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).

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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.encodegenes.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 Bartel 2013; Schmitt 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 (Cabali et al. 2011).

In the present study, we investigated the potential role of a TNBC up-regulated lncRNA, *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 lncRNA 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 lncRNAs were deregulated at least twofold in tumorigenic M3 cells compared to the nontumorigenic M1 cells (Jadaliha et al. 2018). Several of lncRNAs 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 lncRNA, *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 lncRNA, *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 lncRNAs 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

FIGURE 1. Nuclear-enriched and polyadenylated *linc02095* show elevated expression in BC cells. (A) RT-qPCR showing relatively high levels of *linc02095* in M3, as compared to M1 and M4 cells. (B) RT-qPCR in nuclear and cytoplasmic fractionated cell extracts showing relative localization of *linc02095*, *MALAT1*, and *actin* RNA. *MALAT1* (nuclear) and *Actin* (cytoplasm) are used as controls. (C) RT-qPCR analyses in poly(A)⁺ and poly(A)⁻ fractionated M3 cells. *18S*RNA [poly(A)⁻] and *actin* [poly(A)⁺] were used as controls. (D) RT-qPCR showing relative levels of *SOX9* mRNA in M1 and M3 cells. Error bars in A–D represent $\pm$ SD of three independent experiments, that is, biological replicates. *P*-values, (*) *P* < 0.05, (**) *P* < 0.01, were obtained by using Student's *t*-test.

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 suc-

cessfully 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 *SOX9* locus neighborhood, we determined the levels of several other lncRNA transcripts (*SOX9-AS1*, *linc001152*, *linc00511*) that are located near the *SOX9* gene locus (Supplemental Fig. S4A) in control and *linc02095*-depleted cells. Our RNA-seq revealed that *SOX9-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*.

FIGURE 2. *Linc02095* and *SOX9* coregulate 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 $\pm$ SD of three independent experiments. *P*-values, (*) $P < 0.05$, (**) $P < 0.01$, were obtained by using Student's *t*-test.

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 (Supplemental Fig. S4G), implying that *SOX9*-AS1 depletion could not rescue the levels of *SOX9* in *linc02095*-depleted cells. These results suggest that *linc02095* regulates the expression of *SOX9* and *SOX9*-AS1 via independent mechanisms. On the other hand, *SOX9*-AS1 KD could act as a negative regulator of *SOX9*. Next, we determined whether *SOX9* regulates the expression of *linc02095* or *SOX9*-AS1. M3 cells stably depleted of *SOX9* (sh-SOX9) showed reduced levels of both *SOX9* mRNA and *SOX9* protein (Fig. 2D,E). Interestingly, depletion of *SOX9* led to a decrease in *linc02095* and *SOX9*-

AS1 RNA levels (Fig. 2F; Supplemental Fig. S4F). Thus, our results indicated that both *linc02095* and *SOX9* positively regulate the expression of each other in BC cells.

***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 oncogenes (*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

FIGURE 3. *Linc02095* and *SOX9* promoted cell proliferation in BC cells. (A,i,ii). Plastic colony assay showing reduced colony numbers in cells depleted of *linc02095* and *SOX9*. (B,a,b) Quantification of the data presented in A. (C,i) RT-qPCR showing relative levels of *linc02095* in cells over-expressing *linc02095*. (C,ii) Plastic colony assay showing increased cell proliferation upon *linc02095* overexpression. (C,iii) Quantification of data presented in Cb. (C,iv) RT-qPCR showing increased *SOX9* mRNA levels in *linc02095* overexpressed cells. (D,a,b) Anchorage independent growth assay showing reduced number of colonies in *linc02095* and *SOX9* depleted cells. (E) RT-qPCR to show relative expression of several cell cycle genes in *linc02095*-depleted cells. Error bars in B (i,ii), C (i,iii,iv), D (i,ii), and E represent $\pm$ SD of three independent experiments, that is, biological replicates. *P*-values, (*) $P < 0.05$, (**) $P < 0.01$, were obtained by using Student's *t*-test.

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 β -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.

FIGURE 4. SOX9 facilitates the transcription of *linc02095*. (A) RNA polymerase II chip showing reduced association of RNA polymerase II at the promoter and gene body of SOX9 in *linc02095*-depleted M3 cells. B-actin and Ch17NC are used as positive and negative controls, respectively. (B) H3K4me3 chip showing no significant change in its association to the SOX9 promoter in control and *linc02095* depleted cells. (C) RT-qPCR to quantify the stability of SOX9 mRNA in control and *linc02095* depleted M3 cells treated with Flavopiridol (1M) for indicated time points (0, 1, 2, 3 h). (D) Nuclear and cytoplasmic fractionation of control and *linc02095* depleted cells showing increased nuclear retention of SOX9 transcript in the absence of *linc02095*. Error bars in A–D represent $\pm$ SD of three independent experiments, that is, biological replicates. *P*-values, (*) $P < 0.05$, (**) $P < 0.01$, were obtained by using Student's *t*-test.

Besides transcription, lncRNAs 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 μ M) 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 $\pm$ 0.12 as opposed to control 1.29 $\pm$ 0.17). To test whether *linc02095* influenced the lo-

calization 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

FIGURE 5. SOX9 facilitates the transcription of *linc02095*. (A) Promoter sequence of *linc02095*, showing potential consensus binding sites of SOX9 (highlighted in blue), SOX6 (highlighted in green), RUNX1 (highlighted in red) and 5' end of transcript (highlighted in yellow). (B) Genomic position of SOX9 binding sites of *linc02095* promoter. (C) Consensus SOX9 binding motifs, based on previous literature (Shi et al. 2015). (D) QPCR followed by SOX9 ChIP in M1 and M3 cells. Please note the increased association of SOX9 in the promoter region of *linc02095* and *S100P* in M3 cells. *S100P* and *CCNB1* were used as positive controls. The non-coding region from chromosome 17 was used as a negative control showing no binding of SOX9. SOX9 showed binding to *linc02095* promoter in M3 cells as opposed to M1 cells. Data (D) represent mean $\pm$ SD of three independent experiments. *P*-value, (*) $P < 0.05$, was obtained by using Student's *t*-test.

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 trans-

criptional 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

FIGURE 6. Overexpression of *linc02095* increases the cellular levels of SOX9 and promotes cell proliferation. (A) RT-qPCR showing increased levels of *linc02095* in SOX9-depleted M3 cells overexpressing *linc02095*. (B,C) RT-qPCR and immunoblot showing rescue of endogenous SOX9 in SOX9-depleted M3 cells overexpressing *linc02095*. (D,E) Plastic colony assay showing increased cell proliferation in *linc02095* overexpressed M3 cells depleted of endogenous SOX9. Error bars in A,B,E represent $\pm$ SD of three independent experiments, that is, biological replicates. P-values, (*) $P < 0.05$, (**) $P < 0.01$, were obtained by using Student's *t*-test.

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 lncRNA 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. *Linc02095*KD 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 lncRNAs 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 μ g/mL streptomycin, 20 ng/mL EGF (epidermal growth factor), 0.5 μ g/mL Hydrocortisone, 100 ng/mL Cholera toxin, 10 μ g/mL insulin and 5% horse serum. M3 (MCF10CA1h) and M4 (MCF10CA1a.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 (MCF10CA1a.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-HCl 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). C_t values of the nuclear or cytoplasmic fraction were normalized to the total RNA.

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 (53,133, Pierce) for 2 h at 4°C. Immunoprecipitation was performed using specific antibodies against RNA polymerase II (Millipore, cat. # 05-623), H3K9me3 (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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