Induction of a novel isoform of the lncRNA HOTAIR in Claudin-low breast cancer cells attached to extracellular matrix

Miao Li1,2, Xi Li2,3, Yan Zhuang4, Erik K. Flemington5, Zhen Lin5 and Bin Shan2

1 Department of Microbiology and Parasitology, College of Basic Medical Sciences, China Medical University, Shenyang, China
2 Department of Biomedical Sciences, Elson S. Floyd College of Medicine, Washington State University Spokane, WA, USA
3 Department of Sports Medicine and Joint Surgery, The People’s Hospital of Liaoning Province, Shenyang, China
4 Department of Medicine, Tulane University School of Medicine, New Orleans, LA, USA
5 Department of Pathology, Tulane University School of Medicine, New Orleans, LA, USA

Keywords
epigenetics; HOTAIR; lncRNA; three-dimensional organotypic culture

Correspondence
M. Li, China Medical University, 77 Puhe Road, Shenyang North New Area, Shenyang, Liaoning 110122, China
Tel: +86 02431939638
E-mail: sendtolm@126.com
and
B. Shan, Washington State University Elson S Floyd College of Medicine, PO BOX 1495, Spokane, WA 99210-1495, USA
Tel: +1 5093587813
E-mail: bin.shan@wsu.edu

(Eceived 9 May 2017, revised 27 July 2017, accepted 11 August 2017, available online 30 October 2017)

doi:10.1002/1878-0261.12133

Elevated overexpression of the lncRNA HOTAIR mediates invasion and metastasis in breast cancer. In an apparent paradox, we observed low expression of HOTAIR in the invasive Claudin-low MDA-MB-231 and Hs578T cells in two-dimensional culture (2D). However, HOTAIR expression exhibited robust induction in laminin-rich extracellular matrix-based three-dimensional organotypic culture (lrECM 3D) over that in 2D culture. Induction of HOTAIR required intact ECM signaling, namely integrin αv2 and SRC kinase activity. Moreover, invasive growth was suppressed by HOTAIR-specific siRNA. Induction of HOTAIR in lrECM 3D culture resulted from the activation of a novel isoform of HOTAIR (HOTAIR-N) whose transcription is started from the first intron of the HOXC11 gene. The HOTAIR-N promoter exhibited increased trimethylation of histone H3 lysine 4, a histone marker of active transcription, and binding of BRD4, a reader of transcriptionally active histone markers. Inhibition of BRD4 substantially reduced the expression of HOTAIR in lrECM 3D culture. In summary, our results indicate that HOTAIR expression is activated by BRD4 binding to a novel HOTAIR-N promoter in Claudin-low breast cancer cells that are attached to ECM. Induction of HOTAIR is required for invasive growth of Claudin-low breast cancer cells in lrECM 3D culture.

1. Introduction

Human breast cancer is classified into ‘intrinsic subtypes’: luminal A, luminal B, basal, Claudin-low, and HER2-enriched based on gene expression profiles (Perou et al., 2000; Prat and Perou, 2011; Sorlie et al., 2001). This molecular stratification complements the pathological classifiers of breast cancer, namely estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), and is preserved in the established human breast cancer cell lines (Holliday and Speirs, 2011; Parker et al., 2009; Prat and Perou, 2011). Claudin-low subtype correlates with triple-negative (ER-negative, PR-negative, and HER2-negative) invasive ductal carcinomas (Holliday and Speirs, 2011; Prat et al., 2010). The gene expression profile of Claudin-low subtype is enriched with the signaling components that regulate cellular

Abbreviations
BRD4, bromodomain containing 4; ECM, extracellular matrix; H3K4me3, trimethylation of histone H4 lysine 4; HOTAIR, HOX transcript antisense RNA; HOX, homeobox; lrECM, laminin-rich extracellular matrix; TSA, trichostatin A.
responses to extracellular matrix (ECM) (Charafe-Jauffret et al., 2009; Creighton et al., 2009; Hennessy et al., 2009; Prat et al., 2010; Shipitsin et al., 2007; Taube et al., 2010). This feature implies that attachment to ECM has profound impacts on gene expression of Claudin-low breast cancer cells. Laminin-rich ECM (Matrigel) three-dimensional organotypic culture (lrECM 3D), pioneered by Bissell’s group, is an ideal model system to investigate ECM-regulated gene expression because it faithfully recapitulates salient properties of breast cancer cells attached to ECM (Kenny et al., 2007a). Moreover, the gene signatures of breast cancer cells in lrECM 3D culture hold prognostic values for patients with breast cancer (Kenny et al., 2007a; Martin et al., 2008).

One family of novel epigenetic regulators of gene expression is long noncoding RNA (lncRNA) (Rinn and Chang, 2012). The lncRNA genes exhibit tissue-specific expression patterns and regulate expression of the genes that are pivotal to development and cancer (Cabili et al., 2011; Guttman et al., 2009). lncRNA can act as a recruiter and scaffold for assembly of chromatin modifiers on their target genes (Chu et al., 2011; Gupta et al., 2010; Spitale et al., 2011; Tsai et al., 2010; Wang and Chang, 2011). The lncRNA HOX transcript antisense RNA (HOTAIR) is elevated in breast cancer and promotes metastasis (Gupta et al., 2010; Rinn et al., 2007). HOTAIR recruits polycomb repressive complex 2 (PRC2) to their target genes for transcriptional repression (Chu et al., 2011). In accordance, an EMT-like derivative of the human breast cancer MCF-7 cells exhibited a robust increase in HOTAIR expression and such an increase was required for accelerated proliferation and resistance to cell death (Antoon et al., 2012; Zhuang et al., 2015). One intriguing observation in the discovery of HOTAIR in breast cancer is that the RNA levels of HOTAIR in breast cell lines in cell culture are significantly lower than those observed in primary and metastatic breast tumors (Gupta et al., 2010). This observation implies that conventional 2D culture lacks critical factors that stimulate the expression of HOTAIR. One critical factor absent in 2D culture is attachment to ECM. Our recent report suggests a link between collagen and HOTAIR in lung adenocarcinoma cells (Zhuang et al., 2013).

