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Author(s)	Habiba, Umma; Kuroshima, Takeshi; Yanagawa-Matsuda, Aya; Kitamura, Tetsuya; Chowdhury, A. F. M. A.; Jehung, Jumond P.; Hossain, Elora; Sano, Hidehiko; Kitagawa, Yoshimasa; Shindoh, Masanobu; Higashino, Fumihito
Citation	Experimental cell research, 369(2), 218-225 https://doi.org/10.1016/j.yexcr.2018.05.021
Issue Date	2018-08-15
Doc URL	http://hdl.handle.net/2115/75223
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Rights(URL)	http://creativecommons.org/licenses/by-nc-nd/4.0/
Type	article (author version)
File Information	Experimental cell research369(2)218-225.pdf



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

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4 Umma Habiba¹, Takeshi Kuroshima³, Aya Yanagawa-Matsuda¹, Tetsuya
5 Kitamura¹, AFMA Chowdhury², Jumond P. Jehung², Elora Hossain³,
6 Hidehiko Sano², Yoshimasa Kitagawa⁴, Masanobu Shindoh¹, Fumihiro
7 Higashino^{1,4}

8
9 ¹ Department of Oral Pathology and Biology, Hokkaido University Faculty
10 of Dental Medicine and Graduate School of Dental Medicine, Sapporo,
11 Japan.

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

14 ³ Department of Oral Diagnosis and Medicine, Hokkaido University
15 Faculty of Dental Medicine and Graduate School of Dental Medicine,,
16 Sapporo, Japan.

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

1

2 **ABSTRACT**

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

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

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

21

22 **Key words:**

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

1 cyclooxygenase-2.

2 **INTRODUCTION**

3 Human antigen R (HuR) is a ubiquitously expressed member of the embryonic lethal
4 abnormal vision (ELAV) family of RNA binding proteins, most abundantly localized in
5 the nucleus. It shuttles between the nucleus and the cytoplasm in the presence of
6 external stimuli such as extracellular stress (1,2). HuR distribution between the nucleus
7 and the cytoplasm is supported by its associated proteins, transportin 1 and 2 (Tran 1 and
8 2) (3,4), pp32, APRIL, exportin and chromosome maintenance region 1 (CRM1) (5,6).
9 However, the mechanisms underlying the cytoplasmic trafficking of HuR and its
10 consignment mRNA are far less understood.

11

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

23

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

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

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

14
15 Therefore, the current study aimed to analyze human cervical (HeLa) and oral
16 squamous cell (HSC3) carcinoma to unveil whether their intracellular trafficking
17 mechanisms are same or different in other tumors. In an aim to decipher the underlying
18 mechanism, we used normal cells (human foreskin fibroblast; BJ and human gingival
19 fibroblast; HGF1) as control. To analyze the functional role of cytoskeletal element in
20 the nucleo-cytoplasmic HuR redistribution and HuR targeted mRNA stabilization in
21 cancer and normal cells, we directly treated the cells with the microfilaments inhibitors:
22 Latrunculin A (Lat.A) and blebbistatin (blebbistat) after stimulation with vasoactive
23 hormone Angiotensin II (Ang II). The involvement of Ang II in HuR reshuttling was
24 demonstrated in a previous study (29).

25

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

10 **MATERIALS AND METHODS**

11 **Cell culture**

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

18 **Reagents and antibodies**

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

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

5

6 **Western blot analysis**

7 Whole cell lysates were prepared using radio immunoprecipitation assay (RIPA) buffer
8 (150 mM NaCl; 25 mM Tris-HCl, pH 7.6; 1% Nonidet P-40; 1% sodium deoxycholate;
9 0.1% SDS) containing protease inhibitors. Twenty micrograms of each sample were
10 separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis
11 (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Millipore,
12 Billerica, MA, USA). Total protein content was confirmed by β -actin staining. In order
13 to separate cells into cytoplasmic and nuclear fractions, cells were suspended in a
14 fractionating buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1.5 mM MgCl₂, 0.5%
15 Nonidet P-40, protease inhibitor cocktail), followed by vigorous shaking for 5 min and
16 centrifugation at 12,000 rpm for 30 secs. The supernatant was used as the cytoplasmic
17 fraction. To estimate the accuracy of cell fractionation, cytoplasmic (β -tubulin) and
18 nuclear (lamin B) proteins were detected using western blotting. Antibodies used in this
19 study were specific to HuR, (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β -actin,
20 β -tubulin, Lamin B (Sigma-Aldrich, St louis, MO, USA). The secondary antibody was
21 horse radish peroxidase-conjugated IgG (Jackson Immuno Research Laboratories, West
22 Grove, PA, USA). Immunoreactive bands were visualized with chemiluminescence
23 using the Supersignal West Femto Maximum Sensitivity Substrate (Thermo Fisher
24 Scientific, USA).

