Novel mechanisms for osteogenic differentiation of human aortic valve interstitial cells

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ABSTRACT

Objective: Aortic valve calcification is common in aging populations without effective pharmacologic interventions. Our previous in vitro data revealed a critical role for long noncoding RNA metastasis-associated lung adenocarcinoma transcript 1 as a positive regulator of osteogenic differentiation in aortic valve calcification pathogenesis. The current study sought to determine the mechanism by which metastasis-associated lung adenocarcinoma transcript 1 is regulated in aortic valve calcification.

Methods: The stability assay was used to examine the effect of human antigen R on metastasis-associated lung adenocarcinoma transcript 1 expression. Aortic valves from patients with aortic stenosis and normal controls were subjected to determination of RNA-binding protein human antigen R expression. Mineralized bone matrix formation was assessed by Alizarin Red staining. The interaction between metastasis-associated lung adenocarcinoma transcript 1 and miR-191-3p was confirmed via RNA pull-down, luciferase reporter, and RNA-binding protein immunoprecipitation assays.

Results: In cultured human aortic valvular interstitial cells, we found human antigen R enhanced metastasis-associated lung adenocarcinoma transcript 1 stability and thus increased its concentration. Moreover, human antigen R was significantly upregulated in human calcific aortic valves and valvular interstitial cells after osteogenic induction. Human antigen R partly relied on metastasis-associated lung adenocarcinoma transcript 1 to positively regulate osteogenic differentiation of valvular interstitial cells. Luciferase reporter assays validated human antigen R as the direct target of miR-191-3p. Metastasis-associated lung adenocarcinoma transcript 1 positively regulated the expression of human antigen R through sponging miR-191-3p.

Conclusions: This study demonstrates the existence of a regulatory loop between metastasis-associated lung adenocarcinoma transcript 1 and human antigen R during osteogenic differentiation of valvular interstitial cells. Our findings provide novel mechanistic insights into a critical role of human antigen R in the aortic valve calcification progression and shed new light on RNA-binding protein-directed diagnostics and therapeutics in aortic valve calcification. (J Thorac Cardiovasc Surg 2020;159:1742-53)

Central Message

HuR overexpression is linked with aortic valve calcification. A positive feedback loop between HuR and lncRNA MALAT1 promotes osteogenic differentiation of VICs.

Perspective

VICs redifferentiate to osteoblast-like phenotype, which is the key cellular mechanism of AVC. This study demonstrates the existence of a positive feedback loop between MALAT1 and HuR during osteogenic differentiation of VICs, provides novel mechanistic insights into a critical role of HuR in the AVC progression, and sheds new light on RBP-directed diagnostics and therapeutics in AVC.

See Commentaries on pages 1754 and 1756.

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Aortic valve calcification (AVC) is the most common valvular disease with high morbidity and mortality rates. However, there are no effective treatments except for invasive interventional surgery, and pharmacologic therapeutic approaches can only be developed when the cellular and molecular processes behind AVC are identified. During the disease progression, quiescent resident valve interstitial cells (VICs) undergo activation and redifferentiate to osteoblast-like phenotype, which is the key cellular mechanism of AVC. Thus, strategies for preventing osteogenic differentiation may develop efficacious noninterventional approaches to reduce the development of AVC.

Micro-RNAs (miRNAs) are a large class of noncoding RNAs of 18 to 26 nucleotides in length capable of silencing gene expression by inhibiting protein translation from messenger RNA (mRNA) transcripts through imperfect base pairing with their 3' untranslated region (3'-UTR). In the past decade, extensive work has elucidated the integral roles of miRNAs in the regulation of AVC. Long noncoding RNAs (lncRNAs) are another class of noncoding RNAs larger than 200 nucleotides in length. Recently, increasing evidence documents the exact role of lncRNAs in the cardiovascular diseases.

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Recently, RNA-binding proteins (RBPs) have emerged as potent effectors and regulators of cellular function in cardiovascular diseases by guiding a large number of post-transcriptional modifications that greatly affect RNA fate, including stability, alternative splicing, translation, and localization. Human antigen R (HuR) is a ubiquitous member of the embryonic lethal abnormal vision protein family, highly conserved RBPs that act as a regulator of targeted mRNA half-life. HuR has been shown to regulate cellular phenotypic conversions that influence the development of cardiovascular diseases. A pivotal role of HuR in controlling the stability of lncRNAs has been reported. Nonetheless, limited knowledge is available concerning whether HuR could regulate osteoblast-like phenotype conversion of VICs and its role in lncRNA-mediated regulation of AVC, which needs to be well documented.

In this study, we provide the first evidence that HuR is upregulated in AVC and acts as a positive regulator of the osteogenic phenotype of VICs. Mechanistic analysis revealed that HuR interacts and enhances the stability of MALAT1 and thus increased MALAT1 concentration. Notably, HuR is negatively regulated by miR-191-3p, and MALAT1, through sponging miR-191-3p, positively regulates HuR levels. Altogether, these results show the existence of a specific circuitry between MALAT1 and HuR to establish correct osteogenic differentiation of VICs.