Emerging evidence from lrECM 3D culture suggests epigenetic regulation of gene expression by the ECM signaling in cancer cells (Lelievre, 2010; Li et al., 2016; Teoh-Fitzgerald et al., 2014). However, ECM-regulated expression and functions of lncRNA have not been examined in Claudin-low breast cancer cells. Herein, we aimed to understand epigenetic regulation of the lncRNA HOTAIR by ECM in Claudin-low breast cancer cells using lrECM 3D culture.

2. Materials and methods

2.1. Reagents and plasmids

Corning (Bedford, MA, USA) provided Matrigel and Cell Recovery Solution for the extraction of cells from lrECM 3D culture. Trichostatin (TSA), a HDAC inhibitor, was purchased from Cayman Chemical (Ann Arbor, MI, USA). PP2, an Src-specific inhibitor, was purchased from Calbiochem (San Diego, CA, USA). JQ1, a bromodomain containing 4 (BRD4)-specific inhibitor, was a kind gift from James Bradner at Dana-Farber Cancer Institute (Loven et al., 2013). The GAPDH- and BRD4-specific antibodies were purchased from Novus Biologicals (Littleton, CO, USA) and Cell Signaling (Danvers, MA, USA), respectively. An integrin α2β1-neutralizing antibody (clone JBS2) was purchased from Chemicon (Temecula, CA, USA) (Knight et al., 1998). A retroviral vector expressing dominant-negative chicken Src-K295R mutant (dnSrc) was kindly provided by Joan Brugge at Harvard University (Thomas et al., 1991).

2.2. Cell lines and cell culture

Two Claudin-low human breast cancer cell lines MDA-MB-231 and Hs578T were purchased from ATCC (Manassas, VA, USA) (Holliday and Speirs, 2011). The cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) as we previously described (Zhuang et al., 2010).

2.3. lrECM 3D organotypic culture

Overlay lrECM 3D culture was carried out as described elsewhere (Li et al., 2011; Vidi et al., 2013). Briefly, MDA-MB-231 and Hs578T cells were seeded at a density of 2 × 10^5 cells per well in a six-well culture dish that was coated with Matrigel. DMEM culture medium supplemented with 4% of Matrigel was replaced every two days. The morphology of cell clusters was monitored and recorded using an inverse phase contrast microscope linked with a digital camera and fluorescent staining for filamentous actin using Alexa 488-conjugated phalloidin followed by confocal microscopy (Li et al., 2011).

2.4. Retroviral transduction

The integrin α2-specific Mission shRNA lentiviral transduction particles and its matching control were
purchased from Sigma (St. Louis, MO, USA). Retroviral transduction was carried out as described elsewhere (Shan et al., 2007). The stably transduced MDA-MB-231 cells were selected using puromycin. In a similar fashion, we generated the MDA-MB-231 variants that stably expressed a dominant-negative Src mutant chicken Src-K295R (Nguyen et al., 2013; Thomas et al., 1991).

2.5. Transient transfection and RNA interference
Sigma provided the human BRD4-specific Mission siRNA (BRD4-siRNA, SIHK0192, SIHK0193, SIHK0194), the HOTAIR-specific Mission siRNA (HOTAIR-siRNA, Sigma ID SASI_Hs02_00380445), and the control Mission siRNA. The HOTAIR isoform NR_047517 (HOTAIR-N)-specific siRNA were designed using IDT’s siRNA designing tool and purchased from IDT (Coralville, IA, USA). The sequences of the HOTAIR-N-specific siRNA were provided in Table S1. All the siRNA were transfected at 60 nM into MDA-MB-231 and Hs578T cells using RNAiMAX per the reverse transfection protocol (Invitrogen, Carlsbad, CA, USA; Shan et al., 2005). Total RNA and protein were extracted on day 3 after transfection in 2D culture and day 4 after transfection in lrECM 3D culture.

2.6. Cell viability assay
Cell viability in lrECM 3D culture was measured using XTT in vitro Toxicology Assays (Sigma) as we previously described (Shan and Morris, 2005). The values from the control groups were set to 100%.

2.7. RNA extraction and RT-PCR
Total cell RNA was extracted using TRIzol (Invitrogen) from 2D and lrECM 3D cultures at the indicated time points as previously described (Li et al., 2012). Quantitative RT-PCR (qRT-PCR) was carried out to determine the RNA levels of the genes of interest. Each transcript was normalized to a housekeeping gene ribosomal protein large P0 (RPLP0). A fold change of each transcript was obtained by setting the values from the control groups to one.

2.8. Immunoblot
Total cell lysates were extracted from MDA-MB-231 cells exposed to the indicated treatments using 1× Laemmli buffer. In lrECM 3D culture, MDA-MB-231 cells were separated from Matrigel using BD cell recover solution as previously described (Nguyen et al., 2013). Immunoblotting was used to measure the protein levels of integrin α2, BRD4, and GAPDH (Shan et al., 2010).

