LncRNA-mediated regulation of SOX9 expression in basal subtype breast cancer cells

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ABSTRACT

Triple-negative breast cancer (TNBC) is one of the most aggressive breast cancer (BC) subtypes with a poor prognosis and high recurrence rate. Recent studies have identified vital roles played by several lncRNAs (long noncoding RNAs) in BC pathobiology. Cell type-specific expression of lncRNAs and their potential role in regulating the expression of oncogenic and tumor suppressor genes have made them promising cancer drug targets. By performing a transcriptome screen in an isogenic TNBC/basal subtype BC progression cell line model, we recently reported ~1800 lncRNAs that display aberrant expression during breast cancer progression. Mechanistic studies on one such nuclear-retained lncRNA, linc02095, reveal that it promotes breast cancer proliferation by facilitating the expression of oncogenic transcription factor, SOX9. Both linc02095 and SOX9 display coregulated expression in BC patients as well in basal subtype BC cell lines. Knockdown of linc02095 results in decreased BC cell proliferation, whereas its overexpression promotes cells proliferation. Linc02095-depleted cells display reduced expression of SOX9 concomitant with reduced RNA polymerase II occupancy at the SOX9 gene body as well as defective SOX9 mRNA export, implying that linc02095 positively regulates SOX9 transcription and mRNA export. Finally, we identify a positive feedback loop in BC cells that controls the expression of both linc02095 and SOX9. Thus, our results unearth tumor-promoting activities of a nuclear lncRNA linc02095 by facilitating the expression of key oncogenic transcription factor in BC.

Keywords: nuclear lncRNA; basal-like subtype; TNBC; regulation; enhancer

INTRODUCTION

Breast cancer (BC) is a frequently diagnosed malignancy and a leading cause of death among women across the globe (Siegel et al. 2016). Breast cancer, like most other epithelial tumors, is a heterogeneous disease with diverse subtypes (Nguyen and Massagué 2007). These subtypes differ from each other in clinical behavior, therapeutic response profiles, and the presence or absence of receptors such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2). Based on expression of these receptors, BC is classified into different categories: luminal A (ER+, PR+, and HER2−), luminal B (ER+, PR+, and HER2+/−), HER2-positive (ER−, PR−, and HER2+), and triple negative BC (ER−, PR−, and HER2−) (Weigelt et al. 2010; Jadaliha et al. 2016).

Triple negative breast cancer (TNBC) has been further classified into two distinct molecular subtypes; basal-like and claudin-low, based on their unique gene expression profiles (Jadaliha et al. 2016). There are no targeted therapies available for TNBC, and patients are typically treated with chemotherapy. However, TNBC patients display poor outcomes due to disease heterogeneity and chemotherapy resistance (Lv et al. 2016).
Less than 2% of the human genome encodes proteins; ~75% of the human genome encodes noncoding RNAs that are: transcripts with no apparent protein-coding potential, such as microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), and the least understood long noncoding RNAs (lncRNAs) (Prensner and Chinnaiyan 2011). In general, lncRNAs range in size between ~200 bp to 100 kb (Derrien et al. 2012). Genome-wide transcriptome analysis revealed that the human genome harbors >16,000 lncRNA genes (https://www.gencodegenes.org/human/stats.html) (Jalali et al. 2016). Mechanistic studies on a handful of them revealed that lncRNAs along with their interacting protein partners contribute toward the regulation of diverse biological processes, including cell proliferation (Prensner and Chinnaiyan 2011; Ulitsky and Chang 2016). They achieve this by modulating gene expression via different molecular mechanisms such as chromatin modification, transcription, protein activity and localization, and post-transcriptional gene regulation (Xu et al. 2017). In the context of BC, recent studies have identified several hundreds of lncRNAs as plausible prognostic markers of BC (Van Grembergen et al. 2016). The aberrant expression of lncRNAs like CCAT2 (Cai et al. 2015), MALAT1 (Jin et al. 2016), H19 (Matouk et al. 2014), HOTAIR (Gupta et al. 2010), and ZFAS1 (Hansji et al. 2016) has been associated with BC metastasis. Moreover, tissue type- and cell type-specific expression of lncRNAs have made them promising candidates to address BC cancer cell heterogeneity (Cabili et al. 2011).

