Linc-Pint Long Non-Coding RNA Sponges miR-7 and Regulates WNT Signaling Pathway in Breast Cancer Cells

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Research Article

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Abstract

Several non-coding RNAs (ncRNAs) are known to regulate signaling pathways in breast cancer (BC) to date. Looking for the differentially expresses lncRNAs, first, RNAseq data analysis indicated that Linc-Pint (Loc646329) gene is downregulated in BC tissues. Consistently, RT-qPCR analysis confirmed its downregulation in BC specimens compared with normal pairs. Then, overexpression of Linc-Pint in MCF7 cells was followed by elevated *APC*, *APC2*, and *Axin* genes expression (all tumor suppressors of Wnt signaling), while *c-Myc*, *CCND1* and *CCND2* genes were downregulated, detected by RT-qPCR. Consistently, Top/Fop flash assay supported Linc-Pint suppressive effect on Wnt signaling in MCF7 cells. On the other hand, miR-7 was predicted to be sponged by Linc-Pint and clash-Seq data analysis supported their interaction. Furthermore, dual luciferase assay verified their interaction and consistently, overexpression of Linc-Pint ended in elevated expression level of TGFBR2 and IGF1R (as target genes of miR-7). Flow cytometry results indicated that Linc-Pint overexpression in MCF7 cells was followed by cell cycle arrest at G1 phase and scratch test assay supported its inhibitory effect on cell migration. Overall, our results indicated that Linc-Pint tumor suppressor attenuates Wnt signaling at least by sponging miR-7 in MCF7 cells. This makes Linc-Pint transcript as a potential new therapeutic target.

Introduction

Breast cancer is one of the leading causes of annual female's death around the world and despite all of treatment progress; it is still a growing concern globally. Family history of BC, obesity, radiation history and lifestyle are reported as its risk factors(1,2). Early detection of breast cancer boosts the prospects of disease-free survival, hence, understanding the molecular mechanism and recognizing the tumor subtype will assist to decide on proper medication(3). As reported by next-generation sequencing (NGS), an ample amount of human genome are transcribed but not translated. Long non-coding RNAs (LncRNAs), and microRNAs (miRs) are two influential types of non-coding transcriptome (4). Various studies have revealed that LncRNAs have diverse functions in cellular processes and they are of significant roles in tumorigenesis and proliferation inhibition. In addition, deregulation of these RNAs has been associated with many cancers (5–8).

The Wnt signaling is one of the significant intracellular pathways that regulates cell growth, survival, and cell cycle progression. Aberrant activation of this cascade has been proven to be related to several cancers, particularly breast carcinoma (9–11). Numerous researches have shown that LncRNAs, by modulating Wnt signaling, can play key roles in controlling cellular status.

Linc-Pint (Loc646329) is a lncRNA reported to be involved in regulation of Wnt signaling pathway through interaction with miR-29b in colorectal cancer (33). MiR-7 is also reported as a regulator of REGγ in BC (34).

Her, we aimed to explore the expression pattern and clinical significance of Linc-Pint in breast cancer and investigated its interaction with miR-7 and their effect on Wnt signaling. We attribute an anti-cancer

activity to the linc-Pint in BC that is exerted by attenuating Wnt signaling activity and sponging miR-7.

Material And Methods

Patients and samples

Breast cancer tissues and adjacent non-cancerous Tissue samples and clinicopathologic data were obtained from the Breast Cancer Research Center Biobank (BCRC-BB), Iran . All the patients were diagnosed with BC and no chemotherapy or radiotherapy was done before the operation. Specimens were immediately frozen into liquid nitrogen and stored at -80° C. Informed consent forms were provided for each subject and the study protocol was approved by the research committee at Tarbiat Modares University.

Cell culture and transient transfection

Human breast cell lines, namely, MCF7, SKBR3, MDA-MB-231 (obtained from the Pasteur Institute, Iran) were cultured in HDMEM medium (Gibco), containing 10% FBS (Gibco), and 100 U/ml penicillin, 100 µg/ml streptomycin(Sigma) followed by incubation in 37° C in with 5% CO2.

Full-length Linc-Pint cDNA was amplified and cloned into pCDNA3.1(+) expression vector (Promega, Madison, WI, USA) at the provided restriction sites. The accuracy of the recombinant vector was confirmed by sequencing.

