Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 356 (2007) 318–321
BBRC
www.elsevier.com/locate/ybbrc

Stabilization of Snail by HuR in the process of hydrogen peroxide induced cell migration

Rui Dong, Jian-Guo Lu, Qing Wang, Xian-Li He, Yan-Kui Chu, Qing-Jiu Ma *

Department of General Surgery, Tangdu Hospital, Fourth Military Medical University, Xi'an 710038, Shaanxi Province, China

Received 25 February 2007
Available online 5 March 2007

Abstract

Snail functions as a key regulator in the induction of a phenotypic change called epithelial to mesenchymal transition (EMT). Aberrant expression of Snail prevails in the onset and development of tumor. Here, we have observed increased expression of Snail under the treatment of hydrogen peroxide (H_2O_2). Investigation into the underlying mechanisms revealed that stabilization of Snail mRNA contributes partially to this process. H_2O_2-induced the luciferase activity of the reporter construct contains the 3′UTR of Snail. Deletion of the AU-rich elements in the UTR eliminated the response of the reporter to H_2O_2, suggesting the potential role of HuR in the process. Lowering of endogenous HuR levels through knockdown of HuR by siRNA greatly reduced the inducability and half-life of Snail mRNA, which consequently inhibited the downregulation of E-cadherin by H_2O_2. Our findings indicate that HuR plays a major role in regulating H_2O_2-induced Snail expression by enhancing Snail mRNA stability, which in turn enhances cell migrating ability through repressing expression of E-cadherin.

Keywords: HuR; Snail; Hydrogen peroxide; Cell migration

Snail plays a fundamental role in the induction of a phenotypic change called epithelial to mesenchymal transition (EMT). Snail-induced EMT converts epithelial cells into mesenchymal cells with migratory properties that contribute to the formation of many tissues during embryonic development and to the acquisition of invasive properties in epithelial tumours [1]. Snail-induced EMT is partly due to the direct repression of E-cadherin transcription both during development and tumour progression [2].

EMT can be triggered by different signaling molecules, such as by epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor beta (TGFβ), hepatocyte growth factor (HGF), WNTs and Notch. In agreement with the involvement of Snail in all studied processes of EMT, these signalling molecules have been shown to induce Snail genes in different cellular contexts [3]. Previously, ROS were found to facilitate the EMT in certain cell types [4]. However, the expression of Snail gene is still to be elucidated and the underlying mechanism is largely unknown.

In addition to transcriptional events, gene expression programs are strongly influenced by posttranscriptional regulatory processes, such as those controlling mRNA turnover and translation [5,6]. Although the mechanisms determining mRNA turnover are poorly understood, they are generally believed to involve RNA-binding proteins recognizing specific RNA sequences. There has been growing interest in a particular pathway which regulates mRNA stability, and is mediated by AU-rich elements (AREs), usually found in the 3′ untranslated region (UTR) of short-lived mRNAs [7,8].

HuR binds target mRNA subsets bearing AREs through its RNA recognition motifs and has been shown to regulate the expression of many target mRNAs, including those that encode c-*fos*, vascular endothelial growth factor, tumor necrosis factor alpha (TNF-α), β-catenin,

* Corresponding author. Fax: +86 29 83377431.
E-mail address: maqingjiu@yahoo.com.cn (Q.-J. Ma).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.02.145

c-*myc*, cyclooxygenase 2, myogenin, MyoD, several cyclins, granulocyte-macrophage colony-stimulating factor, several interleukins, p21, p27, p53, and hsp70. Accordingly, HuR has been directly implicated in regulating various cellular responses, including cell division, carcinogenesis, muscle cell differentiation, replicative senescence, immune cell activation, and stress responsiveness [9,10].

Interestingly, Snail mRNA bears multiple AREs in the 3′UTR. What's more, H_2O_2 enhance the cytoplasmic localization of HuR [11], suggesting the potential role of HuR in the regulation of Snail under the treatment of H_2O_2.

