MUC16 promotes Triple-Negative Breast Cancer Lung Metastasis by Modulating RNA- Binding Protein ELAVL1/HUR

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Abstract

Background: Triple-negative breast cancer (TNBC) is highly aggressive with an increased metastatic incidence compared to other breast cancer subtypes. However, due to the absence of clinically reliable biomarkers and targeted therapy in TNBC, chemotherapy still remains the standard of care. Hence, there is an urgent need to understand biological mechanisms that lead to identifying novel therapeutic targets for managing metastatic TNBC disease.

Methods: Clinical significance of MUC16 and ELAVL1 were examined using breast cancer TCGA data. Microarray was performed using MUC16 knockdown and scramble breast cancer cells and identified MUC16 associated genes using RNA immunoprecipitation and metastatic cDNA array. Metastastic properties of MUC16 was evaluated in MUC16 knockdown using tail-vein metastastic experiments.

Results: Here, we show that MUC16 is highly expressed in TNBC and correlates with its target gene Hu antigen R (HuR) through global transcriptomic analysis. Depletion of MUC16 showed decreased invasion (P=0.03), migration (P=0.001), and colony formation abilities (P=0.01) of human and mouse TNBC cells. MUC16 depleted cells injected mice showed less likely to develop (P=0.001) lung metastasis. Notably, MUC16 and HuR were highly expressed in the lung tropic TNBC cells and breast cancer patients lung metastases tissues. Mechanistically, we identified MYC as a HuR target gene in TNBC using RNA immunoprecipitation and metastatic cDNA array. Furthermore, MUC16 knockdown and pharmacological inhibition of HuR (MS444 & CMLD-2) in TNBC cells showed the reduction in cMYC expression, suggesting the importance of the MUC16/HuR/cMYC axis in TNBC metastasis.

Conclusions: Overall, our study identified MUC16 as TNBC lung metastasis promoter that acts through HuR/cMyc axis. In future, this study will form the basis to target both MUC16 and MYC amplified aggressive tumors.

Background

Breast cancer (BC) is the most common malignancy and the second leading cause of cancer-related deaths in women[1]. In the United States, about 290, 560 new cases and nearly 43, 780 patients are expected to die in the year 2022[1]. Triple-negative breast cancer (TNBC) is a highly aggressive subtype of breast cancer with a median overall survival of approximately one year compared to five years in other subtypes[2] and is often associated with metastases[3]. Therefore, identifying better prognostic markers and mechanisms associated with the metastasis could help develop targeted therapies and better clinical management of the TNBC patients.

Cancer antigen 125/Mucin 16 (CA125/MUC16) has been associated with cancer progression and metastasis in various cancer types, including BC[4–7]. In addition, we have previously demonstrated that MUC16 is overexpressed in BC and induced a rapid G2/M cell cycle transition to promote cell survival *via* JAK2/STAT3 signaling pathways[8]. MUC16 is also used as a biomarker for ovarian cancer to monitor disease progression, recurrence, and chemotherapeutic response[9–11]. Apart from the critical role of

MUC16 in BC pathogenesis[12–14], it was also the most frequently mutated gene in metastatic breast tumors, followed by TP53[15, 16].

ELAV (embryonic lethal, abnormal vision, Drosophila)-like protein 1 (ELAVL1) or Hu antigen R (HuR) is a RNA binding protein that contains RNA recognition motifs[17] which regulates the stability of various target mRNAs[18]. High levels of HuR expression were observed in many cancers and regulates many cancer-associated transcripts stability during cancer progression and metastasis[19–22]. The function of HuR is dependent on the localization of HuR; for instance, HuR mediates its oncogenic role when it translocates from the nucleus to the cytoplasm[23]. For therapeutic purposes, several small-molecule inhibitors have been developed to target HuR in various manners[24, 25]. MS-444 targets HuR by blocking its dimerization in the nucleus, thus preventing its cytoplasmic trafficking[25, 26]. While another inhibitor, CMLD-2, exhibits its effect by disrupting the interaction between HuR and target mRNAs[24]. In breast cancer, HuR is upregulated in the cytoplasm and correlated with poor clinical outcomes[22, 27, 28]. Y-Box binding protein 1 (YBX-1) is an interaction partner of HUR, which is also overexpressed in many cancers and plays an important role in various cellular functions[29].

Recent studies have shown that cancer cells preferentially spread and metastasize to distant organs under the influence of selective cellular and molecular programs[30–32]. Numerous studies focus on identifying cell-intrinsic determinants for such distinct organtropism during metastasis, including transcription factors, kinases, and cell surface receptors expressed on tumor cells that facilitate such preferential tropism[32–34]. However, the efficient colonization in secondary organs mostly depends on various oncogenic proteins, which modulate the extensive survival signals and colonization activities. In this study, we have demonstrated that MUC16 is specifically overexpressed in TNBC and mediates lung metastasis. We identified the new HuR target cMYC in TNBC that is associated with MUC16. Altogether, we observed that MUC16 regulates the HuR and its target cMyc during TNBC lung metastasis.

Methods

Cell Culture and stable knockdown cell line generation

Breast cancer cell lines: MDA MB 231, and HCC1937 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI1640 medium supplemented with 10% FBS and 1X penicillin and streptomycin. Mouse basal type cell line, 4T1, was cultured in DMEM with the supplements mentioned above. Scramble control and pSUPER-Retro-sh-MUC16 were transfected into phoenix cells using Lipofectamine 2000 to generate viral particles (Invitrogen, Carlsbad, CA, USA). After 48 h, supernatant (viral particles) was collected, centrifuged, and used to infect the MDA MB 231 and HCC1806. Similarly, mouse-specific pSUPER-Retro-sh-Muc16 were used for 4T1 cells. The pooled population of MUC16 knockdown cells was obtained using antibiotic selection (Puromycin 4µg/ml) and was further expanded to confluent levels to obtain stably transfected cells. Then, MUC16 knockdown and scramble cells were used for microarray analysis.

Tissue Microarray, immunohistochemistry, and immunofluorescence

Immunohistochemistry was performed in the commercially available tissue microarray (TMA)- A202V (Accumax array), BR1503 (US Biomax), which included 75 cases/150 cores and normal lung tissues. Briefly, the TMAs or slides were baked overnight at 56°C to remove the excess paraffin and hydrated in the graded alcohol (100 to 20%, 5 min each). Antigen was retrieved in the 0.01M citrate buffer for 15 min in the microwave, endogenous peroxidase activity was quenched by 0.3% H2O2 (1 h/dark) followed by blocking with 2.5% horse serum (Impress reagent kit, Vector Laboratories) at room temperature, 1 h. The slides were incubated overnight with MUC16 (M11 clone, Dako) and HuR at 4°C, washed with PBST, and further incubated with ImmPRESS Universal anti-mouse Ig/rabbit Ig for 30 min. The brown color was developed by using DAB, counterstained with hematoxylin, followed by dehydration with graded alcohol (20 to 100%), air dried, and mounted with PerMount [8]. MUC16 immunostaining was evaluated by a trained pathologist, who was blinded to the clinical information (3). A quantitative assessment of MUC16 protein expression in the xenograft tissues was performed using Fiji-Image J software [35].

