

The Ribonucleic Complex HuR-MALAT1 Represses CD133 Expression and Suppresses Epithelial-Mesenchymal Transition in Breast Cancer

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Abstract

Epithelial-to-mesenchymal transition (EMT) is a core process underlying cell movement during embryonic development and morphogenesis. Cancer cells hijack this developmental program to execute a multi-step cascade, leading to tumorigenesis and metastasis. CD133 (PROM1), a marker of cancer stem cells, has been shown to facilitate EMT in various cancers, but the regulatory networks controlling CD133 gene expression and function in cancer remain incompletely delineated. In this study, we show that a ribonucleoprotein complex including the long noncoding RNA MALAT1 and the RNA-binding protein HuR (ELAVL1) binds the CD133 promoter region to regulate its expression. In luminal nonmetastatic MCF-7 breast cancer cells, HuR silencing was sufficient to upregulate N-cadherin (CDH2) and CD133 along

with a migratory and mesenchymal-like phenotype. Furthermore, we found that in the basal-like metastatic cell line MDA-MB-231 and primary triple-negative breast cancer tumor cells, the repressor complex was absent from the CD133-regulatory region, but was present in the MCF-7 and primary ER+ tumor cells. The absence of the complex from basal-like cells was attributed to diminished expression of MALAT1, which, when overexpressed, dampened CD133 levels. In conclusion, our findings suggest that the failure of a repressive complex to form or stabilize in breast cancer promotes CD133 upregulation and an EMT-like program, providing new mechanistic insights underlying the control of prometastatic processes. *Cancer Res*; 76(9); 2626–36. ©2016 AACR.

Introduction

The misactivation of the embryonic dedifferentiation program, epithelial-to-mesenchymal transition (EMT) increases cancer cell motility and dissemination (1), and transient EMT is considered the first essential step in metastasis formation (2). During this complex process, EMT promotes dedifferentiation, growth arrest, migration and invasion of cancer cells, and directly inhibits proliferation by the activation of specific transcription factors of the TWIST, SNAIL, and ZEB families (1–3). One of the recently identified pathways triggering the EMT program stems from the transmembrane protein CD133, and signals through SRC and ERK1/2 (4–6).

CD133 (PROMININ1) is widely recognized as one of the general cancer stem cell (CSC) marker in several cancer types, including breast (7), together with the CD44^{high}/CD24^{low} signature (8). CD133 has a prognostic role in breast cancer (9, 10) and, additionally, its expression has been associated with vascular mimicry capability of triple-negative breast cancer (TNBC) cell lines (11), correlating its presence to more metastasis-prone basal-like cancers. CD133 gene regulation and function in the EMT is still poorly investigated in breast cancer. In this study, we show that a newly identified molecular complex, scaffolded by the long noncoding (lnc) RNA MALAT1 (metastasis associated lung adenocarcinoma noncoding-transcript 1) and comprising the RNA binding protein HuR, regulates CD133 gene expression in dedifferentiating breast cancer cells, both *in vitro* and *in vivo*.

MALAT1 is one of the most widely studied lncRNA with a strong relation to cancer development and progression. MALAT1 is overexpressed in several solid tumors, and its expression correlates with metastasis in lung cancer (12), but the roles of this lncRNA in the EMT process are not completely defined. MALAT1 is highly abundant in the nucleus (13), and its presence in nuclear speckles has been associated with the activity of RNA polymerase II, recruitment chromatin SR splicing factors (14), and Polycomb-2 protein (15). A genome-wide screening experiment revealed the binding to the lncRNA MALAT1 of the RNA-binding protein HuR (16), but the implications of this binding remain unknown so far.

The human antigen R (HuR; or ELAV1) is a member of the embryonic lethal abnormal vision RNA-binding protein family, known to regulate the half-life of target mRNAs (17). Initially

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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identified as an mRNA export factor (18), its main nuclear functions are regulation of splicing, mRNA processing, and polyadenylation (19). In this work, we identified the chromatin-associated HuR/MALAT1 functional complex, during the dedifferentiation process of cancer cells, which controls CD133 gene expression, both *in vitro* and *in vivo*. HuR/MALAT1 impact on CD133 gene expression can regulate EMT phenotype features, suggesting that the fine regulation of these molecules could control, at least in part, tumor progression.

Materials and Methods

Cell lines

The MCF-7 and MDA-MB-231 breast cancer cell lines, purchased and authenticated (STR profiling) from Interlab Cell Line Collection were kept in culture for no more than 2 months in DMEM, 10% FCS. Mammosphere cells were cultured in F12/DMEM (1:1) supplemented with B27 (Invitrogen), 20 ng/mL recombinant human EGF (R&D Systems), 20 ng/mL recombinant human basic-FGF (R&D Systems), 100 U/mL penicillin, 100 µg/mL streptomycin, and 5 µg/mL heparin. Floating aggregates were considered mammospheres when diameter >60 µm.

Primary cell culture

Human breast carcinoma specimens were obtained from the Oncology Unit, San Paolo Hospital (Milan, Italy). Tumor samples were processed within 1 hour after surgical resection, washed with F12/DMEM (1:1) medium, minced with a sterile scalpel, and placed in digestion medium (DMEM/F-12) supplemented with 200 U/mL collagenase A (Stem Cell Technologies) and 100 U/mL hyaluronidase (Stem Cell Technologies) for 1 hour at 37°C.

Cell growth and invasion

The total number of cells in each culture passage was measured, after mechanical dissociation, by Tali image based cytometer (Life Technologies). Results were displayed as log10 of the total number of cells. Cell invasion ability was assessed by QCM fluorimetric cell migration assay kit (ECM509, Chemicon) as per manufacturer's recommendations.

PKH26 staining

Scrambled and knockdown (KD) HuR MCF-7 cells were cultured under mammosphere culture conditions, washed in PBS, and stained with PKH26 (M0973, Sigma). Epifluorescence was measured by BD FACScanto flow cytometer after 8 culture passages (20).

Immunocytochemistry

After cultivation on acid-washed glass coverslips, cells were treated and fixed. The following antibodies were used: anti-HuNu (Millipore, MAB1281), anti-HuR (SC71290, Santa Cruz Biotechnology), and anti-HDAC6 (AB88494, Abcam)

RNA FISH

FISH was adapted from ref. 21. Sense and antisense AlexaFluor 488-labeled RNA probes (Ulysis Nucleic Acid Labeling Kit; Life Technologies) were synthesized from a MALAT1-amplified sequence. The cDNA sequence was verified by sequencing.

Soft agar assay

Mammospheres obtained from parental and KD HuR MCF-7 were suspended 2,500 cells/cm² in 0.3% agarose with mammosphere culture medium on a 0.8% agar base layer and covered with culture medium. Colonies >50 µm diameter were counted and quantified by ImageJ software.

Quantitative real-time PCR

Total RNA from cultured cells and tissue samples were isolated using TRIzol Reagent (Invitrogen). Total RNA (1 µg) was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. qRT-PCR was performed with an DNA engine OPTICON2 detection system (MJ Research) using iQ SYBR Green Supermix (Bio-Rad).