In the present study, we investigated the potential role of a TNBC up-regulated IncRNA, linc02095, in BC cell proliferation. We observed elevated expression of both linc02095 and its neighboring protein-coding gene SOX9 in TNBC patient samples. Gain- and loss-of-function experiments revealed that linc02095 promotes cell proliferation and BC progression in vitro. Furthermore, we found that both linc02095 and SOX9 regulated the expression of each other, and this coregulation is required for enhanced tumorigenic activities of BC cells. Taken together, our results imply that linc02095 could function as an oncogenic IncRNA in BC via its role in promoting the expression of oncogenic and prometastatic transcription factor SOX9.

**RESULTS**

**Linc02095 is up-regulated in TNBC BC cells and patient samples**

Human breast carcinoma could progress via sequential genetic modifications of benign hyperplasia of mammary duct epithelial cells into atypical ductal hyperplasia, to ductal carcinoma in situ, to invasive tumor localized to the breast or lymph node, ultimately metastasizing to distant organs (Santner et al. 2001). In order to understand the role of lncRNAs during BC progression, we utilized a well-established isogenic mammary epithelial cell line-derived triple negative breast cancer (TNBC or basal subtype) progression model system. This model system consists of three isogenic cell lines (M1, M3, and M4), all of them originally derived from nontumorigenic MCF10A mammary epithelial cells. The cell line series consist of MCF10A (M1), tumorigenic but less metastatic MCF10CA1h (M3) and highly tumorigenic and metastatic MCF10CA1a.c11 (M4) cells. M3 gives predominantly well-differentiated low-grade carcinomas in the xeno-graft models (Tang et al. 2003; Imbalzano et al. 2009; Kadota et al. 2009, 2010; Fu et al. 2010; McKeen Polizzotti et al. 2012).

We recently performed poly A+ deep RNA-seq (~160–250 million paired-end reads/sample) of M1, M2, M3, and M4 cells that were grown as three-dimensional (3D) acinar or organoid-like structures in Matrigel for 7–10 d (Jadaliha et al. 2018). By analyzing the RNA-seq, >1800 IncRNAs were deregulated at least twofold in tumorigenic M3 cells compared to the nontumorigenic M1 cells (Jadaliha et al. 2018). Several of IncRNAs that showed altered expression between M1 versus M3 cells also displayed aberrant expression in BC patient samples (Jadaliha et al. 2018). In the present study, we focused on one such IncRNA, linc02095 or ROCR (Regulator of chondrogenesis) (Barter et al. 2017) (ENSG00000228639.2/AC005152.3) that was consistently elevated in M3 cells as compared to M1 cells (Supplemental Fig. S1A). Linc02095 is a multiexonic gene located on chromosome 17q23 and is transcribed into multiple transcript isoforms of (624 and 323 nt long) (Supplemental Fig. S1A,B). Our RNA-seq data revealed that all the isoforms of linc02095 showed elevated levels in M3 cells (Supplemental Fig. S1A,B). Quantitative real-time PCR (RT-qPCR) analysis confirmed significantly elevated linc02095 levels in M3 (>100-fold) cells (Fig. 1A). RT-qPCR results revealed that linc02095 also displayed enhanced expression even in 2D grown M4 cells (greater than fivefold) over M1 cells (Fig. 1A). RT-qPCR from nuclear and cytoplasmic fractionated RNA revealed that linc02095 was preferentially localized in the nuclear fraction (Fig. 1B), similar to the well-established nuclear IncRNA, MALAT1 (Tripathi et al. 2012, 2013). In addition, linc02095 was found to be a poly A+ RNA (Fig. 1C). Interestingly, the gene encoding the stem cell transcription factor SOX9 is located in close proximity to the linc02095 locus (~95 kb) and is transcribed in the opposite direction (see Supplemental Fig. S1C). Several IncRNAs show coregulated expression with protein-coding genes located in genomic proximity, and some of them also regulate the expression of protein-coding genes in a cis-regulated manner (Schmitt and Chang 2016; Warburton and Boone 2017; Yan et al. 2017). We therefore sought to determine whether SOX9 also displayed coregulated expression with linc02095 in the M1 and M3 cells. Both RNA-seq and RT-qPCR data
demonstrated that SOX9 showed enhanced expression in M3 cells compared to M1 cells (Fig. 1D; Supplemental Fig. S1C). Similar to linc02095, SOX9 also showed elevated levels in M4 cells over M1 (Fig. 1D). Further, immunofluorescence staining of SOX9 in M3 cells revealed that it preferentially enriched in the nucleus in all of the cells (Supplemental Fig. S2). Earlier studies had reported that SOX9 is up-regulated in BC samples, and SOX9 plays an essential role in the induction and maintenance of tumor-initiating capacity of BC cells (Fazilaty et al. 2016; Jeselsohn et al. 2017). We therefore analyzed whether linc02095 also showed elevated expression in BC patient samples of various subtypes using TANRIC (The Atlas of Noncoding RNA In Cancer) (Li et al. 2015a) CRN (Cancer RNA-Seq Nexus)-based analyses of the TCGA data set (Li et al. 2015b). Both linc02095 and SOX9 showed significantly elevated levels in basal subtype (TNBC) BC samples as compared to other BC subtypes (Supplemental Fig. S3A–E). Our results imply that both linc02095 and SOX9 show elevated expression in TNBC or basal-like cell line and in patient samples.