Cell line were cultivated in complete medium without antibiotics overnight, and transfected with TurboFect reagent (Thermo Fisher Scientific), following the manufacturer's instruction after reaching proper confluency.

RNA extraction and cDNA synthesis

Total RNA was extracted from BC tissues and cell lines using RiboEx reagent (GeneAll) and quantified with agarose gel electrophoresis and Nanodrop instrument. In order to remove genomic DNA contamination, DNaseI treatment was performed at 37° C for 30 minutes followed by heat and EDTA inactivation. Reverse transcription was done using 1µg total RNA, Random hexamer, oligo-dT mix primers, and Prime Script II Reverse transcriptase(Takara, Japan) at 42° C for 70 min, subsequently RT inactivation at 72° C for 10 min. Besides, for miR detection, polyA tail was added to the 3’ end of RNAs before cDNA synthesis (polyA polymerase, NEB, UK).

RT-qPCR

RT-qPCR was performed using BioFACT™ 2X Real-Time PCR Master Mix in StepOne system (Applied Biosystems, USA). Transcriptional levels of target genes were measured in duplicate and normalized to Beta-2-Microglobulin (B2M) and U48, as reference genes, following the ΔΔCt formula. Primer sequences are listed in Table1.

Cell cycle assay

The cells were transfected with Linc-Pint-variant D or mock control vectors using TurboFect reagent. The cells were trypsinized and harvested 36 hrs after transfection, and centrifuged at 12000 rpm for 5 minutes, then washed in cold PBS twice. Subsequently, fixing with 70% ice-cold ethanol (Bidestan, Iran) and stained with PI (Sigma, USA)/RNase A (Thermo scientific, USA) staining solution following incubation at 37° C for 30min away from light. The samples were subjected to BD Flow Cytometer (BioScience, San Diego, CA, USA). The test was performed in duplicate and results were analyzed by flowing software version 2.5.0.

Scratch Assay

The MCF7 cells were grown in 24-well plates as 80% confluent. Thereafter, the cells were wounded with a 100-µl pipette tip and then transiently transfected with Linc-Pint containing or empty vectors. The cell migration was photographed at 0, 24, and 48 h after transfection.

Dual-Luciferase Reporter Assay

PsiCHECK-2 dual luciferase vector (Promega) was used to clone the Linc-Pint joined to luciferase ORF sequence. To study direct interaction, HEK293T cells were co-transfected with wild-type (control) reporter plasmid (psi-check2 construct) and miR-7 (mock vector) using TurboFect Reagent (Thermo Scientific). A fragment with no predicted microRNA recognition element (MRE) for miR-7 was used as a control. Forty-eight hours after transfection using dual-glo luciferase reporter system, the activities of the Firefly and Renilla luciferases were measured sequentially from cell lysates, according to the manufacturer's instructions (Promega). Firefly luciferase units were normalized against Renilla luciferase units to control the transfection efficiency.

TOP/FLASH assay

MCF7 cells were transfected with Linc-Pint expressing and 0.2Mg TOPflash vector. After 48 hours MCF7 cells were lysed using Lysis buffer and was used for the measurement of luciferase activities using a luciferase reporter assay kit (Promega) and luminometer.

Statistical analysis

Data are reported as means ± SEM. Graph pad prism version 6 were used for statistical analysis. Unpaired student’s t-test and one way ANOVA were used for comparisons amongst two or multiple groups. P-value < 0.05 was considered as statistically significant.

Results

Bioinformatics analysis of differentially expressed LncRNAs in BC

To identify the differentially expressed genes in BC, transcriptome was investigated using TCGA (The Cancer Genome Atlas) and also multiple GSE dataset analysis was performed. The RNA-seq data were analyzed via DSEq2 packages to extract differentially expressed genes (12). Microarray studies were adopted from GEO and analyzed by the LIMMA package to obtain differential expression (13). The data were quantile normalized and transformed as log2 relative expression. Bioinformatics analysis presented that Long Intergenic Non-Protein Coding RNA, P53 Induced Transcript (Linc-PINT) or loc646329 is downregulated in breast cancer tissues in comparison to normal breast tissues and it is negatively correlated with their invasive phenotype (Fig. 1A). Kaplan-Meier analysis was performed looking for the association of Linc-PINT with clinical features of breast cancer patients. Results indicated that the BRCA patients with low Linc-PINT expression levels had worse overall survival time, compared with patients with high expression levels (log-rank test: p-value = 0.01, Fig. 1B). Equal distribution of stimulated genes and downregulated genes following the volcano plot drawing indicated that RNA-seq data analysis has been correct (Fig. 1C). KEGG pathway analysis introduced “Focal Adhesion” and “WNT signaling pathway” as the main pathways to be correlated with Linc-PINT gene expression (Fig. 1D). UCSC data analysis introduced miR-7 as an interacting molecule to Linc-Pint and their direct interaction was supported by the results of RNA hybrid software (Fig 1E). There was a negative correlation between miR-7 and the Linc-pint gene in colorectal cancer samples extracted from the TCGA database (Fig 1F). Finally, Clip-Seq data analysis indicated a direct interaction between the Linc-Pint and miR-7 (Fig.1G).

