HuR Is Necessary for Mammary Epithelial Cell Proliferation and Polarity at Least in Part via ΔNp63

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Abstract

HuR, a RNA binding protein, is known to function as a tumor maintenance gene in breast cancer and associated with tumor growth and poor prognosis. However, the cellular function of this protein remains largely unknown in normal mammary epithelial cells. Here, we showed that in immortalized MCF10A mammary epithelial cells, HuR knockdown inhibits cell proliferation and enhances premature senescence. We also showed that in three-dimensional culture, MCF10A cells with HuR knockdown form abnormal acini with filled lumen and an aberrant expression pattern of the extracellular matrix protein laminin V. In addition, we showed that HuR knockdown increases ΔNp63, but decreases wild-type p53, expression in MCF10A cells. Moreover, we showed that ΔNp63 knockdown partially rescues the proliferative defect induced by HuR knockdown in MCF10A cells. Consistent with this, we identified two U-rich elements in the 3′-untranslated region of p63 mRNA, to which HuR specifically binds. Finally, we showed that HuR knockdown enhances ΔNp63 mRNA translation but has no effect on p63 mRNA turnover. Together, our data suggest that HuR maintains cell proliferation and polarity of mammary epithelial cells at least in part via ΔNp63.

Introduction

Posttranscriptional regulation, an important process in the control of gene expression, starts with interactions of RNA-binding proteins with cis-acting elements in the regulated transcripts [1,2]. HuR is among the most prominent RNA binding proteins, which modulates mRNA stability and translation, and consequently regulates cell proliferation, angiogenesis, apoptosis, and stress response.

HuR, a member of the Hu family, is ubiquitously expressed and related to Drosophila embryonic lethal abnormal vision protein [3]. The other three members of the Hu family, HuB/HeN1, HuC and HuD, are primarily expressed in the neuronal tissues [4]. HuR contains three RNA-recognition motifs through which it binds to AU- or U-rich sequences in 3′-untranslated regions (3′UTR) of target mRNAs [5]. HuR is predominantly localized in the nucleus under non-stress conditions. Upon stimulation, such as heat shock, HuR is exported to cytoplasm where it regulates mRNA stability and/or translation [6]. The export of HuR is mediated at least by two pathways, transporting by transportins 1 and 2 [7], or by pp32 and APRIL in CRM1-dependent manner [6].

To date, elevated expression of HuR is associated with carcinogenesis in a wide variety of human tumors, including breast, colon, and prostate [8,9,10]. High levels of cytoplasmic HuR are associated with poor differentiation, large tumor size, and short survival in patients with breast ductal carcinoma [11] and non-BRCA1/2 mutated hereditary breast cancer [12]. The biological function of HuR in breast cancer is dependent on the mRNAs to which it is binding [4,13]. Elevated cytoplasmic HuR in breast cancer cells increases cyclin E1 and COX-2 expression and growth potential of cancer cells [8,14]. In addition, ectopic expression of HuR decreases BRCA1 expression [15]. In invasive breast tumors, HuR suppresses Wnt-5a mRNA translation [16], and reduced Wnt-5a expression is known to shorten disease-free survival [17]. Interestingly, miR-125a decreases HuR protein translation in breast cancer cells, and consequently inhibits cell proliferation and promotes apoptosis [18]. As such, HuR is established as a marker for breast cancer aggressiveness and poor prognosis as well as a target for treating breast cancer. Thus, delineation of HuR function in normal mammary epithelial cells is warranted.

P63 is known to be pivotal for the development and maintenance of epithelial tissues. p63−/− mice display gross developmental abnormalities. The most striking defect is complete lack of all stratified epithelia and their derivatives, including epidermis and mammary glands [19]. Recently, we showed that p63 mRNA stability is regulated by RNPC1, a RNA-binding protein, via AU-/U-rich elements in p63 3′UTR [20]. Considering that HuR prefers to bind to AU-/U-rich elements in 3′UTR of its targets, we explored whether HuR regulates p63 expression and cell proliferation in mammary breast epithelial cells.