**Linc02095 and SOX9 coregulate the expression of each other**

To determine whether linc02095 positively regulates the expression of SOX9, we performed linc02095 loss-of-function studies. By stably expressing two independent shRNAs (sh1-02095 and sh3-02095) targeting linc02095, we successfully depleted linc02095 in M3 cells (Fig. 2A). M3 cells depleted of linc02095 showed a significant decrease in the levels of SOX9 mRNA (Fig. 2B). Further, immunoblotting showed that linc02095-depletion resulted in a significant decrease in SOX9 protein (Fig. 2C). To test whether linc02095 positively regulates the expression of other genes located in the SOX9 gene locus (Supplemental Fig. S4A) in control and linc02095-depleted cells. Our RNA-seq revealed that SOX-AS1 also showed elevated expression in M3 cells over M1 (Supplemental Fig. S4B) whereas linc001152, and linc00511 showed low expression in M1 and M3 cells (Supplemental Fig. S4C,D). However, linc02095 depletion in M3 cells resulted in enhanced expression of both SOX9-AS1 and linc001152 (Supplemental Fig. S4E). On the contrary, linc00511 showed no significant change in the levels upon linc02095 depletion (Supplemental Fig. S4E). On the contrary, SOX9 KD led to a decrease on SOX9-AS1 level indicating that SOX9 might regulate SOX9-AS1 expression (Supplemental Fig. S4F). These results imply that linc02095 could positively or negatively regulate the expression of specific genes in the SOX9 gene neighborhood. The up-regulation of SOX9-AS1 upon linc02095 depletion raised the possibility that linc02095 may regulate the expression of SOX9 via modulating SOX9-AS1 expression. To test this model, we determined the levels of SOX9 mRNA in the absence of SOX9-AS1.
SOX9-AS1 KD caused a small but significant increase in SOX9 mRNA level, indicating that it negatively regulates SOX9 expression (Supplemental Fig. S4G). However, both control and SOX9-AS1-depleted M3 cells that were devoid of linc02095 continued to show low levels of SOX9 mRNA and protein in control and linc02095-depleted M3 cells. (D,E) RT-qPCR and immunoblot showing relative levels of SOX9 mRNA and protein in control and SOX9-specific shRNA (sh-SOX9)-transduced M3 cells. (F) RT-qPCR showing relative levels of linc02095 in M3 cells depleted of SOX9. Data (A,B,D,F) represent mean ± SD of three independent experiments. P-values, (*) \( P < 0.05 \), (**) \( P < 0.01 \), were obtained by using Student’s t-test.

**FIGURE 2.** Linc02095 and SOX9 coregulated the expression of each other. (A) RT-qPCR showing relative levels of linc02095 in control and linc02095-specific shRNA-treated (sh1-02095 and sh3-02095) M3 cells. (B,C) RT-qPCR and immunoblot showing relative levels of SOX9 mRNA and protein in control and linc02095-depleted M3 cells. (D,E) RT-qPCR and immunoblot showing relative levels of SOX9 mRNA and protein in control and SOX9-specific shRNA (sh-SOX9)-transduced M3 cells. (F) RT-qPCR showing relative levels of linc02095 in M3 cells depleted of SOX9. Data (A,B,D,F) represent mean ± SD of three independent experiments. P-values, (*) \( P < 0.05 \), (**) \( P < 0.01 \), were obtained by using Student’s t-test.