**MATERIALS AND METHODS**

Expanded methods are available in Appendix E1.

**Human Aortic Valve Samples**

All of the studies involving humans complied with the Declaration of Helsinki and were approved by the Ethic Board of Tongji Medical College of Huazhong University of Science and Technology. Written informed consents were obtained before surgeries from all patients. Calcified aortic valve leaflets (CAVS) were explanted from patients undergoing aortic valve replacement. Control noncalcified aortic valves with normal echocardiographic analyses were obtained during heart transplant procedures.

**Cell Culture and Treatment**

Human aortic VICs were isolated from noncalcified aortic valves by collagenase I digestion method as described previously. All experiments involving VICs were performed on cells from independent batches. VICs between passages 3 and 7 were selected for further studies and incubated with an osteogenic induction medium or the conditioning medium to stimulate osteogenic differentiation.
Transfection

Transfection of VICs was performed as previously described. Short hairpin RNAs targeting HuR and MALAT1 were synthesized in GenePharma (Shanghai, China). Overexpression vectors targeting HuR and MALAT1 were purchased from GeneChem (Shanghai, China) with an empty GV144 vector served as a control. miR-191-3p mimics and inhibitors were obtained from RiboBio (Guangzhou, China).

Messenger RNA Stability Assay

The mRNA stability assays were performed as described previously. Briefly, 24 hours after transfection, VICs were exposed to medium containing Actinomycin D (Sigma, St Louis, Mo) at a final concentration of 5 μg/mL. Total RNA was isolated from each sample at 0, 2, 4, 6, and 8 hours, and qRT-PCR analysis was performed as described earlier.

FIGURE 1. HuR stabilizes and increases MALAT1 concentration. A, Knockdown of HuR decreased MALAT1 level. B, HuR overexpression increased MALAT1 level. C, Whole cell lysate HuR levels and HuR levels after immunoprecipitation as internal controls were examined by Western blot. D, RIP showed that HuR could directly bind to MALAT1. E, HuR silencing or overexpression did not change MALAT1 promoter transcriptional activity. HuR knockdown (F) or overexpression (G) decreased or increased the half-life of MALAT1, respectively. *P < .05 versus sh-NC, empty, or immunoglobulin G.

MALAT1, Metastasis-associated lung adenocarcinoma transcript 1; sh-NC, short hairpin RNA (shRNA) for negative control; HuR, human antigen R; IgG, immunoglobulin G.
FIGURE 2. HuR is highly expressed in human CAVS and association with osteogenic differentiation of VICs. A, qRT-PCR showed that the HuR mRNA level was increased in human CAVS (N = 50) compared with normal control (N = 34). Increased HuR protein level in human CAVS was confirmed by Western blot (B) and immunohistochemical staining (C). N = 10 for each group. Both mRNA (D) and protein (E) levels of HuR exhibited a time-dependent gradual increase after osteogenic induction in VICs. F, HuR mRNA level was positively correlated with the expressions of osteogenesis-specific markers (Runx2, Osteocalcin, and Osteopontin). Scale bar: 50 μm. *P < .05 versus control. HuR, Human antigen R; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAVS, calcified aortic valve leaflets; Runx2, runt-related transcription factor 2; mRNA, messenger RNA.
Pull-Down Assay With Biotinylated Metastasis-Associated Lung Adenocarcinoma Transcript 1

The biotinylated DNA probe complementary to MALAT1 RNA was amplified by PCR using a T7-containing primer and subcloned into the plasmid vector GV394 (GeneChem). The resultant plasmid was linearized by restriction enzyme XhoI. Biotin-labeled RNAs were reversely transcribed using biotin RNA Labeling Mix and T7 RNA polymerase (all from Roche, Indianapolis, Ind). Finally, the bound RNAs were purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and then extracted for further evaluation by qRT-PCR analysis.

RNA-Binding Protein Immunoprecipitation Assay

RNA-binding protein immunoprecipitation (RIP) assays were performed as described. Briefly, VICs were lysed in lysis buffer containing RNase inhibitor and a protease inhibitor cocktail, and then immunoprecipitated with magnetic beads precoated with an anti-HuR antibody or anti-rabbit immunoglobulin G for 1.5 hours at 4°C. RNA was extracted from RNA-protein complexes, bound to the beads, and then analyzed by qRT-PCR.

Luciferase Reporter Assay

Luciferase reporter assay was performed as described previously. The full-length MALAT1 promoter reporter vector and the wild-type HuR 3′-untranslated region (3′-UTR) fragment containing putative miR-191-3p binding sites for reporter vector were all designed and synthesized by RiboBio. The fusion PCR method was applied to generate the mutant of HuR 3′-UTR, which had 3 bases changed, from GUUCUC to GAAGUC, at the putative binding sites for miR-191-3p. MALAT1 wild-type and the mutant derivative devoid of miR-191-3p-binding site (MALAT1-mut) were subcloned downstream of the coding region of luciferase gene.