Materials and methods

Cell culture, reagents, treatments, and transfections. MCF-7 cells, originally obtained from American Type Culture Collection, were maintained in culture in a 37 °C incubator with 5% CO_2 in RPMI 1640 supplemented with 10% fetal bovine serum. Anti-HuR antibody, anti-actin, anti-E-cadherin, anti-snail, and anti-hnRNP C1/C2 were bought from Santa Cruz. Cells were treated with H_2O_2 for indicated time at indicated doses. Synthetic small interfering RNAs (Invitrogen) targeting HuR (si-HuR AAGAGGCAATTACCAGTTTCA), as well as a control small interfering RNA (si-crtl; AATTCTCCGAACGTGTCACGT) were used at 20 nM. Cells were transfected with Oligofectamine (Invitrogen) on day 0 and treated with H_2O_2 and harvested at indicated time.

Construction of pGL3-Snail 3′UTR and pGL3-Δ Snail 3′UTR and Luciferase Assay. For the construction of pGL3-Snail 3′UTR and pGL3-Δ Snail 3′UTR, PCR products were prepared with 5′ primers: 5′-CC TCTAGACCCTCGAGGCTCCCTCTTCCTC-3′ and 5′-CCTCTAGAA TATAAATTAACTGCTTTATTGAATATC-3′, respectively, and the 3′ primer: 5′-CCTCTAGAGAAACAGGTGCCACCCCACC-3′ and cloned into the *Xba*I site of plasmid pGL3-Control vector (Promega). Cells were plated at a density of 10^4/well in a 24-well dish and transiently transfected with 300 ng pGL3-Snail 3′UTR and pGL3-Δ Snail 3′UTR. Fifty nanograms of pBIND vector (Promega) were co-transfected as an internal control.

RT-PCR. RT-PCR experiment was done similar as previously described [12]. At the commencement of each experiment, cells growing in log phase were plated in a 6-well dish at a density of 3×10^5 cells/well. Cells were treated with indicated experiments for indicated time before harvested for extraction of RNA, and 2 μg of total RNA was used to prepare cDNA in 25 μl system. PCR was then performed on 1 μl cDNA with the primers: Snail forward, GGGCAGGTATGGAGAGGAAGA; Snail reverse, TTCTTCTGCGCTACTGCTGCG; E-cadherin forward, CAGC ACGTACACAGCCCTAA; E-cadherin reverse, GCTGGCTCAAGTC AAAGTCC; β-actin forward, AGCGAGCATCCCCCAAAGTT; β-actin reverse, GGGCACGAAGGCTCATCCATT.

Subcellular fractionation and Western blot analysis. Subcellular fractionation was prepared as previously described [13]. To obtain cytoplasmic fractions, cells were trypsinized, rinsed with phosphate-buffered saline, incubated in 200 μl of hypotonic buffer A (10 mM Hepes [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$) supplemented with inhibitors (leupeptin [1 μg/ml], aprotinin [1 μg/ml], and 0.5 mM phenylmethylsulfonyl fluoride) on ice, and lysed by addition of 25 μl of buffer A containing 2.5% Nonidet P-40 plus inhibitors. Nuclei were pelleted (3500 rpm, 4 min, 4 °C), and supernatants were saved, freeze-thawed five times, and centrifuged (10 min, 3500 rpm, 4 °C). Cytosolic fractions were prepared by subjecting cytoplasmic lysates to an additional step of high-speed centrifugation (14,000 rpm for 60 min at 4 °C) and discarding any pelleted material. For preparing nuclear fractions, nuclear pellets were incubated in extraction buffer C (20 mM Hepes [pH 7.9], 0.45 M NaCl, 1 mM EDTA) plus inhibitors and centrifuged (10 min, 14,000 rpm, 4 °C), and supernatants were saved. The efficiency and quality of nuclei preparation were monitored with a hemacytometer at the end of the nucleus isolation procedure. Whole cell, cytoplasmic or nuclear lysates were size fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene difluoride membranes. Equal loading and transferring of samples were confirmed by staining membranes with Ponceau red before hybridization.