2.9. Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChIP) was performed as previously described with minor modifications (Li et al., 2016). Millipore (Darmstadt, Germany) provided EZ ChIP Kit (Cat #17–371). The

Fig. 1. Induction of HOTAIR in lrECM 3D Culture. (A) Total cell RNA was extracted from MDA-MB-231 cells in 2D (day 3) and lrECM 3D cultures at the indicated time points. The RNA levels of HOTAIR were compared between two culture conditions using qRT-PCR. A fold change of HOTAIR at each time point in lrECM 3D culture over 2D culture was obtained by normalizing to the housekeeping gene RPLP0 and setting the values from 2D culture to one. (B) Similar to part (A) except that the RNA levels of HOTAIR were compared between two culture conditions in Hs578T cells at the indicated time point. (C) The morphology of Hs578T cells in lrECM 3D culture (day 6) was visualized by staining for filamentous actin using Alexa 488-conjugated phalloidin (pseudocolored in green). The stellate projections were indicated by red arrowheads. The image was captured at 200× magnification using a confocal microscope. * and ** indicated a P value < 0.05 and 0.01, respectively.
ChIP-grade H3K4me3 antibody was purchased from Active Motif (Carlsbad, CA, USA). Sheared chromatin was prepared from roughly \(1 \times 10^7\) cells using Cell Recovery Solution and then immunoprecipitated using the BRD4- or H3K4me3-specific antibody, or a control antibody. The sequences of the primers specific for the human HOTAIR promoter are provided in Table S1. The input and immunoprecipitated HOTAIR promoter were quantified using qPCR. The ratios of the immunoprecipitated DNA versus its corresponding input were compared between the groups.

2.10. RNA-Seq analysis

Raw RNA-sequencing (RNA-Seq) reads from invasive breast carcinoma samples and their paired normal samples generated through The Cancer Genome Atlas (TCGA) project were obtained from the National Cancer Institute Genomic Data Commons. The datasets were then analyzed using the RSEM algorithm for the quantification of isoforms of HOTAIR transcripts as previously described (Li and Dewey, 2011; Strong et al., 2014).

2.11. Statistical analysis

When presented, means and standard deviations were obtained from at least three independent experiments. A \(P\) value between any two compared groups was determined using unpaired two-tailed Student’s \(t\)-test (GraphPad Prism, version 5, GraphPad Software, Inc., La Jolla, CA USA).

3. Results

3.1. Induction of the lncRNA HOTAIR in IrECM 3D culture of Claudin-low breast cancer cells

We set out to explore ECM-mediated regulation of expression of the lncRNA HOTAIR in Claudin-low...
breast cancer cells using lrECM 3D culture (Kenny et al., 2007b; Prat et al., 2010). We chose Claudin-low MDA-MB-231 and Hs578T cells because of their invasive and metastatic competence (Neve et al., 2006; Prat et al., 2010). We recently reported a widespread induction of the homeobox (HOX) genes in lrECM 3D culture over 2D culture of MDA-MB-231 cells and Hs578T cells (Li et al., 2016). The HOXC cluster-derived HOTAIR exhibited a robust increase in MDA-MB-231 cells from day 2 to day 20 (Fig. 1A). A similar induction of HOTAIR was observed in Hs578T cells on day 6 (Fig. 1B). Induction of HOTAIR was also observed in gene expression profiling (GEO GSE36953) of lrECM 3D culture of MDA-MB-231 (Yotsumoto et al., 2013). Induction of HOTAIR correlated with invasive growth of MDA-MB-231 cells and Hs578T cells (Fig. 1C) (Li et al., 2016). We previously reported invasive growth as evidenced by stellate morphology of MDA-MB231 (Li et al., 2016). Herein, we demonstrated similar stellate morphology of Hs578T cells in lrECM 3D culture using fluorescent staining for filamentous actin (Fig. 1C). Stellate morphology of both cell lines featured irregular cell clusters and projections formed by chains of cells that intersected occasionally as described elsewhere (Fig. 1C, indicated by red arrowheads) (Kenny et al., 2007b).

We questioned whether induction of HOTAIR required ECM signaling in lrECM 3D culture. To this end, we generated an MDA-MB-231 variant in which integrin α2, a major cell surface receptor for ECM, was knocked down by the stably expressed integrin α2-specific shRNA (ITGα2KD). The protein levels of integrin α2 were substantially reduced in ITGα2KD when compared with a matching control variant (CTL) (Fig. 2A).

We measured the RNA levels of HOTAIR in ITGα2KD and CTL variants in lrECM 3D culture using qRT-PCR. The RNA levels of HOTAIR in the ITGα2KD variant were reduced to 26% of that in the CTL variant (Fig. 2B). To confirm an essential role of integrin α2 in the induction of HOTAIR, we inhibited integrin α2 using its neutralizing antibody (clone JBS2) in lrECM 3D culture of MDA-MB-231 and Hs578T cells (Knight et al., 1998). The integrin α2-neutralizing antibody (10 μg·mL⁻¹) reduced the RNA levels of HOTAIR to 32% and 38% of that in the control IgG-treated MDA-MB-231 and Hs578T cells, respectively (Fig. 2C).

Src kinase is a key intracellular signal transducer downstream of integrins in response to ECM and growth factors in lrECM 3D culture (Huang et al., 2011; Nguyen et al., 2013). Thus, we questioned whether Src kinase activity is required for the induction of HOTAIR in lrECM 3D culture. To this end, we exposed MDA-MB-231 and Hs578T cells to PP2 (5 μM), an Src-specific inhibitor in lrECM 3D culture. PP2 reduced the RNA levels of HOTAIR to 36% and 31% of the DMSO groups in MDA-MB-231 and Hs578T cells, respectively (Fig. 3A). To further confirm a requirement of Src kinase activity for the induction of HOTAIR in lrECM 3D culture, we generated two variants of MDA-MB-231 cells that were transduced with either a retroviral vector expressing a dominant-negative Src mutant (MDA-MB-231-dnSrc) or its backbone vector (CTL). As expected, the RNA levels of HOTAIR in MDA-MB-231-dnSrc were reduced to 39% of that in the control group (Fig. 3B). These findings indicated that HOTAIR was induced in lrECM 3D culture via the ECM signaling.