Statistical Analysis

Results were expressed as mean ± standard deviation and compared with the Student t test (when 2 groups were compared) or analysis of variance followed by Bonferroni’s multiple comparison test by SPSS version 18.0 (IBM-SPSS Inc, Armonk, NY) (when >2 groups were compared). The normality of distribution of all continuous variables was confirmed by the Kolmogorov–Smirnov test and visualized by Q-Q plot. MALAT1 remaining at different time points was tested using 2-way

FIGURE 3. HuR partly relies on MALAT1 to act as a positive regulator of osteogenic differentiation of VICs. A, HuR silencing significantly decreased ALP activity. B, Alizarin red staining showed that knockdown of HuR inhibited calcified nodules formation. C, HuR silencing decreased the protein levels of HuR and osteogenesis-specific markers (Runx2, Osteocalcin, and Osteopontin). A-C, MALAT1 could partially reverse HuR-induced positive effects on osteogenic differentiation of VICs. Scale bar: 50 μm. *P < .05 versus empty + sh-NC, #P < .05 versus empty + sh-HuR-1. ALP, Alkaline phosphatase; sh-NC, short hairpin RNA (shRNA) for negative control; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; sh-HuR-1, short hairpin RNA (shRNA-1) for human antigen R; HuR, human antigen R; Runx2, runt-related transcription factor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
analysis of variance followed by Bonferroni post hoc tests, and the half-life was analyzed by unpaired $t$ test. Two-tailed Pearson’s correlation analysis was used to analyze the association of the 2 variables. For the human study and in vitro assays with human aortic VICs, $n$ represents the number of experiments performed in different donors (biological replicates).

Sample Size Estimation

The sample size for 2-sample comparison of mean in the human study was estimated by Stata software version 12.0 (StataCorp, LP, College Station, Tex). Sample size was set as identical in 2 groups. Power was set as 0.8, and alpha was set as 0.05. Results were expressed as mean ± standard deviation in the human study.

RESULTS

Human Antigen R Stabilizes Metastasis-Associated Lung Adenocarcinoma Transcript 1 (but Does Not Promote Metastasis-Associated Lung Adenocarcinoma Transcript 1 Expression)

Our previous study showed that MALAT1 promoted the osteogenic differentiation of VICs.12 Because IncRNAs were previously described to be modulated by RBPs,16 we further studied whether MALAT1 could be regulated by 4 RBPs (Quaking, HuR, Muscleblind, and SRSF1) in AVC. Human normal VICs were isolated from nonmineralized aortic valves (Table E1 shows details on patient characteristics). As shown in Figure 1, A, MALAT1 level was significantly decreased upon HuR silencing (Figure E1, A and B). On the contrary, HuR overexpression (Figure E1, C and D) caused a significant increase in MALAT1 level (Figure 1, B). However, the MALAT1 level was not affected by the other 3 RBPs (Quaking, Muscleblind, and SRSF1; data not shown).

To investigate the precise mechanism by which MALAT1 is regulated by HuR, the RIP assay was performed and showed that higher MALAT1 level was detected in anti-HuR immunoprecipitates relative to control the immunoglobulin G group (Figure 1, C and D). Thus, HuR bound directly to MALAT1, which corresponded well with a genome-wide screening experiment revealing the binding to MALAT1 of HuR.20 Notably, there was no change in MALAT1 promoter transcriptional activity after knockdown or overexpression of HuR (Figure 1, E), suggesting HuR does not promote MALAT1 expression. HuR is known to enhance the stability of IncRNAs by binding to the adenylate-uridylate (AU)-rich RNA motifs.16,18 First, we found that MALAT1 contains 4 AU-rich elements as predicted by the AU-rich element site (Figure E2, A) and predicted the interaction between MALAT1 and HuR using the RBPmap database (Figure E2, B). VICs were incubated with actinomycin D to measure the stability of MALAT1. The results showed that HuR silencing lowered the half-life of MALAT1 (Figure 1, F), whereas HuR overexpression increased the half-life (Figure 1, G). We also collected 50 human CAVS (Table E2 shows details on patient characteristics) and found a positive correlation between HuR and MALAT1 expression levels ($r = 0.5341, P < .05; Figure E3, A$). These results demonstrate that HuR physically interacts with and stabilizes MALAT1 without altering its transcriptional activity.

Human Antigen R Is Highly Expressed in Human Calcific Aortic Valves and Associated With the Osteogenic Differentiation of Valvular Interstitial Cells

Next, we evaluated whether HuR expression was altered in AVC. We collected human CAVS and control noncalcified valves (Table E2 shows details on patient characteristics), and found an increased mRNA level of HuR in CAVS tissues (Figure 2, A). Western blot (Figure 2, B) and immunohistochemical staining (Figure 2, C) confirmed the increased HuR protein level in human CAVS. To investigate whether HuR was associated with the osteogenic differentiation of VICs, we incubated VICs with osteogenic medium and found that both mRNA and protein levels of HuR exhibited a time-dependent gradual increase after osteogenic induction (Figure 2, D and E). To confirm the location of this increased HuR expression in the cells, we extracted both cytosolic and nuclear proteins from the VICs after osteogenic induction. The results from Western blot showed that osteogenic medium treatment significantly increased the cytosolic HuR level, but not the nuclear HuR level (Figure E4). Furthermore, there was a strong positive correlation between the HuR mRNA level and the osteogenesis-specific markers (Runx2, Osteocalcin, and Osteopontin; Figure 2, F). Thus, HuR may be involved in the development and progression of AVC.