Western blot analysis

Whole cell lysates were used for Western blotting performed in denaturing conditions on polyvinylidene difluoride (PVDF) membrane following Laemmli SDS-page procedure. Anti-HuR (SC365816, Santa Cruz Biotechnology), anti-HDAC6 (AB1440, Abcam), anti-CD133 (130105226, Miltenyi Biotec), anti-N-cadherin (610920, BD Biosciences), anti-E-cadherin (3195, Cell Signaling Technology), and anti-histone H3 (AB1791, Abcam), anti-LDH (ABN311, Millipore), ST1011 (Millipore), and anti-β-actin (A5441, Sigma) antibodies were used. Signal detection was performed by HRP-conjugated secondary antibody and ECL solutions (RPN2108, GE Healthcare). Images were taken with Geldoc XR (Bio-Rad).

Cell migration assay by xCELLigence

Cell migration was monitored in real-time using xCELLigence system. Cells were seeded in a CIM-Plate 16 in triplicate and the migration behavior of each was monitored for 48 hours. Cells maintained in serum-free media served as a control.

IHC

Five-micron thick sections representative of the tumor were immunostained using the Super Sensitive Non-Biotin HRP Detection System (34002, Thermo Scientific), with the anti-human Nuclei (HuNu; MAB1281, Millipore). Eosin (230251, Sigma) staining was used as counterstaining. Controls consisting of alternate sections incubated by omitting the primary antibody did not show any detectable immunoreactivity.

RNA immunoprecipitation

RNA was isolated from the different samples by TRIzol as per the manufacturer's recommendations, retrotranscribed into cDNA by MBI-Fermentas kit, and used as a template for PCR analysis (22).

Capture hybridization analysis of RNA targets

Cross-linked and sonicated nuclear cell extract was incubated with C-oligo, sense-oligo, or without probes and hybridized overnight. Hybridized material was captured with magnetic streptavidin resin (Invitrogen). Bound material was washed, eluted with RNase H, and crosslinking was reversed at 65°C over night, and DNA and protein were purified by phenol:chloroform:isoamyl alcohol precipitation or boiled 5' in Laemmli buffer, respectively. MALAT-1 binding and specificity of used C-oligos and all following steps were performed as described previously (23).

ChIP and RT-on-ChIP

Chromatin immunoprecipitation (ChIP) was performed following previously published protocols (24). In reverse transcription PCR on ChIP (RT-on-ChIP) experiments, the immunoprecipitation protocol was performed in the presence of RNase inhibitor and at the end of the elution step, the samples were reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions and analyzed by PCR.

Xenotransplantation

Scrambled and HuR knocked down (HuR-KD) cells were cultured under mammosphere culture conditions, washed with PBS and stained with PKH26 (M0973 Sigma), and resuspended in PBS/Matrigel mixture (1:1 volume). A measure of 5×10^6 cells in 0.1 mL of this mixture was implanted in the mammary fat pad of 5-week-old female athymic nude Fox1nu mice (Harlan Laboratories). The mice received β -estradiol (E2758, Sigma) supplementation (0.4 mg/kg) every 7 days. Animal experimentation was performed according to the protocol 2/2013 approved by the local ethical committee.

Anoikis test

Anoikis assay (25) was performed by seeding MCF-7 cells at 1×10^5 cells/mL in either DMEM complete medium or in mammosphere forming medium. Cyclosporin-A (30024, Sigma) 10 μ mol/L for 24 hours was used as a positive control. Cells were incubated for 24 hours prior to the MTT assay (M5655, Sigma). We tested cell population in adhesion and after 5 days in suspension cultures. MTT signal was normalized to the number of cells and measured by Bradford assay at day 1 and day 5.

Ethical disclosure

The ethical committee of the Department of Health Sciences approved the design of this study, which was carried out according to the guidelines of the University of Milan (Milan, Italy). Specimens from 6 subjects (females between the ages of 66 to 73 years) were kept at the San Paolo Hospital (Milan, Italy). An informed consent was obtained from all the patients. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council, Italy. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Milan (Milan, Italy).

Additional and detailed materials and methods are provided in the "online Supplementary Material" section.

Results

HuR-stable silencing leads to adhesion increase and cell proliferation decrease of mammospheres

To characterize the role of HuR in maintaining the mesenchymal state of cancer cells, we generated mammospheres from MCF-7, in which HuR was stably ablated (HuR-KD; ref. 26; Supplementary Fig. S1A and S1B). Cumulative cell counts of HuR-KD mammospheres at passage 8 revealed a reduced number of cells, compared with the control (scrm). The higher degree of PKH26 staining retention in HuR-KD mammospheres pointed to a slower rate of cell proliferation, and not to a differential resistance to anoikis (Fig. 1A and Supplementary

Fig. S2). Moreover, HuR-KD mammospheres were higher in number but smaller in dimension than control-derived mammospheres (Fig. 1B). We evaluated the ability of HuR-KD mammospheres to readhere to the substrate and migrate or invade. Surprisingly, in cell motility tests, HuR-KD mammospheres reached significantly higher values compared with the parental population, in the absence of serum, (Fig. 1C and Supplementary Fig. S3A). In anchorage-independent condition, HuR-KD mammospheres grew slowly, as smaller colonies than wild-type cells were formed in soft agar at fixed time points (Fig. 2A). However, when HuR-KD cells were allowed to grow until the colonies reached a fixed dimension, we counted the same number of colonies as for the wild-type cells, again suggesting a decrease in proliferation rate, and not in clonogenic survival. To evaluate the tumorigenic potential of disaggregated cells from HuR-KD mammospheres, we injected dilutions of cells (5×10^2 – 5×10^6) orthotopically in the mammary fat pad of the recipient female nude mice and monitored the onset and dimension of the derived tumors (Fig. 2B and Supplementary Fig. S3B–S3D). Cells from both types of mammospheres generated tumors in the 45 days dedicated to the observation, but those from HuR-KD mammospheres gave rise to significantly smaller tumors in comparison with the control, confirming the reduced proliferation rate of HuR-KD cells, but equivalent tumorigenic potential. HuR-KD cells displayed high N-cadherin and CD133 expression levels compared with the wild-type, suggesting that genetic ablation of HuR can affect the EMT outcome. As CD133 regulates N-cadherin expression (6), we silenced CD133 (CD133 KD; Fig. 2C and Supplementary Fig. S4A) in wild-type and HuR-KD MCF-7 cells. Interestingly, CD133 silencing in HuR-KD cells reduced N-cadherin expression to the wild-type level, suggesting a role for HuR in CD133-dependent EMT. In summary, HuR-KD mammospheres show the same tumorigenic potential as wild-type population, but HuR silencing enforces a mesenchymal phenotype by increasing cell migration together with the decrease in cell proliferation.