25

1 **Fluorescence microscopy**

2 Cells were grown on coverslips in 35-mm dishes at 60–80% confluence and were
3 stimulated for 2 h with Angin II without or with microfilament inhibitor either
4 latrunculin A (Lat.A;30 min) or blebbistatin (Blebbistat;4 hour). Immediately following
5 treatment, cells were rinsed once with phosphate buffered saline (PBS), fixed for 20 min
6 at RT with 4% paraformaldehyde in PBS, blocked and permeabilized in 2% BSA plus
7 0.1% Triton X-100 (PBS) at room temperature (RT). Subsequently, the fixed cells were
8 incubated with anti-HuR and myosin II primary antibody for overnight at 4°C followed
9 by Alexa Fluor 488 and Alexa Fluor 568 secondary antibodies for 1 hour at room
10 temperature respectively. F-actin was visualized with rhodamine-conjugated phalloidin.
11 Cell nuclei were counterstained with DAPI before mounted on slides by using Mountant
12 permafluor (Thermo scientific, FM 111212A). Cells were observed using an IX71
13 inverted microscope (Olympus). Image acquisition was performed with the Olympus
14 Fluoview Software (FV10-ASW 4.2 viewer).

15

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

17 Total RNA was extracted using the TRI reagent (Sigma-Aldrich, St louis, MO, USA)
18 and the RNA was subjected to reverse transcription using Rever Tra Ace qPCR RT
19 master mix with genomic DNA remover (Toyobo, Osaka, Japan). Quantitative real-time
20 RT-PCR was performed in the DNA Engine Opticon2 (MJ Research, Watertown, MA,
21 USA), and cDNA was amplified using the following primers:
22 GAPDH, 5'-ATCCTGGGCTACACTGAGCA-3', 5'-TGCTGTAGCCAAATTCGTTG-3',
23 COX-2,
24 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3',5'-AGATCATCTCTGCCTGAGTAT

1 CTT-3' and Cyclin A,5'-ATTAGTTTACCTGGACCCAG-3',

2 5'-CACAAAGCTACTTCTGCTCT

3 -3'. GAPDH was used for normalization.

4 The C(T) values of mRNA levels were normalized to the C(T) values of GAPDH

5 mRNA within the same sample. To evaluate the half-life of total COX-2 mRNA, cells

6 were treated with 5µg/ml actinomycin D for 30, 60, 120 min. Total cellular RNA of

7 each cell was used for quantitative real time RT-PCR.

8

9 **Protein interaction Assay**

10 To search proteins that interact with HuR in cancer and normal cells, HeLa, HSC3 and

11 BJ were lysed in TENT lysis buffer [25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1%

12 NP-40, 1% sodium deoxycholate, 0.1% SDS]. For immunoprecipitation, protein G

13 sepharose beads was equilibrated with lysis buffer. After preclearing the lysates,

14 proteins were immunoprecipitated with anti HuR and mouse normal IgG and incubated

15 for 4 hours at 4°C under gentle rotation. Afterwards,50ul beads were added with 500ug

16 protein and incubated for 4 hours at 4°C under rotary agitation. After centrifugation for

17 5 min at 3000g, the supernatant was discarded followed by washing for several times

18 with lysis buffer. The Ag-Ab complex was eluded by boiling the samples in loading

19 buffer for 5 min. Finally, equal volumes of proteins were subjected to SDS-PAGE and

20 analyzed by immunoblotting.

21 **Statistical analysis**

22 Statistical analysis was performed by using one-way ANOVA. Post-hoc multiple

23 comparisons were done by Tukey's test at 5% level of significance. All statistical

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

3 **RESULTS**

4 **Effects of Actin- myosin cytoskeleton interfering drugs on cancer and normal** 5 **fibroblast cells and actin cytoskeletal organization**

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

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

1 independent experiments.

2

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

6

7 **Modulatory effect of nucleo-cytoplasmic HuR shuttling by microfilament**

8 **inhibitors**

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

21

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

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

13

14 **Down regulation of HuR triggered mRNA expression by microfilament inhibitors**

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

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

2

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

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

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

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

1 (Fig.6).

2 DISCUSSION

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

7 We employed cytoskeleton inhibitors to test the potential role of actin-myosin based
8 cytoskeleton in the constitutive HuR shuttling, HuR dependent mRNA expression and
9 stabilization in cancer and normal cell. Latrunculin A is the marine derived macrolide
10 which reversibly binds the cytoskeleton actin monomers, forming complex with G-
11 actin and disrupt its polymerization (31). Some studies reported that Latrunculin A
12 inhibits microfilament mediated cell process without affecting the organization of the
13 microtubule dependent cytoskeleton and showed potent antitumorigenic properties
14 (32,33). Blebbistatin is a highly selective inhibitor of non-muscle myosin II ATPase,
15 that interfere the myosin II mediated cell motility, migration and adhesion. Additionally,
16 blebbistatin can interfere with the cellular invasiveness of pancreatic adenocarcinoma
17 (34,35).