Human Antigen R Partly Relies on Metastasis-Associated Lung Adenocarcinoma Transcript 1 to Positively Regulate Osteogenic Differentiation of Valvular Interstitial Cells

We next performed in vitro gain- and loss-of-function studies to confirm whether HuR played a regulatory role in AVC. The results showed that short hairpin RNA–mediated silencing of HuR largely negated the osteogenic medium-induced increase in alkaline phosphatase (ALP) activities (Figure 3, A), calcified nodule formation (Figure 3, B), and protein levels of HuR and osteogenesis-specific genes (Figure 3, C), suggesting that HuR could promote the osteogenic medium-induced osteogenic differentiation of VICs. Because HuR stabilizes MALAT1, we next tested whether MALAT1 participated in HuR-mediated effects on osteogenic differentiation of VICs. First, MALAT1 inhibition or overexpression in VICs was confirmed by qRT-PCR (Figure E5, A and B). The inhibition effects of shHuR-1 described were found to be partly reversed by
miR-191-3p silencing promotes osteogenic differentiation of VICs by targeting HuR. A, Putative binding sites of HuR with miR-17-3p, miR-146b-5p, miR-191-3p, miR-371a-3p, and miR-589-5p, as predicted by FINDTAR3. B and C, Dual-luciferase reporter assays indicated a specific inhibitory effect of miR-191-3p on HuR. miR-191-3p overexpression inhibited the mRNA (D) and protein (E) levels of HuR, whereas, knockdown of
MALAT1 overexpression (Figure 3, A-C). In contrast, HuR overexpression resulted in further increases in ALP activities, calcified nodule formation, and protein levels of HuR and osteogenic differentiation markers, which were attenuated by MALAT1 depletion (Figure E6, A-C). Moreover, HuR overexpression was sufficient to induce osteogenic differentiation in a conditioning medium, as evidenced by increased ALP activities, calcified nodule formation, and mRNA levels of osteogenic differentiation markers (Figure E7, A-C). These results provide the concrete evidence that HuR partly relies on MALAT1 to positively regulate osteogenic differentiation of VICs.

miR-191-3p Silencing Promotes Osteogenic Differentiation of Valvular Interstitial Cells by Targeting Human Antigen R

HuR was previously described to be targeted by miRNAs. To explore which miRNAs could target HuR in VICs, we first performed a search using online bioinformatics databases (FINDTAR3 and RNAhybrid) and found 5 potential miRNAs (miR-17-3p, miR-146b-5p, miR-191-3p, miR-371a-3p, and miR-589-5p) (Figure 4, A). The results from dual-luciferase reporter assays showed that overexpression of miR-191-3p, but not the other 4 miRNA candidates, significantly reduced the luciferase activity of the wild-type 3′-UTR of HuR, whereas it had no effect on its mutation 3′-UTR (Figure 4, B). In contrast, the luciferase activity was increased after miR-191-3p knockdown, and the effect was abolished by mutating the predicted miR-191-3p target site in HuR 3′-UTR, indicating a specific inhibitory effect of miR-191-3p on HuR (Figure 4, C). Moreover, miR-191-3p overexpression resulted in decreased HuR mRNA and protein levels (Figure 4, D and E), whereas miR-191-3p downregulation led to increased levels of HuR (Figure 4, F and G). Finally, an inverse correlation between miR-191-3p and HuR levels was found in the 50 human CAVS used (r = −0.4672, P < .05; Figure E3, B). These results support the bioinformatics predictions indicating HuR as a direct target of miR-191-3p. Our results also showed that miR-191-3p level was decreased in human CAVS (Figure E8) and that miR-191-3p knockdown greatly promoted the osteogenic medium-induced increases in calcified nodule formation (Figure 4, H) and the protein levels of osteogenesis-specific genes (Figure 4, I). Thus, miR-191-3p silencing could promote the osteogenic differentiation of VICs by targeting HuR.

Metastasis-Associated Lung Adenocarcinoma Transcript 1 Acts as an miR-191-3p Sponge to Positively Regulate Human Antigen R Expression In Vitro

