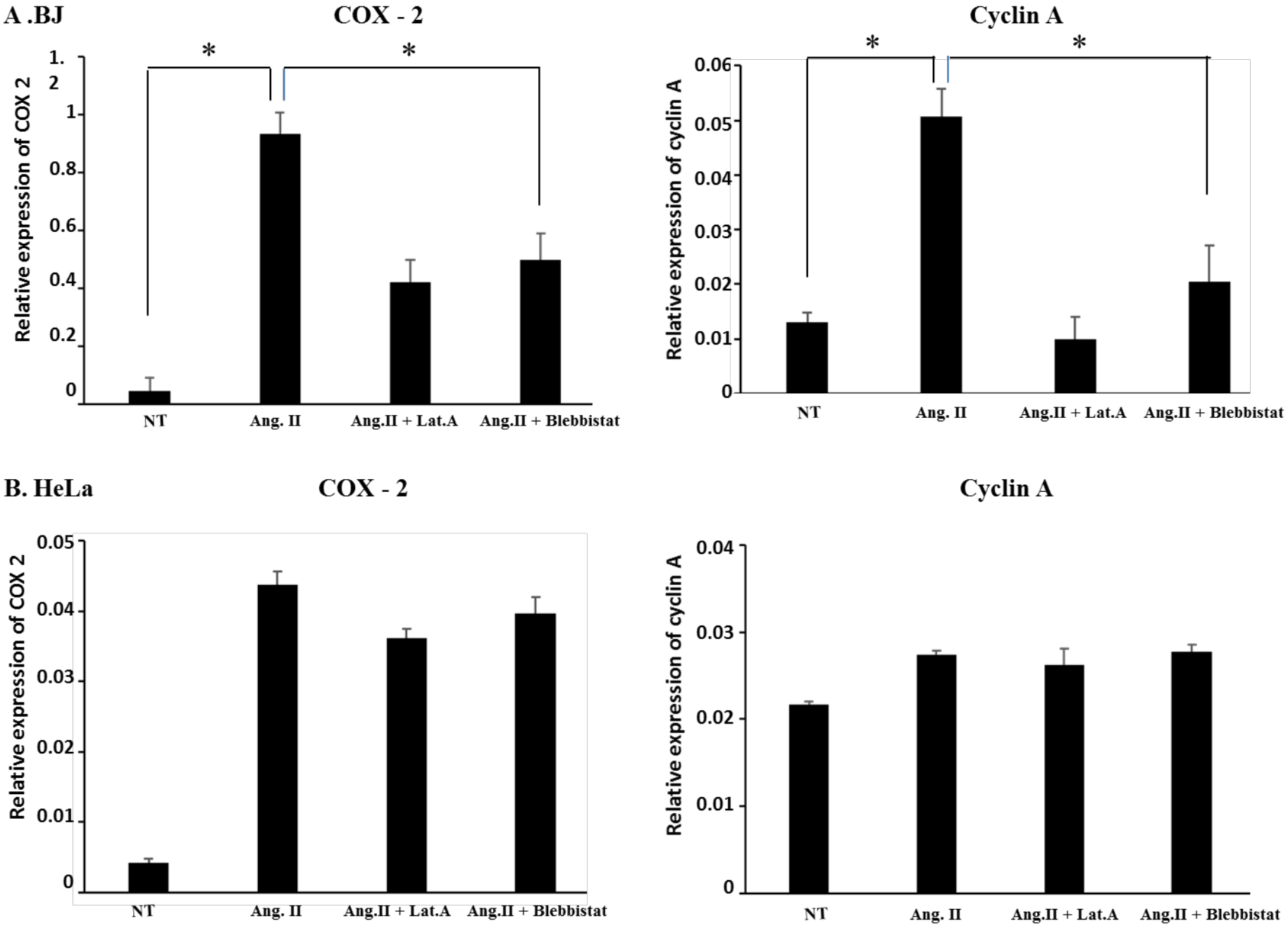
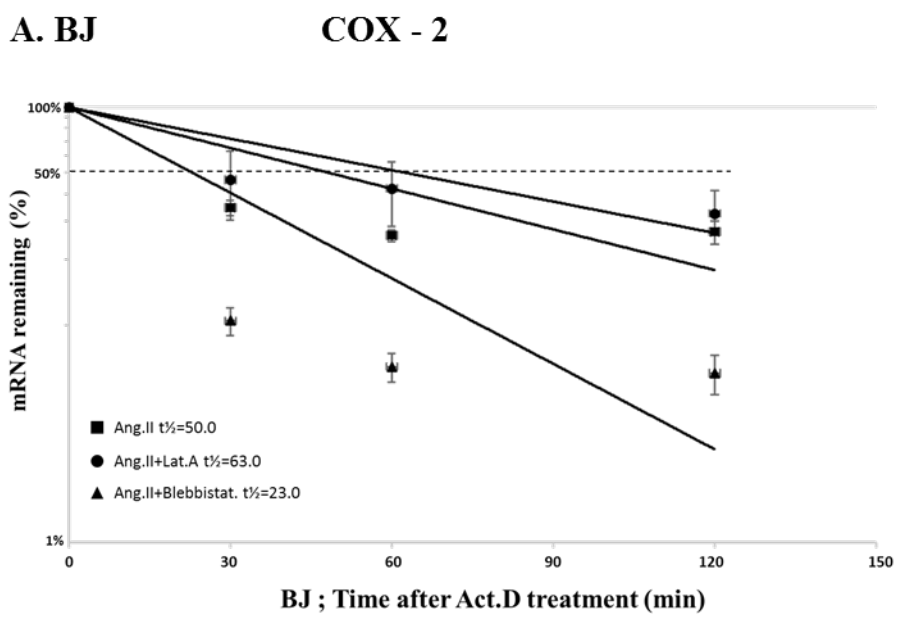