Results

HuR Knockdown Inhibits Proliferation of Normal Mammary Epithelial Cells

Several studies have been performed to examine HuR function in breast tumor tissues and cell lines. These study showed that...
HuR regulates multiple pathways involved in breast carcinoma formation [8,11,12,14,15,21]. However, these systems are relatively intractable for studying HuR function in normal mammary epithelial cells. MCF10A is a spontaneously immortalized, but nontransformed human mammary epithelial cell line [22]. This cell line exhibits features of normal mammary epithelium, such as lack of tumorigenicity in nude mice and requirement of multiple growth factors and hormones for proliferation and survival [22]. Importantly, MCF10A cells form acinar structures in three-dimensional culture, a characteristic of normal glandular epithelium in vivo [23,24]. Furthermore, ectopic expression of oncoproteins in MCF10A cells, such as mutant p53 and ErbB2, disrupts this morphogenetic process [25,26]. Thus, in this study, MCF10A cells were used to address the implication of HuR in normal mammary epithelial cells. First, we generated multiple MCF10A cells in which HuR can be stably knocked down. Two representative HuR-KD MCF10A cell lines were shown in Fig. 1A (no. 6 and 26). We found that the level of HuR protein was significantly reduced in MCF10A cells with HuR knockdown. The level of actin protein was examined as a loading control. As a negative control, LacZ-knockdown MCF10A cell line was generated (Fig. 1B, no. 4). Next, to examine whether HuR knockdown has any effect on cell proliferation, MCF10A-HuR-KD cell lines no. 6 and 26 were used for long-term colony formation assay. We found that HuR knockdown markedly inhibited cell proliferation because the numbers of colonies formed by MCF10A-HuR-KD cell lines no. 6 and 26 were reduced to 61% and 28% of the control cells, respectively (Fig. 1C, top panel, and 1D). Camptothecin, an inhibitor of DNA topoisomerase I, is known to inhibit cell proliferation and induce senescence in low concentration [27,28]. Thus, we tested whether HuR knockdown made MCF10A cells more sensitive to camptothecin treatment. We found that MCF10A cells with HuR knockdown were highly sensitive to short-term treatment of 50 nM camptothecin because the numbers of colonies formed by MCF10A-HuR-KD cell lines no. 6 and 26 were reduced to 48% and 21% of the control cells, respectively (Fig. 1C, bottom panel, and 1D). To further confirm the requirement of HuR for proliferation of mammary epithelial cells, we found that HuR knockdown promoted premature senescence in MCF10A cells (Fig. 1E and 1G). In addition, HuR knockdown sensitized MCF10A cells to camptothecin-induced premature senescence (Fig. 1F–G). This result suggests that HuR is necessary for normal cell proliferation of MCF10A mammary epithelial cells.

HuR Knockdown Disrupts the Formation of Polarized Acinus-like Architecture

To determine whether HuR knockdown regulates cell polarity, we examined acinar structures of MCF10A cells in 3-D culture. We found that compared to control cells (Fig. 2A), MCF10A cells with HuR knockdown formed smaller acinar structures (Fig. 2B–C). In addition, we found that control LacZ-KD MCF10A cells formed polarized acinar architectures along with hollow lumen (Fig. 2E, top), consistent with previous reports [23,29,30]. However, MCF10A cells with HuR knockdown formed an acinus with filled lumen (Fig. 2E, middle and bottom).

To further demonstrate that HuR is required for the normal architecture of MCF10A acini, we immunostained laminin V, a principal extracellular matrix protein, in acini formed by MCF10A-LacZ-KD-4 and MCF10A-HuR-KD-26 cells in 3-D culture. We found that laminin V was deposited at the basal surface of the spheroid formed by MCF10A-LacZ-KD-4 cells (Fig. 2F, top). In contrast, in MCF10A-HuR-KD-26 cells, laminin V was secreted into the lumen of the acini and cell-cell junctions (Fig. 2F, bottom). This result suggests that HuR is required for development of a polarized acinus-like architecture in 3-D culture of MCF10A cells.