HuR depletion increases CD133 expression without binding its mRNA

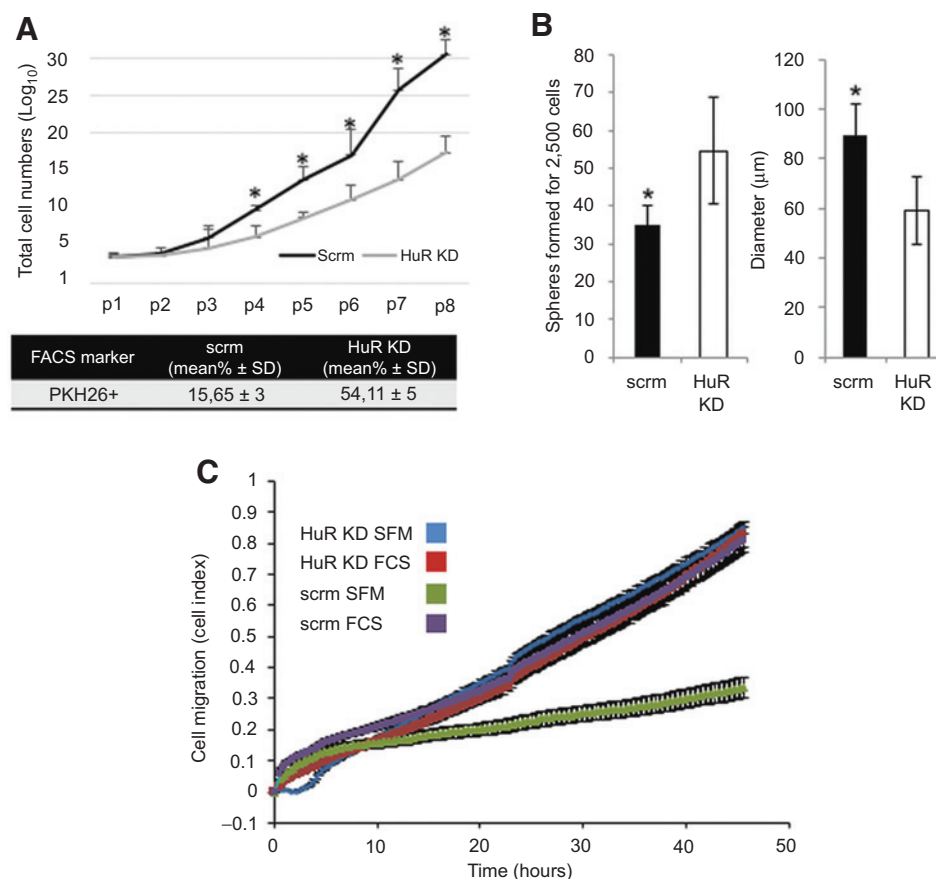
As the overexpression of N-cadherin was CD133 dependent in HuR-KD mammospheres, we investigated whether HuR was directly regulating CD133 mRNA. A significant increment of CD133 mRNA and protein expression was observed in the entire HuR-KD population, compared with control (Fig. 2D). HuR is known for its ability to bind the 3'UTR of mRNA molecules regulating their half-life and translation (27), but, by RNA immunoprecipitation assay (RIP), we did not find any significant fold increase of CD133 mRNA in the HuR-bound material (Supplementary Fig. S4B).

HuR binding to CD133 promoter region in MCF-7 mammospheres requires MALAT1

We then wondered whether HuR was binding to the CD133 gene, using ChIP both in adherent cells and in mammospheres. We probed four different positions (A, B, C, and D region) and found HuR bound to the one (D), within the second intron of the CD133 gene, but only in mammospheres originating from MCF-7. We did not observe this binding in mammospheres derived from MDA-MB-231 breast cancer cells, where CD133 gene is transcribed at a higher level than MCF-7 cells (Fig. 3A). The

Figure 1.

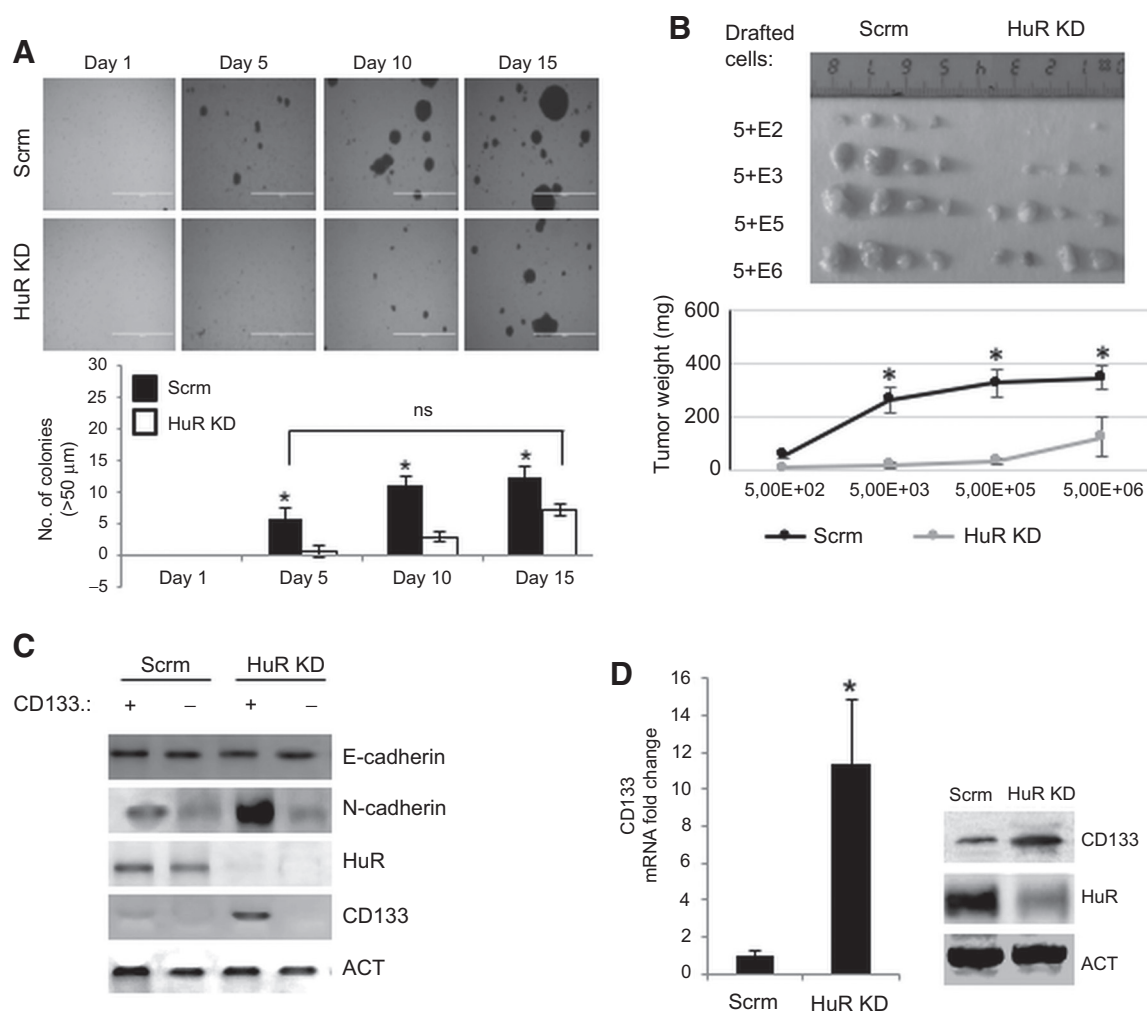
HuR-KD mammospheres cellular are less proliferating but more migrating than wild-type mammospheres. A, cell growth curves of scrambled wild-type mammospheres (scrm) and HuR silenced (HuR-KD) mammospheres reported as total cell number counted at each culture passage (p1–p8, every four days; top). Bottom, PKH26 staining of MCF-7 mammospheres as a percentage of positive cells. *, $P < 0.01$ in t test. B, mammosphere number and diameter of HuR-KD (white bar) and control scrm (black bar) MCF-7 cells after 15 culture days by 2,500 seeded cells. *, $P < 0.01$ in t test. C, xCELLigence migration/adhesion plot of HuR-silenced (HuR-KD) and scrambled control (scrm) mammospheres. Cells were cultured and monitored for surface adhesion (cell index) for 45 hours in the presence (FCS) or absence (SFM) of serum. The HuR-KD cells in the presence (red dot) or absence (blue dot) of serum adhere to the surface, instead scrm adhere only in the presence of serum (purple dot) and fail adhesion in serum-free condition (green dot).