RT-qPCR analysis indicated downregulation of Linc-PINT in human breast cancer

Looking for the expression status of Linc-Pint in breast cancer tissue samples, RT-qPCR was performed against 29 pairs of normal and tumor breast tissues (Fig. 2A). Overall results indicated that Linc-PINT expression level is significantly lower in breast cancerous tissues compared with normal adjacent pairs (Fig. 2B). These RT-qPCR results were also analyzed based on tumor subtypes, and it seemed less Linc-PINT expression level in more invasive tumor samples, where lower expression level was detected in TNBC and her2-enriched tumors relative to Luminal types (Fig. 2C). Also, RT-qPCR analysis against linc-PINT expression in BC originated cell lines indicated lower expression of it in more aggressive cell lines such as MDA-MB-231 and SKBR3 in comparison to the less aggressive MCF-7 cells (Fig. 2D).

Experimental evidences for Linc-PINT effects against WNT signaling pathway in BC

Linc-Pint cDNA was amplified from SW480 cells and cloned in PCDNA3.1 vector in order to overexpress it in MCF7 cell line (Fig. 3A). Then, RT-qPCR results indicated that *APC*, *APC2*, and *AXIN* (all Wnt signaling tumor suppressors) were considerably upregulated at the mRNA level in Linc-PINT overexpressing cells (Fig. 3B to D). Inversely, *C-myc*, *Cyclin D1* and *Cyclin D2* expression level was significantly decreased under the same condition (Fig. 3E To G). Furthermore, Top/flash assay was performed and results indicated that WNT signaling pathway has been suppressed at the condition of Linc-Pint overexpression in MCF7 cells (Fig. 3H).

Experimental evidences for direct interaction of Linc-PINT with miR-7

UCSC and clshseq data analysis indicated that miR-7 is sponged by Linc-Pint transcripts. Bioinformatics data indicated there are two miR- 7 recognition sites within the first and second exons of LOC646329 transcript (Fig. 1E). Therefore, a direct interaction between miR-7 and Linc-Pint transcript was investigated through dual luciferase assay. To this aim, 719 bp length Linc-Pint related cDNA was cloned downstream to the Renilla luciferase in the pSicheck2 vector and was co-transfected in HEK293Tcells along with pEGFP vector ensuring miR-7 overexpression. Then, results were compared with the cells that mock control vector was co-transfected along with pSicheck2 recombinant vector. Results of dual luciferase assay supported a direct interaction between miR-7 and Linc-Pint transcript (Fig. 4A).

Following the supportive evidences showing LOC646329 sponge's miR-7, overexpression effect of Linc-Pint against the target genes of miR-7 (TGFBR2, IGF1R) was investigated through RT-qPCR. Results indicated that these target genes were upregulated following the Linc-Pint overexpression consistent to its role as sponger of miR-7 expression (Fig. 4B).

Linc-PINT effect on cell-cycle progression in BC originated cell line

Linc-Pint was overexpressed in MCF7 cells and its effect on the cell cycle progression was investigated through flow cytometry (Fig. 5). Results indicated that G1 cell population was significantly increased following the overexpression of Linc-Pint in these cells. Consistently S phase cell population was reduced at the same condition.

Linc-PINT overexpression effect on breast cancer cell migration

To investigate the effect of Linc-Pint overexpression on cell migration, wound-healing assay was performed against transfected MCF7 cell line. As it is shown in figure 6, linc-PINT overexpression was followed by decreased cell migration in MCF7 cells in comparison to the cells which were transfected with mock vector. Relative ratios of wound closure were remarkably lower in overexpressing cells.