LncRNAs could act as endogenous miRNA sponges to interact with miRNAs and modulate the local concentration of miRNAs, leading to a reduced binding to target genes of miRNAs. We predicted the interaction between MALAT1 and miR-191-3p using starBase v2.0 and found a target site of miR-191-3p by MALAT1 (Figure 5, A). As shown in Figure 5, B, a luciferase construct of MALAT1 RNA (Luc-MALAT1 wild type) and a mutated form (Luc-MALAT1-mut) were generated. We confirmed that miR-191-3p overexpression inhibited the luciferase activity of the MALAT1 RNA in VICs, whereas it had significantly less effect on the mutated form compared with the wild-type, revealing a specific interaction of MALAT1 with miR-191-3p in this putative binding site (Figure 5, B). We next performed a RIP assay and found that higher MALAT1 and miR-191-3p RNA levels were detected in Ago2 immunoprecipitates compared with the control immunoglobulin G group (Figure 5, D), suggesting that MALAT1 and miR-191-3p were in the same RNA-induced silencing complex. Together, these results support the bioinformatics predictions and indicate that MALAT1 can directly sponge miR-191-3p. Our data also indicated that miR-191-3p expression was upregulated after knockdown of MALAT1 (Figure 5, E). In contrast, MALAT1 overexpression led to a decrease in the miR-191-3p level (Figure 5, F). However, MALAT1 expression was not affected after knockdown or overexpression of miR-191-3p (Figure E10, A and B). Finally, we examined whether MALAT1 could positively regulate HuR expression through sponging miR-191-3p. As shown in Figure 5, G, the luciferase reporter assay was conducted to show that MALAT1 overexpression counteracted the inhibitory effect of miR-191-3p on HuR. Moreover, MALAT1 attenuated the effect of miR-191-3p on HuR mRNA and protein expressions (Figure 5, H and I). We also found an inverse correlation between miR-191-3p and MALAT1 levels in the 50 human CAVS (r = −0.3941, P < .05; Figure E3, C). Collectively, these
results indicate that MALAT1 sponges miR-191-3p and positively regulates HuR expression in vitro.

In this study, we provide the first evidence that a positive feedback loop exists between MALAT1 and HuR during osteogenic differentiation of human aortic VICs. HuR interacts and enhances the stability and concentration of MALAT1. Meanwhile, HuR is negatively regulated by miR-191-3p, and MALAT1, through sponging
miR-191-3p, positively regulates HuR levels (Figure 6 and Video 1).

**DISCUSSION**

At present, it is an unmet medical need to discover new potential therapeutic targets for treating AVC. In this study, our in vitro assays showed that HuR promoted osteogenic differentiation of VICs through the stabilization of MALAT1, and thereby promotes osteogenic differentiation of human aortic VICs in progression of AVC. Meanwhile, HuR is negatively regulated by miR-191-3p, and MALAT1, through sponging miR-191-3p, positively regulates HuR levels. (Central Image: A positive feedback loop between MALAT1 and HuR promotes AVC.) HuR, Human antigen R; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; 3′-UTR, 3′-untranslated region; VIC, valvular interstitial cell.

Recently, the emerging roles for RBPs as pivotal regulators of cardiovascular diseases have gained more acceptance and may open up the opportunity for the generation of novel therapeutic targets. In this study, we reported a novel, to date unknown function of the RBP HuR in AVC, and showed that HuR, highly expressed in CAVS, could promote osteogenic differentiation of VICs as evidenced by increased ALP activities, calcified nodule formation, and expressions of osteogenesis-specific markers. In fact, HuR also plays crucial roles in neuronal differentiation, muscle differentiation, and T-cell differentiation. HuR was shown to control the processing and stability of IncRNAs. In this study, HuR directly interacted with MALAT1, which was consistent with a previous study. Moreover, we described that HuR enhanced MALAT1 stability and thus increased its concentration. However, we cannot exclude the possibility that HuR may also stabilize other lncRNAs, whose function in cellular differentiation and homeostasis is unknown. Thus, whether the potential therapeutic modulation of HuR (with the scope to control AVC progression) could be limited to MALAT1 without affecting other cellular functions requires further study.

LncRNAs may act as endogenous decoys for miRNAs; such activity would in turn affect the distribution of miRNAs on their targets. HuR was shown to be negatively regulated by miRNAs. We validated HuR as the direct target of miR-191-3p, which was consistent with a previous...
study.\textsuperscript{29} Several lines of evidence exist to support MALAT1 as an endogenous miR-191-3p sponge that regulated HuR expression in the present study. First, MALAT1 depletion increased miR-191-3p expression, whereas the expression of miR-191-3p was decreased upon MALAT1 overexpression. Second, we predicted the interaction between MALAT1 and miR-191-3p, and found that MALAT1 contains a target site of miR-191-3p. Third, miR-191-3p overexpression partially reversed the upregulation of HuR caused by MALAT1. Finally, MALAT1 could directly interact with miR-191-3p. One sponge lncRNA could modulate several protein-coding genes.\textsuperscript{30} Our previous study has already demonstrated that MALAT1 acts as a competing endogenous RNA to regulate Smad4 expression in VICs.\textsuperscript{12} Moreover, individual protein-coding transcripts could be regulated by multiple sponge lncRNAs.\textsuperscript{31} Thus, whether MALAT1 could act as a sponge lncRNA to modulate more key driver genes and HuR could be regulated by other sponge lncRNAs in AVC remain to be clarified. Recently, delivery of miR-199a through an adeno-associated virus (AAV) vector was shown to stimulate uncontrolled cardiac repair after myocardial infarction in pigs.\textsuperscript{32} Moreover, a previous study has confirmed the safety of gene therapy through infusion of AAV1 in patients with heart failure and reduced ejection fraction.\textsuperscript{33} Thus, delivery of MALAT1 or HuR inhibitors through an AAV vector may provide a novel and promising gene therapy approach to AVC treatment.