For immunofluorescence studies, the tissues were processed according to the above-mentioned antigen retrieval step. The tissues were then blocked with 10% goat serum (Jackson Immunoresearch Labs, Inc., West Grove, PA, USA) for 30 min followed by incubation with primary antibodies:- MUC16 (Mouse, 1:750) and HuR (rabbit, 1:500) for overnight at 4°C. The next day, the cells were washed with PBS (5 min, 3X) and incubated with fluorescein isothiocyanate-conjugated anti-mouse and Texas red-conjugated anti-rabbit secondary antibodies (Jackson Immunoresearch Labs, Inc.) for 30 min at room temperature in the dark. Next, the tissues were washed with PBS (5 min, 3X) with gentle shaking and mounted with an anti-fade vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). Images were acquired with an LSM710 microscope (Carl Zeiss GmbH, Jena, Germany).

Quantitative real-time PCR

Quantitative real-time PCR was performed as previously described [8]. Total RNA was isolated using Qiagen Kit (Germantown, MD, USA). Total RNA (two micrograms) was used for cDNA synthesis using reverse transcriptase SuperScript®II (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed using SYBER Green, and β-actin was used as an internal control.

Immunoblot

Total protein was isolated using the RIPA buffer (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors (1mM phenylmethyl sulphonyl fluoride, 1mg/ml aprotinin, 1mg/ml leupeptin). About 20-40 µg protein was run in 10% SDS-PAGE gel for the signaling studies. While for a very high molecular weight glycoprotein, MUC16, 2% agarose gel was run. The gels were transferred to PVDF membrane, blocked with 5% skimmed milk, and probed with respective primary antibodies- MUC16 (M11 clone, Mouse 1: 1000, Fisher Scientific), HuR (Rabbit, 1:1000, Abcam), MMP1 (Rabbit, 1:1000, Cell Signaling), pSrcY-416 (rabbit, 1:1000, Cell Signaling), Src (rabbit, 1:1000, Cell Signaling), c-MYC (Mouse 1:1000, Santa Cruz biotechnology), β-catenin (rabbit, 1:2000, Cell Signaling), Twist 1 (rabbit, 1:1500, Santa Cruz biotechnology), YBX1 (rabbit, 1:1000, Cell Signaling), and anti-β-actin for overnight at 4˚C. The membranes were then washed (3X, 10min) in PBST at room temperature, probed

with the appropriate secondary antibodies (1:5000 dilutions) for 1 h, and washed (3X, 10min) with PBST. The signal was detected with the ECL chemiluminescence kit (Amersham Bioscience, Buckinghamshire, UK).

Invasion and cell motility assay

For invasion and migration assays, about 1 million scramble and MUC16 knockdown cancer cells (MDAMB231, HCC1806, and 4T1) were seeded (serum-free condition) in the 8 µm pore size six-well inserts (Becton Dickinson, Franklin Lakes, NJ, USA). 10-20% serum was used as an attractant in the bottom chamber. After 24h, the cancer cells that migrated to the lower chamber were stained with the Quick-Diff kit staining solution and then counted in eight different random fields, and the average number of motile cells per representative field was calculated.

Tail vein injection

About 1 million viable GFP-labeled MDAMB-231(SCR. and shMUC16) in 50 µl PBS was injected via the tail vein of nude mice (n=6/group). The mice were monitored every week for metastasis by IVIS imaging. After 30 days, the mice were sacrificed, and tissues were collected for further investigation. The mouse studies were performed in accordance with the US Public Health Service 'Guidelines for the Care and Use of Laboratory Animals' under an approved protocol by the Institutional Animal Care and Use Committee, University of Nebraska Medical Center.

RNA Immunoprecipitation (RIP) and PCR array

RIP was performed using an anti-HuR antibody (1, 2). HCC1806-SCR and HCC1806-shMUC16 were cultured and lysed using polysome lysis buffer (1000 mM KCl, 50 mM MgCl2, 100 mM HEPES-NaOH pH 7, 5 % NP40) supplemented with RNase and protease inhibitors. Lysates were pre-cleared by adding 30 µg of IgG1 (BD Bioscience) and 50 µl of Protein-A/G Sepharose beads swollen in NT2 buffer with 5% BSA. Beads were coated by adding either IgG1 (BD Biosciences, San Diego, CA) as control or anti-HuR antibody and incubated overnight at 4 °C. After extensive washes of pre-coated Protein-A/G sepharose beads, the pre-cleared lysate was added and incubated for 4 h at 4 °C, and then 30 µg of proteinase K was added to digest protein by incubation at 55 °C for 30 min. The extracted RNA was reverse transcribed into cDNA and performed cDNA array (PAHS-028Z, QIAGEN) to identify the HuR target mRNAs.

MS-444 and CMLD treatment and cell viability assays

MDA MB 231 and HCC1806 cells were seeded at subconfluent levels in 96-well cell culture plates and treated with increasing concentrations of MS-444 and CMLD for 48 h at 37°C [24, 25]. The cell viability of MS-444 and CMLD were determined using MTT assays [8]. Based on the IC_{50} concentration of MS-444 and CMLD, MDA MB 231 and HCC1806 cells were treated for 48 h, and lysates were collected for western blot analysis.

Data analysis

Statistical significance was evaluated with the Student t-test using GraphPad Prism 8.1.2 software. P values less than 0.05 were considered statistically significant. All experiments were performed in triplicates.

Results

MUC16 expression and poor survival outcomes in breast cancer.

To assess the clinical impact of MUC16 in breast cancer, we performed immunohistochemistry (IHC) in the tissue microarray (TMA), which consisted of moderately differentiated (n=25) and poorly differentiated (n=36) tumors. We observed that MUC16 expression was significantly higher in poorly differentiated tumors (P=0.02) compared with moderately differentiated tumors **(Fig. 1A)**. Further analysis in the BRCA METABRIC cohort also indicated that MUC16 expression was significantly (P=1.118e-5) high in advanced breast tumor (N=1198) tissues (grade 3) compared to grade 1 (N=214) or grade 2 BC tumors (N=976) **(Fig. 1B)**. Furthermore, Kaplan-Meier survival curve analysis in the Tang 2018 cohort depicted that high MUC16 (Q8WXI7) protein expression (N=17) was significantly (P=0.046) associated with poor overall survival compared to low MUC16 (N=48) expressing BC patients[36], suggesting that MUC16 is associated with BC pathogenesis and poor survival (**Fig. 1C**). We next analyzed the expression of MUC16 in BC patients in the context of the chemotherapy regime using the TCGA BRCA cohort. To our surprise, we found that MUC16 is one of the top significantly (P<1.52e-13) upregulated gene in chemotherapy-treated BC patients compared to treatment-naive patients (**Fig. 1D**). This finding was further corroborated in BC patients who had failed to respond to chemotherapy and also showed an increased MUC16 expression compared to responders with an AUC curve of 0.573 (P= 0.0026) (**Fig. 1E & F**). These findings indicated that MUC16 is associated with BC aggressiveness, cancer differentiation status, worst survival, and therapy resistance.

Overexpression of MUC16 in TNBC subtype.