3.2. Requirement of HOTAIR for invasive growth of Claudin-low breast cancer cells in lrECM 3D culture

Claudin-low MDA-MB-231 and Hs578T cells exhibited invasive growth in lrECM 3D culture (Fig. 1C; Kenny et al., 2007b; Li et al., 2016). A correlation between induction of HOTAIR and invasive growth prompted us to examine the role of HOTAIR in invasive growth in lrECM 3D culture. We transfected MDA-MB-231 cells with either the HOTAIR-specific siRNA (HOTAIRsiRNA) or control siRNA (CTLsiRNA). RNAi-mediated knockdown of HOTAIR was confirmed by a decrease to 33% of the control transfection (Fig. 4A). In accordance, the HOTAIRsiRNA-
transfected group was void of invasive growth pattern, whereas the CTLSiRNA-transfected group exhibited invasive growth (Fig. 4B). We also noticed an apparent decrease in number of cells (Fig. 4B). Thus, we examined cell viability of MDA-MB-231 and Hs578T cells transfected with either the HOTAIRsiRNA or CTLSiRNA using XTT assays. The HOTAIRsiRNA-transfected groups exhibited a substantial decrease in cell viability, to 58% in MDA-MB-231 and 43% in Hs578T cells, respectively (Fig. 4C,D).

3.3. Induction of HOTAIR-N in IrECM 3D culture of Claudin-low breast cancer cells

We speculated that induction of HOTAIR expression resulted from activation of the HOTAIR promoter in IrECM 3D culture. We initially focused on the promoter of the canonical transcript NR_003716 (HOTAIR-C) that was first discovered in breast cancer (Gupta et al., 2010; Rinn et al., 2007). We examined trimethylation of histone 3 lysine 4 (H3K4me3) that has been used as a marker of the transcriptionally active IncRNA (Guttman et al., 2009). To our surprise, we observed minimal difference in the promoter region proximal to the transcription start site of HOTAIR-C (~287 to −404 relative to the transcription initiation site) between 2D and IrECM 3D cultures (Fig. 5A). We were aware that the primers for qRT-PCR can amplify all three HOTAIR isoforms as listed in the latest RefSeq release 81 (Fig. 5B) (O’Leary et al., 2016). Thus, induction of HOTAIR in IrECM 3D culture could result from the activation of a novel isoform other than HOTAIR-C. We speculated that NR_047517 (HOTAIR-N) and its promoter accounted for the induction of HOTAIR in IrECM 3D culture of Claudin-low breast cancer cells because its
transcription start site is located in the first intron of the HOXC11 gene and distant from the promoters for the other two HOTAIR isoforms (Fig. 5B). We examined the RNA levels of HOTAIR-N by qRT-PCR using a pair of HOTAIR-N-specific primers. In congruence to induction of HOTAIR using the nonisoform-specific primers, we observed a robust five- and fourfold increase in the RNA levels of the novel HOXC11-overlapping HOTAIR transcript (HOTAIR-N) in lrECM 3D culture over 2D culture (Fig. 5C). To determine whether HOTAIR-N accounted for increase in HOTAIR in lrECM 3D culture, we transfected the HOTAIR-N-specific siRNA into MDA-MB-231 cells. The RNA levels of total HOTAIR and HOTAIR-N were substantially reduced by the HOTAIR-N-specific siRNA to 40% and 20% (Fig. 5D). Consistently, we observed a 4.7-fold increase in H3K4me3-associated HOTAIR-N promoter (−139 to −247 relative to the transcription initiation site) in Hs578T and MDA-MB-231 cells in lrECM 3D culture over 2D culture (Fig. 5E). The increase in H3K4me3 led us to question whether inhibition of histone deacetylases (HDAC) was able to increase HOTAIR expression in 2D culture. Exposure to a pan-HDAC inhibitor TSA (500 nM) for 72 h increased the RNA levels of HOTAIR to 108-fold over the DMSO group (Fig. 5F). These findings indicated a correlation between H3K4me3 of the HOTAIR-N promoter and induction of HOTAIR in lrECM 3D culture of Claudin-low breast cancer cells.

** and *** indicated a P value < 0.01 and 0.001, respectively.
Table 1. Expression of HOTAIR isoforms in human breast cancer specimens. The TCGA invasive breast carcinoma RNA-Seq data were surveyed for expression of HOTAIR isoforms using RSEM. Fourteen samples were selected because they exhibited substantial increase in HOTAIR expression in the tumor tissues over their paired normal tissues. Amount of each HOTAIR isoform in each tumor tissue was determined using transcript per million reads (TPM). Percentage of each isoform (IsoPct) in each tumor tissue was also determined. NR_047517 corresponds to HOTAIR-N, NR_003716 corresponds to HOTAIR-C.

| Tumor ID | NR_047517 TPM | NR_047517 IsoPct | NR_47518 TPM | NR_47518 IsoPct | NR_003716 TPM | NR_003716 IsoPct |
|----------|----------------|------------------|--------------|----------------|----------------|----------------|
| T73      | 33.72          | 91.83            | 2.46         | 6.71           | 0.54           | 1.46           |
| T25      | 7.99           | 90.44            | 0.63         | 7.11           | 0.22           | 2.45           |
| T85      | 11.32          | 90.01            | 1.02         | 8.14           | 0.23           | 1.85           |
| T87      | 15.99          | 75.01            | 1.79         | 8.37           | 3.54           | 16.62          |
| T46      | 15.40          | 69.47            | 3.44         | 15.53          | 3.33           | 15.01          |
| T102     | 9.32           | 67.17            | 0.88         | 6.32           | 3.68           | 26.51          |
| T54      | 15.79          | 62.60            | 2.34         | 9.27           | 7.10           | 28.13          |
| T71      | 8.69           | 57.84            | 1.23         | 8.16           | 5.11           | 34.00          |
| T110     | 3.84           | 48.46            | 0.57         | 7.22           | 3.51           | 44.32          |
| T77      | 5.44           | 42.11            | 2.90         | 22.42          | 4.58           | 35.47          |
| T80      | 3.90           | 39.71            | 0.60         | 6.06           | 5.33           | 54.23          |
| T19      | 8.39           | 29.24            | 5.13         | 17.87          | 15.18          | 52.89          |
| T98      | 2.52           | 19.63            | 3.01         | 23.43          | 7.31           | 56.94          |
| T2       | 1.59           | 14.04            | 1.73         | 15.23          | 8.02           | 70.72          |
| Average  | 10.28          | 56.97            | 1.98         | 11.56          | 4.83           | 31.47          |