**SOX9 and linc02095 promote cell proliferation in TNBC cells**

Earlier studies have documented the involvement of SOX9 in breast tumor cell proliferation (Fazilaty et al. 2016; Lei et al. 2016). Since linc02095 positively regulated SOX9 expression in BC cells, we determined the involvement of linc02095 in cell proliferation. Long-term cell proliferation assessed by anchorage-dependent growth assay revealed that depletion of either linc02095 or SOX9 significantly inhibited cell proliferation (Fig. 3Ai,ii, Bi,ii). Furthermore, BrdU-PI flow cytometry analyses revealed that linc02095-depleted cells showed defective S-phase progression (Supplemental Fig. S5A). Next, we transiently overexpressed one of the isoforms of linc02095 (323 nt) in M3 cells and determined the effect on cell proliferation. Colony formation assays revealed that linc02095-overexpressing cells showed enhanced cell proliferation (Fig. 3Ci–iii). Linc02095-overexpressing cells also showed enhanced levels of SOX9 mRNA (Fig. 3Civ). SOX9 has been implicated in the formation and growth of tumors in various tissues, including breast (Fazilaty et al. 2016; Jeselsohn et al. 2017). We tested the ability of linc02095 and SOX9 to control the tumorigenicity of BC cells by performing anchorage-independent soft-agar colony formation assays. We observed that depletion of linc02095 or SOX9 reduced anchorage-independent growth of M3 cells as observed by the reduced number of colonies, implying that linc02095 and SOX9 promote clonogenicity in vitro (Supplemental Fig. S5B,C). Defects in cell proliferation and tumorigenicity upon linc02095 depletion could be attributed to changes in the expression of genes controlling the cell cycle. RT-qPCR studies confirmed that linc02095-depleted cells show reduced expression of several oncosgenes (Aurora kinase, Bub1b, cyclin A and Cenp E), controlling cell cycle progression (Fig. 3E). Based on these results, we conclude that linc02095 promotes cell proliferation and tumor progression in BC, potentially by
influencing the expression of genes controlling cell cycle progression.

Linc02095 modulates SOX9 expression

To further elucidate the mechanism by which linc02095 might regulate SOX9 expression, we determined the status of RNA pol II occupancy on the SOX9 promoter and gene body by performing RNA polymerase II (Pol II) ChIP in control and linc02095-depleted cells. Linc02095-depleted cells showed decreased enrichment of RNA Pol II at the promoter and gene body of SOX9 gene (Fig. 4A). On the other hand, Pol II displayed no significant difference in association to b-actin promoter in control and linc02095-depleted cells. Our data illustrate that knock down of linc02095 affects SOX9 expression at the transcription level. We next determined whether linc02095-depletion has any impact on the chromatin organization at the SOX9 regulatory region such as the promoter. Toward this, we determined potential changes in H3K4me3 modification, an epigenetic mark present at the 5′ end of the gene surrounding the transcription start site (TSS) (Barski et al. 2007), at the SOX9 promoter upon linc02095 depletion. ChiP-qPCR did not show any significant change in H3K4me3 occupancy at the SOX9 promoter in the presence or absence of linc02095 (Fig. 4B). Based on this, we conclude that linc02095 modulates SOX9 transcription, potentially by altering the loading or elongation of RNA polymerase II, without having an impact on the chromatin structure.
Besides transcription, IncRNAs are also known to regulate posttranscriptional events, including RNA stability and export. We next determined whether linc02095 modulates RNA stability and export of SOX9 mRNA. We treated control and linc02095-depleted M3 cells with RNA Pol II transcription inhibitor Flavopiridol (1 µM) for indicated time points, and determined the turnover rate (half-life) of SOX9 mRNA. Our data illustrated that knock down of linc02095 did not impact the turn-over rate of SOX9 mRNA (Fig. 4C; t\(_{1/2}\) 1.15 ± 0.12 as opposed to control 1.29 ± 0.17). To test whether linc02095 influenced the localization of SOX9 mRNA, we performed RT-qPCR analyses of nuclear and cytoplasmic RNA fractions from control and linc02095-depleted cells. Interestingly, linc02095-depleted cells showed a significant reduction in the cytoplasmic pool of SOX9 mRNA compared to control cells (Fig. 4D). The reduction in the cytoplasmic pool of SOX9 mRNA could contribute to lower levels of SOX9 protein in linc02095-depleted M3 cells. These results indicate that linc02095 regulates SOX9 transcription and export of the SOX9 mRNA. Future studies will focus on determining the precise molecular mechanism by which linc02095 regulates the SOX9 expression.