Discussion

Long non-coding RNAs are a class of ncRNAs and they have particular functions in cellular processes including transcriptional regulation of mRNAs, affecting cell signaling networks, RNA stability and chromosomal interaction. Thus, aberrant expression of LncRNAs leads to multiple diseases involving cancer(18–21). As they are highly tissue-specific molecules, they have been investigated to be potential biomarkers to prevent cancer progression. For example, HULC (highly upregulated in liver cancer) has been introduced as a biomarker in hepatocellular carcinoma(22), or SNHG7 serves the same purpose in multiple cancers(23,24).

Some LncRNAs act as signaling pathway modulator; thereby they can alter cell proliferation, cell migration, and growth.

LINC-PINT is located on chromosome 7 and seems to encode several transcript variants. It is suggested to be a tumor suppressor while its inhibitory effect has been observed in various cancers such as CRC

(14) and glioblastoma (17). Linc-PINT is downregulated in ovarian cancer (15), and lung cancer as well (16). NO bona fide ORF has been introduced for this ncRNA and no protein or peptide has been attributed to it yet.

In this study we aimed to investigate the role of Linc-pint in breast cancer where DEGs exploring indicates its differential expression in breast cancerous tissues in comparison to normal pairs (Fig. 1). Consistently, RT-qPCR results against 29 breast normal/cancer pairs revealed its lower expression in cancerous tissues (Fig. 2A, B) while the lowest expression belonged to the HER2-enriched subtype(Fig. 2C). To examine the Linc-pint's mechanism of effect, we probe its expression in different breast cancer cell lines. MCF7 cell line was opted for further investigations because of its mildest expression level (Fig. 2D). Gene Ontology analysis was performed where WNT signaling was recommended as one of the highest correlated pathways (Fig. 1D). WNT cascade is involved in cell proliferation, motility, survival and metabolism. It is the most frequently altered pathway in human cancers (25). Deviant activation of this pathway has been confirmed to be related to several cancers such as breast (26), CRC (24), leukemia (27), prostate cancer (28), NSCLC (29) and ovarian cancer(30). Successful overexpression of Linc-pint in MCF7 led to the upregulation of *APC*, *APC2*, *AXIN* and downregulation of *C-myc* and *CCND1* and *CCND2* detected by RT-PCR (Fig. 3B to F). These results pointed that linc-pint exercises its suppressive effect through Wnt pathway. Then, Linc-PINT effect on Wnt signaling was further verified thorough performing TOP/flash assay analysis in transfected MCF7 cells (Fig. 3H).

CLIP-seq data indicated that miR-7 directly targets the APC gene and it has been shown that it is an important regulator of the Wnt signaling pathway (32). On the other hand, our bioinformatics analysis indicated that miR-7 might be sponged by Linc-Pint (Fig. 1). Then, we verified the interaction between Linc-Pint and miR-7 by performing dual reporter assay which is a powerful strategy for evidencing direct interaction between two RNAs (Shanet al. 2018). Consistently, when Linc-Pint was overexpressed in MCF7 cells, known target genes of miR-7 including TGFBR2 and IGF1R were upregulated (Fig. 4B). All of these results could be interpreted based on the sponging effect of *Linc-Pint* against *miR-7.*

Due to CCND2 downregulation, which is a subG1 inducer arrest (31), we consider probing its effect on cell cycle progression using flow cytometry, where it was observed cell cycle dearest in G1 in overexpressing cells (Fig. 5). Also, the impact of Linc-pint on cell migration was explored by wound-healing assay and a remarkable decrease was observed in cellular motility while overexpressing LINC-PINT. In summary, downregulation of Linc-pint in breast cancer tumors was confirmed experimentally, and its interaction with miR-7 was supported by experimental evidences. Since here we confirmed Linc-Pint effect on Wnt signaling in BC originated cell line, and also showed its cell cycle arrest effect, final suggestion is that Linc-Pint exerts its negative effect on Wnt signaling and cell cycle progression in BC cell line, at least through sponging miR-7. More details of Linc-Pint effect on cell cycle progression through Wnt signaling in summarized in Figure 7.

