Intestinal Epithelial HuR Modulates Distinct Pathways of Proliferation and Apoptosis and Attenuates Small Intestinal and Colonic Tumor Development

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Abstract

HuR is a ubiquitous nucleocytoplasmic RNA-binding protein that exerts pleiotropic effects on cell growth and tumorigenesis. In this study, we explored the impact of conditional, tissue-specific genetic deletion of HuR on intestinal growth and tumorigenesis in mice. Mice lacking intestinal expression of HuR (HurIKO mice) displayed reduced levels of cell proliferation in the small intestine and increased sensitivity to doxorubicin-induced acute intestinal injury, as evidenced by decreased villus height and a compensatory shift in proliferating cells. In the context of Apcmin mice, a transgenic model of intestinal tumorigenesis, intestinal deletion of the Hur gene caused a three-fold decrease in tumor burden characterized by reduced proliferation, increased apoptosis, and decreased expression of transcripts encoding antiapoptotic HuR target RNAs. Similarly, HurIKO mice subjected to an inflammatory colon carcinogenesis protocol [azoxymethane and dextran sodium sulfate (AOM-DSS) administration] exhibited a two-fold decrease in tumor burden. HurIKO mice showed no change in ileal Asbt expression, fecal bile acid excretion, or enterohepatic pool size that might explain the phenotype. Moreover, none of the HuR targets identified in Apcmin HurIKO were altered in AOM-DSS–treated HurIKO mice, the latter of which exhibited increased apoptosis of colonic epithelial cells, where elevation of a unique set of HuR-targeted proapoptotic factors was documented. Taken together, our results promote the concept of epithelial HuR as a contextual modifier of proapoptotic gene expression in intestinal cancers, acting independently of bile acid metabolism to promote cancer. In the small intestine, epithelial HuR promotes expression of prosurvival transcripts that support Wnt-dependent tumorigenesis, whereas in the large intestine epithelial HuR indirectly downregulates certain proapoptotic RNAs to attenuate colitis-associated cancer. Cancer Res; 74(18); 5322–35. ©2014 AACR.

Introduction

HuR is a ubiquitously expressed member of the Elav family of RNA-binding proteins (1), whose regulation of target mRNA stability and/or translation reflects binding to cis-acting AU-rich elements within the 3′-untranslated region (UTR) of selected mRNAs (2). Transcriptome-wide analysis using RNA-protein crosslinking of unstimulated HeLa cells identified approximately 26,000 HuR binding sites, mainly localized in 3′-UTR regions but that also included intronic sites (3). Those studies further suggested that HuR interacts with more than 4,800 genes, corresponding to approximately half of all HeLa transcripts (3). siRNA knockdown of HuR demonstrated significantly decreased mRNA expression and concomitantly reduced protein synthesis of the majority of these candidate targets, placing HuR as a central player in a large network of factors that modulate posttranscriptional gene expression (3). This vast range of HuR targets identified in HeLa cells raises the question of whether there exists a cell autonomous and/or developmental-stage–specific hierarchy that modulates HuR-dependent pathways of gene expression in vivo and in particular how HuR might influence the development and progression of disease (4).

Several studies have correlated elevated cytoplasmic HuR expression with cancer initiation and progression in various types of human cancer cells, including lung (5), ovarian, breast, gastric, pancreatic, and colon cancers (6–8). HuR analyzes in paired human normal and colon cancer revealed increased total HuR expression in tumor samples with increased cytoplasmic staining compared with the corresponding normal
tissue (6). Similar findings of increased cytoplasmic HuR staining were observed in squamous esophageal cancer, where this pattern of expression was associated with decreased survival (9). These findings strongly suggest that HuR may play an important role in cancer initiation and progression and underscore the need for greater understanding of its cell- and tissue-specific roles in vivo.

Germline HuR deletion in mice leads to embryonic lethality as a result of impaired placental development (10), necessitating the use of conditional deletion strategies to examine the loss-of-function phenotype. However, global HuR deletion in adult mice also led to rapid lethality, associated with critical defects in hematopoietic progenitor cell production as well as defective intestinal stem cell dynamics and spontaneous villus atrophy (11). Those findings suggested that HuR plays a key role in intestinal epithelial growth and maintenance. More recent work used a conditional macrophage-specific HuR deleter line to reveal increased sensitivity to systemic inflammation and chemical colitis with enhanced secretion of pro-inflammatory cytokines (TNF, IL6, IL1b, IL12) followed by progression to colitis-associated cancer (12).

Here we generated conditional intestine-specific HuR knockout mice (HuR<sup>fl</sup>fl<sup>C</sup>) and demonstrate that epithelial HuR is important for intestinal homeostasis. We generated compound Apc<sup>Min</sup>+/<sup>HuR</sup>fl<sup>C</sup> mice and demonstrate that intestinal HuR deletion attenuates small intestinal polyposis. We further show that HuR<sup>fl</sup>fl<sup>C</sup> mice exhibit 2-fold reduced colon tumor burden following azoxymethane and dextran sodium sulfate (AOM-DSS) administration. Both models reveal that intestinal HuR deletion protects against tumorigenesis by promoting apoptosis, but via regulation of cell-type-specific transcripts.