SOX9 promotes the expression of linc02095

The significant decrease in linc02095 mRNA levels in SOX9-depleted cells further intrigued us to test the possibility of a positive-feedback mechanism. To assess whether SOX9 induces the transcription of linc02095, we looked for SOX9 binding motifs in the linc02095 promoter region. We found two consensus SOX9 binding motifs in the promoter region of linc02095 (CCAAT, ACAAAG; highlighted in blue boxes) (Fig. 5A–C; Shi et al. 2015). SOX9 recognizes specific promoter regulatory elements by forming heterodimeric complexes with other transcription factors, including SOX6 (Liu and Lefebvre 2015) and SOX6 (Ohba et al. 2015). We also found consensus SOX6 and RUNX1 motifs in the promoter region of linc02095 (Fig. 5A; highlighted in green and red). To examine the binding of SOX9 to the linc02095 promoter, we performed SOX9 ChIP-qPCR on the linc02095 promoter region in M1 (cell expressing low levels of SOX9 and linc02095) and M3 (cell type expressing elevated levels of SOX9 and linc02095) cells (Fig. 5D; Supplemental Fig. S6). We found significant enrichment of SOX9 at the linc02095 promoter in M3 as compared to M1 cells (Fig. 5D). Other known SOX9 target genes, including S100P (highly expressed in M3) and CCNB1 (highly expressed in M1) were used as a positive control; a noncoding region from chromosome 5A; highlighted in green and red). To examine the binding of
17 was used as a negative control to rule out nonspecific binding of SOX9 (Fig. 5D). In summary, we demonstrated significant enrichment of SOX9 at the promoter of linc02095, coregulated expression of linc02095 and SOX9 in BC cells, and reduced expression of linc02095 in SOX9-depleted cells. Based on these results, we conclude that SOX9 is a bona fide transcription activator of linc02095. Additionally, linc02095 and SOX9 positively regulate each other’s expression in BC cells as part of a positive feedback mechanism in order to promote cell proliferation and tumor progression.

**Linc02095 rescues cell proliferation defects observed in SOX9-depleted cells**

We further investigated whether linc02095 acts as a driver of SOX9 expression. We exogenously overexpressed linc02095 in BC cells stably depleted of endogenous SOX9 and determined the effect on SOX9 expression and cell proliferation (Fig. 6A; Supplemental Fig. S7). Linc02095 overexpressed cells showed consistent increase in the levels of SOX9 mRNA and SOX9 protein (Fig. 6B,C). At present, it is not clear how transient overexpression of linc02095 resulted in enhanced levels of SOX9. Linc02095 could potentially enhance SOX9 transcription in trans or SOX9 mRNA export to the cytoplasm ensuring their translation leading to an increase in protein levels (Fig. 6B,C). Alternatively, elevated expression of SOX9 could result in enhanced cell proliferation upon linc02095 OE. We observed that linc02095 overexpressing cells rescued the cell proliferation defects observed upon SOX9 depletion (Fig. 6D,E), implying that linc02095 promotes BC cell proliferation.

**DISCUSSION**

In the present study, we observed elevated expression of linc02095 in basal BC cells. High expression of linc02095 in tumorigenic cells reflects its potential as a regulator of cell proliferation and tumor progression in basal-like BC cells (Supplemental Fig. S3). Moreover, we also found that linc02095 positively regulates the expression of SOX9 both at transcriptional and posttranscriptional levels. In support of our observation, a very recent study reported that depletion of ROCR or linc02095 in mesenchymal stem cells (MSC) disrupted MSC chondrogenesis by decreasing SOX9 expression (Barter et al. 2017). Together, these results indicated that linc02095 regulated the expression of SOX9 in different cell or tissue types under physiological and pathological conditions.