HuR Knockdown Increases ΔNp63, but Decrease Wild-type p53, Expression in MCF10A Cells

It is well-known that p53 plays a pivotal role in cell proliferation and premature senescence [31], and MCF10A cells carry a wild-type p53 [25]. Therefore, we tested whether p53 is correlated with premature senescence and deficient proliferation in MCF10A cells with HuR knockdown. We found that compared to control cells, the level of p53 protein was moderately decreased in MCF10A cells with HuR knockdown (Fig. 3A). This was consistent with previous reports that HuR contributes to induction of p53 expression via direct association with AU-rich element in 3’UTR of p53 mRNA [32,33]. Likewise, HuR knockdown slightly decreased the protein level of p21, the transcript of which is also a target of HuR [34]. However, HuR knockdown had little if any effect on PUMA expression in MCF10A cells (Fig. 3A). These results were further confirmed in MCF10A with transient HuR knockdown (Fig. 3B). Thus, due to lack of correlation between cell proliferation and levels of p53 and p21, we conclude that both p53 and p21 are unlikely to play a role in premature senescence and deficient cell proliferation in MCF10A cells with HuR knockdown.

Previously, we showed that ΔNp63 isoforms, especially ΔNp63β, possess a remarkable ability to transactivate target genes and suppress cell proliferation [35,36]. MCF10A cells express a high level of ΔNp63 [23]. Thus, we examined whether ΔNp63 plays a role in HuR-mediated premature senescence and cell proliferation. We found that the protein levels of ΔNp63α and ΔNp63β were increased in MCF10A cells with HuR knockdown (Fig. 4A). Interestingly, we found that expression of GADD45, a target of ΔNp63 and a mediator for growth suppression, was also increased in MCF10A cells upon HuR knockdown (Fig. 4A). Consistent with this, we found that transient HuR knockdown obviously increased the expression of ΔNp63 and GADD45 in MCF10A cells (Fig. 4B).

To assess whether ΔNp63 is sufficient to mediate HuR function in cell proliferation, we tested several concentrations of p63 siRNA to counteract the upregulation of ΔNp63 induced by HuR knockdown in MCF10A-HuR-KD-26 cells. We found that 30 nM p63 siRNA efficiently reduced the levels of ΔNp63α and ΔNp63β proteins in MCF10A-HuR-KD-26 cells to the basal levels of ΔNp63α and ΔNp63β proteins in control cells (Fig. 4C). Thus, 30 nM p63 siRNA was chosen for colony formation assay in MCF10A-HuR-KD-26 cells. We found that ΔNp63 knockdown partially rescued the proliferative defect induced by HuR knockdown in MCF10A-HuR-KD-26 cells. The number of colonies formed by MCF10A-HuR-KD-26 cells with ΔNp63 knockdown was only reduced to 76% of the control cells (Fig. 4D), compared to 28% in MCF10A-HuR-KD-26 cells without ΔNp63 knockdown (Fig. 4C-D). Furthermore, we found that ΔNp63 knockdown made MCF10A-HuR-KD-26 cells resistant to camptothecin treatment. The number of colonies was only reduced to 60% of the control cells (Fig. 4D), compared to 20% in MCF10A-HuR-KD-26 cells without ΔNp63 knockdown (Fig. 1C–D). Together, these data suggest that ΔNp63, but not p53, may at least in part mediate HuR-knockdown-induced growth suppression and premature senescence in MCF10A mammary epithelial cells.
HuR Binds to U-rich Elements in 3' UTR of ΔNp63 Transcript

It is known that HuR functions as a RNA binding protein and binds to AU-/U-rich elements in 3' UTRs of its target mRNAs [5]. Thus, we explored whether HuR regulates ΔNp63 expression via directly binding to its transcript. For this purpose, RNA electrophoretic mobility shift assay (REMSA) was performed to identify potential HuR binding regions in ΔNp63 transcript. First,
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A. LacZ-KD-4  
B. HuR-KD-6  
C. HuR-KD-26  

D.  

E.  