To determine whether HOTAIR-N accounts for increased expression of HOTAIR in breast cancer patient biopsies, we surveyed the TCGA Invasive Breast Carcinoma RNA-Seq data. We selected 14 paired samples that exhibited higher increase in expression of HOTAIR in tumor over their paired normal tissues. We analyzed HOTAIR transcripts by transcript per million (TPM) and percentage of each isoform in the tumor samples using RSEM (Li and Dewey, 2011; Strong et al., 2014). In nine tumor samples, HOTAIR-N exhibited the highest expression among all three isoforms (Table 1). The average contribution of HOTAIR-N and HOTAIR-C was that HOTAIR-N accounted for 57% and HOTAIR-C accounted for 31.5% of HOTAIR in breast cancer (Table 1). These findings indicated that HOTAIR-N is a major isoform in invasive breast carcinoma.

3.4. Requirement of BRD4 for induction of HOTAIR expression in lrECM 3D culture

BRD4 binds histone markers for active transcription to promote gene expression (Loven et al., 2013; Zuber et al., 2011). Because we observed an increase in H3K4me3 associated with the HOTAIR-N promoter, we speculated that BRD4 mediated induction of HOTAIR in lrECM 3D culture of Claudin-low breast cancer cells. To test this hypothesis, we treated MDA-MB-231 and Hs578T cells in lrECM 3D culture with a BRD4-specific inhibitor JQ1 for 4 days. JQ1 (50 and 250 nm) substantially reduced the RNA levels of HOTAIR in lrECM 3D culture of both cell lines (Fig. 6A, B). To confirm the requirement of BRD4 for the activation of HOTAIR, we transfected MDA-MB-231 cells with the BRD4-specific siRNA or control siRNA. The protein levels of BRD4 were substantially reduced by BRD4 siRNA (two individual siRNA and a pool of three siRNA) when compared with the control siRNA group (Fig. 6C). HOTAIR RNA levels were also substantially reduced by the BRD4 siRNA when compared with the control siRNA group in lrECM 3D culture (Fig. 6D). We postulated that BRD4 was also required for the activation of HOTAIR by the HDAC inhibitor TSA in 2D culture. We exposed MDA-MB-231 cells to TSA alone (500 nM) with or without JQ1 in 2D culture for 72 hrs. As expected, JQ1 (250 nM) reduced the RNA levels of HOTAIR in the presence of TSA to 7% of that in the TSA-alone group (Fig. 6E).

To determine the association between BRD4 binding and HOTAIR expression, we carried out ChIP assays to compare BRD4 binding to the HOTAIR-N promoter in 2D and lrECM 3D cultures of MDA-MB-231 cells. We observed a 5.5-fold increase in the BRD4-bound HOTAIR-N promoter (47517 to 47518 relative to the transcription initiation site) in lrECM 3D culture over 2D culture (Fig. 7A). We then questioned whether JQ1 disrupted BRD4 binding to the HOTAIR-N promoter because JQ1 inhibited the induction of HOTAIR by the HDAC inhibitor TSA in 2D culture. We exposed MDA-MB-231 cells to TSA alone (500 nm) with or without JQ1 in 2D culture for 72 hrs. As expected, JQ1 (250 nm) reduced the RNA levels of HOTAIR in the presence of TSA to 7% of that in the TSA-alone group (Fig. 6E).

4. Discussion

Herein, we demonstrate that the lncRNA HOTAIR is induced in lrECM 3D culture of Claudin-low breast cancer cells over conventional 2D culture. Such
induction is mediated by epigenetic activation of a novel isoform of HOTAIR.

Claudin-low breast cancer cells are enriched with the genes that are critical to cellular responses to ECM (Charafe-Jauffret et al., 2009; Prat et al., 2010). Therefore, understanding of cancer biology of Claudin-low breast cancer cells attached to ECM is particularly important. We have reported miRNA that are only differentially expressed between Claudin-low (MDA-MB-231) and luminal A (MCF-7) breast cancer cells in lrECM 3D culture (Nguyen et al., 2012). Our previous report implies a critical role of noncoding RNA in cell responses to ECM. Our current study strengthens and expands this notion. Our results indicate that HOTAIR, a classic example of tumor-promoting lncRNA, is induced in lrECM 3D culture of Claudin-low breast cancer cells, and such induction requires the canonical ECM signaling pathway, namely integrins and Src kinase (Figs 1–3). We are aware that inhibition of integrin α2 and Src kinase results in substantial, but incomplete inhibition of HOTAIR expression in lrECM 3D culture (Figs 2 and 3). These findings likely reflect the complexity in composition of ECM and the signaling pathways in response to ECM. Interference of other integrin receptors and kinases individually and in combination, such as integrin α6 (major receptors for laminin), is still needed to determine the role of each individual ECM and its responsive cellular signaling molecules in the activation of HOTAIR expression (Kenny et al., 2007b; Lee et al., 2007; Lu et al., 2012; Weaver et al., 1997; Ziober et al., 1999). More importantly, our results indicate that HOTAIR expression is required for invasive growth of Claudin-low breast cancer cells, particularly cell viability (Fig. 4). These findings likely reflect that a broad spectrum of cellular responses to ECM are regulated by lncRNA.