Since BC is highly heterogeneous at the molecular level, we wanted to analyze the expression variation of MUC16 in different molecular subtypes of BC. Our IHC data indicated that MUC16 is significantly high in TNBC tumor tissues (N=65) as compared to other subtypes, namely, luminal A (N=21, P=0.027), luminal B (N=13, P=0.001), and HER2 (N=23, ns) enriched tumor tissues **(Fig. 1G)**. Similarly, TCGA BRCA analyses showed a significantly higher expression of *MUC16* (P<2.2e-16) in the TNBC subtype (N=171) compared to other subtypes HER2 (78), Luminal A (499), and Luminal B (N=197) **(Fig. 1H)**. We have previously shown that MUC16 is not observed in healthy breast tissues[8]. Overall, this finding indicates that MUC16 overexpression is molecular subtype-specific (TNBC) and may associate with disease progression and aggressiveness.

MUC16 targets RNA bio-synthetic and metastasis pathways in TNBC

To investigate the MUC16 associated molecular mechanism(s) and pathways of MUC16 promoting TNBC advancement, gene expression studies were performed in the MUC16 knockdown MDA MB 231

cells (MDA MB 231-shMUC16) and corresponding scramble (MDA MB 231-shRNA) transduced cells. Transcriptome analysis indicated that HuR (also known as ELAVL1), MMP1, and MMP3 genes were significantly downregulated in MUC16 knockdown cells compared to control **(Fig. 2A)**. Next, we have performed the pathway analysis (Gene ontology) using top downregulated genes affected due to MUC16 knockdown (MDA MB 231-shMUC16). We noticed that RNA biosynthesis, RNA metabolism, apoptosis, and regulation of programmed cell death, cell proliferation, and metastasis pathways were significantly affected by MUC16 stable suppression in TNBC cells **(Fig. 2B)**. Further analysis of the transcriptomic data by qRT-PCR also confirmed significant downregulation of *HuR, MMP1*, and *MMP3* in the MUC16 knockdown cells compared to scramble (**Fig. 2C, Supplementary Fig. 1A-C**). Western blot analysis also indicated that expression of HuR has drastically decreased in MUC16 knockdown TNBC MDA MB 231 and HCC1806 cells **(Fig. 2D and E)**. Like MUC16, high HuR protein expression (Q15717) was significantly (P=0.055) associated with poor overall survival compared to low HUR expression BC patients[36] **(Fig. 2F)**. Furthermore, HuR was also significantly overexpressed in the TNBC subtype as compared to other subtypes **(Fig. 2G)**. Despite, MMP1 and MMP3 are overexpressed in breast cancer but not high in TNBC (**Supplementary Fig. 1D-G)**. Thus, overall , MUC16 and HuR are significantly overexpressed in TNBC and associated with TNBC development.

Effect of MUC16 on invasion, migration, and colony formation ability of human and mouse TNBC cells

Next, to determine the functional role of MUC16 in TNBC *in vitro* metastasis, MDA MB 231-shMUC16 and scramble cells were seeded in the Matrigel-coated Boyden chamber transwell inserts for 24 h. The results show that MUC16 knockdown cells showed a significant decrease in invasion (P=0.03) **(Fig 3A)**, migration (P<0.001) **(Fig 3B),** and colony formation (P=0.01) abilities **(Fig. 3C)** compared to the scramble controls. At the molecular level, our western blot analyses showed decreased invasion and migration were due to the reduction of phosphorylated Src (Y416), β-catenin, and Twist 1 in MUC16 knockdown cells but no change in total Src compared to the scramble control **(Fig. 3D)**. Conversely, there was an increased expression of epithelial marker (E-cadherin) and decreased expression of mesenchymal marker (N-cadherin) in MUC16 knockdown cells (**Supplementary Fig. 2A** and **B**). Similarly, MUC16 knockdown HCC1806 **(Fig. 2E)** cells also showed decreased migratory properties as compared to scramble cells **(Fig. 3E).** Additionally, Muc16 knockdown in mouse basal type cell line, 4T1 (**Fig. 3F**), revealed that *HuR* expression was decreased in Muc16 knockdown cells compared to scrambled control **(Fig. 3G)**. Further, Muc16 knockdown cells (4T1-shMuc16) also showed a significant reduction in migration compared to control **(Fig. 3H)**. In support of these, mesenchymal marker Zeb-1 was decreased in Muc16 knockdown cells compared to scramble control cells (**Supplementary Fig. 2C**). Overall, these findings suggest that MUC16 is involved in TNBC metastasis.

MUC16 is associated with lung metastasis of TNBC

MUC16 associated molecular pathways and *in vitro* analyses indicated that MUC16 induced TNBC metastasis. To prove this concept, we performed a tail-vein injection experiment using MUC16 knockdown and scramble control cells (1 X 10^6 RFP labeled cells/mouse) in athymic nude mice. The

organ metastases in the athymic nude mice were routinely monitored by IVIS imaging **(Fig. 4A)**. We observed that MUC16 knockdown cells (N=5) injected mice develop less lung metastasis as compared to scramble control (N=6) cells injected mice. Subsequent histological analysis (H & E) of the lung tissues also showed a reduction in metastases with the presence of micrometastases in MUC16 knockdown cells injected lung xenograft (P=0.001), while there were higher levels of macrometastasis in the scramble control cells injected xenograft **(Fig. 4B & C).** Next, we wanted to determine the expression of MUC16 and HuR in metastatic lung tumor xenograft. The IHC analyses demonstrated that the expression of MUC16 and HuR were low in MUC16 knockdown cells injected lung tumors compared to scramble control xenograft tissues, respectively **(Fig. 4D & E)**. We next performed a confocal analysis of MUC16 and HuR in the lung tumor xenografts and found that HuR is highly distributed in the cytoplasmic region in scramble cells injected lung tumor tissues compared to MUC16 knockdown cells injected tumors **(Fig. 4F)**. Of note, lung tropic cells MDA MB 231-LM2 also showed the elevated expression of MUC16 **(Fig. 4G)** and HuR **(Fig. 4H)** compared to parental MDA MB 231. Overall, these findings suggested that MUC16 induces TNBC lung metastasis by modulating HuR and its target genes.

Expression of MUC16 in breast cancer lung metastatic tissues: To investigate the clinical impact of MUC16 on breast cancer metastasis, we analyzed MUC16 expression in publicly available patient datasets of metastatic breast cancer. The GSE14020 (N=36) dataset was used for various metastasis samples such as bone (n=8), brain (n=7), liver (n=5) and lung (n=16). The datasets were individually Robust Multichip Average (RMA) normalized and further z-normalized to reduce experiment or platform-induced bias. MUC16 is highly expressed in the lung, brain, and bone metastatic tissues, indicating that MUC16 may have a role in metastasis (**Supplementary Fig. 2D**). Like MUC16, elevated levels of HuR were observed in lung and other metastatic organs (**Supplementary Fig. 2E**), indicating that the MUC16/HuR axis is required for breast cancer metastasis.