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

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

16

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

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

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

17
18 To the best of our knowledge, this is the first report to demonstrate that
19 nucleo-cytoplasmic shuttling of HuR can occur without actin in oral and cervical cancer.
20 Our observations point to the possible differential molecular transport system of HuR in
21 individual cancer types. We hypothesized that molecular transport system of HuR is
22 different in individual cancer and cancer cells acquire an alternative HuR trafficking
23 strategy when actin-myosin cytoskeleton is disrupted. However further studies are
24 needed to elucidate specific cancer types. In addition, discovery of cancer types that

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

3

4 **ACKNOWLEDGEMENTS**

5 The authors thank Mr. Yohei Murayama (Support Section for Education and Research,
6 Hokkaido University Graduate School of Dental Medicine) for the technical assistance.

7 The authors are grateful to the faculty members for helpful discussion.

8

9 **FUNDING**

10 This work was supported by the Japan Society for the Promotion of Science (JSPS)
11 [17H04401 and 17F17123].

12

13 **CONFLICT OF INTEREST STATEMENT**

14 The authors declare no conflict of interests.

15

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15
16

17 **Figure Legends**

18 **Fig.1 Expression of morphological changes after application of microfilament**

19 **inhibitor.** BJ (A) and HeLa (B) Cells were treated with Ang II (2 h) followed by in the
20 absence or presence of either latrunculin A (Lat.A) or blebbistatin (Blebbistat), which
21 were treated for 30 min (Lat. A) or 4 h (Blebbistat) respectively. Treatment with
22 Angiotensin II and Angiotensin II+Blebbistatin resulted in cell-cell separation and a
23 stellate appearance in some cells compared with control cells. Cells treated with
24 Angiotensin II+Latrunculin A showed the greatest degree of change in cell morphology

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

4 **Fig.2 Cytoplasmic HuR abundance in different human fibroblast cells is modulated**
5 **by microfilament inhibitors but cancer cells show microfilament independent**
6 **exportation.** Human fibroblasts (A) BJ (human foreskin fibroblast; left panel) (B)
7 HGF1 (human gingival fibroblast; left panel), and cancer cells (C) HeLa (human
8 cervical cancer; right panel) (D) HSC3 (human oral squamous cell carcinoma; right
9 panel) were stimulated for 2 h with Angin II without or with either latrunculin A (Lat.A;
10 30 min) or blebbistatin (Blebbistat 4 hour) as indicated. Equal amount (20 μ g) of total
11 protein and cytoplasmic and nuclear fractions were subjected to SDS-PAGE and
12 immunoblotted with anti-HuR, anti- β actin, anti- β tubulin and anti-lamin B respectively.
13 The level of HuR protein was monitored in total cell lysates (TP) and cytoplasmic (CP)
14 and nuclear extracts (NP). The data shown are from a single experiment representative
15 for three independent experiment giving similar results.

16

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

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

8

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

17

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

1 levels are quantified by quantitative real time RT-PCR experiments using GAPDH as a
2 normalization control. Graphs depict the percentage of remaining COX-2 mRNA levels
3 in relation to GAPDH mRNA and compared with the levels of normalized mRNA
4 species measured immediately after the addition of actinomycin D and which are set as
5 100%. The half-lives of the mRNAs (min) are indicated as $t_{1/2}$. The plot shows the mean
6 of three independent experiments.

7

8 **Fig.6 Protein interaction with HuR in cancer and normal cells.** To search protein
9 that interact with HuR in cancer (HeLa and HSC3) and normal cell (BJ), cells were
10 lysed with TENT lysis buffer. Protein G sepharose beads was equilibrated with lysis
11 buffer. After preclearing the lysates, proteins were immunoprecipitate with anti HuR
12 and mouse normal IgG and incubate for 4 hours at 4°C. 50ul beads were added with
13 500ug protein and incubate for 4 hours at 4°C followed by centrifugation for 5 min at
14 3000g. Supernatant was discarded and the Ag-Ab complex was eluded by boiling the
15 samples in loading buffer for 5 min. Finally, equal volumes of proteins were subjected
16 to SDS-PAGE and analyzed by immunoblotting.

17

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

1 the Alexa-Fluoro 488 coupled (green) secondary antibody. F-actin was visualized with
2 rhodamine conjugated phalloidin (Upper panel). Cells were also treated as mentioned
3 above and immunostained for myosin IIA (green), HuR (red) using either Alexa-Fluoro
4 488 secondary antibodies (Lower panel). In all cases, cell nuclei were visualized by
5 staining with DAPI (40,60-DAPI). Bars,20 μ m. Data shown are from a single
6 experiment representative of three repeats giving similar results.

