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

Elisa Latorre1,2, Stephana Carelli2, Ivan Raimondi3, Vito D’Agostino1, Ilaria Castiglioni1, Chiara Zucal1, Giacomina Moro4, Andrea Luciani4, Giorgio Ghilardi2,4, Eleonora Monti2,4, Alberto Inga3, Anna Maria Di Giulio2, Alfredo Gorio2, and Alessandro Provenzani1

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.

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+ /CD24low 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 (Inc) RNA MALAT1 (metastasis associated lung adenocarcinoma non-coding 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 IncRNA 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 IncRNA 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 IncRNA 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
EMT Can Be Modulated by Fine Tuning MALAT1 and HuR

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 (Qness). Results were displayed as log10 of the total number of cells. Cell invasion ability was assessed by QCM (Qness).

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). Fluorescence 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 (Ulyssys 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 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-CDC133 (130105226, Milltenyi Biotech), 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 (RP2108, GE Healthcare). Images were taken with GelDoc XR (Bio-Rad).

Cell migration assay by xCELLingence

Cell migration was monitored in real-time using xCELLingence 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 cul-
tured under mammosphere culture conditions, washed with PBS
and stained with PKH26 (M0973 Sigma), and resuspended in
PBS/Matrix mixture (1:1 volume). A measure of 5 and stained with PKH26 (M0973 Sigma), and resuspended in
PBS/Matrix mixture (1:1 volume). A measure of 5 × 106 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 Labora-
tories). The mice received β-estradiol (E2758, Sigma) supplemen-
tation (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 ×
105 cells/ml in either DMEM complete medium or in mamos-
sphere forming medium. Cyclosporin-A (30024, Sigma) 10
µmol/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 sus-
pension 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). Speci-
mens 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 Com-
mittee 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-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 immu-
noprecipitation assay (RIP), we did not find any significant fold
increase of CD133 mRNA in the HuR-bound material (Supple-
mental 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

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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 bound by HuR (Fig. 4D). 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.
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 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 (form 5 x 10^2 to 5 x 10^6). The graph shows the tumor weight (mg) originated from the two cell types. *, P < 0.01. 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^(-ΔΔCt), where the housekeeping genes were 18S and GAPDH. Values were plotted as mean ± 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.
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.
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. Molecularily, 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 IncRNA molecules as linc-MD1 and lincRNA-p21 and posttranscriptionally regulate their function in the cytoplasm (38, 39). 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. We showed that genetic downregulation of any components of the complex led to an upregulation of the CD133 gene, affecting EMT phenotype. Importantly, we did not detect HuR, and its complex, on the other hand, on 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 it 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 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). 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 ± SD from three experimental replicates. *, P < 0.05; **, 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 enrichment 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 IncRNA 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 linc-RNA-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 it 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
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 cancer 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

<table>
<thead>
<tr>
<th>Dataset samples</th>
<th>Pearson coefficient, r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal, adjacent tissue</td>
<td>-0.36</td>
</tr>
<tr>
<td>ER+ Her2– tissue</td>
<td>-0.34</td>
</tr>
<tr>
<td>Normal adjacent tissue plus ER+ Her2– tissue</td>
<td>-0.44</td>
</tr>
<tr>
<td>TNBC</td>
<td>0.016</td>
</tr>
</tbody>
</table>

NOTE: The inverse correlation is moderate for normal, ER+, and for the sum of these two datasets but absent in TNBC cancer tissues.
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
Conception and design: E. Latorre, S. Carelli, G. Ghilardi, A. Inga, A. Gorio, A. Provenzani
Development of methodology: E. Latorre, V. D’Agostino, G. Ghilardi
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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