MUC16 associated HuR targets in TNBC

To determine the MUC16 mediated HuR target genes in TNBC, we performed RNA-immunoprecipitation (RIP) with HuR antibody, followed by a tumor metastasis PCR array. First, HuR target genes were pull-down using HuR antibody from scramble control (HCC1806-SCR) cells and MUC16 knockdown (HCC1806-shMUC16) cells. Then, immunoprecipitated HuR transcripts were used for PCR analysis (human tumor metastasis cDNA array) and observed enrichment of several genes, namely Proto-Oncogene, BHLH Transcription Factor (*MYC*), EWS RNA Binding Protein 1 (*EWSR1*), Ribosomal Protein Lateral Stalk Subunit (*RPLP*), Phosphatase and Tensin Homolog (*PTEN*), MDM2 Proto-Oncogene (*MDM2*), EWS RNA Binding Protein 1 (*EWSR1*) and Neurofibromin 2 (*NF2*) in HuR immunoprecipitated scramble, while ETS Variant Transcription Factor 4 (*ETV4*), C-X-C Motif Chemokine Receptor 2 (*CXCR2*), and CD82 Molecule (*CD82*) were downregulated in HuR immunoprecipitated MUC16 knockdown cells (HCC1806-shMUC16) **(Fig. 5A)**. These molecules were validated by western blot analysis, we observed a drastic reduction of cMYC expression in the MUC16 knockdown cells (HCC1806-shMUC16 and MDA MB231-shMUC16) compared to scramble controls (**Fig. 5B & C)**. These data suggest that MUC16 regulates cMYC through HuR, and these genes may be required for TNBC growth and metastasis. Of note,

HuR interaction partner YBX-1 protein was also drastically reduced in MUC16 knockdown cells as compared to scramble control cells (**Fig. 5B & C)**, suggesting that MUC16 regulates HuR and YBX-1 during post transcription of cMyc in TNBC cells.

Pharmacological inhibition of HuR inhibits cMYC expression in TNBC cells

We next used two pharmacological inhibitors of HuR-MS-444 and CMLD-2 to investigate the effect of HuR on cMYC expression. MS-444 was shown to block HuR dimerization in the nucleus and thus prevent its cytoplasmic trafficking[37], while CMLD-2, an inhibitor of HuR-ARE (adenine-uridine rich elements) interaction, binds to HuR protein, thereby disrupts its interaction with ARE containing mRNAs[24]. Initially, we performed the cell viability assay using HuR inhibitors, MS-444 **(Fig. 5D),** and CMLD-2 (**Supplementary Fig. 3A**) to identify the IC_{50} concentration. Then, MDA MB 231 and HCC1806 cells were treated with MS-444 and CMLD-2 drugs for 48 h. Western blot analyses showed that MS-444 inhibitor (10 μM) effectively inhibited the cMYC expression in both the cell lines, MDA MB 231 and HCC1806 **(Fig. 5E & F)**. Similarly, decreased cMYC expression was observed in the CMLD-2 treated MDA MB 231 cells as compared to untreated cells **(Supplementary Fig. 3B)**. Furthermore, we observed that MUC16 is significantly co-expressed with HuR in BC patients samples (**Fig. 5G)**. Similarly, HuR is also strongly co-expressed with cMyc in the same BC patient cohort (**Fig. 5H**). These findings suggest that cMyc is a direct target of HuR in TNBC cells.

Discussion

TNBC subtype represents approximately 15% of all breast cancer subtypes[38]. Among all subtypes, BC patients with TNBC phenotype are more likely to occur in younger women with an abysmal disease prognosis[2, 39, 40]. TNBC frequently metastasizes to the lungs (36.9%), brain (25%), and bone (40%) and is associated with poor and shorter survival outcomes[3]. The median overall survival of metastatic TNBC is approximately one year compared to five years for the other BC subtypes[2]. The poor survival of TNBC is due to the lack of clinically relevant biomarkers, targeted therapies, and chemotherapy is the standard of care available for localized and metastatic patients[2, 3]. Our previous reports indicated that MUC16 could induce the G2/M transition of BC cells by interacting with Janus kinase 2 (JAK2), which in turn enhances the phosphorylation of STAT3 (Y705) and Aurora Kinase A[8]. However, the role of MUC16 in BC sub-types and the mechanism associated with metastasis in BC has not been explored before. Thus, identifying a potential link between MUC16 overexpression in BC to cause organ-specific metastasis will help the physicians to identify MUC16 high-expressing patients in high-risk BC metastsic patients.

Breast tumor data indicated that MUC16 is overexpressed in poorly differentiated breast tumors and overexpressed in the TNBC subtype as compared to other subtypes. Further database analysis indicated that MUC16 is significantly elevated in the breast cancer patients who underwent chemotherapy and also indicated increased MUC16 expression in non-responders patients to any chemotherapy suggesting the role of MUC16 in therapy resistance and relapse in BC. In addition, we and others have demonstrated that MUC16 is involved in chemoresistance in various cancers[8, 9]. A recent pan-cancer analysis using the

GEPIA web server demonstrated overexpression of MUC16 in several solid malignancies such as LUAD, OV, PAAD, UCEC, and UCS [41].

Previous studies demonstrated the overexpression of MUC16 and its impact on migration and invasion of different cancer cells[6, 42, 43]. Our *in vitro* metastatic experiment demonstrated that MUC16 is required for the invasion, migration and colonization of TNBC cells. In support of these findings, the phosphorylation of Src (Y416), Twist 1, MMP1, and β-catenin were drastically decreased in MUC16 knockdown cells, suggesting that MUC16 play a role critical role in TNBC metastasis. Similarly, metastatic investigation revealed that MUC16 knockdown cells were less likely to develop lung metastasis than scramble cells injected into mice; these findings clearly indicated that MUC16 induced TNBC lung metastasis. Further, MUC16 is significantly overexpressed in lung tropic cells, and patient data also revealed that MUC16 is elevated in breast cancer lung cancer metastatic tissues, suggesting that MUC16 is involved in breast cancer lung metastasis.

Our previous study has demonstrated that MUC16 promotes pancreatic cancer metastasis by activating the Epithelial to mesenchymal phenotype through FAK activation [44]. Further, the molecular mechanisms through which MUC16 promotes lung cancer migration *via* STAT3/glucocorticoid receptor (GR)/testis-specific protein Y-encoded-like 5 (TSPYL5) axis. In the current study, we identified that MUC16 regulates RNA binding protein HuR in BC and is associated with BC metastasis. MUC16 was found to linked to RNA biosynthesis, cell death, and cell migration pathways, suggesting that MUC16 may mediate these pathways during breast cancer progression. Following that our RNA-IP and cDNA array experiment demonstrated that HuR is directly binding with Myc in TNBC cells. Further, cMyc expression was drastically decreased in MUC16 Knockdown TNBC cells. In addition, we also observed HuR interaction partner YBX-1 was drastically reduced in MUC16 knockdown cells, suggesting that MUC16 activates HuR/YBX-1 axis during post-transcriptional regulation of various oncogenes, including cMyc. Furthermore, pharmacological inhibition of HuR (MS-444 and CMLCD) drastically reduced cMyc expression indicating that HuR regulates cMyc in TNBC cells.

Conclusion

Overall, our study has demonstrated that MUC16 regulates the HuR for cMYc expression that mediates TNBC cell invasion and lung metastasis **(Fig. 6)**. Altogether, targeting MUC16 may prevent TNBC lung metastasis and improve the patient's outcomes.