Fig 1

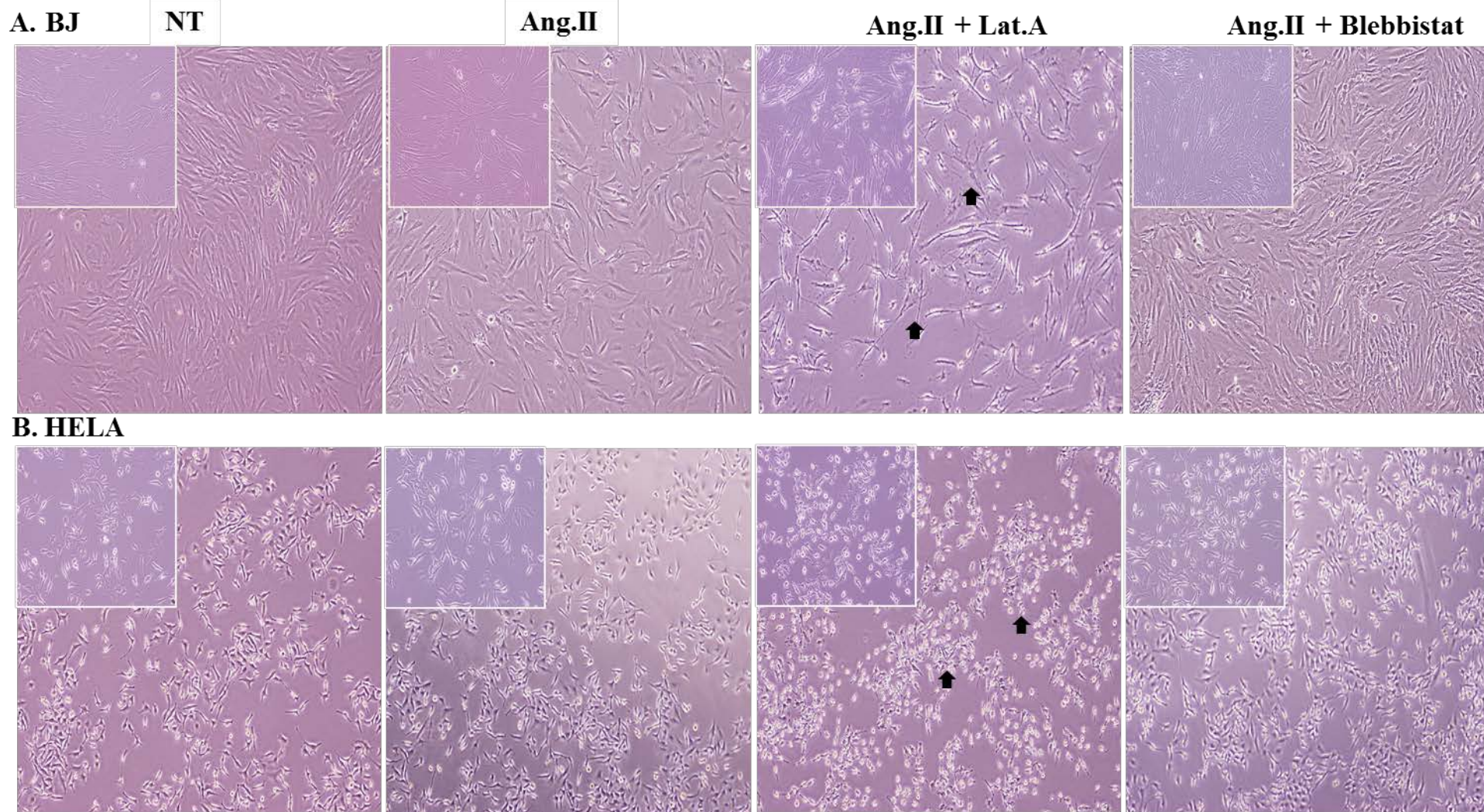


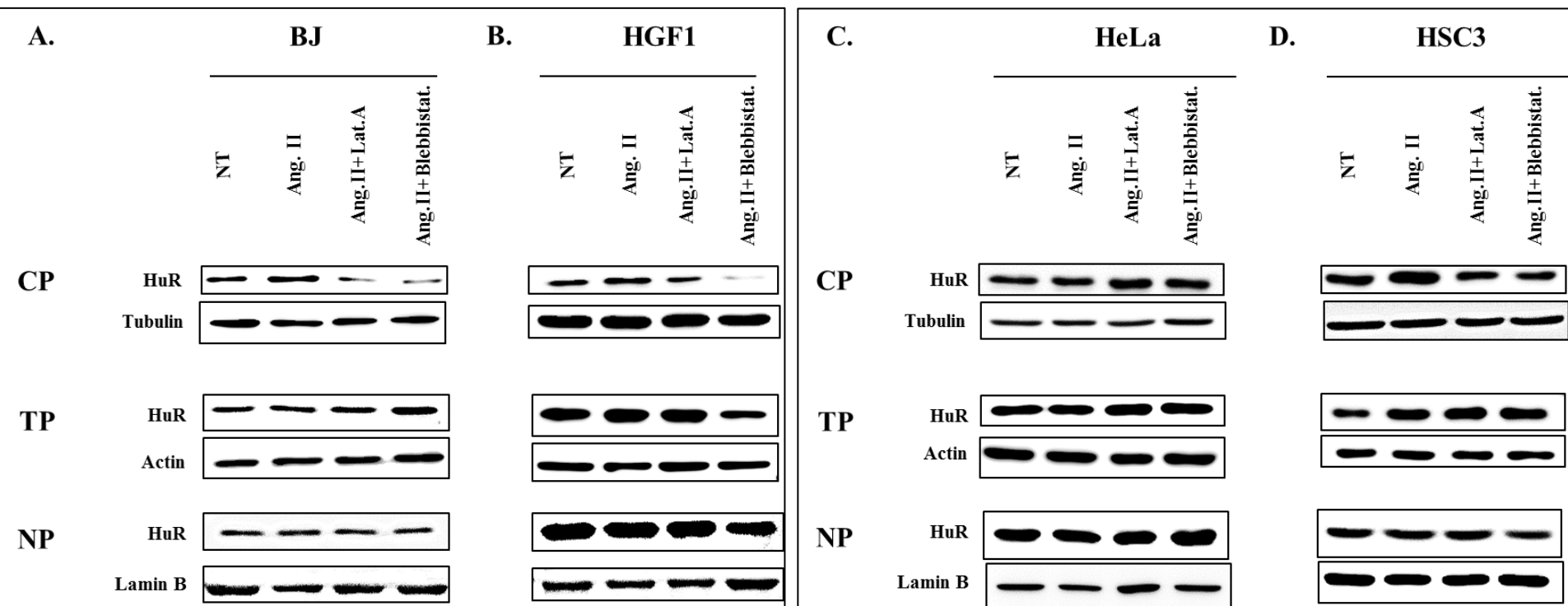
Fig 2