Cell migration assay. Cell migration assay was done as previously described [14]. MCF-7 cells were grown to 80% confluence. Cells were transfected with siRNA against HuR or the control for 36 h and trypsinized and washed in serum-free DMEM before plating into a 12-well Transwell plate (12 mm diameter, 8 μm pore size, Corning Incorporated Costar). 12-well transwell chambers were incubated with IMEM containing 0.01% bovine serum albumin and 0.01% FBS overnight. Cells (5×10^4) were added to the upper well, which was placed into a lower well containing DMEM, 10% FBS, and various agents indicated in the results part. After incubation for 24 h, the filter was removed, and ASMC on the upper side of the filter were scraped off. MCF-7 cells that had migrated to the lower side of the filter were fixed in methanol at 4 °C for 15 min, stained with Toluidin blue and counted under a microscope for quantification of MCF-7 migration.

Results and discussion

H_2O_2 increased Snail mRNA partially through enhanced stability

Previous study had suggested that H_2O_2 could facilitate the process of tumor cell migration by increasing the expression of Snail [4]. As to the common role of HuR in the tumor development [5,15], we asked whether HuR functions in the regulation of Snail. To directly investigate this, MCF-7 cells were transfected with synthetic siRNA against HuR or the control siRNA. Knockdown efficiency was detected by the Western blotting 36 h after transfection. As shown in Fig. 1A, 70% of the endogenous HuR was knocked-down. Cells 36 h after transfection with either

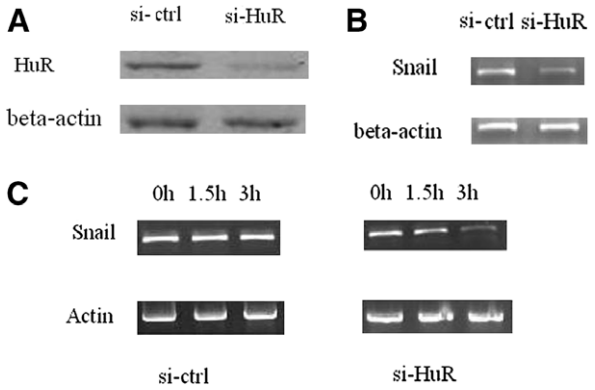

Fig. 1. H_2O_2 increased Snail mRNA partially through enhanced stability. (A) siRNA against HuR efficiently decreased the endogenous expression of HuR in MCF-7 cells MCF-7 cells were transfected with 20 nM siRNA against HuR or the control and harvested for Western blotting 36 h after transfection. (B) Decreased expression of Snail mRNA in si-HuR MCF-7 cells 36 h after transfection of siRNA against HuR or the control, cells were treated with H_2O_2 for additional 12 h before extraction of RNA. (C) Shorter half-life of Snail mRNA in si-HuR MCF-7 cells 36 h after transfection of siRNA against HuR or the control, cells were treated with H_2O_2 for 6 h. Then, cells were incubated with 5 mg/L actinomycin D (transcription inhibitor) for additional 0 h, 1.5 h, 3 h, and harvested for extraction of RNA. Data presented here represents two times of experiments.

the control or the siRNA against HuR were treated with 50 μM H_2O_2 for 12 h before harvested for RNA extraction. Decreased mRNA level of Snail was seen in the si-HuR group (Fig. 1B), suggesting a role of HuR in the process. Consistent changes of Snail at protein level were also observed (data not shown). As a common role of HuR in the regulation of the mRNA stability of target genes, we asked whether HuR increased the expression of Snail in the same way. To test the possibility, we observed the half-life of Snail in the HuR knocked down cells and the control cells. Actinomycin D was added 12 h after H_2O_2 treatment to block the transcription. Shorter half-life of Snail was found in si-HuR group (Fig. 1C), pointing out that HuR increased the stability of Snail.