Abbreviations

BC, Breast cancer; FAK, Focal Adhesion Kinase; HuR-Hu Antigen or ELAVL1- embryonic lethal, abnormal vision, Drosophila-like protein 1; IHC, Immunohistochemistry; IVIS, non-invasive in vivo live imaging; KD, Knockdown; KO, Knockout; MUC, Mucins; PCR, Polymerase chain reaction; RNA-IP, RNA-Immunoprecipitation; TCGA, The cancer genome atlas; TMA, Tissue microarray; TNBC,Triple-negative

breast cancer; TPM, Transcripts per million; UNMC, University of Nebraska Medical Center; YBX-1,Y box protein 1.

Declarations

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Author contributions

IL, SKB conceived and designed the experiments. IL and SC performed the experiments. SC, PS, SKM, assisted with *in vivo* experiments. Data were collected and analyzed by IL, SC, SKM, PN, SM, PS, AS, PA, CVR, MMP. The manuscript was written by IL with input from SKB, MPP and reviewed by all authors. IHC scoring were done by SML. All authors have read and approved the final manuscript.

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Availability of data and materials

The microarray data and materials associated with the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The mouse studies were performed in accordance with the US Public Health Service ‘Guidelines for the Care and Use of Laboratory Animals’ under an approved protocol by the Institutional Animal Care and Use Committee, University of Nebraska Medical Center.

Consent for publication

Not applicable.

Competing interests

SKB is the co-founder of Sanguine Diagnostics and Therapeutics, Inc. None of the other authors has any conflict of interest.

“The authors declare that there are no competing interests”

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Figures

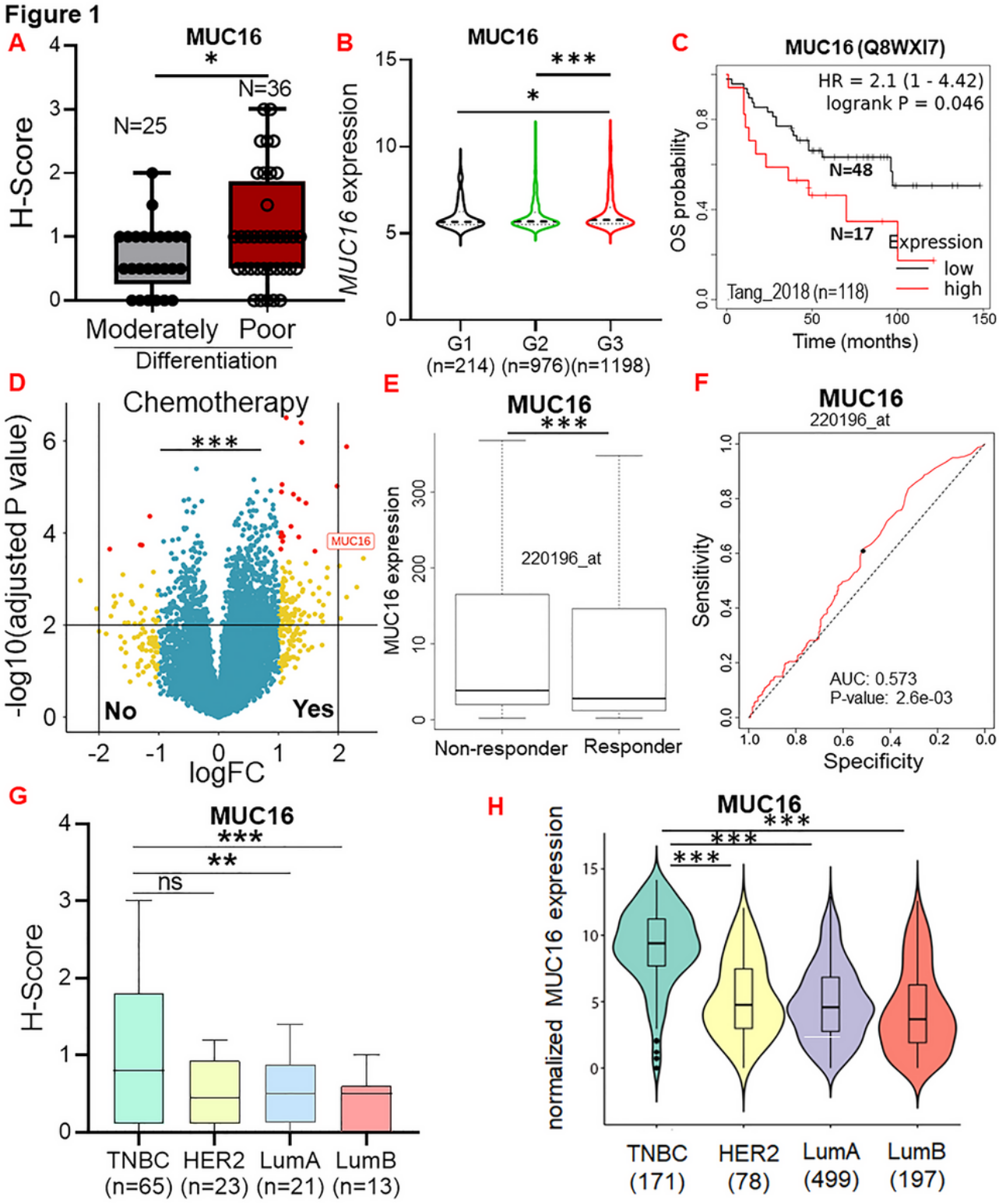

Figure 1

Overexpression of MUC16 in TNBC patients. **A.** Expression of MUC16 is highly upregulated in the poorly differentiated patients (n=36) compared to moderately differentiated samples (n=25). **B.** In silico TCGA-BRCA analysis indicating grade-wise expression of MUC16 transcript. **C.** Kaplan Mier survival plot showing significant reduction in overall survival of breast cancer patients with high MUC16 (Q8WXI7) protein expression (https://kmplot.com). **D**. Volcano plot of TCGA-BRCA dataset indicating high levels of

MUC16 mRNA in breast cancer patients that have undergone chemotherapy compared to chemotherapy-naive patients. **E**. Box plot showing increased MUC16 expression in non-responders (any chemotherapy) compared to responders in breast cancer. **F**. AUC curve of MUC16 (220196_at) based on the 5-year relapse-free survival of breast cancer after any chemotherapy (http://www.rocplot.org). **G.** Box plots of IHC scores showing upregulation of MUC16 expression in TNBC patients compared to other breast cancer subtypes. **H.** Similarly, in silico dataset (TCGA-BRCA) indicating upregulation of MUC16 in TNBC patients (n=171) compared to HER2 (n=78), Lum A (n=499), and Lum B (n=197). Statistical significance * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