primers used to probe region D amplify a 234 bp long fragment from base 16077104 to base 16077337 of chromosome 4 (GRCh37), within the second intron of the *CD133* gene. Notably, in adherent cells, HuR was bound to a promoter region of the *CD133* gene (A). We evaluated whether the binding detected by ChIP was RNA-dependent (28): RNAse digestion abrogated HuR binding to both regions A (in adherent cells) and D (in mammospheres) of the *CD133* gene. HuR binds to several lncRNAs, such as linc-p21, linc-MD1, and MALAT1 (16, 29, 30). MALAT1 has been shown to scaffold ribonucleoprotein complexes on the chromatin to regulate gene expression (31, 32) and is overexpressed in mammospheres compared with adherent cells while linc-p21 expression was not affected by the adhesion condition (Fig. 3B). According to this, we hypothesized that the RNA molecule responsible for HuR binding is MALAT1. Strikingly, genetic ablation by siRNA against MALAT1 abolished HuR binding to the *CD133* D region in mammospheres, but not to the *CD133* A region in adherent cells (Fig. 3A). MALAT1 is mainly a nuclear lncRNA molecule (Fig. 3C; refs. 33, 34) and is bound by HuR (Fig. 3D). To validate the presence of the complex on the *CD133* D region, we performed capture hybridization analysis of RNA targets (CHART; ref. 35). MALAT1 was bound to the regulatory region of its gene (32), our positive control, and, on the *CD133* gene A region, but weakly on the *CD133* gene D region, and not on the *ACTIN* gene, used as negative control (Fig. 4A, top). In mammospheres, MALAT1 appeared to be bound to the D region and not to the A region (Fig. 4A, bottom). Moreover,

analyzing MALAT1 pull-down protein fraction during CHART, we observed the presence of HuR, validating its binding to MALAT1 on the chromatin, only in mammospheres (Supplementary Fig. S5). This result is in agreement with ChIP data and supports the copresence of HuR and MALAT1 on the D chromatin region in mammospheres, and in A region in adherent cells. MALAT1 was present alone on the D region, but with much less confidence and to a lower extent than to the A region, in adherent condition (Fig. 4A). Therefore, the additional contribution of HuR is important for stabilizing MALAT1 binding to this region, and, at the same time, its ability in binding the chromatin also in the absence of HuR, possibly together with other trans-factors. HuR binding to the A region in adherent cells is MALAT1 independent, but RNA-dependent, indicating additional mechanisms of position-specific recruitment of HuR on the *CD133* locus. In mammospheres, we also checked for the presence of MALAT1 RNA in the HuR ChIP samples by RT-PCR (RT-on-ChIP) showing the existence of the HuR-MALAT1 assembly in the nucleus (Fig. 4B). Either HuR or MALAT1 silencing induced the upregulation of the *CD133* protein expression level (Figs. 2D and 4C), and of the downstream molecule N-cadherin (Fig. 4D) showing the functional relevance of the HuR/MALAT1 complex. Moreover, MALAT1 downregulation did not affect HuR expression level (Fig. 4D). Therefore, HuR and MALAT1 recruited within the second intron of *CD133* gene to the chromatin exclusively in MCF-7 mammospheres, limiting the expression of the gene.

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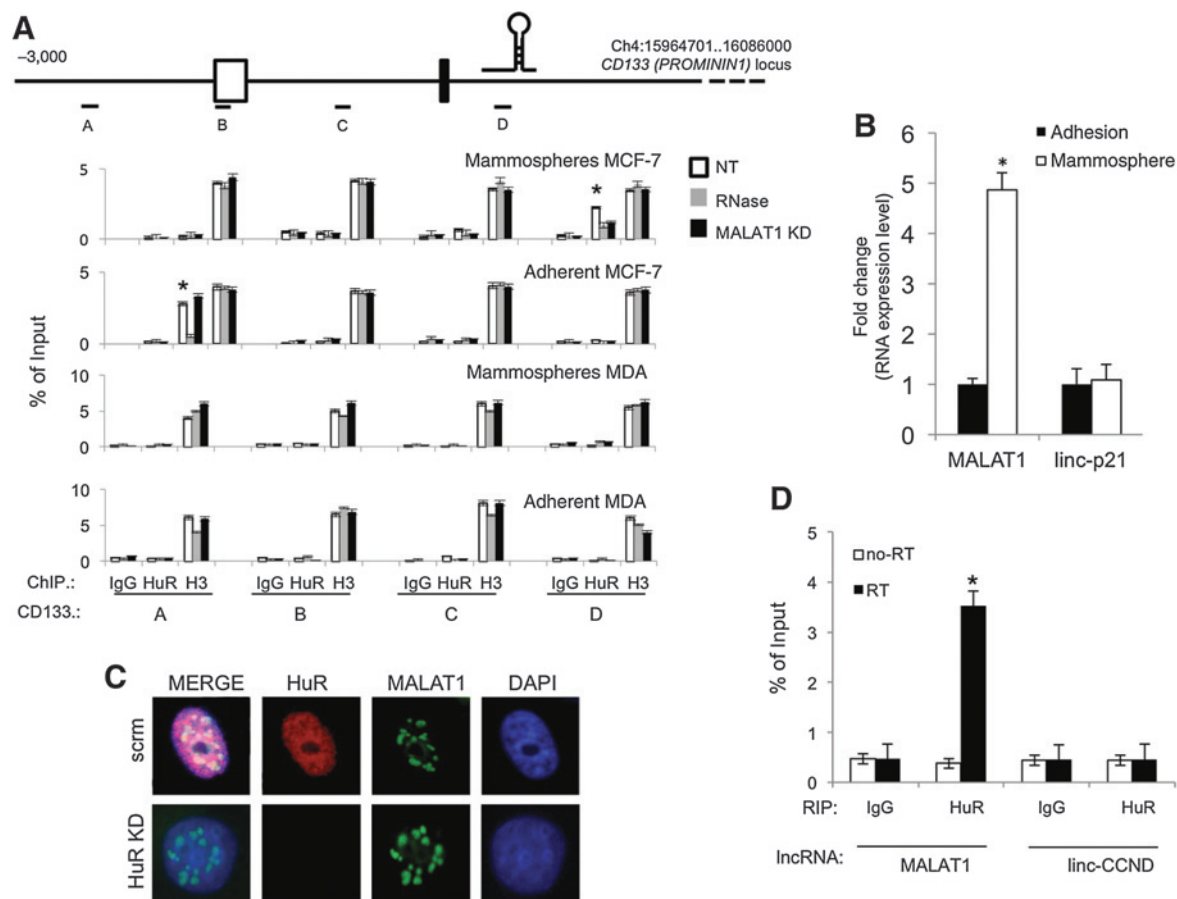
**Figure 2.**