HuR functions through the AREs located in the 3′UTR of Snail

Since HuR usually functions through the ARE located in the 3′UTR of target genes, we analyzed the 3′UTR of the Snail mRNA. The UTR region is highly AU rich and there are three typical AREs (Fig. 2A). We sought to directly test whether HuR was involved in the regulation of 3′UTR of Snail through the supposed AREs. Both the UTR containing the AREs and the deletion without the AREs were cloned to pGL3-Control vector and transfected into si-HuR and the control MCF-7 cells. As shown in Fig. 2B, the construction had lower activity compared with

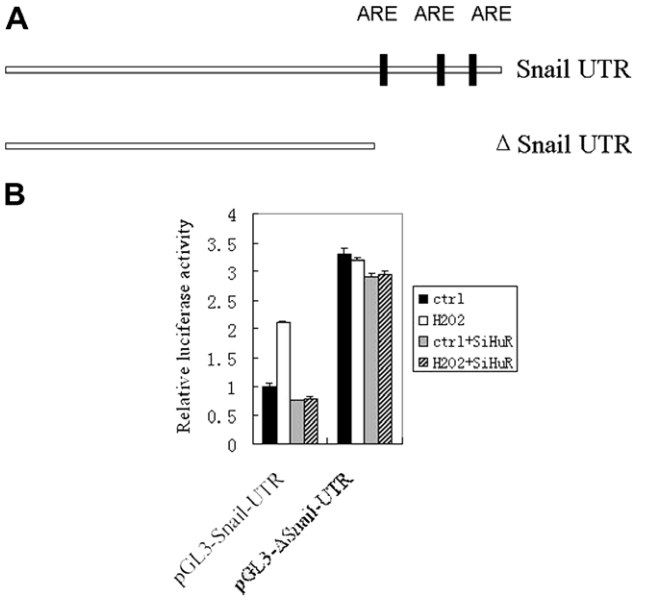

Fig. 2. HuR functions through the AREs located in the 3′UTR of snail. (A) Schematic representation of the 3′UTR of Snail AREs in the UTR was demonstrated as black bars. The deletion in which AREs were all deleted was also indicated. These two fragments were amplified from the cDNA and cloned into the pGL3 repoter. (B) MCF-7 cells were transfected with 20 nM siRNA against HuR or the control plus 200 ng of indicated reporter constructs and 50 ng pBIND. Forty-eight-hour later cells were harvested for luciferase assay. The results are means ± SE and represent more than three different experiments.

the deletion in MCF-7 cells without H_2O_2 treatment and HuR knockdown, suggesting the AREs functions as a element for instability. In addition, only the construct with AREs were responsive to H_2O_2 (about 2-fold under the treatment of H_2O_2) in MCF-7 cells without HuR knockdown, suggesting that both AREs and HuR are necessary for H_2O_2 induced Snail upregulation. In addition, in si-HuR MCF-7 cells, even the construction containing AREs rarely responded to H_2O_2. Together these results suggested that H_2O_2 increased the expression of Snail by the interaction of ARE and HuR.

Subcellular re-distribution of HuR after H_2O_2 treatment

HuR is predominantly localized in the nucleus while shuttled to the cytoplasm under stress conditions to function as an mRNA stabilizer or a translational regulator. To assess whether H_2O_2 treatment caused any changes in relative distribution of HuR, subcellular fraction were studied by western blot in untreated or H_2O_2-treated cultures. As shown in Fig. 3A, HuR were predominantly nuclear, as reported earlier [10,15,16]. By 6 h of H_2O_2 treatment, HuR were found to enrich in the cytoplasm. In addition, no obvious changes of the total level of HuR were found. Together these results pointing out that H_2O_2 increased Snail expression at least partially through the posttranscriptional regulation by enriching HuR in the cytoplasm. Of note, our study can not rule out the importance of transcriptional regulation. In fact, Snail was rarely expressed in cells without H_2O_2 treatment in MCF-7 cells. Thus, we can draw the conclusion that combination of transcriptional and posttranscriptional regulation contributes to the upregulation of Snail under the treatment of H_2O_2 in MCF-7 cells.