F. To-Pro-3  
Laminin V  
Merged  

HuR-KD-4  
HuR-KD-26  

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HuR Knockdown Enhances \(\Delta Np63\) mRNA Translation but has no Effect on mRNA Turnover

HuR protein is best-known for its function in the regulation of mRNA stability. Thus, we explored whether HuR knockdown has an effect on the level of \(\Delta Np63\) mRNA. We found that the level of \(\Delta Np63\) mRNA was not significantly altered by HuR knockdown in MCF10A cells (Fig. 6A). HuR protein is also known to suppress translation of its targets without affecting mRNA turnover, such as Wnt-5a [16] and BRCA1 [15]. Thus, we examined whether HuR knockdown increases the rate of \(\Delta Np63\) mRNA translation. To test this, we labeled newly synthesized \(\Delta Np63\) protein with \(^{35}\)S-methionine for a short period of 30 min in MCF10A-LacZ-KD and MCF10A-HuR-KD cells. We found that the level of newly synthesized \(\Delta Np63\) protein was obviously increased in MCF10A cells with HuR knockdown (Fig. 6B, compare lane 5 with 6). Together, our data suggest that HuR regulates \(\Delta Np63\) expression via mRNA translation in MCF10A cells.

Discussion

Here, we found that in MCF10A cells, HuR knockdown inhibits cell proliferation and enhances premature senescence. In addition, we found that in 3-D culture, MCF10A cells with HuR knockdown develop abnormal acinar architectures. Furthermore, we showed that HuR knockdown increases \(\Delta Np63\), but decreases wild-type p53, expression in the MCF10A cells. Correspondingly, we showed that \(\Delta Np63\) knockdown partially rescues the proliferative defect induced by HuR knockdown in MCF10A cells. Consistent with this, we found that HuR can specifically bind to two U-rich regions in 3’ UTR of p63 mRNA and consequently inhibits \(\Delta Np63\) expression via translation. Thus, our data suggest that HuR is necessary for maintaining cell proliferation and polarity of MCF10A cells at least in part via regulating \(\Delta Np63\) expression.

p63, a member of the p53 family, is expressed as two major groups, the TA and \(\Delta N\) variants [37,38]. The TA variant, which is expressed from the upstream promoter, contains an activation domain similar to that in p53 [38]. Thus, \(\Delta Np63\) has a strong transcriptional activity and is capable of inducing cell cycle arrest and apoptosis when overexpressed [37,38]. In contrast, the \(\Delta Np63\) variant, which is expressed from a promoter in intron 3, lacks such an activation domain [38] but obtains 14 unique residues at N terminus. These 14 amino acids together with the adjacent proline-rich region constitute an activation domain for the \(\Delta N\) variant [35]. \(\Delta Np63\) isoforms have been highlighted to possess oncogenic potential and act as dominant-negative molecules against both TA\(p63\) isoforms and p53 [38]. However, many studies also suggest that \(\Delta Np63\) acts as a tumor suppressor. \(\Delta Np63\), especially \(\Delta Np63\)B, possesses a remarkable ability to transactivate target genes and suppress cell proliferation [35,36]. In mammary progenitor cells, knockdown of \(\Delta Np63\) results in genomic instability and increased cell proliferation [39]. In addition, depletion of both \(\Delta Np63\) and \(\Delta Np63\)B results in epithelial to mesenchymal transition in MCF10A cells, which can be rescued by expression of \(\Delta Np63\)B [40]. Consistent with this,
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A. MCF10A

|            | LacZ-KD-4 | HuR-KD-6 | HuR-KD-26 |
|------------|-----------|----------|-----------|
| ΔNp63α     | 1.0       | 2.6±0.2* | 4.5±1.1*  |
| ΔNp63β     | 1.0       | 2.3±0.1* | 5.4±0.8*  |
| GADD45     | 1.0       | 2.1±0.4* | 4.1±1.1*  |
| Actin      | 1.0       | 2.0       | 4.1±1.1*  |