HuR ablation in mammospheres decreases tumor dimension but increases CD133-dependent N-cadherin expression. A, soft agar colony formation of scrambled (scrm) and HuR-silenced (HuR-KD) mammospheres. Top, direct light pictures of the cultures at different time points (day 1–15). Bottom, histogram gives a quantification of the colony formed by scrm (black bar) and HuR-KD (white bar). Scrm colonies at day 5 reached a similar size as HuR-KD cells at day 15. *, $P < 0.01$, t test. B, the picture shows the tumors surgically removed from female nude mice orthotopically xenotransplanted in the mammary fat pad with scrambled (scrm) and HuR-silenced (HuR-KD) mammospheres. Surgery was performed with different dilution of cells (from 5×10^2 – 5×10^6). The graph shows the tumor weight (mg) originated from the two cell types. *, $P < 0.01$, t test. C, Western blots showing the expression of E-cadherin, N-cadherin, HuR, and CD133 in parental (scrm) or HuR-KD MCF-7 mammospheres in which CD133 expression was transiently ablated (CD133 KD). β -Actin was used as a loading control. HuR-KD displayed higher CD133 and N-cadherin compared with the parental. CD133 silencing reverted N-cadherin expression to the parental level in HuR-KD. D, the histogram shows CD133 mRNA expression levels in scrambled (scrm) and HuR-silenced (HuR-KD) mammospheres. mRNA levels were determined by qRT-PCR and are reported as $2^{-\Delta(C_T)}$, where the housekeeping genes were 18S and GAPDH. Values were plotted as mean \pm SD from three independent experimental replicates. *, $P < 0.01$ in t test. Right, Western blotting showing CD133 protein expression level in HuR-KD mammospheres in comparison with control (scrm). Actin was used as loading control.

Differential association of the HuR/MALAT1 complex on the CD133 gene in ER⁺ and TNBCs

We compared MCF-7 cells, a model for luminal (epithelial) breast cancer, with MDA-MB-231 cells, defined as basal-like invading (mesenchymal) breast cancer (35). The protein level of EMT markers, in mammospheres derived from MDA-MB-231 cells, showed high N-cadherin and low E-cadherin expression, compared with MCF-7 mammospheres (scrm). The same was observed with mammospheres derived from primary ER⁺ (luminal) or TNBC (basal-like) tumor cells (Fig. 5A; Supplementary Table S1). CD133 was higher in MDA-MB-231 and TNBC primary

cells, as in the HuR-KD MCF-7 cells, compared with the parental MCF-7 and the ER⁺ primary cells (Fig. 5A). HuR expression was comparable between MCF-7 and MDA-MB-231 or ER⁺ and TNBC primary cells (Fig. 5A). CD133 was overexpressed in mammospheres derived from MDA-MB-231 cells, and in TNBC when compared with the MCF-7 cells and ER⁺ samples. Notably, MALAT1 had an opposite trend of expression to CD133 while HuR levels did not change (Fig. 5B and C). We ectopically overexpressed MALAT1 in MDA-MB-231, observing CD133 and N-cadherin downregulation (Fig. 5E and F) according to the HuR-MALAT1 complex repression activity.

**Figure 3.**

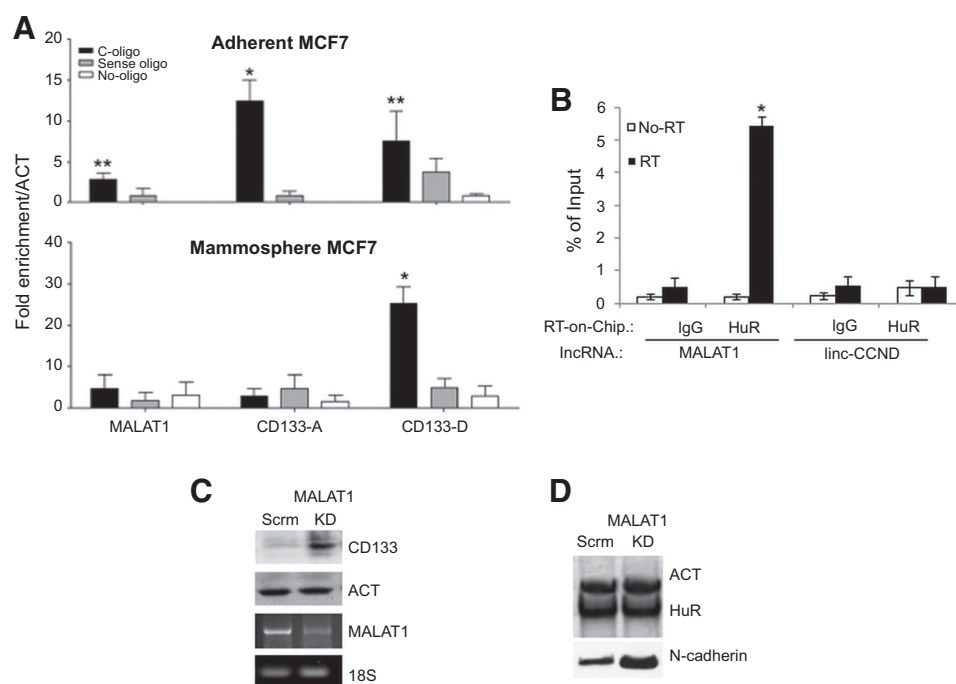
HuR interacts with MALAT1 and binds to the CD133 gene in mammospheres only. A, top, map showing prominin1 locus. Black and white (5'UTR) boxes, exons. The small black bars named from A-D refer to the regions. The RNA hairpin is representative of MALAT1. Bottom, ChIP was performed with MCF-7 and MDA-MB-231 as mammospheres or in adhesion. HuR binding to CD133 gene regions named A, B, C, and D was tested. Results are reported in the histogram as input percentage (% of input) of the fold enrichment measured by densitometry. PCR was performed with (RNase, gray bar) or without RNase treatment (NT, white bar) or after MALAT1 RNA silencing (MALAT, black bar). Preimmunization serum (IgG) was used as precipitation negative control. Anti-histone-H3 was used as positive control. Densitometric values were plotted as mean \pm SD from independent experimental triplicates. *, $P < 0.01$ in t test. B, the histogram shows MALAT1 and linc-p21 RNA expression level in MCF-7 cells grown in adhesion (adhesion, black bar) in comparison with mammospheres (mammosphere, white bar). Values were plotted as mean \pm SD from three independent experimental replicates. *, $P < 0.01$ in t test. C, immunofluorescence and RNA FISH against HuR (red) and MALAT1 RNA (green) on MCF-7 cells silenced (HuR-KD) or not (scrm) for HuR expression. DAPI was used as counterstaining. D, RIP assay was performed with MCF-7-derived mammospheres to evaluate MALAT1 RNA binding to HuR. The results are reported in the histogram as input percentage (% of input) of the fold enrichment, measured by densitometry. PCR was performed with (black bar) or without retrotranscription (no RT) as amplification negative control (white bar). Preimmunization serum (IgG) was used as precipitation negative control. linc-CCND was used as a negative control. Densitometric values were plotted as mean \pm SD from three independent experimental replicates. *, $P < 0.01$ in t test.