Materials and Methods

**Animals and experimental procedures**

All mice protocols were approved by the Washington University Animal Studies Committee and conformed to criteria outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All mice were housed in a specific pathogen-free animal facility under standard 12:12-hour light–dark cycle and were fed standard rodent Lab Chow (no. 5001; Purina Mills) and tap water ad libitum. Conditional intestine-specific HuR deleter mice (HuR<sup>fl</sup>/<sup>HuR</sup>fl<sup>C</sup>) were generated by intraperitoneal injection of 35-day-old mice with 1 mg tamoxifen (T5648; Sigma-Aldrich) per g body weight doxorubicin (AOM; A5486; Sigma-Aldrich). One week later, 2.5% DSS (MW 40000–50000 9011-18-1; Affymetrix Inc.) was provided in drinking water for 5 days, followed by 15 days of regular water. This cycle was repeated two more times; each time the DSS was provided for 7 days. Mice were sacrificed 12 weeks after the last DSS cycle. Tissues were collected and flash frozen for further RNA and protein analysis. Intestines were pinned and fixed in 10% formalin for scoring with a Nikon SMZ800 dissecting microscope and photographed using a Photometrics CoolSNAPcf camera (Imaging Processing Services, Inc.). Measurements including width, length, and total area were performed using Metavue software (Molecular Devices). Mice were injected intraperitoneally 2 hours before sacrifice with 200 µL bromodeoxyuridine (BrdUrd) solution (20 mg/mL; B5002; Sigma-Aldrich).

**Determination of bile acid pool size and fecal bile acid output**

Bile acid pool size was determined from the total content of the entire small intestine, gallbladder, and liver (14). Total bile acid mass was determined enzymatically (431-15001; Wako Chemicals) and fecal bile acid output was determined from stool collected from individually housed mice for 72 hours (14, 15). Where indicated, mice of different genotypes were fed a diet containing 2% cholestyramine (C4650; Sigma-Aldrich) for 10 days, and fecal bile acid excretion determined.

**Immunohistochemical analysis**

Immunohistochemical analysis for H&E, HuR, OLFM4, BrdUrd, TUNEL, and Alcian blue/potent acid-Schiff’s reagent (AB/PAS) were conducted on formalin-fixed paraffin-embedded tissues. Sections (4 µm) were stained with the following antibodies: anti-HuR primary antibody 1:200 (SC 5261; Santa Cruz Biotechnology); rabbit anti-OLFM4 1:100 (ab96280; Abcam Inc.) rat anti-BrdUrd 1:300 (Accurate Chemical & Scientific Corp.). Apoptosis was analyzed by TUNEL staining following the ApopTag Peroxidase In Situ Oligo Ligation (ISOL) Apoptosis Detection Kit (S7200; Chemicon International). Intestinal proliferation was determined by scoring full longitudinal sections of crypts and reported as the number of BrdUrd-positive cells normalized to the total number of cells per crypt. Similarly, the apoptotic index evaluates the number of TUNEL-positive cells normalized to the total number of cells in crypt and villi. Goblet cells were visualized using AB/PAS and counterstained with hematoxylin. For each animal, the number of Goblet cells was determined as the percentage of total number of cells per crypt. Similarly, the number of AB/PAS-stained Goblet cells per villus was counted and expressed as the percentage of the total villus cell number. For scoring cell position, each crypt was divided in half and cells were numbered sequentially from crypt base to crypt–villus junction, with cell position one being occupied by the first cell at the base of each half crypt (13). Villus length and crypt depth were evaluated on hematoxylin and eosin (H&E)-stained sections using Axios Imager software on images captured with an Axios Imager A1 and an AxiosCam MRC 5 high-resolution camera (Carl Zeiss Microimaging).

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Electron microscopy

Two-millimeter segments of the jejunum were flushed with PBS followed by immersion in 3% glutaraldehyde (EM grade), 1% formaldehyde (EM grade) in 0.1 mol/L sodium cacodylate (pH 7.4), 2 mmol/L CaCl₂, and 2 mmol/L MgCl₂. Specimens were stained in 2% aqueous uranyl acetate, and viewed on a Hitachi H-600 electron microscope (Hitachi High Technologies). Three animals per genotype were examined to obtain representative images.