The SOX9 gene is located at 17q23 in a gene desert ~2 Mb region surrounded by several lncRNA genes. SOX9 is an HMG-box transcription factor that plays an essential role in several cellular processes, including lineage specification and chondrocyte development (Akiyama 2008; Pritchett et al. 2011). Several enhancer elements have been identified, which are located upstream of SOX9 gene (~70, ~84, ~195, and ~250 kb), and these enhancer elements control tissue or cell type-specific
expression of SOX9 (Leipoldt et al. 2007; Gordon et al. 2009; Mead et al. 2013; Yao et al. 2015). Furthermore, chromosome conformation capture-on-chip (4C) analysis in testicular Sertoli cells and peripheral lymphoblasts revealed potential interaction between SOX9 promoter region and the flanking upstream (Smyk et al. 2013) and downstream enhancer elements as a consequence of chromatin looping (Gordon et al. 2009; Smyk et al. 2013; Yao et al. 2015). Chromosomal rearrangements with breakpoints mapping up to 1.6 Mb up- or downstream from SOX9 have been identified in patients with campomelic dysplasia (CD), a skeletal malformation syndrome often associated with sex reversal (Leipoldt et al. 2007). This suggests that aberrant activation of one or more of these enhancers could contribute to altered expression of SOX9 and disease pathology. An earlier study reported that several regulatory elements of SOX9 also overlap with IncRNA genes. Linc02095 is transcribed from one of the enhancer regions, located ~95 kb upstream of SOX9 gene (Supplemental Fig. S1). A recent bioinformatics study reported a positive correlation between SOX9 and linc02095 expression (AC005152.3 or ROCR; R-value 0.42) in basal BC samples further supporting our observation that both linc02095 and SOX9 are coregulated in BC (Supplemental Fig. S8; Su et al. 2014; Tang et al. 2017). Our knockdown experiments demonstrated that linc02095 plays a functional role in regulating SOX9 expression thereby modulating cell proliferation. SOX9 displays strong nuclear localization in TNBC cells as opposed to other BC subtypes (Pomp et al. 2015). Furthermore, the nuclear localization of linc02095 (Fig. 1C) and the enrichment of H3K4me1 and H3K27Ac marks across linc02095 gene body (Supplemental Fig. S9) raised the possibility that linc02095 could act as an enhancer RNA. Our RNA Pol II Chip analyses further support the model that linc02095 promotes SOX9 transcription. Linc02095KD leads to an increase in nuclear localization of SOX9 mRNA. Such nuclear retention of SOX9 mRNA could also result in reduced levels of SOX9 protein. Based on the results, we propose that linc02095 is one of several regulatory IncRNAs that are transcribed from SOX9 regulatory elements and is involved in promoting the expression of SOX9 in TNBC cells. The genomic positioning of linc02095 at the SOX9 enhancer region and its up-regulation in basal BC reflects a potential interplay of these candidates in regulating BC progression.

**MATERIALS AND METHODS**

**Cell culture**

The benign and nontumorigenic MCF10A (M1) cells were cultured in DMEM/F12 medium containing 5% horse serum supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 20 ng/mL EGF (epidermal growth factor), 0.5 µg/mL Hydrocortisone, 100 ng/mL Cholera toxin, 10 µg/mL insulin and 5% horse serum. M3 (MCF10A-1h) and M4 (MCF10A-1a,cl1) cells are H-ras transformed MCF10A cells derivatives, which were isolated from tumors followed by xenografting for several generations. M3 cells possess high tumorigenicity with low metastatic potential (Dawson et al. 1996). M4 (MCF10A-1a,cl1) cells tend to
be highly tumorigenic and metastatic in nature. M3 and M4 cells were cultured DMEM/F12 medium containing 5% horse serum supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin.

Cell fractionation

The nuclear and cytoplasmic fractionation was performed as described previously (Anantharaman et al. 2016, 2017). Briefly, M3 cells were cultured for 36 h to reach 80% confluence. Cells were initially washed with ice cold phosphate buffer saline (PBS) and lysed in the RSB buffer (10 mM Tris-Cl pH 7.4, 100 mM NaCl, 2.5 mM MgCl₂, RNase Inhibitor, Digitonin (8 µg/mL) on ice. The supernatant (cytoplasmic fraction) was collected after centrifugation (2000 rpm, 4°C, 10 min). The pellet (nuclear fraction) was washed with RSB buffer as described above. The nuclear RNA and cytoplasmic RNA were extracted by using TRIzol LS and TRIzol reagent, respectively (Invitrogen).