To evaluate the generalization of the CD133-MALAT1 inverse correlation between ER⁺ and TNBC tumors, we checked public datasets derived from high-throughput sequencing expression profiles (RNA-seq) performed on primary tumors (116 patients, 28 breast cancer cell lines, 42 TNBC primary tumors, 42 ER⁺ and HER2-negative breast cancer primary tumors and 30 uninvolved breast tissue samples). TNBC patients display significantly higher CD133 expression levels if compared with the ER⁺ ones (GSE58135; Fig. 5D). MALAT1 was upregulated in tumor samples compared with healthy tissues and was downregulated in TNBC compared with ER⁺ tumor samples. However, the difference was not highly significant, suggesting that other parameters can play a role in defining the CD133 expression. HuR was not found to change in the overall analysis. Normal breast tissue showed a Pearson coefficient (r) = -0.36, analogous to the ER⁺ breast

cancer tissue samples (r = -0.34) showing a moderate inverse correlation of MALAT1 and CD133 expression levels. Combining the two datasets, the inverse correlation is even stronger (r = -0.44). TNBC samples lost this inverse correlation (r = 0.016), in good agreement with the absence of MALAT1/HuR on the CD133 gene, and consequent dysregulation of CD133 expression level (Table 1).

We performed RIP, RT-on-ChIP, and ChIP analyses on mammospheres derived from three ER⁺ breast cancer patients' primary cells. Indeed, we found the same pattern of chromatin association as in MCF-7 cells: HuR was binding to MALAT1 RNA (Fig. 6A), and the complex was present on the chromatin (Fig. 6B) at the level of the CD133 D region in an RNA-dependent manner (Fig. 6C). Coherently, the HuR/MALAT1 inhibiting ribonucleic complex was not found to be associated with the CD133 D region by ChIP

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**Figure 4.**

Differential binding sites of MALAT1 in mammospheres and MALAT1-dependent CD133 expression. A, MALAT1 ChART enrichment of MALAT1, CD133 locus A, and CD133 locus D DNA assessed by qPCR from adherent MCF-7 cells or MCF-7-derived mammospheres. The readout is expressed as chromatin fold enrichments normalized for the negative control Actin gene. ChART sheared DNA was precipitated in three different conditions with MALAT1-specific RNA probe (C-oligo, black bar), with RNA probe not complementary to MALAT1 (sense-oligo, gray bar) and without probe as a further control (no-oligo, white bar). *, $P < 0.01$; **, $P < 0.05$ in t test. B, RT-on-ChIP was performed on MCF-7-derived mammospheres to detect MALAT1 RNA binding the chromatin bound to HuR. The results are reported in the histogram as input percentage (% of input) of the fold enrichment. PCR was performed with (black bar) or without retrotranscription (no RT) as negative control (white bar). Preimmunization serum (IgG) was used as precipitation negative control. linc-CCND was used as a negative control. Densitometric values were plotted as mean \pm SD from three experimental replicates. *, $P < 0.01$ in t test. C, Western blots showing CD133 protein expression level during MALAT1 silencing (MALAT) in mammospheres in comparison with scrambled control (scrm). Actin was used as loading control. MALAT1 expression level was measured by semi-qRT-PCR using 18S as loading control. D, Western blots showing HuR and N-cadherin protein expression level during MALAT1 silencing (MALAT) in mammospheres in comparison with scrambled control (scrm). Actin was used as loading control.

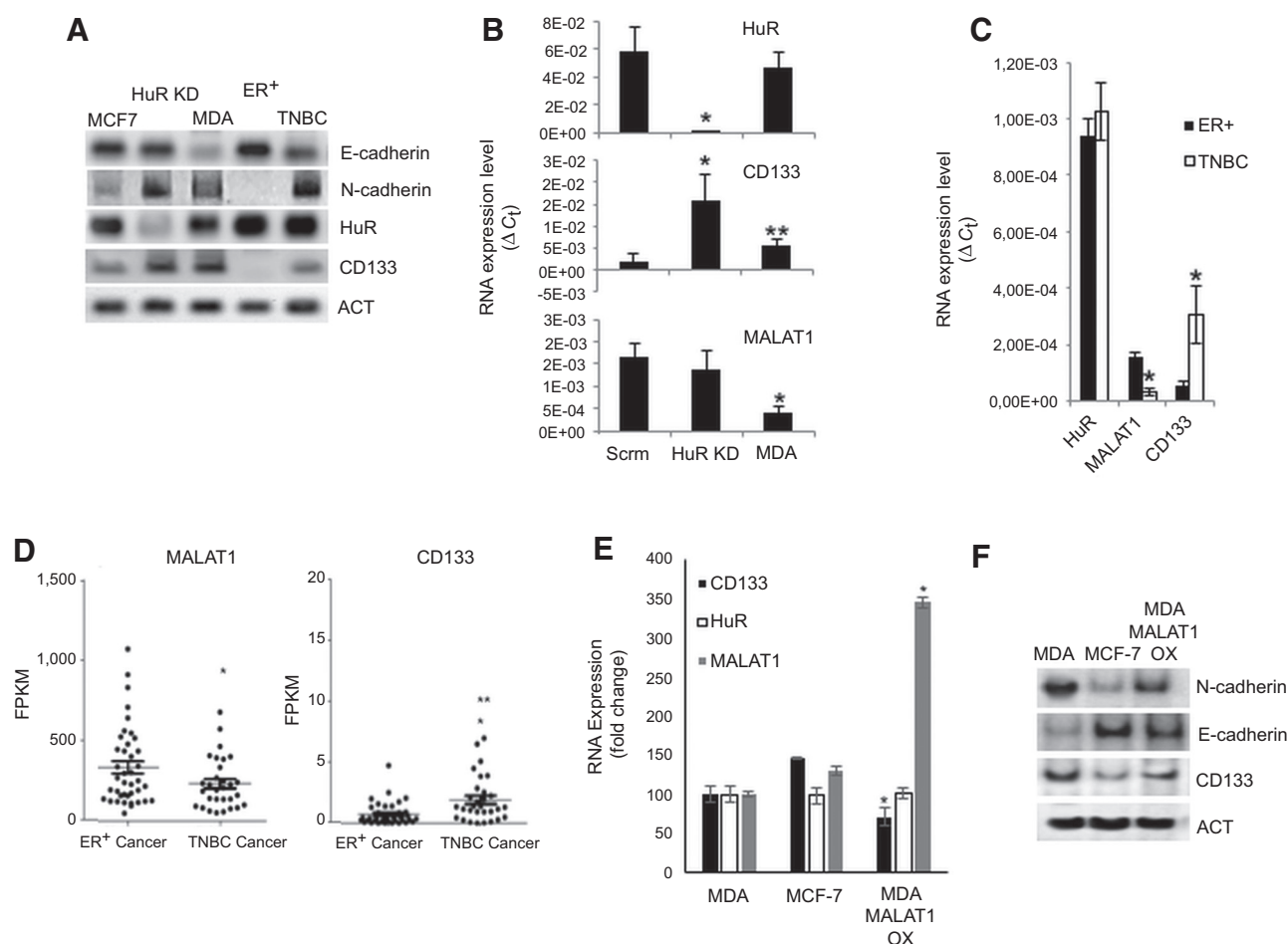
analysis in MDA-MB-231 and TNBC primary cells derived mammospheres (Fig. 6D). Therefore, CD133 is upregulated in TNBC samples, and the reason for this overexpression partially resides in the downregulation of MALAT1 in TNBC compared with ER⁺ samples. This supports the idea that the fine-tuning of the complex components regulates the expression level of CD133 gene, affecting EMT phenotype.