Protein extraction and Western blotting

Scraped mucosa was homogenized in tissue lysis buffer containing 20 mmol/L Tris (pH 8); 0.15 mol/L NaCl; 2 mmol/L EDTA; 1 mmol/L sodium vanadate; 0.1 mol/L sodium fluoride; 50 mmol/L β-glycerophosphate; 5% glycerol, 2× protease inhibitor (Roche Applied Science); 1% Triton; and 0.1% SDS. Homogenate (60-μg protein) were resolved by 10% SDS–PAGE, transferred to PVDF membrane, and probed with mouse anti-HuR antibody (3A2; Sc5261; Santa Cruz Biotechnology Inc.), mouse anti-cyclin D1 (Mouse MAb; NeoMarkers), rabbit anti-C–Myc (Sc-764; Santa Cruz Biotechnology Inc.), rabbit anti-OLFM4 (ab96280; Abcam Inc.), and a rabbit anti–β-catenin antibody (9582; Cell Signaling Technology). Equal loading was verified using a rabbit anti–α-tubulin antibody (A2066; Sigma-Aldrich) or rabbit anti-Hsp40 (SPA-400; Assay designs).

RNA extraction and quantitative PCR analysis

Total RNA was isolated from scraped mucosa (proximal, middle, distal intestine, and colon) using TRIzol (Ambion), and used to synthesize cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR (qPCR) was conducted in triplicate on an ABI Step One Plus Detection System (Applied Biosystems) using SYBR GreenER qPCR SuperMix (Invitrogen).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software, Inc.) and Microsoft Excel. Data are reported as mean ± SEM. All data were subjected to one-way ANOVA and Mann–Whitney test. All data underwent two-tailed Student t testing as deemed appropriate. For all comparisons, statistical significance was set at a P value of <0.05.

Results

Conditional intestinal HuR deletion alters intestinal epithelial proliferation

HuR expression was attenuated throughout the small intestine and colon in 10 to 12 weeks old HurKO mice with approximately 90% reduction in protein expression compared with HuR+/C2 mice (Fig. 1A). Immunohistochemical analysis showed HuR expression in nuclei of crypt, mature villus, and stromal cells of HuR+/C2 mice, with epithelial deletion in HurKO mice (Fig. 1B). HurKO mice exhibited shorter villi and crypts (Fig. 1C and D) and reduced microvillus length (Fig. 1E). HurKO mice also exhibited reduced proliferation, evidenced by decreased BrdUrd-positive epithelial cells throughout the entire small intestine (Fig. 1F). There was no change in intestinal apoptosis by genotype (data not shown). To understand the pathways that might be altered in this phenotype, we surveyed expression of RNAs encoding factors involved in cell growth and survival. We demonstrated upregulation of Tp53, C-myc, Cnd1, CcnB1, and CcnE1 mRNAs in HurKO mice (Fig. 1G) and a corresponding increase of CCND1 and C-Myc protein compared with HuR+/ mice (Fig. 1H and I). Together these observations suggest that intestinal epithelial HuR deletion is associated with alterations in the expression of genes regulating proliferation and cell-cycling programs.

Conditional intestinal HuR deletion alters intestinal epithelial morphology

AB staining revealed reduced goblet cells in the small intestine (Fig. 2A and C) and colon (Fig. 2B and C) of HurKO mice. These changes were associated with a 2-fold increase in Musashi1 (Msi1) and Hes-1 mRNA in HurKO mice (Fig. 2D), both of which have been implicated in regulating mammalian intestinal development (16, 17). The findings with Msi1 mRNA expression were unexpected, because HuR has been demonstrated to stabilize Msi1 RNA and promote its translation (18). However, because of the key role of Notch signaling in intestinal differentiation (19), we analyzed the effect of Hur deletion on the expression of both Notch1 receptor and of RBP-Jk, its downstream target. Both RNAs showed a trend toward increased expression in HurKO mice (Fig. 2D), suggesting that Notch-dependent regulation cannot be invoked as an explanation of the upregulation of Msi1 and Hes-1 expression in HurKO animals, implying that yet other pathways must also be in play. HurKO mice also showed a >60-fold increase in mRNA abundance of Olfm4 in small intestine (and a similar trend in colon), with approximately a 3-fold induction of Lgr5 RNA (Fig. 2D). The induction of Olfm4 mRNA was also confirmed in isolated enterocytes from HurKO mice (Fig. 2E). The induction of Olfm4 expression in HurKO mice was further confirmed by verifying increased Olfm4 protein in HurKO small intestine (Fig. 2F and G). These findings together suggest that intestinal HuR deletion alters intestinal homeostasis and Lgr5-dependent pathways of differentiation, most likely through Notch-independent mechanisms (20).