Knockdown of HuR decreased the migration induced by H_2O_2

The fundamental role of Snail in the regulation of E-cadherin and thus regulating migration of tumor cells,

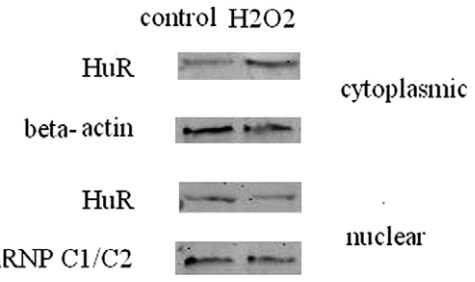

Fig. 3. Cytoplasmic enrichment of HuR under the treatment of H_2O_2. Western blot analysis of HuR levels in cytoplasmic and nuclear lysates prepared from MCF-7 cells with or without the treatment of H_2O_2 for 6 h. The levels of β-actin (a cytoplasmic protein) and hnRNP C1/C2 (a nuclear protein) in the same samples were assessed by Western blotting in order to ascertain the quality of the fractionation procedure and to detect loading differences.

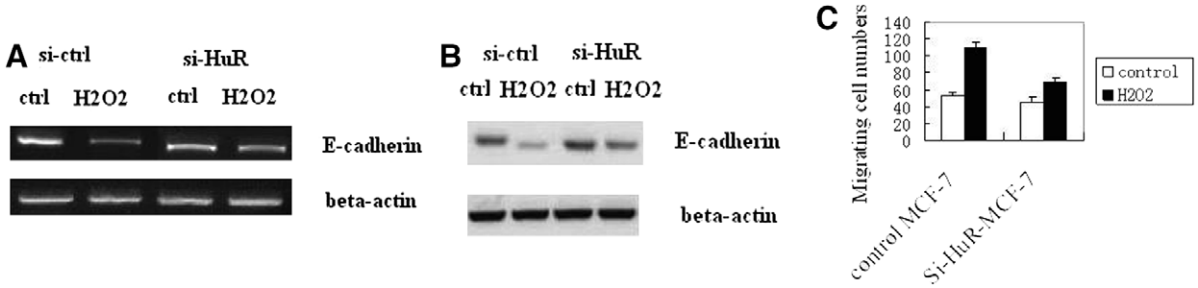

Fig. 4. Decreased migrating cells induced by H_2O_2 in si-HuR MCF-7 cells. (A) Effects of knockdown of HuR or H_2O_2 on the mRNA level of E-cadherin 36 h after transfection of siRNA against HuR or the control, cells were treated with H_2O_2 or H_2O as a control for additional 12 h before extraction of RNA. Data presented here represents two times of experiments. (B) Effects of knockdown of HuR or H_2O_2 on the mRNA level of E-cadherin 36 h after transfection of siRNA against HuR or the control, cells were treated with H_2O_2 or H_2O as a control for additional 18 h before harvested for Western blotting assay. Data presented here represents two times of experiments. (C) In Boyden chamber assays, cells transfected with siRNA against HuR or the control was treated 36 h after transfection with 50 μM H_2O_2 or the same volume of H_2O as a control for 24 h. Migrating cells was counted. Experiments were done in triplicates and data are expressed as means ± SE. Significant difference was found in si-HuR group compared with the control under the treatment of H_2O_2.

raises the possibility that knockdown of HuR would change the expression pattern of E-cadherin and thus result in insufficient migrating ability. To this end, first we observed the expression of E-cadherin both at mRNA level and protein level under H_2O_2 induction either in MCF-7 cells with or without HuR knockdown. As shown in Fig. 4A, H_2O_2 treatment reduced the transcription of E-cadherin in MCF-7 cells without HuR knockdown (about 4-fold). And this repression effect was only partially rather than totally eliminated in HuR knockdown MCF-7 cells (about 2-fold downregulation induced by H_2O_2 treatment), suggesting that HuR only functions partially in the induction of Snail and the consequent inhibition of E-cadherin. In addition, HuR knockdown itself seemed to have no effect on the expression of E-cadherin. Consistent with the mRNA data, the protein level of E-cadherin displayed similar changes (Fig. 4B). Then we observed the migrating ability in si-HuR cells and the control cells. As expected, decreased migrating ability was found in the si-HuR cells under the treatment of H_2O_2 (Fig. 4C). Thus, besides the anti-apoptotic role of HuR [15–18], here we suggest a role of HuR in the migrating process.

In summary, our study here has proposed a role of HuR in the migration of tumor cells, which is consistent with its previously supposed tumorgenic role. It is important to note that it is still needed to confirm the direct interaction of HuR and the exact ARE(s) of Snail by RNA-EMSA and RNA-IP assay, which is now undergoing.