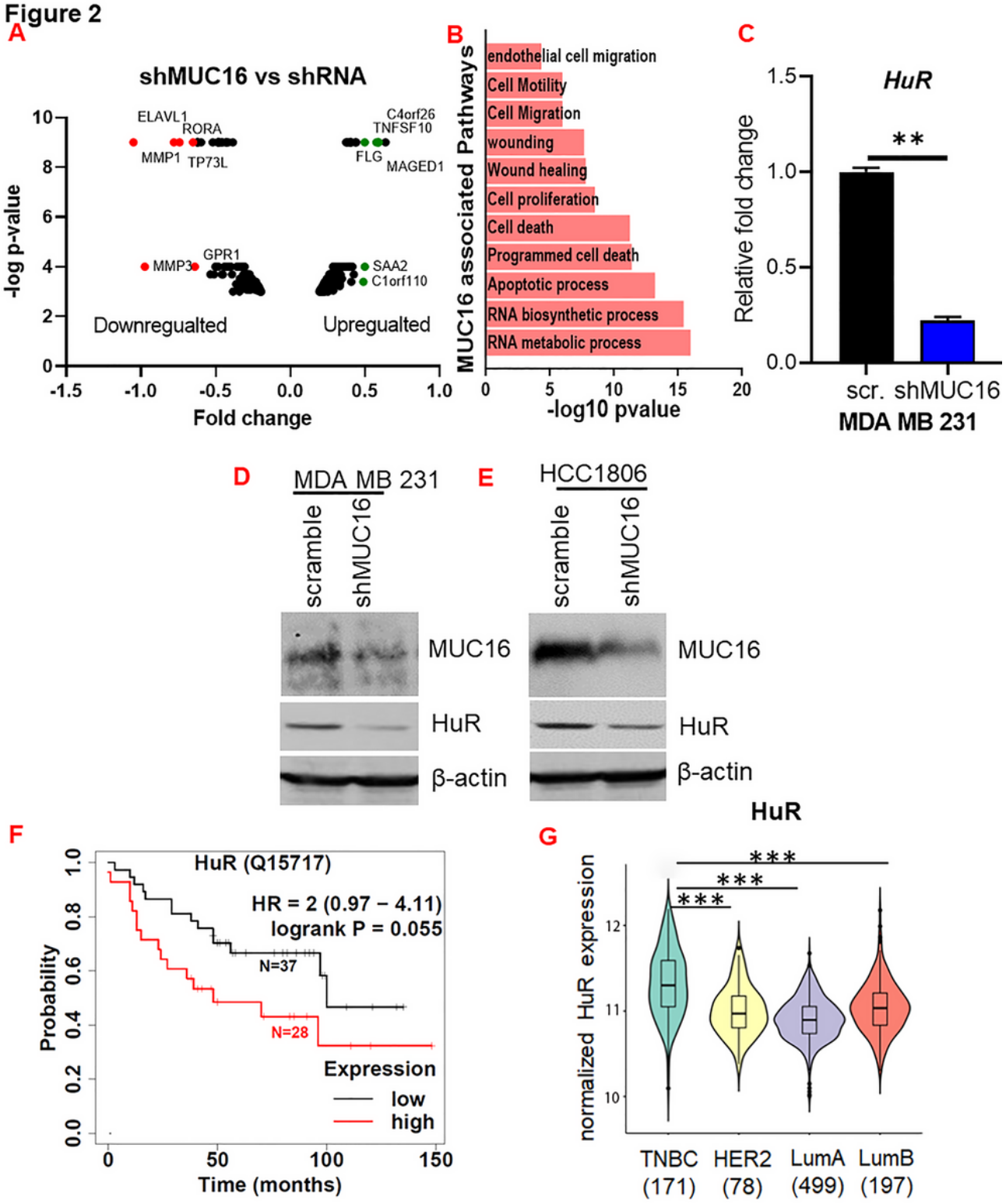

Figure 2

MUC16 regulates HuR in TNBC cells. A. Volcano plot demonstrated decreased expression of ELAVL1/HuR, MMP1, and MMP3 in MUC16 knockdown TNBC cells. **B.** Gene ontology analysis of MUC16 associated pathways suggesting its role in RNA biosynthesis, apoptotic and cell motility, and migration pathways. **C.** Bar diagram of qRT-PCR showing decreased HuR expression in MUC16 knockdown cells (MDA MB 231) compared to scramble. Actin was used as an internal control. **D.** Western blot results

indicated that HuR was downregulated in MUC16 knockdown (MDA MB 231 and HCC1806). **F**. Kaplan Mier survival plot (Q15717) showing HuR is associated with poor survival in breast cancer patients. **G.** HuR expression in different breast cancer subtypes.