Aged HurKO mice exhibit normal intestinal proliferation and no alteration of cell growth–related factors

Because the effect of epithelial HuR deletion in young (~12-week-old) mice were rather subtle, we asked whether prolonged HuR deletion of over 1 year might lead to a more dramatic phenotype. However, this turned out not to be the case. Aged HurKO mice grew comparably to littermate controls (Fig. 3A). Also, despite evidence that intestinal HuR protein expression was still attenuated (Fig. 3B), small intestinal proliferation was no longer reduced in aged HurKO mice (Fig. 3C). Furthermore, the expression of RNAs encoding factors involved in intestinal homeostasis, cell growth, and survival—all of which were upregulated in young mice (Fig. 1G)—were unchanged at 1 year (Fig. 3D). These findings suggest that there is a temporal component to the adaptive effects of intestinal epithelial HuR deletion in modulating compensatory pathways involved in intestinal integrity.
HurIKO mice exhibit increased injury following doxorubicin administration

The findings to this point suggest that intestinal HuR deletion results in subtle alterations in basal small intestinal homeostatic regulation. We next examined the response of HurIKO mice to an acute chemical injury, in which intestinal morphology was examined at 72 (peak of injury) and 120 hours (regenerating phase) following doxorubicin injection (13). The findings reveal subtle yet significant increased injury in HurIKO mice, with decreased villus length compared with baseline.
Figure 2. Conditional intestinal HuR deletion alters intestinal epithelial morphology. HurIKO mice show reduced number of goblet cells and increased expression of stem cell markers. Representative photographs of AB-stained sections of ileum (A) and colon (B) from HurIKO and Hur<sup>ft</sup> mice. C, the number of Goblet cells was scored by surveying 4 to 12 small intestinal villi, 2 to 8 small intestinal crypts, and 3 to 9 colonic crypts. Results, mean ± SE and expressed as the percentage of AB-positive cells over total number of cells in villi and crypt, respectively (n = 4 to 7 mice/genotype). ***P < 0.0001; **P < 0.01. D, qRT-PCR analysis of expression of intestinal stem cells markers. RNA was prepared from distal scraped mucosa. Data, mean ± SE (n = 6 animals/genotype); *P < 0.05; **P < 0.001. E, quantitative analysis of Olfm4 RNA expression in enterocytes isolated from HurIKO and Hur<sup>ft</sup> mice. Data, mean ± SE (n = 5 Hur<sup>ft</sup> and 6 HurIKO); *P < 0.05. F, scraped mucosa protein extracts from three separate HurIKO and three separate controls; Hur<sup>ft</sup> were separated on SDS-PAGE and probed with anti-OLFM4 antibody. OLFM4 expression was normalized to actin and shown as OLFM4/actin ratio (right); *P < 0.05. G, immunohistochemical evaluation of OLFM4 shows increased expression in epithelial compartment from HurIKO small intestine (distal section) compared with Hur<sup>ft</sup> controls.
polyps/mouse in Apcmin 5B). Chemical validation of epithelial-specific HuR expression was confirmed by Western blot analysis of intestinal scraped mucosa from three individual HuR+/ and four HuR−/− mice. HuR was used as loading control.

C, quantitation of BrdUrd incorporation in the small intestine. Data, mean ± SE (n = 4 animals/genotype). D, qRT-PCR analysis of Olfm4 and cell growth–related factors. RNA was extracted from distal scraped mucosa. Data, mean ± SE (n = 4 animals/genotype).

Numerous studies in cancer cell lines have shown that RNAs encoding several antiapoptotic factors are targets of HuR and that RNA binding promotes mRNA stability and increases mRNA expression (11, 22–27). Accordingly, we hypothesized that HuR deletion may be associated with decreased expression of these candidates RNAs, which might in turn lead to activation of apoptotic pathways. qPCR analysis of antiapoptotic RNA (reported targets of HuR) revealed reduced

Intestinal HuR deletion attenuates polyposis in Apcmin/+ mice

The aforementioned results showing altered expression of cell-cycle genes and an increased proliferative response to acute injury in HuR−/− mice raised the question of whether intestinal HuR deletion would promote or attenuate intestinal tumorigenesis. To address this question, we initiated conditional HuR deletion in 5-week-old compound Apcmin/+ HuR−/− mice and examined spontaneous intestinal polyposis at 103 days, that is, after 68 days of HuR deletion. Using this protocol, we observed reduced polyp formation in Apcmin/+ HuR−/− mice compared with controls (Fig. 5A and B), with immunohistochemical validation of epithelial-specific HuR expression (Fig. 5B). Apcmin/+ HuR−/− mice exhibited a 60% reduction in tumor number (mean of 42 polyps/mouse Apcmin/+ HuR−/− vs. 102 polyps/mouse in Apcmin/+ HuR+/+ ) and a 70% decrease in tumor burden (estimated from total area) across the entire small intestine (Fig. 5C). The tumors in Apcmin/+ HuR−/− mice were also significantly smaller (Fig. 5C). Colonic polyp burden was also significantly reduced (~3-fold) in HuR−/− mice (data not shown). Further analysis revealed a trend toward decreased high-grade dysplasia in polyps from Apcmin/+ HuR−/− mice (Fig. 5D). These observations collectively suggest that deletion of intestinal epithelial HuR attenuates tumorigenesis. We further investigated whether perinatal deletion of epithelial HuR would alter tumor initiation. In this approach, we undertook conditional intestinal HuR deletion in 2-day-old Apcmin/+ HuR−/− mice and analyzed tumor burden 100 days later. Surprisingly, perinatal HuR-deleted animals showed no difference in tumor burden (or dysplasia) compared with control animals (Supplementary Fig. S1C and S1D), indicating that early postnatal epithelial HuR is not essential for small intestinal adenoma initiation.