**Study Limitations**

Our study has some limitations. First, only in vitro experiments were conducted in this study to elaborate the role of HuR on the osteogenic phenotype. Whether HuR promotes AVC progression in preclinical animal models still needs further investigation. Second, we only revealed a link among MALAT1, miR-191-3p, and HuR to enhance the osteogenic differentiation of VICs. Whether MALAT1 sponges miR-191-3p to affect expressions of other key regulators, which play a critical role in AVC, needs to be further explored. Third, our study revealed an increased HuR level in human CAVS and VICs after osteogenic induction. However, its upstream induction mechanisms still need to be explored. Moreover, how HuR affects the expression of those critical transcription factors promoting the osteogenic transition remains largely unknown. Nonetheless, the present findings in aortic tissues and cell assays generate novel hypotheses about this positive feedback loop between HuR and MALAT1 in AVC.

**CONCLUSIONS**

To the best of our knowledge, our work is the first study to reveal a novel mechanism of the HuR-mediated regulatory network in AVC. The positive feedback loop between HuR and MALAT1 could indicate an important potential therapeutic target and provides a strong rationale for the development of HuR-based therapeutic strategies for AVC.

**Conflict of Interest Statement**

Authors have nothing to disclose with regard to commercial support.

**References**


Key Words: RNA-binding proteins, long noncoding RNAs, osteogenic differentiation, aortic valve calcification
**APPENDIX E1**

**Histopathology and Immunohistochemistry**

Human aortic valve tissues were rapidly harvested, rinsed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde in PBS, and embedded in paraffin for histopathologic analyses. Immunohistochemical staining was performed using the following antibodies: HuR (1:200 dilution) from Santa Cruz (Santa Cruz, Calif). At least 2 of the 3 aortic valve cusps for each patient were analyzed.

**Cell Culture and Treatments**

To isolate human aortic VICs, nonmineralized aortic valves were collected from patients undergoing heart transplant procedures (Table E3 shows details on patient characteristics). Primary human aortic VICs were isolated from noncalcified aortic valves by collagenase I digestion method as described previously. \(^{E1}\) In brief, aortic valves were digested in 1 mg/mL type I collagenase for 30 minutes at 37°C, vortexed to remove valvular endothelial cells, and further incubated with a fresh solution of 1 mg/mL collagenase for 4 to 6 hours at 37°C. After repeated aspirations to break up the tissue fragments, the cell suspension was gently spun for 10 minutes at 1000 rpm to precipitate cells. Finally, isolated VICs were resuspended, seeded, and cultured in Dulbecco’s modified Eagle’s medium (Gibco, Invitrogen Corporation, Carlsbad, Calif) containing 100 μg/mL streptomycin (Gibco), 10% fetal bovine serum, and 100 U/mL penicillin (Gibco) at 37°C supplied with 5% CO2 atmosphere. All experiments involving VICs were performed on cells from independent batches. To induce osteogenic differentiation, VICs were grown in the osteogenic induction medium (growth medium supplemented with 50 mg/mL ascorbic acid, 0.1% fetal bovine serum, 5 mmol/L β-glycerophosphate, 50 mg/mL bone morphogenetic protein-2, and 100 nmol/L dexamethasone; all purchased from Sigma-Aldrich Ltd, St Louis, Mo), \(^{E2}\) or the conditioning medium (growth medium supplemented with 10 mmol/L β-glycerophosphate, 10 mmol/L dexamethasone, 4 μg/mL cholecalciferol, and 8 mmol/L CaCl2; all purchased from Sigma). \(^{E3}\)

**Quantitative Real-time Polymerase Chain Reaction Analysis**

qRT-PCR analysis was conducted as previously described. \(^{E2,E4}\) Total RNA was isolated from cultured cells or aortic valve tissues using Trizol reagent (Invitrogen, Carlsbad, Calif) and then reverse transcribed using the reverse transcription kit PrimeScript RT reagent Kit from Takara (Otsu, Japan) or commercial miRNA reverse transcription PCR kit (RiboBio). qRT-PCR was performed with SYBR Premix Ex Taq kit from Takara on the ABI Step1 Plus system (Applied Biosystems, Foster City, Calif). The primer sequences are listed in Table E3.

**Immunoblotting Analysis**

Cell lysates were subjected to immunoblotting analysis, as previously described. \(^{E4,E5}\) Briefly, equal amounts of total protein were loaded onto 10% sodium dodecyl sulfate polyacrylamide gels followed by electrophoresis, and then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, Calif). After blocking in 5% nonfat milk and 0.05% Tween 20, the membranes were blotted using the primary and secondary antibodies, and then visualized using clarity western enhanced chemiluminescence substrate (BioRad, Ontario, Toronto, Canada). Images were acquired and quantification analyses were performed using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, Md). Antibodies for HuR, Runx2, Osteocalcin, Osteopontin, glyceraldehyde-3-phosphate dehydrogenase, and horseradish peroxidase–conjugated goat-anti-rabbit secondary antibody were all purchased from Santa Cruz Biotechnology, Inc.