RNA extraction and RT-qPCR analysis

Total RNA was extracted with Tri-reagent (Sigma) followed by DNase I digestion (Sigma). One microgram of total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega). Quantitative RT-PCR was performed using the Step One Plus system. (Applied Biosystems) and primers used are listed in Supplemental Table S1. The relative fold change in mRNA level was determined by using the 2^−ΔΔCT method (Livak and Schmittgen 2001).

Linc02095 knockdown/overexpression experiments

Linc02095 and SOX9 were stably depleted in BC cells using lentiviral-mediated transduction of shRNAs followed by puromycin selection (Supplemental Table S2). For overexpression, full-length human linc02095 was expressed using lentivirus-mediated transduction, and stable lines were selected using puromycin selection. Empty vector transduced cells were used as a control.

Soft agar anchorage-independent and anchorage-dependent plastic colony formation assays

Anchorage-independent colony formation (5 × 10³ cells) and plastic colony formation (1 × 10³ M3 and 2 × 10³ M3 cells) assays were performed as described previously (Jadaliha et al. 2016, 2018).

Chromatin-immunoprecipitation (ChIP)

ChIP was performed using the previously published study from our laboratory (Khan et al. 2015). Cells in biological triplicates were cross-linked in 1% formaldehyde (Sigma) for 10 min at room temperature, followed by glycine (0.125 M final concentration) treatment to quench crosslinking. Chromatin was prepared in two subsequent extraction steps (10 min at 4°C) with Buffer 1 (50 mM HEPES/ KOH pH 7.5; 140 mM NaCl; 1 mM EDTA; 10% glycerol; 0.5% NP-40; 0.25% Triton) and Buffer 2 (200 mM NaCl; 1 mM EDTA; 0.5 mM EGTA; 10 mM Tris pH 8). The nuclei were resuspended in Buffer 3 (50 mM Tris pH 8; 0.1% SDS; 1% NP-40; 0.1% Na-Deoxycholate; 10 mM EDTA; 150 mM NaCl). The pellet was then subjected to sonication with Bioruptor Power-up (Diagenode) resulting in the genomic DNA fragments within size range of 150–300 bp. Preclearing of the chromatin was done with Protein A/G ultralink beads (S3,133, Pierce) for 2 h at 4°C. Immunoprecipitation was performed using specific antibodies against RNA polymerase II (Millipore, cat. # 05–623), HP1 (Millipore 07–523) and SOX9 (Millipore Cat # AB–5535) overnight at 4°C. Immuno-complexes were recovered by adding preblocked protein G Dynabeads (Life Technologies) and incubated for 2 h at RT. The unbound DNA fragments were removed by washing the beads twice with low salt buffer (0.1% SDS; 1% Triton; 2 mM EDTA; 20 mM Tris pH 8; 150 mM NaCl) and twice with high salt buffer (0.1% SDS; 1% Triton; 2 mM EDTA; 20 mM Tris pH 8; 500 mM NaCl). The beads were further washed once with LiCl wash buffer (10 mM Tris pH 8.0; 1% Na-deoxycholate; 1% NP-40, 250 mM LiCl; 1 mM EDTA), and twice with TE + 50 mM NaCl. Beads were eluted in TE + 1% SDS at 65°C, and was reverse cross-linked overnight at 65°C. The eluted DNA was used for QPCR. ChIP primers are detailed in Supplemental Table S2. ChIP-qPCR results were calculated as percentage of IP/input signal (% input).

Flow cytometry

Flow cytometric analysis to quantify S-phase progression was done by using BrdU and PI as labels for the control and linc02095 depleted cells, as described previously (Tripathi et al. 2013).

RNA stability assay

To measure RNA stability, cells were treated with Flavopiridol (1 µM) for different durations as indicated. Total RNA was extracted at each of the time points and subjected to RT-qPCR. An exponential curve ($y = e^{-bt}$) was then fitted to the data points. The half-life was then calculated using the equation $t_{1/2} = \ln(2)/b$ from each of the experiments. The average half-life with SD is shown in the graph (Fig. 4C).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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