We next turned to an examination of the potential pathways by which intestinal HuR deletion modifies the tumor susceptibility phenotype. Intestinal tissue (both normal, uninjured, and polyp) from Apcmin/+ HuR−/− mice demonstrated a 2-fold reduction in proliferation compared with control (Fig. 6A). In addition, TUNEL staining revealed a significant (~1.8-fold) increased apoptosis in normal tissues from Apcmin/+ HuR−/− mice compared with Apcmin/+ HuR+/+ controls (Fig. 6B). Apoptosis was significantly elevated (~5 fold) in HuR−/− polyps compared with HuR+/+ polyps (Fig. 6B). These data suggest that small intestine epithelial HuR influences both enterocyte proliferation and apoptosis, which in turn attenuates tumor susceptibility.

Numerous studies in cancer cell lines have shown that RNAs encoding several antiapoptotic factors are targets of HuR and that RNA binding promotes mRNA stability and increases mRNA expression (11, 22–27). Accordingly, we hypothesized that HuR deletion may be associated with decreased expression of these candidates RNAs, which might in turn lead to activation of apoptotic pathways. qPCR analysis of antiapoptotic RNA (reported targets of HuR) revealed reduced
expression of Bcl2l2, Xiap, Hif1α, mRNAs in both normal and tumor tissues from Apc<sup>min−/−</sup> Hur<sup>IKO</sup> mice compared with Apc<sup>min−/−</sup> Hur<sup>ff</sup> animals (Fig. 6C). Apc<sup>min−/−</sup> Hur<sup>IKO</sup> mice also demonstrated reduced β-catenin RNA and a corresponding decrease in protein expression (Ctnnb1; Fig. 6C and D). The downstream β-catenin target, survivin (Birc5) RNA was significantly downregulated in Hur<sup>IKO</sup> polyps (Fig. 6C), consistent with attenuated activation of Wnt-dependent signaling.

On the basis of the findings (above, Figs. 2 and 4) that expression of Olfm4 mRNA was upregulated in Hur<sup>IKO</sup> small intestine, we analyzed Olfm4 RNA expression in normal and polyp tissue from Apc<sup>min−/−</sup> Hur<sup>IKO</sup> mice. These findings revealed a 3-fold increase in Olfm4 mRNA in normal tissue from Apc<sup>min−/−</sup> Hur<sup>IKO</sup> compared with Hur<sup>ff</sup> mice (Fig. 6E). Olfm4 mRNA expression was decreased 5-fold in Hur<sup>IKO</sup> tumor, but was unchanged in polyp tissue from Apc<sup>min−/−</sup> Hur<sup>IKO</sup> mice.

Figure 4. Hur<sup>IKO</sup> mice exhibit increased injury following doxorubicin administration. Time-dependent changes in villus length and crypt depth following cytotoxic small intestinal injury. For all measurements, numbers of animals per genotype and per time points are as followed: 72 hours, n = 5 animals/genotype; 120 hours, 3 animals/genotype. Evaluations shown here are from the mid small intestine. A, villus length was measured 72 and 120 hours following doxorubicin injection (20–40 villi were measured at 72 and 120 hours postxorubicin injection). Measurements, mean ± SE; *, P < 0.05; **, P < 0.001. B, crypt depth was evaluated in 10 to 20 crypts from mice sacrificed 72-hour postxorubicin injection and in 15 to 20 crypts from mice sacrificed 120-hour postxorubicin. Crypt depth is significantly reduced in Hur<sup>IKO</sup> mice compared with Hur<sup>ff</sup> mice. Data, mean ± SE; **, P < 0.001. C, BrdUrd incorporation was evaluated 72 and 120 hours postxorubicin injection. Ten and 20 crypts per animals were analyzed at 72 and 120 hours postxorubicin, respectively. Proliferative cells counts are expressed as number of BrdUrd-positive cells per crypt (mean ± SE); *, P <0.05; **, P < 0.0001. D, the position of proliferative cells in the crypt was recorded during the damage phase (72 hours) and during repair phase (120 hours). Data, the percentage of BrdUrd-positive cells at each position relative to total number of cell in the crypts. E, qRT-PCR analysis of mid-jejunum mRNA expression at peak damage and repair phases. Data were normalized to Gapdh mRNA expression and reported as mean ± SE (n = 3 animals/genotype); *, P < 0.05.
further magnifying the differential induction of Olfm4 mRNA by genotype (Fig. 6E). In addition, the expression of Lgr5 mRNA was increased 4-fold in Hurf/f polyp compared with normal tissue. In contrast, Lgr5 mRNA was induced 1.4-fold in HurIKO tumors with attenuated mRNA expression compared with Hurf/f tumor (Fig. 6F). These findings collectively suggest that in a genetic background of spontaneous intestinal tumorigenesis (i.e., ApcMin/+) epithelial HuR may regulate actively proliferating stem cells, and in turn function to promote growth via induction of antiapoptotic factors.