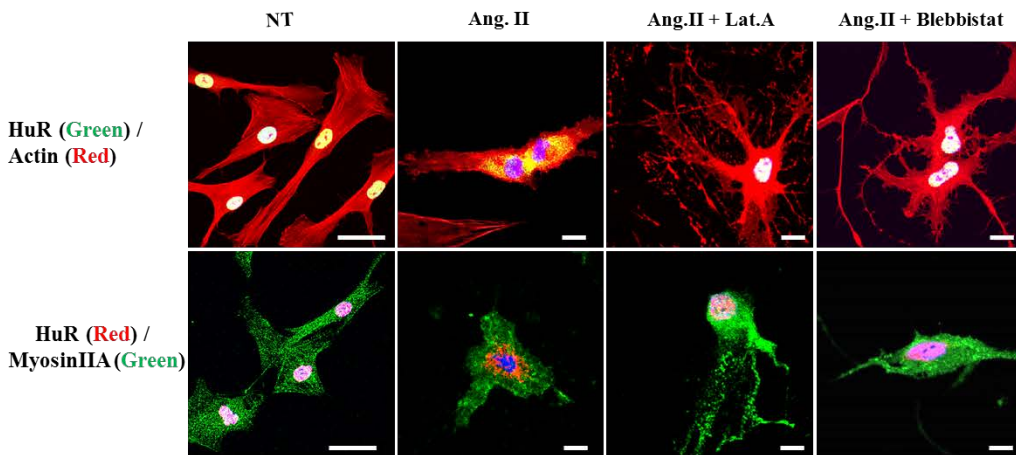
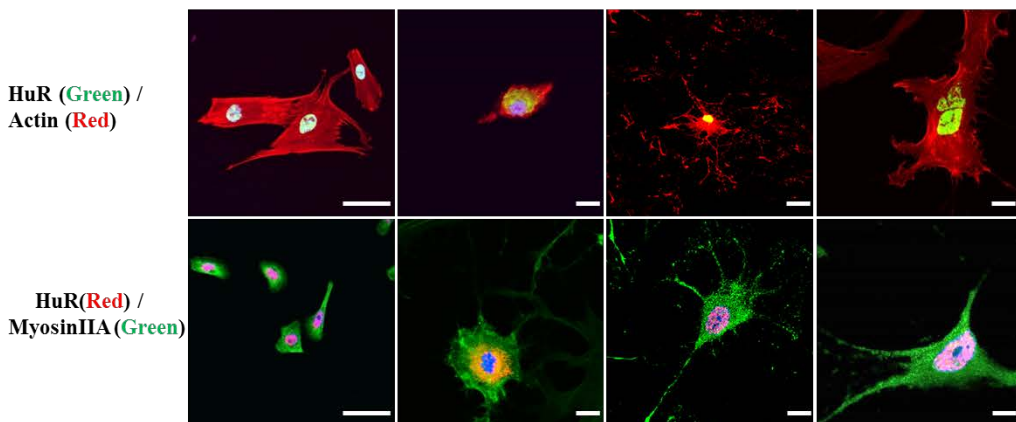
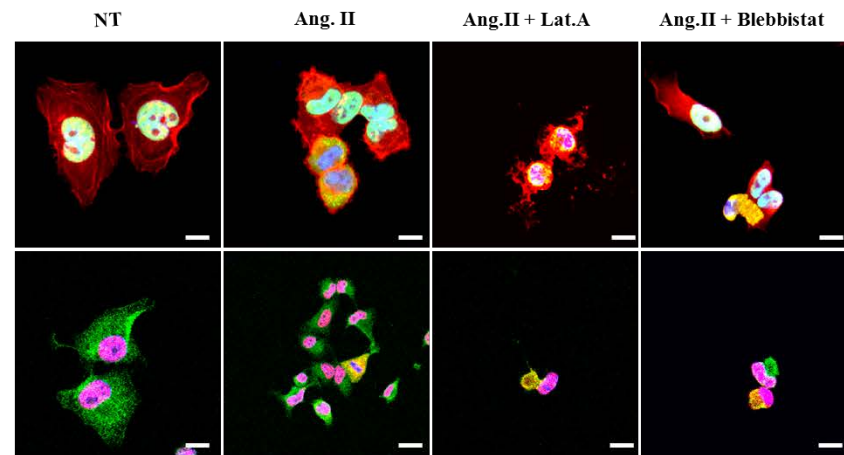