References

1. Becker S. A historic and scientific review of breast cancer: The next global healthcare challenge. Int J Gynecol Obstet [Internet]. 2015;131:S36–9. Available from: http://dx.doi.org/10.1016/j.ijgo.2015.03.015
2. Kamińska M, Ciszewski T, Łopacka-Szatan K, Miotła P, Starosławska E. Breast cancer risk factors. Prz Menopauzalny. 2015;14(3):196–202.
3. Maiz C, Silva F, Domínguez F, Galindo H, Camus M, León A, et al. Mammography correlates to better survival rates in breast cancer patients: A 20-year experience in a University health institution. Ecancermedicalscience. 2020;14:1–7.
4. Ma L, Bajic VB, Zhang Z. On the classification of long non-coding RNAs. RNA Biol. 2013;10(6):924–33.
5. Jafarzadeh M, Tavallaie M, Soltani BM, Hajipoor S, Hosseini SM. LncRNA HSPC324 plays role in lung development and tumorigenesis. Genomics [Internet]. 2020;112(3):2615–22. Available from: https://doi.org/10.1016/j.ygeno.2020.02.012
6. Jafarzadeh M, Soltani BM, Soleimani M, Hosseinkhani S. Epigenetically silenced LINC02381 functions as a tumor suppressor by regulating PI3K-Akt signaling pathway. Biochimie. 2020;171–172:63–71.
7. Schmitt AM, Chang HY. Long Noncoding RNAs in Cancer Pathways. Cancer Cell [Internet]. 2016;29(4):452–63. Available from: http://dx.doi.org/10.1016/j.ccell.2016.03.010
8. Xu Y, Chen M, Liu C, Zhang X, Li W, Cheng H, et al. Association Study Confirmed Three Breast Cancer-Specific Molecular Subtype-Associated Susceptibility Loci in Chinese Han Women. Oncologist. 2017;22(8):890–4.
9. Cidado J, Park BH. Targeting the PI3K/Akt/mTOR pathway for breast cancer therapy. J Mammary Gland Biol Neoplasia. 2012;17(3–4):205–16.
10. Noorolyai S, Shajari N, Baghbani E, Sadreddini S, Baradaran B. The relation between PI3K/AKT signalling pathway and cancer. Gene [Internet]. 2019;698(November 2018):120–8. Available from: https://doi.org/10.1016/j.gene.2019.02.076
11. Fresno Vara JÁ, Casado E, de Castro J, Cejas P, Belda-Iniesta C, González-Barón M. P13K/Akt signalling pathway and cancer. Cancer Treat Rev. 2004;30(2):193–204.
12. Anders S, Huber W. Differential expression analysis for sequence count data. 2010;
13. Data M. limma: Linear Models for Microarray Data. :2–3.
14. Reza A, Sadat J, Mehrdad D, Bahram B. TGFβ signaling in colorectal cancer. 2020;(0123456789).
15. Gao C, Zhao D, Zhao Q, Dong D, Mu L, Zhao X, et al. Microarray pro fi ling and co-expression network analysis of lncRNAs and mRNAs in ovarian cancer. Cell Death Discov [Internet]. 2019; Available from: http://dx.doi.org/10.1038/s41420-019-0173-7
16. Marín-béjar O, Mas AM, González J, Martinez D, Athie A, Morales X, et al. The human lncRNA LINC-PINT inhibits tumor cell invasion through a highly conserved sequence element. 2017;1–15.