Intestinal HuR deletion does not influence ASBT expression or bile acid homeostasis

Among the candidate intestinal mRNAs for which HuR has been implicated in regulating expression is the ileal sodium-dependent bile acid transporter (Asbt), with in vitro data suggesting that intestinal epithelial HuR may stabilize Asbt mRNA, increasing its expression and promoting bile acid absorption and recycling (28). This possibility is important because studies have linked altered bile acid metabolism with colorectal cancer (29, 30). However, we found no changes in ASBT gene expression or any parameter of enterohepatic bile acid metabolism in HurKO mice (Supplementary Fig. S2).

Intestinal HuR deletion protects against AOM-DSS cancer-associated colitis

We extended the phenotypic characterization of intestinal HuR deletion to yet another model of tumorigenesis, namely colitis-associated cancer. These studies were prompted in part by work indicating the key role of inflammatory cytokines in oncogenesis, including many cytokine mRNAs identified as targets of HuR (31, 32). In addition, other work demonstrated that myeloid-specific HuR expression modulates intestinal inflammation and carcinogenesis (12). Those findings demonstrated that myeloid-lineage-specific HuR deletion dramatically increased susceptibility to colitis-associated cancer, whereas myeloid transgenic HuR overexpression was protective (12). We deleted HuR in 35-day-old mice and exposed the HurKO mice to AOM followed by 3 cycles of DSS. HurKO mice began to die after the first cycle of DSS (1 animal out of 25; Fig. 7A). Five of 25 (20%) HurKO mice died after the third cycle of DSS. At the time of sacrifice (12 weeks after the last cycle of DSS), 48%
(12/25) of the treated HurKO mice died, a 52% survival rate. In contrast, 11% (3/27) of Hurf/f controls died 6 weeks after the last cycle DSS (Fig. 7A). At time of sacrifice, 10/27 Hurf/f mice died, a 63% survival rate. These findings suggest that HurIKO mice might be more sensitive to DSS exposure because they seem to die earlier than Hurf/f controls, despite a similar overall survival rate for the entire experiment (i.e., 52% vs. 63%).

Nevertheless, at the time of sacrifice (i.e., 161 days after Hur deletion), the surviving HurIKO mice manifested a reduced colonic tumor burden compared with Hurf/f mice (Fig. 7B and C). Western blot analysis of colon tissue verified...
Figure 7. Intestinal HuR deletion protects against AOM-DSS cancer-associated colitis. A, Kaplan–Meier survival curves of HurIKO and Hurff after exposure to AOM-DSS (n = 27 Hurff and 25 HurIKO mice). Arrows, tamoxifen-induced Hur knockout (week 5) followed by one injection of AOM (week 8) and three consecutive exposures to DSS (weeks 9, 12, and 15). Log-rank test (Mantel–Cox) was not significant (P = 0.137). B, gross morphology photographs of colon from Hurff and HurIKO 20 weeks after exposure to AOM-DSS. C, pinned colons were analyzed under dissecting microscope and surveyed for total polyp number and size relative to total colon area. HurIKO mice show a significantly reduced tumor burden; ***; P < 0.001. D, Western blot analysis of HuR in protein extracts prepared from full thickness normal (N) and tumor (T) tissues isolated from Hurff and HurIKO mice treated with AOM-DSS. Note the increased expression of HuR in tumor isolated from control animals. E, representative HuR staining of polyps in colon from Hurff and HurIKO mice. Note the complete absence of HuR in epithelial cells with staining confined to the stroma; scale bars, 50 μm. F, HurIKO mice treated with AOM-DSS show no alteration of cell proliferation. BrdUrd-positive epithelial cells were counted and data are represented as total number of BrdUrd-positive cells per high magnification power (×40). On average, 16 fields were evaluated per mouse, (n = 3–4 mice/genotype). Data, mean ± SE; ***; P < 0.001. G, HurIKO mice show increased apoptosis 20 weeks after AOM-DSS administration. Apoptotic cells were visualized by TUNEL staining and counted. Data, total number of TUNEL-positive epithelial cells per high-resolution field (magnification, ×40). Six to 10 fields were analyzed per mouse (n = 3–5 mice/genotype). Data, mean ± SE; *; P < 0.05; ***; P < 10−3. H, Following AOM-DSS exposure, HurIKO mice showed elevated expression of a subset of RNAs encoding proapoptotic factors. RNA was extracted from normal and tumor tissues. RNA expression was evaluated by qPCR and normalized to Gapdh RNA. Data, mean ± SE (n = 3–5/genotype); *; P < 0.05.
attenuated HuR expression in HurIKO mice (Fig. 7D). There was a subtle but reproducible increase in HuR protein expression in HurIKO tumor tissue compared with normal (Fig. 7D), consistent with the increased HuR expression previously reported in colorectal and other cancers (6, 7). Immunohistochemical analysis of HuR expression in HurIKO mice revealed staining restricted to stromal cells (Fig. 7E). Epithelial proliferation was increased in tumor tissue compared with normal colon in both genotypes but there was no difference in tumor proliferation by genotype (Fig. 7F). In contrast, HurIKO mice showed increased TUNEL-positive epithelial cells compared with AOM-DSS-treated HurIKO controls (Fig. 7G). These findings suggest that increased apoptosis in tumors from HurIKO mice may contribute to the attenuated tumor burden in colitis-associated cancer.