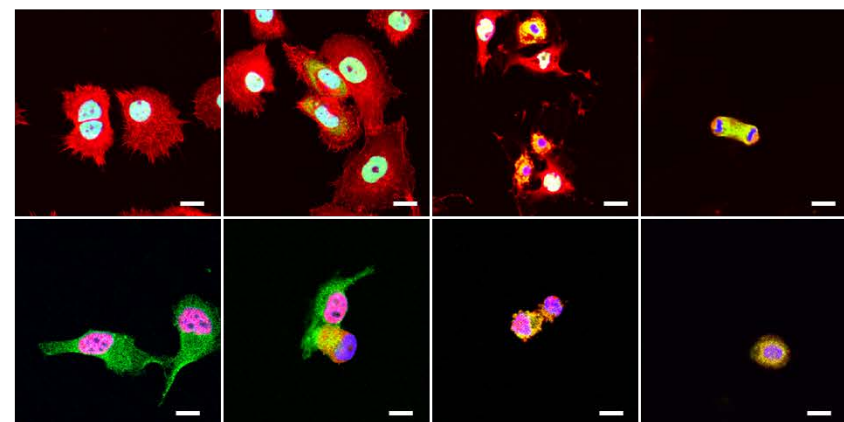
Fig 3**A. BJ****B. HGF1****C. HeLa****D. HSC3**