B. MCF10A

|            | Scr | HuR siRNA (3d) |
|------------|-----|----------------|
| ΔNp63α     | 1   | 3.6±0.9*       |
| GADD45     | 1   | 2.3±0.6*       |
| Actin      | 1   | 2.0             |

C. 10 nM 20 nM 30 nM

|            | Scr | Scr | p63 | Scr | Scr | p63 | Scr | Scr | p63 | siRNA |
|------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-------|
| LacZ-KD-4  |     |     |     |     |     |     |     |     |     |       |
| HuR-KD-26  |     |     |     |     |     |     |     |     |     |       |
| HuR-KD-26  |     |     |     |     |     |     |     |     |     |       |
| LacZ-KD-4  |     |     |     |     |     |     |     |     |     |       |
| HuR-KD-26  |     |     |     |     |     |     |     |     |     |       |
| HuR-KD-26  |     |     |     |     |     |     |     |     |     |       |
| LacZ-KD-4  |     |     |     |     |     |     |     |     |     |       |
| HuR-KD-26  |     |     |     |     |     |     |     |     |     |       |
| HuR-KD-26  |     |     |     |     |     |     |     |     |     |       |
| HuR        |     |     |     |     |     |     |     |     |     |       |
| ΔNp63α     |     |     |     |     |     |     |     |     |     |       |
| ΔNp63β     |     |     |     |     |     |     |     |     |     |       |
| Actin      |     |     |     |     |     |     |     |     |     |       |

D. LacZ-KD-4 Scr siRNA HuR-KD-26 p63 siRNA

|            | No treatment | No treatment CPT |
|------------|--------------|------------------|
| LacZ-KD-4  | Scr siRNA    |                  |
| HuR-KD-26  | Scr siRNA    |                  |
| p63 siRNA  |              |                  |

The number of colonies > 1 mm

No treatment: 76% ± 2%
No treatment CPT: 60% ± 2%

Legend:
- LacZ-KD-4 + Scr siRNA
- HuR-KD-26 + p63 siRNA
ΔNp63 expression is found to be repressed in breast tumors [41]. Studies showed that ΔNp63 is the predominant isoform in MCF10A mammary epithelial cells, whereas TAp63s are expressed at very low levels and undetectable by immunoblot [23,42]. Here, we found that HuR knockdown obviously increases ΔNp63, but moderately decreases wild-type p53, expression in MCF10A cells. In addition, knockdown of both ΔNp63α and ΔNp63β partially rescues the proliferative defect induced by HuR knockdown in MCF10A cells. Thus, upregulation of ΔNp63, especially ΔNp63β, in MCF10A mammary epithelial cells may actually transactivate growth-suppressing genes, such as GADD45, to suppress cell proliferation, rather than act as dominant-negative molecules against TAp63 and p53.

HuR has numerous functions mostly related to cell stress response. However, emerging evidence showed that HuR plays a role in the processes of differentiation and development, such as lung branching morphogenesis, placental branching morphogenesis, spermatogenesis, myogenesis and adipogenesis [43,44,45,46,47]. In this study, we found that in 3-D culture, MCF10A cells with HuR knockdown formed an abnormal acinus with filled lumen and an aberrant expression pattern of laminin V, suggesting that HuR is required for development of a polarized acinus-like architecture in mammary epithelial cells. This effect of...