To investigate the pathways accounting for the increased apoptosis with HuR deletion, we analyzed the expression of pro- and antiapoptotic HuR RNA targets (11, 22–27). Two RNAs encoding factors with antiapoptotic activity, Sirt1 and Vegf, both of which have been demonstrated to undergo HuR-dependent modulation in other settings (33–35), were downregulated in normal tissue from HurIKO mice (Fig. 7H). Vegf-A RNA was also recently verified as an HuR target in mouse macrophages (36). Other proapoptotic factors (Tp53, Cdkn1a, Casp9, and Fas) showed unchanged RNA expression in normal tissues from HurIKO versus HurKO mice, but these mRNAs were significantly upregulated in HurIKO tumor tissues (Fig. 7H). The expression of these RNAs is also presented by genotype, comparing mRNA levels in tumor versus normal tissue (Supplementary Fig. S3). These observations together suggest that in the background of AOM-DSS–induced colitis-associated cancer, HuR deletion promotes tumor apoptosis by activation of pathways including the p53-dependent intrinsic apoptotic pathway as well as Casp9, Cdkn1a, and Fas.

Discussion

The central findings of this study are that conditional, intestinal epithelial–specific HuR deletion in adult mice produces a subtle and temporally dependent growth phenotype, with an exaggerated response to cytotoxic injury and attenuation of both spontaneous intestinal polyposis and colitis-associated cancer. The findings collectively highlight the role of HuR in modulating tissue- and cell-specific pathways of small intestinal and colonic epithelial homeostasis and also the role of epithelial HuR expression in the initiation and progression of intestinal tumorigenesis. Several elements of these overarching conclusions merit additional discussion.

Among the key observations was that conditional intestinal epithelial–specific HuR deletion in adult mice is not critical for survival. Earlier studies using tamoxifen-inducible Rosa26/Cre-mediated HuR deletion in young adult (8-week-old) mice demonstrated marked abnormalities in the gastrointestinal tract within 2 to 4 days of (global) HuR deletion and lethality within 10 days (11). In particular, those studies demonstrated villus atrophy, decreased crypt proliferation and goblet cell loss, findings reminiscent of those observed in this study (11). However, a major distinction in the observations from this study and those earlier findings is that intestinal atrophy with villus blunting in HurIKO mice was more modest and not progressive, compared with the global knockout model. These findings suggest that the gross morphologic disruption of both small intestine and colonic mucosal architecture following global HuR deletion may reflect the loss of stromal HuR expression in addition to the loss of enterocyte-specific HuR expression. It is worth noting that epithelial-specific HuR deletion virtually eliminated HuR protein expression (Fig. 1A and B) from scraped mucosal samples of small intestine and full thickness colonic extracts (both of which would include epithelial and stromal compartments), suggesting that even a small population of mesenchymal or stromal cells expressing HuR must play a crucial role in epithelial maintenance. The exaggerated response to acute cytotoxic small intestinal injury mediated by doxorubicin exposure underscores the role of HuR in maintaining intestinal homeostasis by controlling stem cell proliferation and subcellular lineage. Among the pathways and mediators implicated in these adaptive processes, we observed upregulation of TPS3 as observed earlier in the global HuR deletion experiments (11). However, we did not observe a change in Mdm2 expression in small intestine or colon of HurIKO mice (data not shown) as noted in early time points (48 hours) following global HuR deletion. In contrast, we observed increased expression of Musashi 1 in the small intestine of HurIKO mice whereas no changes were observed at 48 hours following global HuR deletion (11). Our observations contrast with the changes noted in the setting of global HuR deletion and strongly suggest that there is a temporal as well as a cell-specific (i.e., spatial) component to the adaptive pathways and phenotypes observed following intestinal HuR deletion.