**Alkaline Phosphatase Activity and Alizarin Red Staining in Valvular Interstitial Cells**

Determination of ALP activity and Alizarin Red staining in cultured cells was performed as described previously. \(^{E2}\) ALP activity in the cell lysates was assayed using the ALP assay kit (Wako, Japan) and expressed as relative ALP activities normalized to those of the control cells. For Alizarin Red staining, cells were washed twice in PBS, fixed in 4% formaldehyde (Sigma) for 30 minutes, and stained with 0.2% alizarin red solution. The red staining indicates calcified nodule formation. For quantification of calcium deposition, the stained cells were incubated for 30 minutes in 10% acetic acid, and the absorbance was read at 405 nm with a spectrophotometer.

**E-References**


FIGURE E1. The mRNA (A) and protein (B) levels of HuR were decreased after silencing of HuR with 2 short hairpin RNAs (sh-HuR-1 and sh-HuR-2) in VICs. The mRNA (C) and protein (D) levels of HuR were increased after HuR overexpression. *P < .05 versus sh-NC or empty. HuR, Human antigen R; mRNA, messenger RNA; sh-NC, short hairpin RNA (shRNA) for negative control; sh-HuR-1, short hairpin RNA (shRNA-1) for human antigen R; sh-HuR-2, short hairpin RNA (shRNA-2) for human antigen R; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

FIGURE E2. MALAT1 contains 4 AU-rich elements as predicted by AU-rich element site (A). The interaction between MALAT1 and HuR was predicted by the RBPmap database (B). MALAT1, Metastasis-associated lung adenocarcinoma transcript 1; 3'-UTR, 3'-untranslated region.
FIGURE E3. The associations among HuR, MALAT1, and miR-191-3p levels in human CAVS. Correlation analysis between MALAT1 and HuR (A), miR-191-3p and HuR (B), and MALAT1 and miR-191-3p (C) among human CAVS (n = 50) as indicated by 2-tailed Pearson’s correlation analysis. HuR, Human antigen R; MALAT1, metastasis-associated lung adenocarcinoma transcript 1.

FIGURE E4. This increased HuR expression was located in the cytosol. Western blot analysis showed that osteogenic medium treatment significantly increased the cytosolic HuR level, but not the nuclear HuR level. *P < .05 versus control. HuR, Human antigen R; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; n.s., not significant.
FIGURE E5. MALAT1 inhibition (A) or overexpression (B) in VICs was confirmed by qRT-PCR. *P < .05 versus sh-NC or empty. MALAT1, Metastasis-associated lung adenocarcinoma transcript 1; sh-NC, short hairpin RNA (shRNA) for negative control.

FIGURE E6. HuR partly relies on MALAT1 to act as a positive regulator of osteogenic differentiation of VICs. HuR overexpression increased ALP activity (A), calcified nodules formation (B), and protein levels of HuR and osteogenesis-specific markers (Runx2, Osteocalcin, and Osteopontin) (C). A-C, MALAT1 could partially reverse HuR-induced positive effects on osteogenic differentiation of VICs. Scale bar: 50 μm. *P < .05 versus sh-NC + Empty. #P < .05 versus sh-NC + GV144-HuR. ALP, Alkaline phosphatase; sh-NC, short hairpin RNA (shRNA) for negative control; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; HuR, human antigen R; Runx2, runt-related transcription factor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
FIGURE E7. HuR induced osteogenic differentiation of VICs in a conditioning medium. HuR overexpression increased ALP activity (A), calcified nodules formation (B), and the mRNA levels of osteogenesis-specific markers (Runx2, Osteocalcin, and Osteopontin) (C). Scale bar: 50 μm. *P < .05 versus empty. ALP, Alkaline phosphatase; HuR, human antigen R; mRNA, messenger RNA; Runx2, runt-related transcription factor 2.

FIGURE E8. miR-191-3p was downregulated in human CAVS. qRT-PCR showed that the miR-191-3p level was reduced in human CAVS (N = 50) compared with normal control (N = 34). *P < .05 versus control. CAVS, Calcified aortic valve leaflets.

FIGURE E9. Detections of MALAT1 using qRT-PCR in the same sample pulled down by biotinylated MALAT1 and negative control probe. Input was used for normalization. *P < .05 versus Bio-NC-probe. MALAT1, Metastasis-associated lung adenocarcinoma transcript 1; NC, negative control.
FIGURE E10. MALAT1 expression was not affected by miR-191-3p knockdown or overexpression. The expression of MALAT1 after miR-191-3p knockdown (A) or overexpression (B) in VICs was determined by qRT-PCR. MALAT1, Metastasis-associated lung adenocarcinoma transcript 1; NC, negative control; n.s., not significant.