**Figure 4.** HuR knockdown increases ΔNp63 expression in MCF10A cells. (A) Western blots were prepared using extracts from MCF10A-LacZ-KD cells (lane 1) and MCF10A-HuR-KD cells (lanes 2 and 3). The blots were probed with antibodies against p63, GADD45, and actin, respectively. Experiments were performed in triplicates. The basal levels of ΔNp63α, ΔNp63β, and GADD45 were arbitrarily set at 1.0 and the fold change was shown below each lane. Asterisk indicates a significant difference (P<0.05). (B) Western blots were prepared using extracts from MCF10A cells transiently transfected with scrambled siRNA or siRNA to knock down HuR for 3 days, and then probed with antibodies against p63, GADD45, and actin, respectively. Quantification and statistical analysis were performed as in (A). (C) Western blots were prepared using extracts from MCF10A-LacZ-KD-4 and MCF10A-HuR-KD-26 cells, which were transiently transfected with various concentrations of scrambled siRNA or p63 siRNA for 3 days, and then probed with antibodies against HuR, p63, and actin, respectively. (D) Left panel, colony formation assay was performed with MCF10A-LacZ-KD-4 and MCF10A-HuR-KD-26 cells, which were transfected with 30 nM of scrambled siRNA and p63 siRNA, respectively. After 1 day, the cells was split into 6-well plates, and then treated with or without 50 nM camptothecin for 4 h at day 2. Right panel, quantification of colonies shown in left panel from three separate experiments. Asterisk indicates a significant difference (P<0.05).

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**Figure 5.** HuR binds to U-rich elements in 3'UTR of ΔNp63 transcript. (A) Schematic presentation of p63 3'UTR and probes used for REMSA. (B) REMSA was performed by mixing 32P-labeled RNA probe a, b, or c with recombinant GST or GST-fused HuR. The bracket indicates RNA-protein complexes. (C) REMSA was performed by mixing 32P-labeled RNA probe c1, c2, c3, or c4 with recombinant GST or GST-fused HuR. The bracket indicates RNA-protein complexes. (D) For competition assay, an excess amount of unlabeled p21 cold probe was added to a reaction mixture containing HuR and probe c. The bracket indicates RNA-protein complexes. (E) For supershift assay, 3 μg of control IgG or anti-HuR was added to a reaction mixture containing probe c with or without GST-HuR.

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Figure 6. HuR Knockdown enhances ΔNp63 mRNA translation without affecting mRNA turnover. (A) RT-PCR was performed with total RNAs isolated from MCF10A cells transiently transfected with scrambled siRNA or siRNA to knock down HuR for 3 days. (B) HuR knockdown increased the newly synthesized ΔNp63 protein. Cell extracts were prepared from MCF10A-LacZ-KD or MCF10A-HuR-KD cells labeled with [35S]methionine for 30 min. The levels of ΔNp63 protein were measured by scintillation counter and an equal amount of 35S-labeled proteins was used for immunoprecipitation. 35S-labeled proteins were measured by scintillation counter and an equal amount of ΔNp63 protein was immunoprecipitated with mouse anti-p63 monoclonal antibody or a control IgG and visualized by autoradiography. Input was used as a loading control. Experiments were performed in triplicates. The level of ΔNp63 in MCF10A-LacZ-KD-4 cells was arbitrarily set at 1.0 and the fold change in MCF10A-HuR-KD-26 cells was shown below the lane.

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Materials and Methods

Reagents

Growth factor-reduced Matrigel was purchased from BD (Franklin Lakes, NJ). Donor horse serum, DMEM/F12 media and To-Pro-3 were purchased from Invitrogen (Carlsbad, CA). Recombinant human epidermal growth factor was purchased from Peprotech (Rocky Hill, NJ). Hydrocortisone, insulin and cholera toxin were purchased from Sigma (St. Louis, MI). Rabbit anti-p53(FL-393), mouse anti-p63(A44), rabbit anti-GADD45α(C-20) and rabbit anti-p21(C-19) were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Rabbit anti-PUMA was purchased from ProSci Incorporated (Poway, CA). Rabbit anti-actin was purchased from Sigma (St. Louis, MI); Mouse Anti-laminin V (D4B5) antibody was purchased from Millipore (Temecula, CA). A small interference RNA (siRNA) against HuR, 5′-GGG AUU AAU AAG UAG CAC A dTdT-3′, a siRNA against p63, 5′-CGA CAG UCU UGU ACA AUU U dTdT-3′, and a scrambled siRNA, 5′-GCA GUG UCU CGU ACU A dTdT-3′, were purchased from Dharmacon RNA Technologies (Chicago, IL).