Among the most highly altered mRNAs detected following intestinal HuR deletion, we observed a striking increase in olfactomedin4 (Olfm4) expression in both small intestine and colon. Olfm4 encodes a glycoprotein implicated in promoting cell proliferation as well as cell adhesion and migration (reviewed in ref. 37). Of particular relevance to this study, Olfm4 overexpression was detected in 90% of human colon cancer tumors compared with normal, unaffected tissue with increased expression in a variety of other solid tumors (38). However, yet other studies observed that Olfm4 also functions as a tumor suppressor and impairs metastasis in an orthotopic model of metastatic mouse melanoma, suggesting that the role of Olfm4 in cancer may be cell specific (39). We observed increased Olfm4 mRNA expression both at baseline and also in the adapting small intestine following doxorubicin-mediated injury, raising the important question of whether this transcript is a direct or indirect target of HuR and what (if any) effects are observed on the expression of its cognate protein product. A survey of the RNA array datasets from gain- and loss-of-HuR knockdown in HeLa and RKO cells did not report changes in Olfm4 RNA (3, 6). However, computational analysis of Par-Clip HuR RNA targets revealed a preference for AU-rich motifs and both mouse and human Olfm4 3'-UTR contain canonical
HuR motifs (UUUUUUU, UUUGUUU, UUUAUUU) as well as preferred AU-rich motifs (AUAUUAUA, AUUAAUUU) raising the possibility that Olfm4 may be an HuR RNA target. However, although Olfm4 mRNA was upregulated in intestine from young (12-week-old) HuRKO mice, there was no change in aged HuRKO mice, suggesting that there may be indirect, temporally regulated, mechanisms of Olfm4 regulation in the setting of HuR deletion.

The loss of goblet cells observed in HuRKO mice, coupled with the increased proliferation observed at 72 hours in response to doxorubicin injury (Fig. 3D), raised the possibility that HuRKO mice might be more tumor prone in the background of altered Wnt signaling (i.e., in the ApcMin/+ background). However, this turned out not to be the case. The findings demonstrated reduced small intestinal tumor burden in HuRKO mice, in association with reduced proliferation and increased apoptosis. These findings are consistent with earlier observations from global HuR deletion where decreased number of progenitor cells was also coupled with decreased proliferation and increased apoptosis (11). The observation that tumor burden in perinatal ApcMin/+ HuRKO mice was indistinguishable from control mice suggest that perinatal small intestinal epithelial HuR is not essential for adenoma initiation. Further studies focusing on mesenchymal/stromal HuR deletion will be required to examine the contribution of non-epithelial intestinal HuR in tumorigenesis and the cross talk between stromal and epithelial compartments in tumor initiation and progression (40–42).

These findings also begin to address our understanding of the role of intestinal HuR in colonocyte homeostasis and in colitis-associated cancer. HuR has been implicated in stabilizing Asbt mRNA, increasing its expression postnatally in rodents (43). However, we found no change in Asbt mRNA or protein expression and no changes in fecal bile acid output or enterohepatic pool size in HuRKO mice, under either basal conditions or with increased fecal bile acid losses. These findings make it extremely unlikely that any of the intestinal phenotypes observed in HuRKO mice are in any way related to altered bile acid metabolism.

We next turned to an examination of the role of intestinal HuR expression in the setting of colitis-associated cancer. Those studies were prompted by the results of studies in which transgenic overexpression of HuR in macrophages and myeloid cells attenuated colon inflammation whereas myeloid-specific HuR deletion enhanced endotoxemia and exaggerated the severity of colitis-associated cancer (12). These findings demonstrate that HuRKO mice manifest attenuation of colitis-associated cancer. The molecular mechanisms likely include reduced expression of the anti-apoptotic factors Vegf and Sirt1 in normal tissue, as well as enhanced expression of proapoptotic factors involved in the p53-dependent and death receptor pathways (Tp53, Casp9, Fas). The decreased expression of Vegf and Sirt1 mRNAs in HuRKO mice may be a direct effect because HuR is reported to stabilize these RNAs (33, 36, 44). In addition, previous studies have shown a positive correlation between cytoplasmic accumulation of HuR and Vegf proteins with increased angiogenesis and tumor size (45). The mechanisms accounting for the upregulation of candidate proapoptotic factors (caspase-9, Vegf, and TP53) are likely to include indirect and possibly multicomponent interactions in vivo, as alluded to above. However, such suggestions will require further validation, particularly because of the well-recognized discordance between the stabilizing effects of HuR on target mRNA half-life in cell lines versus the effects noted upon transgenic overexpression or targeted HuR deletion in vivo (12).

In summary, these studies illustrate the important role of enterocyte-specific HuR expression in small intestinal epithelial homeostasis and in 2 models of tumorigenesis. The finding that intestinal HuR deletion is protective in these 2 distinct models of intestinal tumorigenesis implies the need for greater understanding of the pathways involved in order to exploit these observations in a therapeutic context. Such understanding will need to include approaches to dissect the molecular mechanisms involved in a tissue- and cell-type–dependent manner and should take into consideration the important temporal role of epithelial HuR in preserving intestinal integrity. That being said, our findings imply that targeting epithelial HuR within a critical time window might be an approach to attenuate intestinal tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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