Discussion

CD133 plays a crucial role in cancer metastasis acting at the level of EMT in pancreatic ductal adenocarcinoma (4, 6) while its functional role in breast cancer is not well defined. We observed that HuR-stable downregulation in dedifferentiating, nonmetastatic breast cancer cell line (luminal ER⁺ cell model, MCF-7) induced the acquisition of EMT traits, such as a more adhesive and migrating phenotype and, concomitantly, a reduction in proliferation rate. Molecularly, the increase of N-cadherin was explained by the upregulation of CD133, which is caused by the detachment of a transcriptional inhibitory complex containing MALAT1/HuR from a CD133 gene regulatory region in intron 2. MALAT1 is a well characterized predictive marker for metastasis development in lung cancer, where it specifically promotes EMT transition, by regulating the expression level of key prometastatic

genes as *LPHN2*, *ROBO1*, *ABCA1*, and *GPC6* (34, 36). However, much less is known regarding its role in breast cancer EMT, where MALAT1 downregulation is suggested to trigger EMT by regulating *LPHN2*, *ROBO1*, and *GPC6* genes and increasing N-cadherin protein expression in breast cancer cell lines (37). HuR is known to bind lncRNA molecules as linc-MD1 and lincRNA-p21 and posttranscriptionally regulate their function in the cytoplasm (29, 30). We showed that HuR was required in the formation of a chromatin regulatory complex, bearing a lncRNA core with a transcriptional repressive function. Other RBPs, such as the hnRNPK or TLS/FUS proteins (38–40) that form repressive chromatin complexes, in association with lincRNA-p21 and ncRNA-CCND1, respectively, on gene promoters. In our case, the complex (HuR/MALAT1) is bound to a region within the CD133 gene second intron, suggesting a variation of the chromatin state, via recruitment of epigenetic modulators, or a different chromatin looping between the adherent and mammosphere cell state.

We showed that genetic downregulation of any components of the complex led to an upregulation of the CD133 gene, and to the following switch of the ER⁺ breast cancer cells to a more basal-like phenotype. Interestingly, CD133 is epigenetically regulated and highly expressed in basal-like TNBC (41, 42), and is a concurring cause of the aggressiveness of this breast cancer subgroup (11, 43). Importantly, we did not detect HuR, and its complex, on the

**Figure 5.**

TNBCs show different expression level of MALAT1, CD133, and N-cadherin in comparison with ER⁺ cancers. A, Western blots showing the expression level of E-cadherin, N-cadherin, HuR, and CD133 in mammospheres derived from parental MCF-7 cells (scrm), HuR knocked down MCF-7 cells (HuR-KD), MDA-MD-231 cells, and primary cells from ER⁺ (Patient ID:ER+1) and TNBC (PatientID: TNBC) tumors. β -Actin was chosen as loading control. HuR-KD displayed higher CD133 and N-cadherin like MDA-MB-231 and TNBC. B, qRT-PCR to evaluate HuR, CD133, and MALAT1 expression level in mammospheres derived from MCF-7 cells HuR silenced (HuR-KD) or not (scrm) and MDA-MB-231 cells. *, $P < 0.01$ in t test. C, qRT-PCR to detect MALAT1 RNA expression level in primary cells from ER⁺ (black bar) and TNBC (white bar) tumors (All patients). *, $P < 0.01$ in t test. D, MALAT1 and CD133 expression levels expressed as fragments per kilobase of exon per million fragments mapped (FPKM) obtained for GSE58135 RNA-seq dataset. TNBC patients display an overall lower MALAT1 and a higher CD133 expression levels if compared with the ER⁺ ones. *, $P = 0.0421$; ***, $P = 0.0006$ in Wilcoxon signed-rank test. E, qRT-PCR to evaluate HuR, CD133, and MALAT1 expression level in mammospheres derived from MCF-7 and MDA-MB-231 cells overexpressing MALAT1 (MDA MALAT1 OX) or not (MDA) and MCF-7 cells. Values are expressed as fold change relative to the MDA. *, $P < 0.01$ in t test. F, Western blots showing the expression level of E-cadherin, N-cadherin, and CD133 in mammospheres derived from parental MCF-7 cells and MDA-MD-231 overexpressing or not MALAT1 (MDA MALAT1 OX). β -Actin (ACT) was chosen as loading control. MALAT1 overexpression reduces CD133 and N-cadherin expression in MDA-MB-231.

CD133 gene regulatory region in TNBC cell model (MDA-MB-231) and primary TNBC tumor cells, as we did in ER⁺ cell model (MCF-7) and primary ER⁺ mammosphere tumor cells. Indeed, mammospheres represent a dedifferentiated cell state in which