TABLE E1. Clinical characteristics of patients for cell cultures

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control valves (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>56 ± 4</td>
</tr>
<tr>
<td>Male (%)</td>
<td>69</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.6 ± 3.8</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>49</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.60 ± 0.28</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>1.72 ± 0.35</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.25 ± 0.36</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>38</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>80</td>
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<tr>
<td>Bicuspid aortic valves (%)</td>
<td>0</td>
</tr>
<tr>
<td>Statins (%)</td>
<td>70</td>
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<tr>
<td>β-Blockers</td>
<td>68</td>
</tr>
<tr>
<td>ACEi/ARB</td>
<td>75</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>55.9 ± 4.1</td>
</tr>
<tr>
<td>Transvalvular gradient (mm Hg)</td>
<td>18.6 ± 5.87</td>
</tr>
<tr>
<td>AVA (cm²)</td>
<td>3.5 ± 0.41</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation or %. BMI, Body mass index; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; LVEF, left ventricular ejection fraction; AVA, aortic valve area.
TABLE E2. Clinical characteristics of patients for quantitative reverse transcription-polymerase chain reaction analysis

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n = 34)</th>
<th>CAVS (n = 50)</th>
<th>P value</th>
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<tbody>
<tr>
<td>Age, y</td>
<td>57 ± 6</td>
<td>61 ± 4</td>
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<tr>
<td>Male (%)</td>
<td>74</td>
<td>66</td>
<td>ns</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.20 ± 3.76</td>
<td>23.10 ± 2.52</td>
<td>ns</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>60</td>
<td>46</td>
<td>ns</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.42 ± 0.21</td>
<td>1.58 ± 0.29</td>
<td>ns</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>1.91 ± 0.53</td>
<td>1.99 ± 0.32</td>
<td>ns</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.42 ± 0.31</td>
<td>1.48 ± 0.28</td>
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</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>35</td>
<td>23</td>
<td>ns</td>
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<tr>
<td>Smoking (%)</td>
<td>80</td>
<td>65</td>
<td>ns</td>
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<tr>
<td>Statins (%)</td>
<td>68</td>
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<tr>
<td>β-Blockers</td>
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<tr>
<td>ACEi/ARB</td>
<td>74</td>
<td>58</td>
<td>ns</td>
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<tr>
<td>LVEF (%)</td>
<td>51.90 ± 2.96</td>
<td>58.90 ± 3.49</td>
<td>ns</td>
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<tr>
<td>Transvalvular gradient (mm Hg)</td>
<td>16.80 ± 5.97</td>
<td>83.80 ± 6.53</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>AVA (cm²)</td>
<td>3.22 ± 0.69</td>
<td>0.82 ± 0.19</td>
<td>&lt;.05</td>
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</tbody>
</table>

Values are mean ± standard deviation or %. CAVS, Calcific aortic valve stenosis; BMI, body mass index; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; LVEF, left ventricular ejection fraction; AVA, aortic valve area.

TABLE E3. Primers for quantitative real-time polymerase chain reaction

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<thead>
<tr>
<th>Genes</th>
<th>Primer sequence (5’ to 3’)</th>
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<tr>
<td>ALP forward</td>
<td>TTGTGCCAGAGAAAGAGAGAGA</td>
</tr>
<tr>
<td>ALP reverse</td>
<td>GTTTCAAGGGCATTTCACAGGT</td>
</tr>
<tr>
<td>Runx2 forward</td>
<td>AGCTTCTGTCTGTGCTTCTGG</td>
</tr>
<tr>
<td>Runx2 reverse</td>
<td>GGAGTAGAGAGGCAAGAGTTT</td>
</tr>
<tr>
<td>Osteocalcin forward</td>
<td>CTCTCTCTGCTCACTCTGCT</td>
</tr>
<tr>
<td>Osteocalcin reverse</td>
<td>TTTGAGGCGGTTTCCTCAAGC</td>
</tr>
<tr>
<td>Osteopontin forward</td>
<td>GAAGTTTCGCAGACCTGACAT</td>
</tr>
<tr>
<td>Osteopontin reverse</td>
<td>GTATGCACCATTCAACTCCTCG</td>
</tr>
<tr>
<td>HuR forward</td>
<td>CCGTCAACCAATGTGAAAGTG</td>
</tr>
<tr>
<td>HuR reverse</td>
<td>TCGGGGCTTCTCTCATAGTTT</td>
</tr>
<tr>
<td>MALAT1 forward</td>
<td>AAAGCAAAGGCTCCCCACAGAG</td>
</tr>
<tr>
<td>MALAT1 reverse</td>
<td>GGTCCTGTGCTAGATCAAAAAGGCA</td>
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<tr>
<td>GAPDH forward</td>
<td>ACGTGTCTCAGTGTTGACCTG</td>
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<tr>
<td>U6 forward</td>
<td>CTGCGCTTCGGCAGCACATA</td>
</tr>
<tr>
<td>U6 reverse</td>
<td>AAGCGTCCTACGAATTTCGGT</td>
</tr>
</tbody>
</table>

ALP, Alkaline phosphatase; Runx2, runt-related transcription factor 2; HuR, human antigen R; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.