References

[1] J.P. Thiery, Epithelial–mesenchymal transitions in tumour progression, Nat. Rev. Cancer. 2 (2002) 442–454.
[2] I.R. Beavon, The E-cadherin-catenin complex in tumour metastasis: structure, function and regulation, Eur. J. Cancer 36 (2000) 1607–1620.
[3] M.A. Huber, N. Kraut, H. Beug, Molecular requirements for epithelial–mesenchymal transition during tumor progression, Curr. Opin. Cell Biol. 17 (2005) 548–558.
[4] D.C. Radisky, D.D. Levy, L.E. Littlepage, H. Liu, C.M. Nelson, J.E. Fata, D. Leake, E.L. Godden, D.G. Albertson, M.A. Nieto, Z. Werb, M.J. Bissell, Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability, Nature. 436 (2005) 123–127.
[5] J. Cherry, V. Karschner, H. Jones, P.H. Pekala, HuR, an RNA-binding protein, involved in the control of cellular differentiation, In Vivo 20 (2006) 17–23.
[6] M.J. Moore, From birth to death: the complex lives of eukaryotic mRNAs, Science 309 (2005) 1514–1518.
[7] C.-Y. Chen, R. Gherzi, S.E. Ong, E.L. Chan, R. Raijmakers, G.J. Pruijn, G. Stoecklin, C. Moroni, M. Mann, M. Karin, AU binding proteins recruit the exosome to degrade ARE-containing mRNAs, Cell 107 (2001) 451–464.
[8] C.Y. Chen, A.B. Shyu, AU-rich elements, characterization and importance in mRNA degradation, Trends Biochem. Sci. 20 (1995) 465–470.
[9] M. Gorospe, HuR in the mammalian genotoxic response: post-transcriptional multitasking, Cell Cycle 2 (2003) 412–414.
[10] C.M. Brennan, J.A. Steitz, HuR and mRNA stability, Cell Mol. Life Sci. 58 (2001) 266–277.
[11] W. Wang, H. Furneaux, H. Cheng, M.C. Caldwell, D. Hutter, Y. Liu, N.J. Holbrook, M. Gorospe, HuR regulates p21 mRNA stabilization by ultraviolet light, Mol. Cell. Biol. 20 (2000) 760–769.
[12] R.E. Bachelder, S.O. Yoon, C. Franci, A.G. de Herreros, A.M. Mercurio, Glycogen synthase kinase-3 is an endogenous inhibitor of Snail transcription: implications for the epithelial–mesenchymal transition, J Cell Biol. 168 (2005) 29–33.
[13] Tomoko Kawai, Ashish Lal, Xiaoling Yang, Stefanie Galban, Krystyna Mazan-Mamczarz, Myriam Gorospe, Translational control of cytochrome *c* by RNA-binding proteins TIA-1 and HuR, Mol. Cell Biol. 26 (2006) 3295–3307.
[14] P.A. Pennisi, V. Barr, N.P. Nunez, B. Stannard, D . Le Roith, Reduced expression of insulin-like growth factor I receptors in MCF-7 breast cancer cells leads to a more metastatic phenotype, Cancer Res. 62 (2002) 6529–6537.
[15] J.D. Keene, Why is Hu where? Shuttling of early-response-gene messenger RNA subsets, Proc. Natl. Acad. Sci. USA 96 (1999) 5–7.
[16] W. Wang, S. Lin, C.M. Caldwell, H. Furneaux, M. Gorospe, HuR regulates cyclin A and cyclin B1 mRNA stability during the cell division cycle, EMBO J. 19 (2000) 2340–2350.
[17] W. Wang, X. Yang, V.J. Cristofalo, N.J. Holbrook, M. Gorospe, Loss of HuR influences gene expression during replicative senescence, Mol. Cell. Biol. 21 (2001) 5889–5898.
[18] A. Lal, T. Kawai, X. Yang, K. Mazan-Mamczarz, M. Gorospe, Anti-apoptotic function of RNA-binding HuR effected through prothymosin α, EMBO J. 24 (2005) 1852–1862.