To generate a construct expressing HuR short hairpin RNA (shRNA), one pair of oligonucleotides (sense, 5′-TCG AGG TCC GGG ATA AAG TAG CAC GTA ATT CAA GAG GAT TGG TGC TAC TAT TAT ATC CCT TTT TG-3′, and antisense, 5′-GAT GCC AAA AGG GAT AAA GTA GGA GGA CAT CCT TTG AAT GTG CTC GTG CTA CTT TAT CCC GGA CC-3′; shRNA targeting region is underlined) was synthesized and cloned into pBabe-U6 at BamHI and XhoI sites. The plasmid is designated pBabe-U6-shHuR. Similarly, a construct expressing LacZ shRNA, pBabe-U6-shLacZ, was generated with one pair of oligonucleotides (sense, 5′-TCG AGG TCC GGG ATA AAG TAG CAC GTA ATT CAA GAG GAT TGG TGC TAC TAT TAT ATC CCT TTT TG-3′, and antisense, 5′-GAT GCC AAA AGG GAT AAA GTA GGA GGA CAT CCT TTG AAT GTG CTC GTG CTA CTT TAT CCC GGA CC-3′; shRNA targeting region is underlined).

HuR knockdown on acinus formation is at least in part mediated by increased ΔNp63 expression, because p63 has an essential role in epithelial development [19,48]. p63<sup>−/−</sup> mice lose all stratified epithelia and their derivatives, including epidermis and mammary glands [19]. Significantly, germline p63 mutations in human are also associated with similar developmental syndromes [49]. In addition, p63 is a key regulator of cell adhesion in mammary epithelial cells. Down- or up-regulation of ΔNp63 caused a profound dysregulation of adhesion-related genes [42,50]. Thus, the ability of p63 to regulate matrix adhesion could play an important role in maintenance of polarity and integrity of mammary epithelial cells and tissues [51]. However, we can not exclude possibility that other pathways are also involved in formation of abnormal acini in HuR-knockdown MCF10A cells, since HuR regulates other targets [5].

HuR is capable of enhancing or suppressing mRNA translation, such as p53 [32], cytochrome c [52], Wnt-3a [16] and BRCA1 [15]. In this study, we found that HuR binds to two U-rich elements in p63 3′ UTR and inhibits p63 mRNA translation. However, the precise mechanism is still not understood. Recently, we showed that p63 mRNA stability can be regulated by RNPC1, a RNA-binding protein, which specifically bound to AU-U-rich elements in p63 3′ UTR [20]. Considering that both HuR and RNPC1 prefer to bind to U-rich elements in the distal region of p63 3′ UTR, it is possible that HuR may cooperate with other RNA binding proteins, such as RNPC1, to regulated p63 expression at different posttranscriptional levels. Thus, future studies are warranted to explore how HuR regulates p63 expression by modulating translation complexes or cooperating with other RNA binding proteins.
immunostaining of laminin V, the fixed 3-D structures were
temperature. The 3-D structures were mounted under glass
and quenched with 100 mM glycine in PBS. Then, the 3-D
growth medium with 2% Matrigel and allowed to grow for 19
days. The medium containing 2% Matrigel was refreshened every
Matrigel-coated chamber slides at 3500 cells/well in complete
KD.

antibody. The positive cell lines were designated MCF10A-LacZ-
shRNA were confirmed by Western blot analysis with anti-HA
pBabe-U6-shLacZ was transfected into MCF10A cells. Puromy-
shHuR was transfected into MCF10A cells. HuR knockdown cell
RT-PCR Assay
Total RNAs were isolated with Trizol reagent (Invitrogen).
cDNAs were synthesized using Iscript™ cDNA synthesis kit (Bio-
Rad). To measure the level of ΔNp63 mRNA, RT-PCR was
performed with forward primer 5'-TGG CAA AAT CCT GGA
GCC AG-3' and reverse primer 5'-GTC TGT GTT ATA GGG
AAT GGA GG-3'. Actin mRNA was amplified with forward primer 5'-
TGA TCC ACA TCT GCT GGA AG-3'.