A .BJ
COX - 2
Relative expression of COX 2
1.2
1
0.8
0.6
0.4
0.2
0
*
*
NT
Ang. II
Ang.II + Lat.A
Ang.II + Blebbistat
Cyclin A
Relative expression of cyclin A
0.06
0.05
0.04
0.03
0.02
0.01
0
*
*
NT
Ang. II
Ang.II + Lat.A
Ang.II + Blebbistat
B. HeLa
COX - 2
Relative expression of COX 2
0.05
0.04
0.03
0.02
0.01
0
NT
Ang. II
Ang.II + Lat.A
Ang.II + Blebbistat
Cyclin A
Relative expression of cyclin A
0.04
0.03
0.02
0.01
0
NT
Ang. II
Ang.II + Lat.A
Ang.II + Blebbistat

Fig 5


A. BJ
COX - 2
100%
50%
1%
mRNA remaining (%)
Ang.II t½=50.0
Ang.II+Lat.A t½=63.0
Ang.II+Blebbistat. t½=23.0
0
30
60
90
120
150
BJ ; Time after Act.D treatment (min)

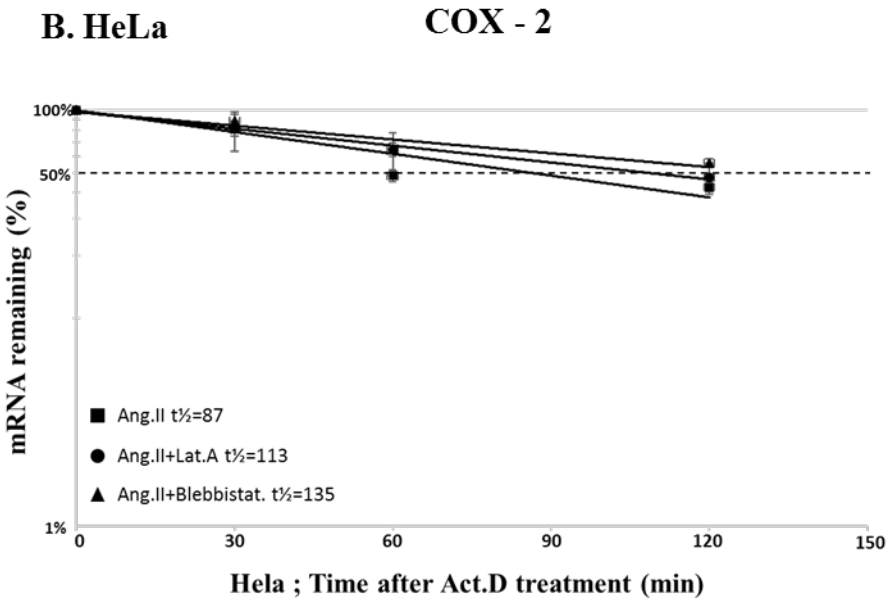
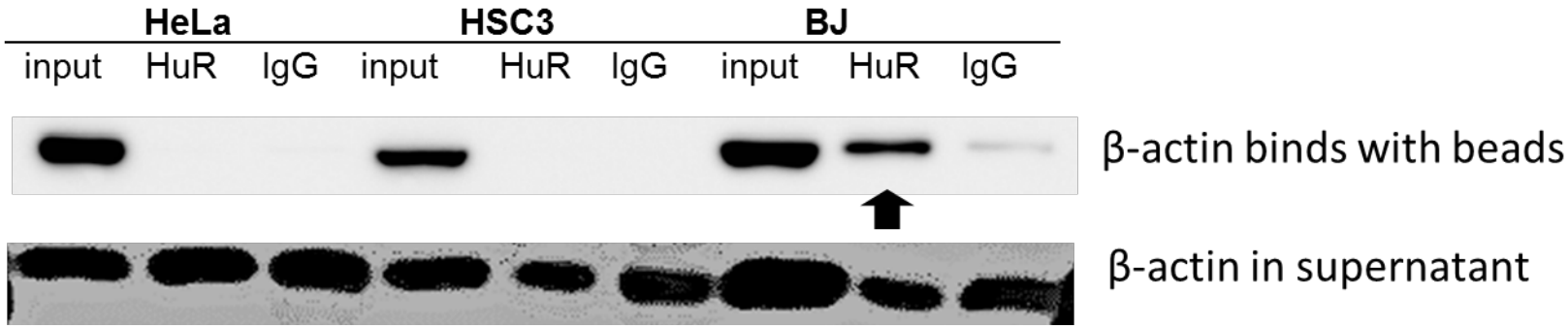

B. HeLa
COX - 2
100%
50%
1%
mRNA remaining (%)
Ang.II t½=87
Ang.II+Lat.A t½=113
Ang.II+Blebbistat. t½=135
0
30
60
90
120
150
Hela ; Time after Act.D treatment (min)

Fig 6


HeLa
HSC3
BJ
input
HuR
IgG
input
HuR
IgG
input
HuR
IgG
β-actin binds with beads
β-actin in supernatant

Supplementary 1