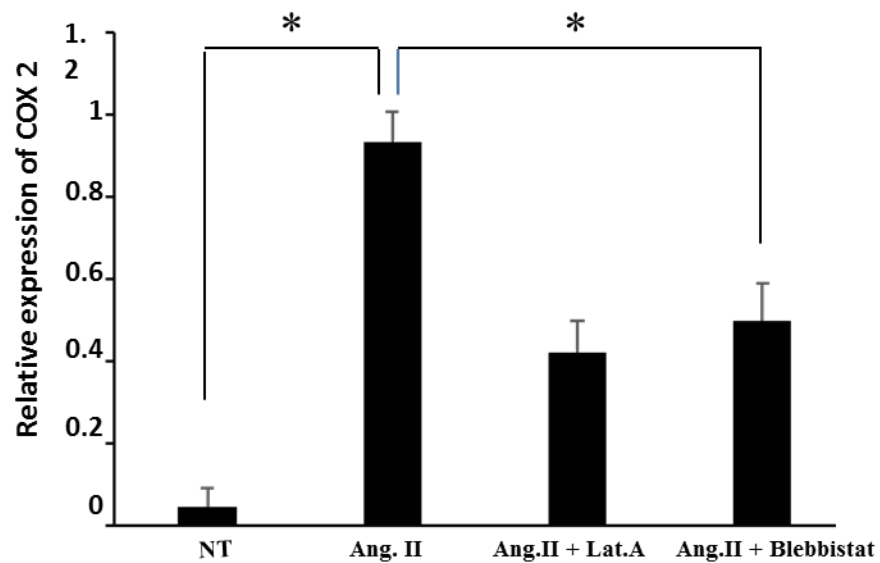
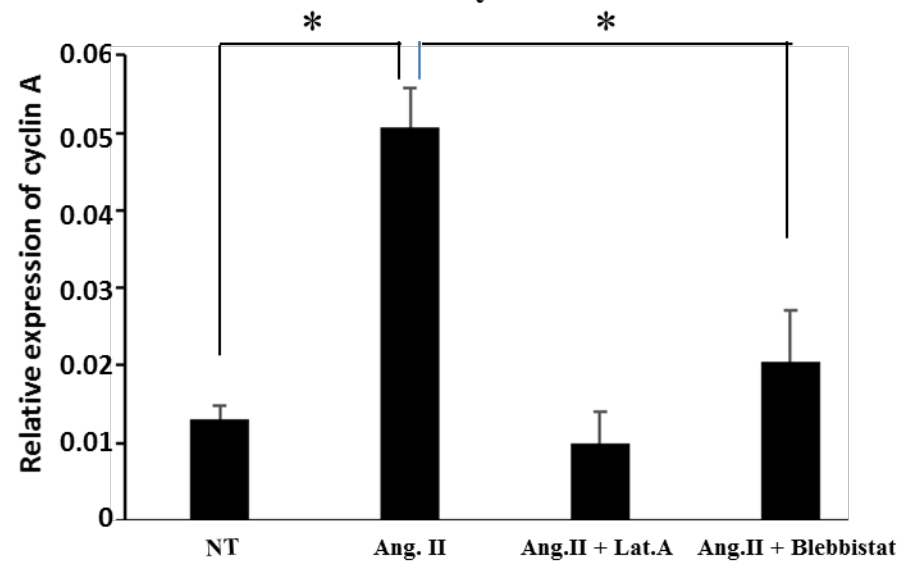
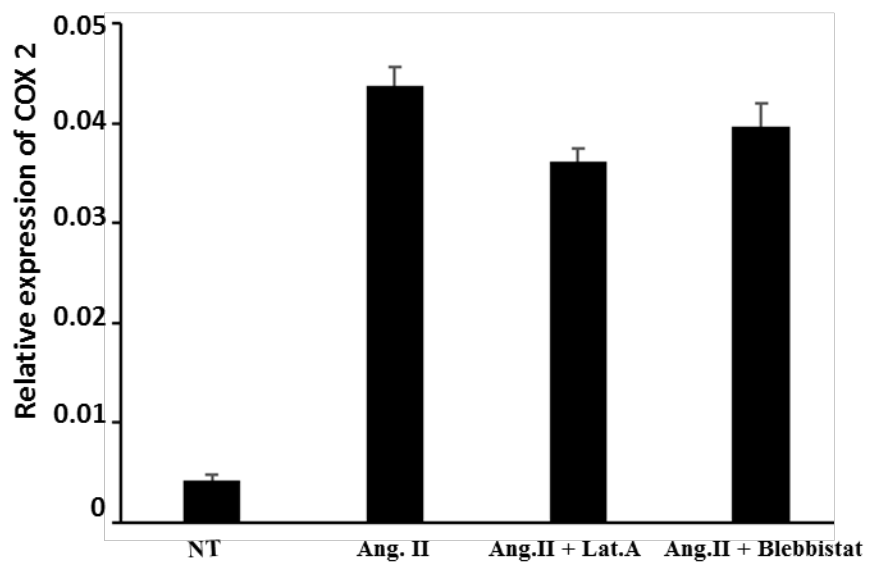
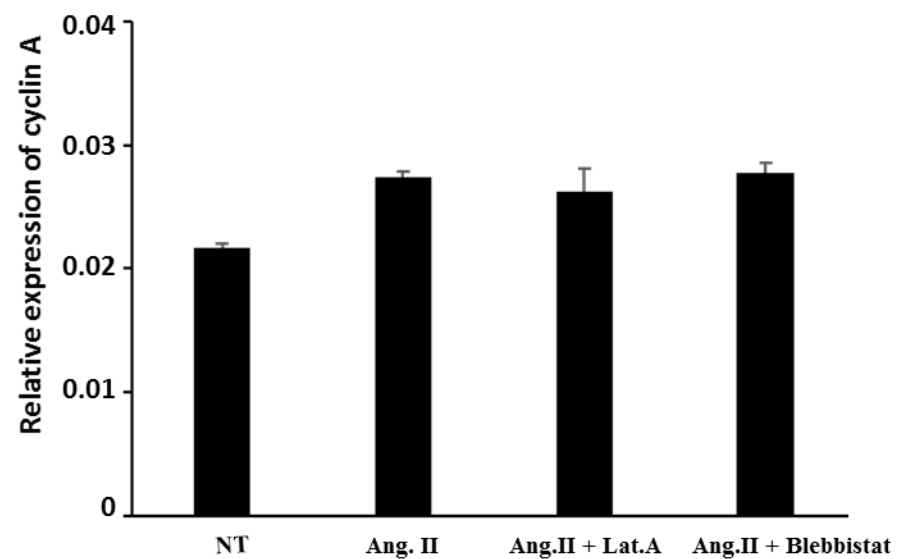
Fig 4**A. BJ****COX - 2****Cyclin A****B. HeLa****COX - 2****Cyclin A**