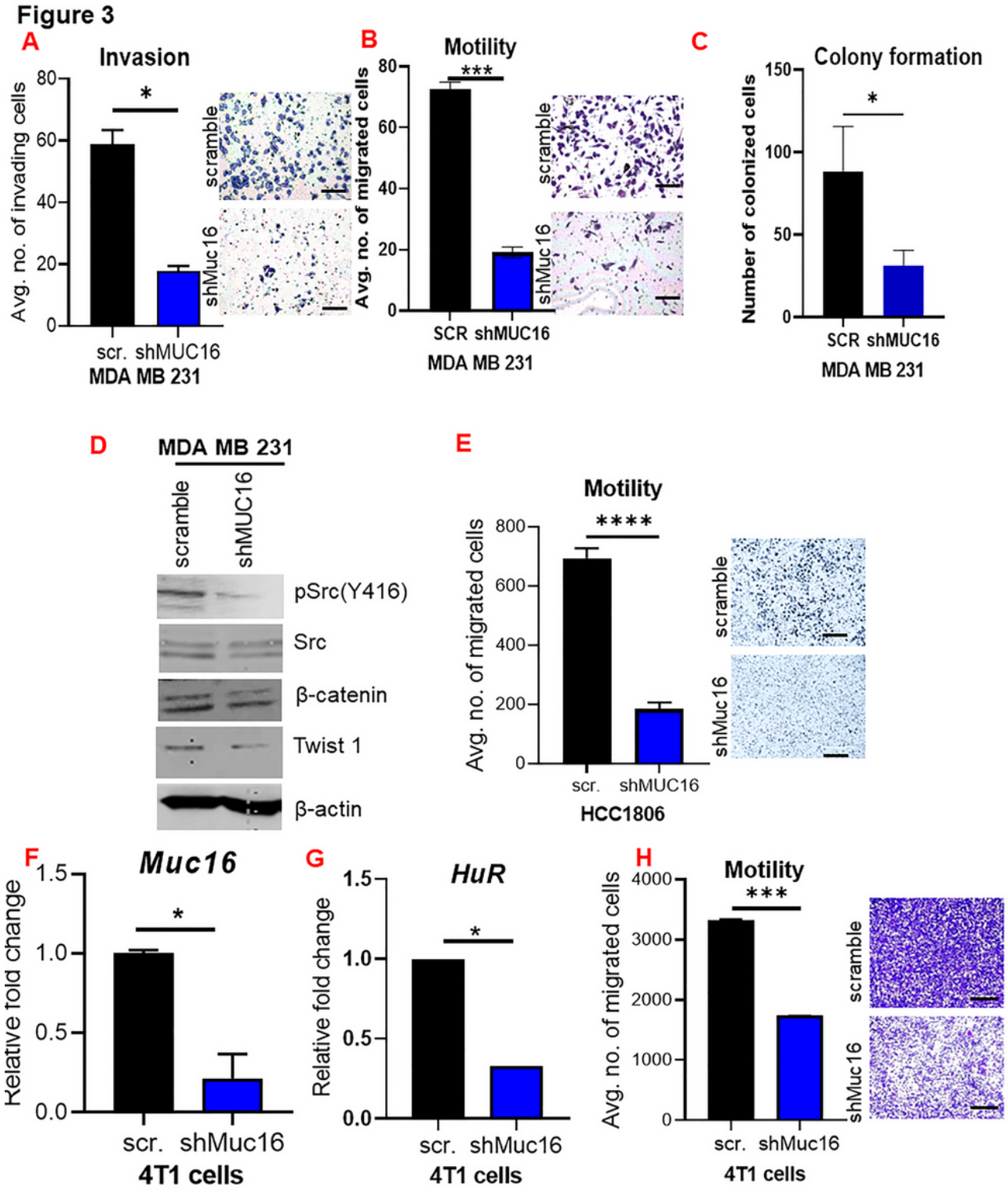

Figure 3

Effect of MUC16 on breast cancer cell invasion and motility. A-B. Boyden chamber transwell assay showed decreased invasion and migration upon MUC16 knockdown cells compared to control cells. **C.** Colony formation ability of MUC16 knockdown cells. **D.** Western blot showing decreased β-catenin, Twist, and pSrc (Y416) in MUC16 knockdown cells (MDA MB 231) compared to scramble cells. β-actin was used as an internal control. **E.** Motility assay indicated that motility of MUC16 knockdown HCC1806-shMUC16 cell was significantly reduced compared to SCR cells (HCC1806-shRNA). **F & G.** Bar diagram showing Muc16 knockdown and its impact on decreased HuR expression in mouse TNBC 4T1 cells. **H.** Boyden chamber motility assay indicating decreased migration capacity after Muc16 knockdown in 4T1 cells. Statistical significance * $P < 0.05$; *** $P < 0.001$.

Figure 4

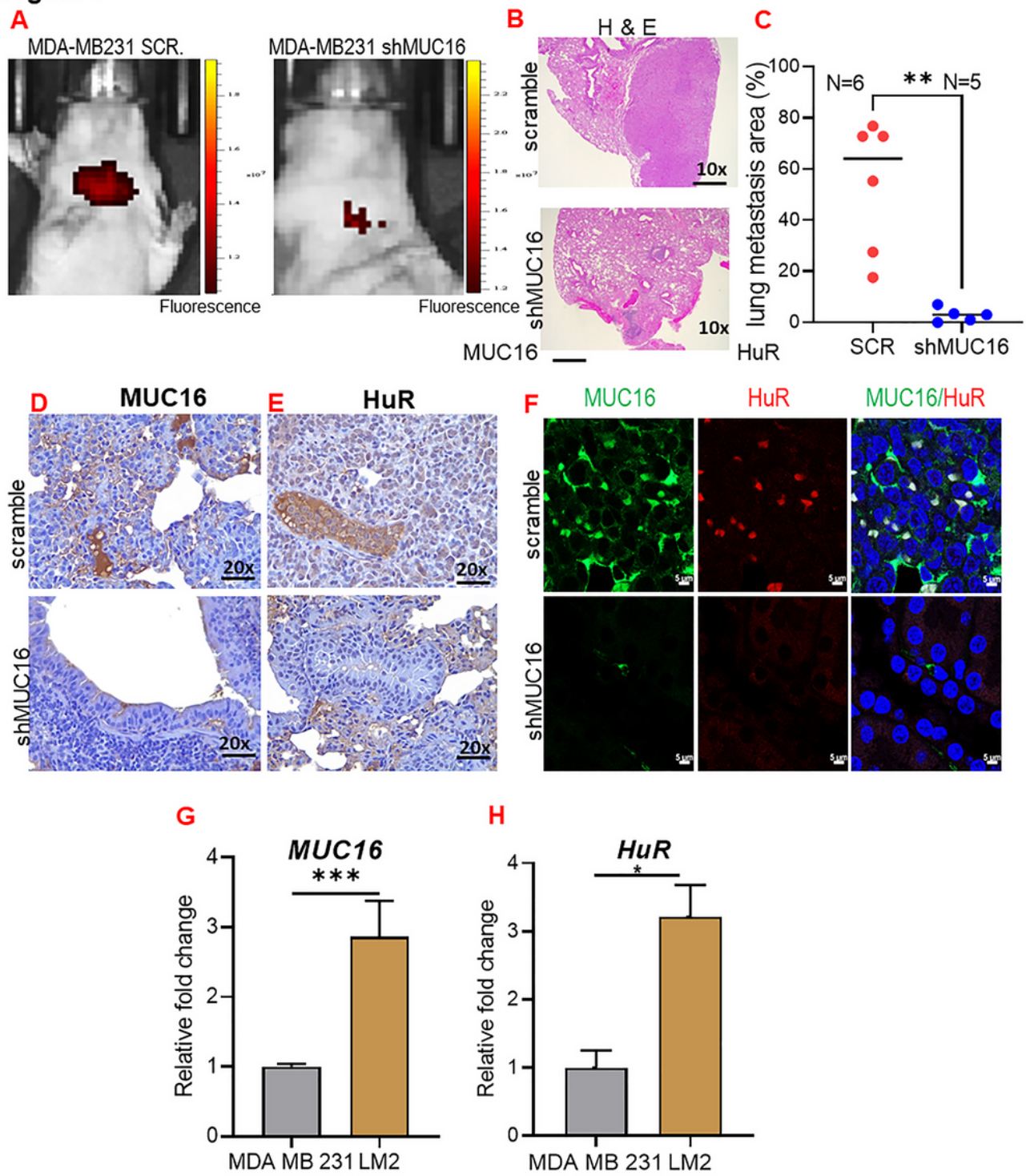

Figure 4

Effect of MUC16 knockdown on TNBC lung metastasis. A. Representative IVIS image of scramble and MUC16 knockdown cells injected mice. **B.** Representative histological images show metastatic lung tumors. **C**. Bar diagram showing decreased percentage area of lung metastasis in nude mice injected with MUC16 knockdown (n=5) as compared to scramble controls (n=6). **D & E.** IHC images showing decreased expression of MUC16 and HuR in MUC16 knockdown lung xenograft compared to control (20X

magnification). **F.** Confocal image indicating decreased MUC16 and HuR expression in the MUC16 knockdown cells injected xenograft as compared to scramble control xenografts. **G & H.** qRT-PCR data showing upregulation of MUC16 and HuR in the MDA MB 231 lung tropic cells (LM2) as compared to parental MDA MB 231 cells. Statistical significance ** $P < 0.01$; *** $P < 0.001$.