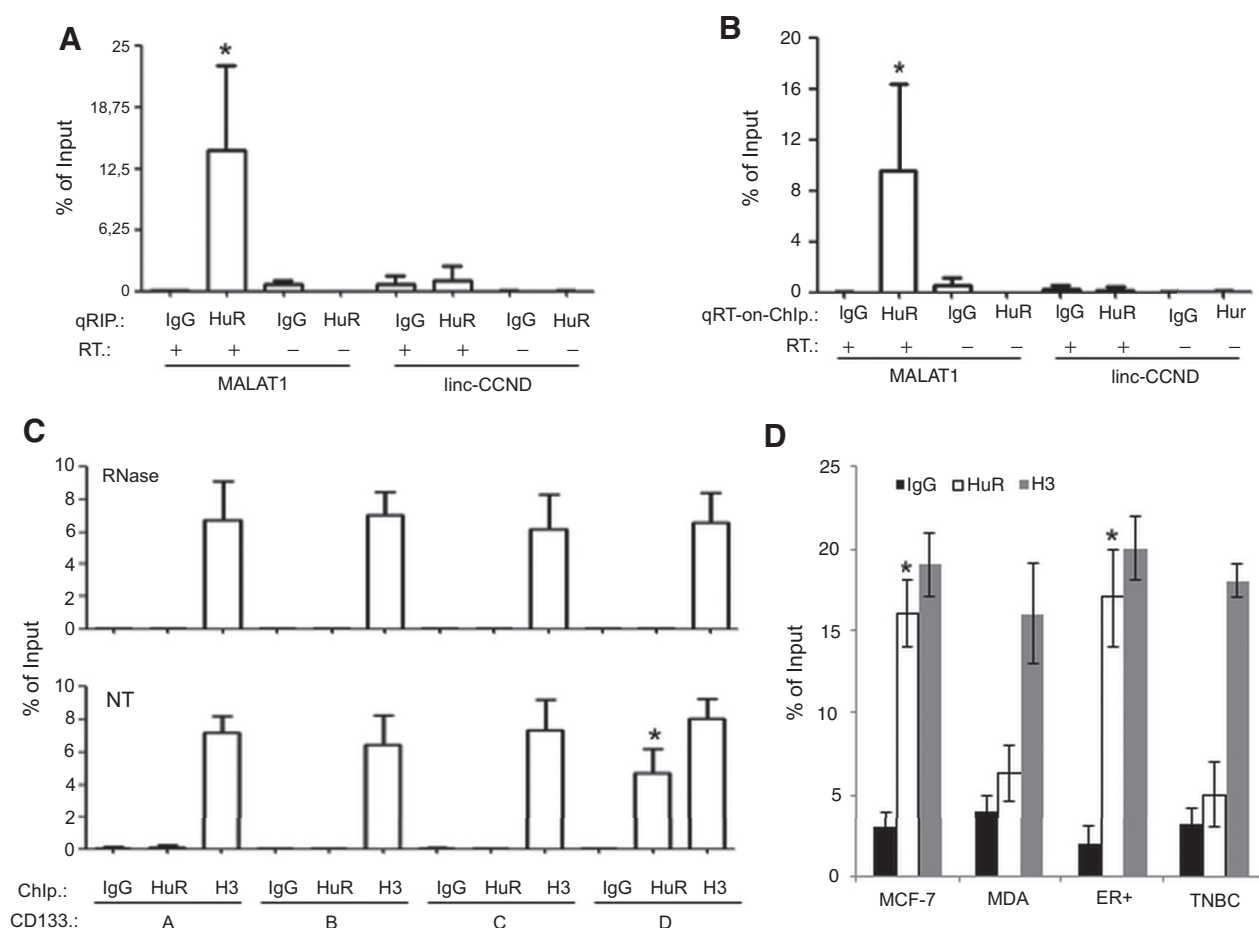
EMT and migration of cancer cells is significantly favored (42, 44). A possible explanation resides in the loss of complex stability due to the downregulation of MALAT1 RNA molecule, in TNBC cells with respect to ER⁺ cells. Our results are in good agreement with the RNA-seq public dataset GSE58135, of breast cancer samples. In this dataset, we observed a significantly higher relative expression of CD133 in TNBC samples compared with ER⁺ samples, while, MALAT1 expression, although higher in comparison with normal tissues (not shown), exhibited a clear downregulating trend in TNBC tumors in comparison with the ER⁺ samples. In addition, the inverse correlation between MALAT1 and CD133 in ER⁺ samples was lost in TNBC samples, further supporting the functional repressive role of the MALAT1/HuR assembly on the D region of the CD133 gene. In conclusion,

Table 1. The Pearson coefficient for CD133 and MALAT1 expression of the indicated datasets

Dataset samples	Pearson coefficient, r
Normal, adjacent tissue	-0.36
ER ⁺ Her2- tissue	-0.34
Normal adjacent tissue plus ER ⁺ Her2- tissue	-0.44
TNBC	0.016

NOTE: The inverse correlation is moderate for normal, ER⁺, and for the sum of these two datasets but absent in TNBC cancer tissues.

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**Figure 6.**

HuR-MALAT1 ribonucleic complex is present on CD133 gene D region in mammospheres derived from ER⁺ primary cells. A, RIP assay was performed on mammospheres derived from ER⁺ primary tumor cells (all patients) to evaluate MALAT1 RNA binding to HuR. The results are reported in the histogram as input percentage (% of input) of the fold enrichment. PCR was performed with or without retro transcription (RT) as amplification negative control. Preimmunization serum (IgG) was used as precipitation negative control. The densitometry values were plotted as mean \pm SD from three different tumors. *, $P < 0.01$ in t test. B, RT-on-ChIP was performed on mammospheres derived from ER⁺ primary tumor cells (all patients) to evaluate MALAT1 RNA binding the chromatin bound to HuR. The results are plotted as input percentage (% of input) of the fold enrichment. PCR was performed with or without retrotranscription (RT) as amplification negative control. Preimmunization serum (IgG) was used as precipitation negative control. linc-CCND was used as negative control. The densitometric values were plotted as mean \pm SD from three different tumors. *, $P < 0.01$ in t test. C, ChIP assay was performed to amplify four CD133 gene regions, named A, B, C, and D bound to HuR or histone H3, on mammospheres derived from ER⁺ primary tumor cells (all patients). The results are reported in the histogram as input percentage (% of input) of the fold enrichment. PCR was performed with (RNase) or without RNase treatment (NT). Preimmunization serum (IgG) was used as precipitation negative control. Anti-histone H3 was used as positive control. Densitometric values were plotted as mean \pm SD from three different tumors. *, $P < 0.01$ in t test. D, ChIP was performed on mammospheres derived from ER⁺ primary tumor cells (all patients), TNBC primary tumor cells (all patients), MCF-7 and MDA-MB-231 cell lines to amplify CD133 gene D region bound to HuR (white bar) or histone H3 (gray bar). The results are reported in the histogram as input percentage (% of input) of the fold enrichment. Preimmunization serum (IgG) was used as precipitation negative control (black bar). Anti-histone H3 was used as positive control. Densitometric values were plotted as mean \pm SD from three different tumors. *, $P < 0.01$ in t test.

two novel observations can be gathered. The first one is the identification of a molecular mechanism underlying the CD133-dependent breast cancer EMT, in which the fine-tuning regulation of MALAT1 expression level modulates the insurgence of EMT traits with different outcome in TNBC cells and in ER⁺ cells. The second one is the description of a novel nuclear role for the RNA-binding protein HuR that, in association with the lncRNA molecule MALAT1, regulates the expression level of a key gene related to the EMT process. Clearly, to generalize this observation, high-throughput investigation combining HuR and MALAT1 association to the chromatin are warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Latorre, C. Zucal, G. Moro, A. Luciani, G. Ghilardi, E. Monti

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Latorre, S. Carelli, I. Raimondi, V. D'Agostino, E. Monti, A.M. Di Giulio, A. Gorio

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Gorio

Study supervision: G. Ghilardi, A.M. Di Giulio, A. Provenzani

Other (performed part of the presented experiments): I. Castiglioni

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The Ribonucleic Complex HuR-MALAT1 Represses CD133 Expression and Suppresses Epithelial–Mesenchymal Transition in Breast Cancer

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