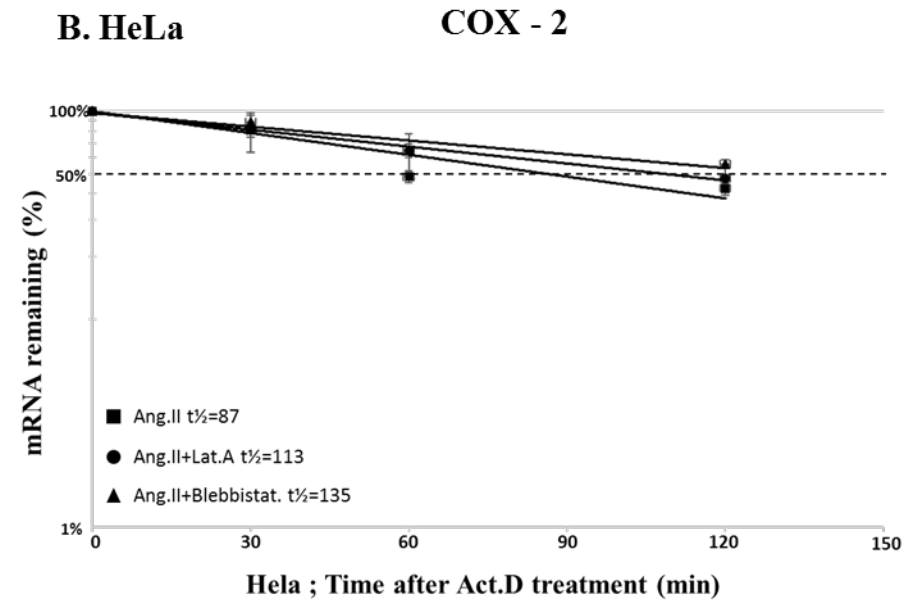
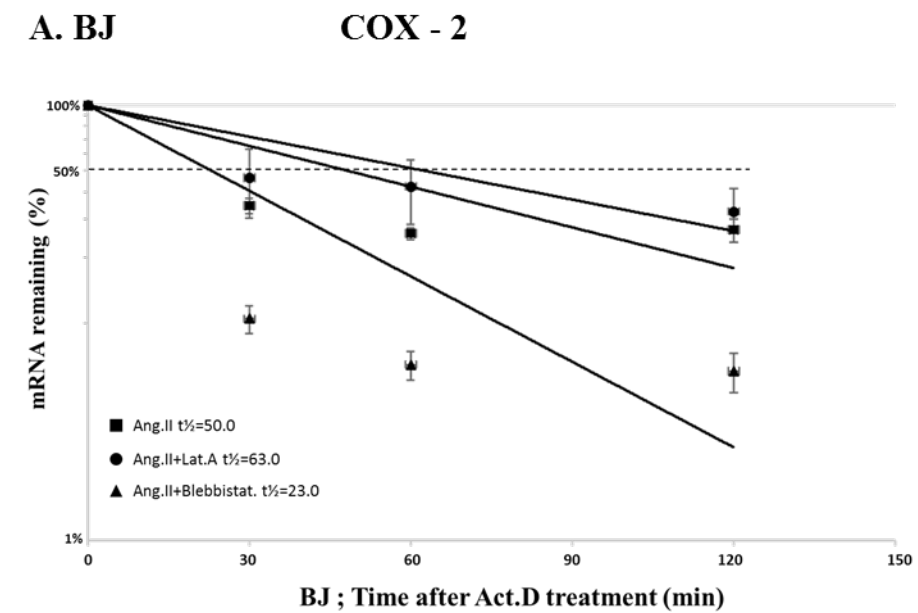
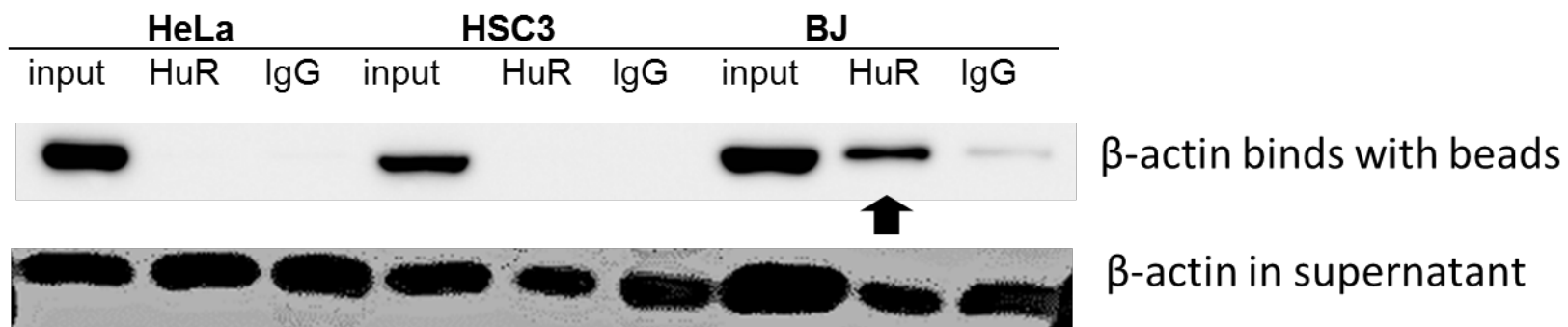
Fig 5

Fig 6



HEPG2