Colony Formation Assay
To determine whether HuR knockdown affects the proliferation
of MCF10A cells, MCF10A-HuR-KD or MCF10A-LacZ-KD
cells (600 cells/well) were cultured in 6-well plates for the 9 days,
and then fixed with methanol/glacial acetic acid (7:1) and stained
with 0.1% of crystal violet.

Confocal Microscopy
The 3-D structures in Matrigel were fixed with 4% parafor-
malddehyde at room temperature for 20 min. The nuclei were
stained with 5 µg/ml of To-Pro-3 in PBS for 15 min at room
temperature. The 3-D structures were mounted under glass
coverslips with 0.1% PDD and 90% glycerol in PBS. For
immunostaining of laminin V, the fixed 3-D structures were
permeablized with 0.5% Triton X-100 in PBS for 30 min at 4°C
and quenched with 100 mM glycine in PBS. Then, the 3-D
structures were blocked with buffer A (130 mM NaCl, 7 mM
Na2HPO4, 3.5 mM NaH2PO4, 0.1% BSA, 0.2% Triton X-100,
0.05% Tween 20, and 10% normal goat serum) for 2 h and
further blocked with buffer B (buffer A plus 20 mg/ml goat anti-
mouse F(ab')2 fragments) for 1 h. The 3-D structures were
incubated with anti-laminin V antibody overnight at 4°C. After
washed, the 3-D structures were stained with FITC-conjugated
secondary antibody for 1 h. The nuclei staining and mounting of
slides were performed as above. The images of acinar structures
were captured by the Z-stacking function for serial confocal
sectioning at 2 µm intervals (LSM-510 Carl Zeiss Laser Scanning
Microscope) and then analyzed by Carl Zeiss software.

RNA Electrophoretic Mobility Shift Assay (REMSA)
Various regions in p63 3’ UTR were amplified by PCR with
primers containing T7 promoter sequence (5’-GGA TCC TAA
TAG GAC TCA GTA TAG GGA G-3’). REMSA was performed
as previously described [34]. Briefly, RNA probes were made from
in vitro transcription by T7 RNA polymerase in the presence of
[α-32P]-UTP. REMSA was performed with 200 nM of recombinant
protein, 1 mg/ml of yeast tRNA and 50000 CPM [32P]-labeled
RNA probe in a reaction buffer (10 mM Tris-Cl, pH 7.5, 25 mM
KCl, 5 mM MgCl2, 1 mM DTT) for 20 min at 37°C. RNA/ protein complexes were digested with 100 U R Nase T1 for
10 min at 37°C and then separated in 6% of native PAGE. RNA-
protein complexes were visualized by autoradiography. For
supershift assay, 3 µg of anti-HA antibody was pre-incubated
with HA-tagged proteins for 30 min on ice prior to incubation
with a RNA probe.

SA-β-galactosidase Staining Assay
This assay was performed as described previously [53]. Cells
were washed with phosphate-buffered saline and fixed with 2%
formaldehyde and 0.2% glutaraldehyde for 10 min at room
temperature. Cells were then washed twice with phosphate-
buffered saline and stained with fresh SA-β-galactosidase staining
solution at 37°C. The SA-β-galactosidase staining solution
contains 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyra-
noside, 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM
potassium ferriyanide, 5 mM potassium ferrocyanide, 150 mM
NaCl, and 2 mM MgCl2.

Measurement of Newly Synthesized ΔNp63
To measure newly synthesized ΔNp63 protein, immunoprecip-
itation was performed with extract from MCF10A-LacZ-KD and
MCF10A-HuR-KD cells, which were culture for 24 h, and then
labeled with [35S]-methionine for 30 min.

Statistics
All experiments were performed at least in triplicates. Numer-
ical data were expressed as mean ± SD. Two group comparisons
were analyzed by two-sided Student’s t test. P values were
calculated and P<0.05 was considered significant.

Author Contributions
Conceived and designed the experiments: WY XC. Performed
the experiments: WY YZ JZ. Analyzed the data: WY XC. Contributed
reagents/materials/analysis tools: YZ JZ SC. Wrote the paper: WY XC.

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