Figure 5

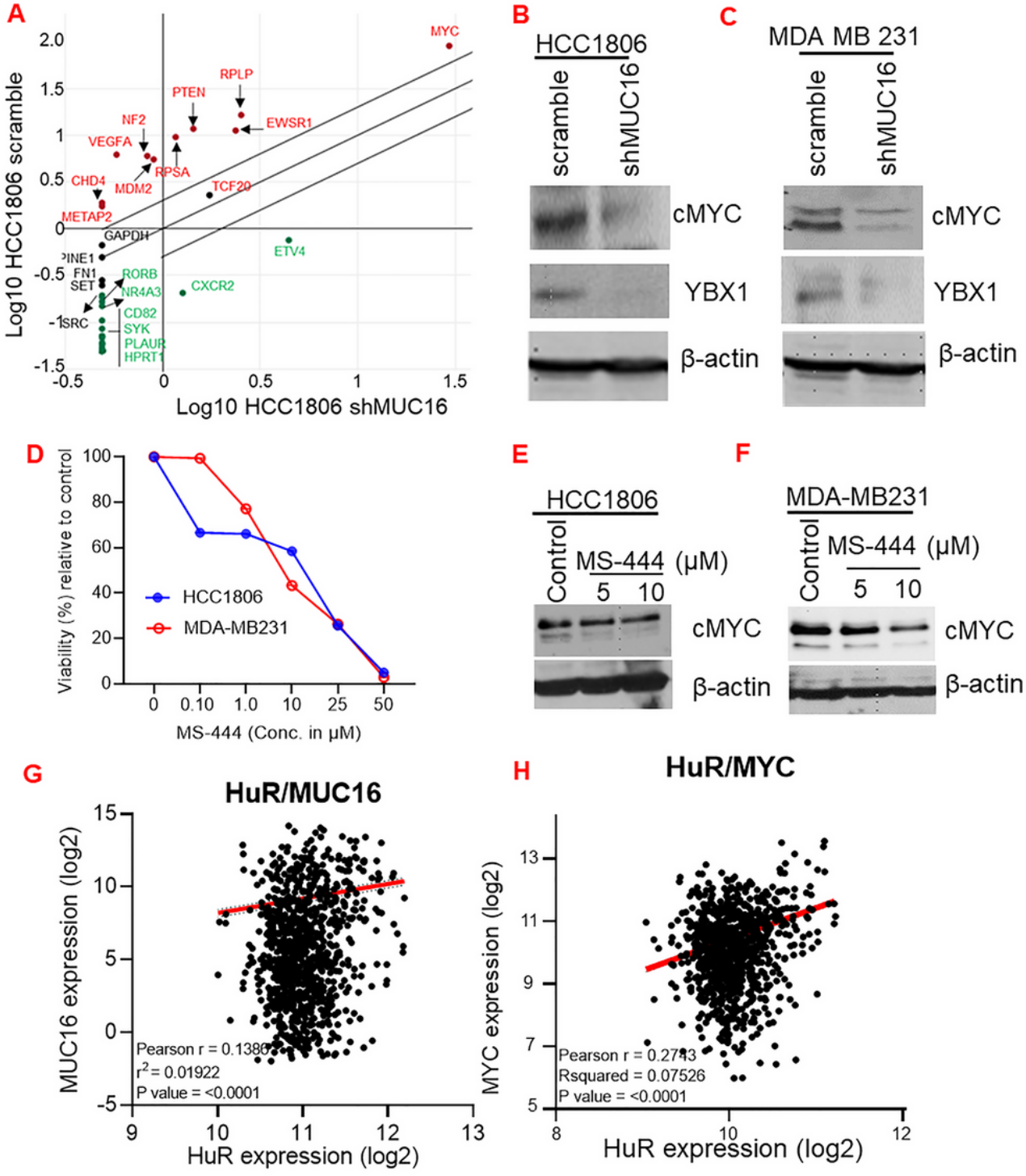

Figure 5

MUC16 associated HuR targets in TNBC. A. Scatter plot of human metastasis array after RIP assay with HuR antibody indicating the MUC16 associated HuR target genes in TNBC cells. Scatter plot showing upregulation of *MYC*, *EWSR1*, *VEGFA*, *RPLP*, *NF2*, and *CHD4* in scramble (HCC1806-SCR) cells; while *ETV4*, *CXCR2, RORB*, and *CD82* genes were downregulated in the MUC16 knockdown (HCC1806-shMUC16) cells. **B & C**. Western blot showing decreased levels of cMYC and YBX-1 expression upon MUC16 knockdown. **D.** MTT assay demonstrating the effect of HuR inhibitor, MS-444, on cell viability of HCC1806 and MDA MB 231 cells. **E & F.** Western blot showing decreased expression of cMYC upon MS-444 treatments in HCC1806 and MDA MB 231 cell lines. **G & H**. TCGA-BRCA analysis indicated co-expression of MUC16 and HuR and HuR and Myc in breast cancer.

Figure 6

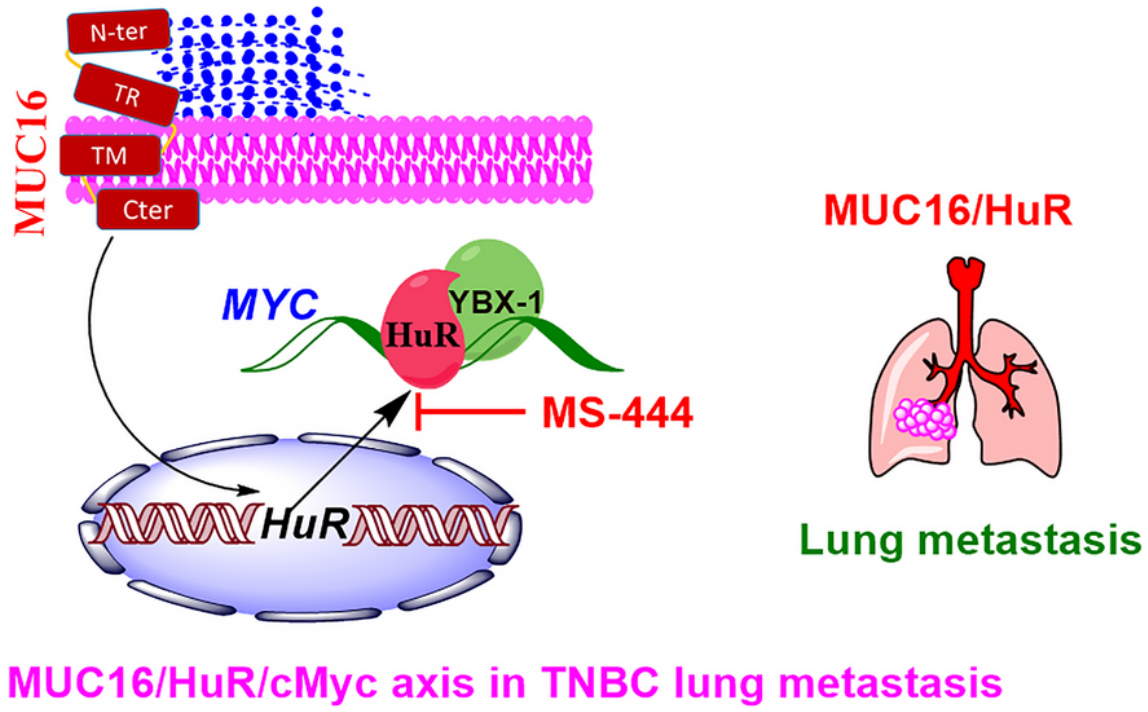

Figure 6

MUC16/HuR signaling in TNBC lung metastasis. The schematic diagram represents that MUC16 regulates the HuR that leads to TNBC lung metastasis through cMyc signaling. Targeting MUC16 could prevent the MUC16 mediated TNBC metastasis.

Supplementary Files

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