Our current knowledge of epigenetic regulation of gene expression in breast cancer cells is largely obtained using conventional 2D culture. However, two critical factors, attachment to ECM and three-dimensional growth, are absent in 2D culture. Our results

Fig. 6. Reduced Expression of HOTAIR by Inhibition of BRD4. (A) Total cell RNA was extracted from lrECM 3D cultures of MDA-MB-231 cells treated with either a BRD4-specific inhibitor JQ1 (50 and 250 nM) or DMSO for 4 days. The RNA levels of HOTAIR were measured using qRT-PCR. A fold change of the HOTAIR RNA was obtained by normalizing to the housekeeping gene RPLP0 and setting the values from the DMSO-treated group to one. (B) Similar to part (A) except that the RNA levels of HOTAIR were measured in Hs578T. (C) MDA-MB-231 cells were transfected with either BRD4-specific siRNA (BRDRsiRNA) or control siRNA (CTlsiRNA) and seeded in lrECM 3D culture. Total cell RNA was extracted on day 4 in lrECM 3D culture. The protein levels of BRD4 were assessed using immunoblots. (D) Similar to part (C) except that the RNA levels of HOTAIR were assessed using qRT-PCR. (E) Total cell RNA was extracted from 2D culture of MDA-MB-231 cells treated with either TSA (500 nM) alone or a combination of TSA and JQ1 (250 nM) for 72 h. The RNA levels of HOTAIR were measured as in part (A). *, **, and *** indicated a P value < 0.05, 0.01, and 0.001, respectively.
suggest distinct epigenetic regulation of lncRNA gene expression between 2D and lrECM 3D cultures (Figs 5–7). As a reader of transcriptionally active histone markers, BRD4 binding is likely a consequence of increased H3K4me3 in the HOTAIR-N promoter because BRD4 binding was required for the induction of HOTAIR in lrECM 3D culture and TSA-induced HOTAIR expression in 2D culture (Dey et al., 2003). Our results warrant further investigation of epigenetic regulation of gene expression by ECM and three-dimensional culture in breast cancer cells. The insight obtained from lrECM 3D culture can provide more reliable and accurate guidance for in vivo studies of epigenetic mechanisms in breast cancer.

HOTAIR is intensely studied in cancer (Loewen et al., 2014). However, its isoforms have not been characterized although aberrant activation of the promoter region upstream of HOTAIR-N has been implied in breast cancer (Milevskiy et al., 2016). Our results indicate that the novel isoform HOTAIR-N is activated in lrECM 3D culture of Claudin-low breast cancer cells (Fig. 4). More importantly, a survey of the TCGA invasive breast carcinoma RNA-Seq data suggests that HOTAIR-N is the predominant isoform in invasive breast carcinoma (Table 1). Recognition of HOTAIR-N as a major isoform in breast cancer can initiate several new frontiers. Firstly, HOTAIR-N is transcribed from the first intron of HOXC11 and forms sense–antisense gene pair with HOXC11 (Fig. 5B). This overlapping implies in cis action of HOTAIR in breast cancer besides its established in trans action via binding to PRC2 (Gupta et al., 2010; Zhuang et al., 2015). This notion is appealing because HOXC11 promotes breast cancer and the importance of PRC2 to HOTAIR functions has been challenged recently (McIlroy et al., 2010; Portoso et al., 2017). Secondly, the HOTAIR-N-HOXC11 region contains a CpG island with a CpG count of 160 assigned by the UCSC Genome Browser (Kent et al., 2002). The locus presents an ideal platform to investigate dysregulation of a sense–antisense gene pair in the context of a CpG island. This is particularly important because of global dysregulation of sense–antisense gene pairs in breast cancer (Maruyama et al., 2012).

5. Conclusions

We demonstrate that HOTAIR expression is activated by increased H3K4me3 and BRD4 binding to a novel HOTAIR-N promoter in Claudin-low breast cancer cells attached to ECM. Thus, we propose an emphasis on the overlooked interactions between ECM, lncRNA, and epigenetic coding in breast cancer. We also propose a focus on the novel HOTAIR-N isoform in the overlapping HOTAIR-HOXC11 locus.

Acknowledgements

This work is supported by Washington State University Startup Fund awarded to BS. XL is supported by Grant No. 81401334 from the National Nature Science Foundation of China awarded to XL.

Author contributions

BS conceived the study and wrote the manuscript. ML, XL, and YZ carried out the experiments. ML analyzed the results and prepared the figures. ZL and EKF analyzed the RNA-Seq data from TCGA.
Induction of HOTAIR by extracellular matrix

M. Li et al.

References

Antoon JW, Lai R, Struckhoff AP, Nitschke AM, Elliott S, Martin EC, Rhodes LV, Yoon NS, Salvo VA, Shan B et al. (2012) Altered death receptor signaling promotes epithelial-to-mesenchymal transition and acquired chemoresistance. Sci Rep 2, 539.

Cabrera-Jauffret E, Ginestier C, Iovino F, Wicinski J, Chu C, Qu K, Zhong FL, Artandi SE and Chang HY (2017) Molecular Oncology 11, 1698–1710 © 2017 The Authors. Published by FEBS Press and John Wiley & Sons Ltd.

Charafe-Jauffret E, Ginestier C, Iovino F, Wicinski J, Cervera N, Finetti P, Hur MH, Diebel ME, Monville F, Dutcher J et al. (2009) Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. Cancer Res 69, 1302–1313.

Chu C, Qu K, Zhong FL, Artandi SE and Chang HY (2011) Genomic maps of long noncoding RNA occupancy reveal principles of RNA-chromatin interactions. Mol Cell 44, 667–678.

Creighton CJ, Li X, Landis M, Dixon JM, Neumeister VM, Sjolund A, Rimm DL, Wong H, Rodriguez A, Herschkowitz JJ et al. (2009) Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. Proc Natl Acad Sci U S A 106, 13820–13825.

Dey A, Chitsaz F, Abbasi A, Misteli T and Ozato K (2003) The double bromodomain protein Brd4 binds to acetylated chromatin during interphase and mitosis. Proc Natl Acad Sci U S A 100, 8758–8763.

Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai MC, Hung T, Argani P, Rinn JL et al. (2010) Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 464, 1071–1076.

Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, Huarte M, Zuk O, Carey BW, Cassidy JP et al. (2009) Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature 458, 223–227.

Hennery BT, Gonzalez-Angulo AM, Stemke-Hale K, Gilcrease MZ, Krishnamurthy S, Lee JS, Fridlyand J, Sahin A, Agrawal R, Joy C et al. (2009) Characterization of a naturally occurring breast cancer subset enriched in epithelial-to-mesenchymal transition and stem cell characteristics. Cancer Res 69, 4116–4124.

Holliday DL and Speirs V (2011) Choosing the right cell line for breast cancer research. Breast Cancer Res 13, 215.

Huang C, Park CC, Hilsenbeck SG, Ward R, Rimawi MF, Wang YC, Shou J, Bissell MJ, Osborne CK and Schiff R (2011) beta1 integrin mediates an alternative survival pathway in breast cancer cells resistant to lapatinib. Breast Cancer Res 13, R84.

Kenny PA, Lee GY and Bissell MJ (2007a) Targeting the tumor microenvironment. Front Biosci 12, 3468–3474.

Kenny PA, Lee GY, Myers CA, Neve RM, Semeiks JR, Spellman PT, Lorenz K, Lee EH, Barcellos-Hoff MH, Petersen OW et al. (2007b) The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. Mol Oncol 1, 84–96.

Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM and Haussler D (2002) The human genome browser at UCSC. Genome Res 12, 996–1006.

Knight CG, Morton LF, Onley DJ, Peachey AR, Messent AJ, Smethurst PA, Tuckwell DS, Farndale RW and Barnes MJ (1998) Identification in collagen type I of an integrin alpha2 beta1-binding site containing an essential GERP sequence. J Biol Chem 273, 33287–33294.

Lee GY, Kenny PA, Lee EH and Bissell MJ (2007) Three-dimensional culture models of normal and malignant breast epithelial cells. Nat Methods 4, 359–365.

Lelievre SA (2010) Tissue polarity-dependent control of mammary epithelial homeostasis and cancer development: an epigenetic perspective. J Mammary Gland Biol Neoplasia 15, 49–63.

Li B and Dewey CN (2011) RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 12, 323.

Li M, Li X, Zhuang Y, Wang Y, Burow ME, Collins-Burrow B, Xue M, Song C and Shan B (2016) Induction of HOXA9 expression in three-dimensional organotypic culture of the Claudin-low breast cancer cells. Oncotarget 7, 51503–51514.

Li C, Nguyen HT, Zhuang Y, Lin Y, Flemington EK, Guo W, Guenther J, Burrow ME, Morris GF, Sullivan D et al. (2011) Post-transcriptional up-regulation of miR-21 by type I collagen. Mol Carcinog 50, 563–570.

Li C, Nguyen HT, Zhuang Y, Lin Z, Flemington EK, Zhuo Y, Kantrow SP, Morris GF, Sullivan DE and Shan B (2012) Comparative profiling of miRNA expression of lung adenocarcinoma cells in two-dimensional and three-dimensional cultures. Gene 511, 143–150.

Loewen G, Zhuo Y, Zhuang Y, Jayawickramarajah J, Shan B (2014) lincRNA HOTAIR as a novel promoter of cancer progression. J Cancer Res Updates 3, 7.

Loven J, Hoke HA, Lin CY, Lau A, Orlando DA, Vakoc CR, Bradner JE, Lee TI and Young RA (2013) Selective inhibition of tumor oncogenes by disruption of super-enhancers. Cell 153, 320–334.

Lu P, Weaver VM and Werb Z (2012) The extracellular matrix: a dynamic niche in cancer progression. J Cell Biol 196, 395–406.
Martin KJ, Patrick DR, Bissell MJ and Fournier MV (2008) Prognostic breast cancer signature identified from 3D culture model predicts clinical outcome across independent datasets. *PLoS One* 3, e2994.

Maruyama R, Shipitsin M, Choudhury S, Wu Z, Protopopov A, Yao J, Lo PK, Bessarabova M, Ishkin A, Nikolsky Y *et al.* (2012) Altered antisense-to-sense transcript ratios in breast cancer. *Proc Natl Acad Sci U S A* 109, 2820–2824.

Mcllroy M, McCartan D, Early S, O Gaora P, Pennington S, Hill AD, Young LS (2010) Interaction of developmental transcription factor HOXC11 with steroid receptor coactivator SRC-1 mediates resistance to endocrine therapy in breast cancer [corrected]. *Cancer Res* 70, 1585–1594.

Milevskiy MJ, Al-Ejeh F, Saunus JM, Northwood KS, Bailey PJ, Betts JA, McCart Reed AE, Nephew KP, Stone A, Gee JM *et al.* (2016) Long-range regulators of the lncRNA HOTAIR enhance its prognostic potential in breast cancer. *Hum Mol Genet* 25, 3269–3283.

Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F *et al.* (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* 10, 515–527.

Nguyen HT, Li C, Lin Z, Zhuang Y, Flemington EK, Burow ME, Lin Y and Shan B (2012) The microRNA expression associated with morphogenesis of breast cancer cells in three-dimensional organotypic culture. *Oncol Rep* 28, 117–126.

Nguyen HT, Zhuang Y, Sun L, Kantrow SP, Kolls JK, You Z, Zhuo Y and Shan B (2013) Src-mediated morphology transition of lung cancer cells in three-dimensional organotypic culture. *Cancer Cell Int* 13, 16.

O’Leary NA, Wright MW, Brister JR, Ciufio S, Haddad D, McVeigh R, Rajput B, Robbertse B, Smith-White B, Ako-Adjei D *et al.* (2016) Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. *Nucleic Acids Res* 44, D733–D745.

Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, Davies S, Fauzon C, He X, Hu Z *et al.* (2009) Supervised risk predictor of breast cancer based on intrinsic subtypes. *J Clin Oncol* 27, 1160–1167.

Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA *et al.* (2000) Molecular portraits of human breast tumours. *Nature* 406, 747–752.

Portoso M, Ragazzini R, Brencic Z, Moiani A, Michaud A, Vassilev I, Wassef M, Servant N, Sargueil B and Margueron R (2017) PRC2 is dispensable for HOTAIR-mediated transcriptional repression. *EMBO J* 36, 981–994.

Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JJ, He X and Perou CM (2010) Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res* 12, R68.

Prat A and Perou CM (2011) Deconstructing the molecular portraits of breast cancer. *Mol Oncol* 5, 5–23.

Rinn JL and Chang HY (2012) Genome regulation by long noncoding RNAs. *Annu Rev Biochem* 81, 145–166.

Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E *et al.* (2007) Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* 129, 1311–1323.

Shan B, Hagood JS, Zhuo Y, Nguyen HT, MacEwen M, Morris GF and Lasky JA (2010) Thy-1 attenuates TGF-alpha-activated gene expression in mouse embryonic fibroblasts via Src family kinase. *PLoS One* 5, e11662.

Shan B and Morris GF (2005) Binding sequence-dependent regulation of the human proliferating cell nuclear antigen promoter by p53. *Exp Cell Res* 305, 10–22.

Shan B, Morris CA, Zhuo Y, Shelby BD, Levy DR and Lasky JA (2007) Activation of proMMP-2 and Src by HHV8 vGPCR in human pulmonary arterial endothelial cells. *J Mol Cell Cardiol* 42, 517–525.

Shan B, Zhuo Y, Chin D, Morris CA, Morris GF and Lasky JA (2005) Cyclin-dependent kinase 9 is required for tumor necrosis factor-alpha-stimulated matrix metalloproteinase-9 expression in human lung adenocarcinoma cells. *J Biol Chem* 280, 1103–1111.

Shipitsin M, Campbell LL, Argani P, Weremowicz S, Bloushatin-Qimron N, Yao J, Nikolskaya T, Serebryiskaya T, Beroukhim R, Hu M *et al.* (2007) Molecular definition of breast tumor heterogeneity. *Cancer Cell* 11, 259–273.

Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS *et al.* (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* 98, 10869–10874.

Spitale RC, Tsai MC, Chang HY (2011) RNA templating the epigenome: long noncoding RNAs as molecular scaffolds. *Epigenetics* 6, 539–543.

Strong MJ, Baddoo M, Nanbo A, Xu M, Puetter A and Lin Z (2014) Comprehensive high-throughput RNA sequencing analysis reveals contamination of multiple nasopharyngeal carcinoma cell lines with HeLa cell genomes. *J Virol* 88, 10696–10704.

Taube JH, Herschkowitz JJ, Komurov K, Zhou AY, Gupta S, Yang J, Hartwell K, Onder TT, Gupta PB, Evans KW *et al.* (2010) Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and...
metaplastic breast cancer subtypes. *Proc Natl Acad Sci U S A* **107**, 15449–15454.

Teoh-Fitzgerald ML, Fitzgerald MP, Zhong W, Askeland RW and Domann FE (2014) Epigenetic reprogramming governs EcSOD expression during human mammary epithelial cell differentiation, tumorigenesis and metastasis. *Oncogene* **33**, 358–368.

Thomas JE, Soriano P and Brugge JS (1991) Phosphorylation of c-Src on tyrosine 527 by another protein tyrosine kinase. *Science* **254**, 568–571.

Tsai MC, Manor O, Wan Y, Mosammaparast N, Wang JK, Lan F, Shi Y, Segal E and Chang HY (2010) Long noncoding RNA as modular scaffold of histone modification complexes. *Science* **329**, 689–693.

Vidi PA, Bissell MJ and Lelievre SA (2013) Three-dimensional culture of human breast epithelial cells: the how and the why. *Methods Mol Biol* **945**, 193–219.

Wang KC and Chang HY (2011) Molecular mechanisms of long noncoding RNAs. *Mol Cell* **43**, 904–914.

Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C and Bissell MJ (1997) Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. *J Cell Biol* **137**, 231–245.

Yotsumoto F, Tokunaga E, Oki E, Maehara Y, Yamada H, Nakajima K, Nam SO, Miyata K, Koyanagi M, Doi K et al. (2013) Molecular hierarchy of heparin-binding EGF-like growth factor-regulated angiogenesis in triple-negative breast cancer. *Mol Cancer Res* **11**, 506–517.

Zhuang Y, Nguyen HT, Burow ME, Zhuo Y, El-Dahr SS, Yao X, Cao S, Flemington EK, Nephew KP, Fang F et al. (2015) Elevated expression of long intergenic non-coding RNA HOTAIR in a basal-like variant of MCF-7 breast cancer cells. *Mol Carcinog* **54**, 1656–1667.

Zhuang Y, Nguyen HT, Lasky JA, Cao S, Li C, Hu J, Guo X, Burow ME and Shan B (2010) Requirement of a novel splicing variant of human histone deacetylase 6 for TGF-beta1-mediated gene activation. *Biochem Biophys Res Commun* **392**, 608–613.

Zhuang Y, Wang X, Nguyen HT, Zhuo Y, Cui X, Fewell C, Flemington EK and Shan B (2013) Induction of long intergenic non-coding RNA HOTAIR in lung cancer cells by type I collagen. *J Hematol Oncol* **6**, 35.

Ziober BL, Chen YQ, Ramos DM, Waleh N and Kramer RH (1999) Expression of the alpha7beta1 laminin receptor suppresses melanoma growth and metastatic potential. *Cell Growth Differ* **10**, 479–490.

Zuber J, Shi J, Wang E, Rappaport AR, Herrmann H, Sison EA, Magoon D, Qi J, Blatt K, Wunderlich M et al. (2011) RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. *Nature* **478**, 524–528.

**Supporting information**

Additional Supporting Information may be found online in the supporting information tab for this article:

**Table S1.** Sequences of the primers and siRNAs.