17. Zhu H, Chen Z, Shen L, Tang T, Yang M, Zheng X. Long Noncoding RNA LINC-PINT Suppresses Cell Proliferation, Invasion, and EMT by Blocking Wnt / β -Catenin Signaling in Glioblastoma. 2021;11(January):1–12.
18. Balas MM, Johnson AM. Non-coding RNA Research Exploring the mechanisms behind long noncoding RNAs and cancer. Non-coding RNA Res [Internet]. 2018;3(3):108–17. Available from: https://doi.org/10.1016/j.ncrna.2018.03.001
19. Schmitt AM, Chang HY. Perspective Long Noncoding RNAs in Cancer Pathways. 2016;(Table 1).
20. Kim M. Non-coding RNA Research Long non-coding RNAs in cancer. Non-coding RNA Res [Internet]. 2019;4(2):45. Available from: https://doi.org/10.1016/j.ncrna.2019.02.003
21. Arun G, Diermeier SD, Spector DL. Therapeutic Targeting of Long Non-Coding RNAs in Cancer. Trends Mol Med [Internet]. 2018;xx:1–21. Available from: http://dx.doi.org/10.1016/j.molmed.2018.01.001
22. Distefano JK. Non-coding RNA Research Long noncoding RNAs in the initiation, progression, and metastasis of hepatocellular carcinoma. Non-coding RNA Res [Internet]. 2017;2(3–4):129–36. Available from: https://doi.org/10.1016/j.ncrna.2017.11.001
23. Cheng D, Fan J, Ma Y, Zhou Y, Qin K, Shi M, et al. LncRNA SNHG7 promotes pancreatic cancer proliferation through ID4 by sponging miR – 342 – 3p. Cell Biosci [Internet]. 2019;1–11. Available from: https://doi.org/10.1186/s13578-019-0290-2
24. Li Y, Zeng C, Hu J, Pan Y, Shan Y, Liu B, et al. Long non-coding RNA-SNHG7 acts as a target of miR-34a to increase GALNT7 level and regulate PI3K / Akt / mTOR pathway in colorectal cancer progression. 2018;1–17.
25. Lee JJX, Loh K, Yap Y. PI3K / Akt / mTOR inhibitors in breast cancer. 2015;4:342–54.
26. Ortega MA, Fraile-mart O, Buj J, Coca S. Signal Transduction Pathways in Breast Cancer: The Important Role of PI3K / Akt / mTOR. 2020;2020.
27. Nepstad I, Hatfield KJ, Grønningsæter IS, Reikvam H. The PI3K-AKT-MTOR signaling pathway in human acute myeloid leukemia (AML) cells. Int J Mol Sci. 2020;21(8).
28. Shorning BY, Dass MS, Smalley MJ, Pearson HB. The PI3K-AKT-mTOR pathway and prostate cancer: At the crossroads of AR, MAPK, and WNT signaling. Int J Mol Sci. 2020;21(12):1–47.
29. Tan AC. Targeting the PI3K/Akt/mTOR pathway in non-small cell lung cancer (NSCLC). Thorac Cancer. 2020;11(3):511–8.
30. Ediriweera MK, Tennekoon KH, Samarakoon SR. Role of the PI3K/AKT/mTOR signaling pathway in ovarian cancer: Biological and therapeutic significance. Semin Cancer Biol. 2019;59(April):147–60.
31. Mende N, Kuchen EE, Lesche M, Grinenko T, Kokkaliaris KD, Hanenberg H, et al. CCND1-CDK4-mediated cell cycle progression provides a competitive advantage for human hematopoietic stem cells in vivo. J Exp Med. 2015;212(8):1171–83.
32. Kishore S, Jaskiewicz L, Burger L, Hausser J, Khorshid M, Zavolan M. A quantitative analysis of CLIP methods for identifying binding sites of RNA-binding proteins. Nat Methods. 2011 May 15;8(7):559 – 64. doi: 10.1038/nmeth.1608. PMID: 21572407.

33. Javanmard AR, Dokanehiifard S, Bohlooli M, Soltani BM. LOC646329 long non-coding RNA sponges miR-29b-1 and regulates TGFβ signaling in colorectal cancer. Journal of cancer research and clinical oncology. 2020 May;146(5):1205–15.
34. Shi Y, Luo X, Li P, Tan J, Wang X, Xiang T, Ren G. miR-7-5p suppresses cell proliferation and induces apoptosis of breast cancer cells mainly by targeting REGγ. Cancer letters. 2015 Mar 1;358(1):27–36.

Tables

Table 1

Primer name	sequence
Forward real Linc-Pint	CATCTGAACTGTCACGGCAGA
Revers real Linc-Pint	GTGAACGGGCGTACAGAGG
Forward real B2M	CCACTGAAAAAGATGAGTATGCCT
Revers real B2M	CCAATCCAAATGCGGCATCTTCA
Forward real APC	CGCACCCGTGAGGACTACAGGC
Revers real APC	GATCATCTTGTGCTTGGAGTGCACC
Forward real AXIN	ATGCAGGAGAGCGTGCAGGTC
Revers real AXIN	TGACGATGGATCGCCGTCCTC
Forward real C-myc	CTCCTACGTTGCGGTCACAC
Revers real C-myc	CGGGTCGCAGATGAAACTCT
Forward real CCND2	AGAACACCCCATGCGTGCTGAG
Revers real CCND2	TGTGTGCCCCTGACCTGGCT
Forward real TGFBR2	TTTGGATGGTGGAAGGTCTC
Revers real TGFBR2	GCAACAGCTATTGGGATGGT
Forward real IGF1R	GGC ACA ATT ACT GCT CCA AAG AC
Revers real IGF1R	CAA GGC CCT TTC TCC CCA C

Figures

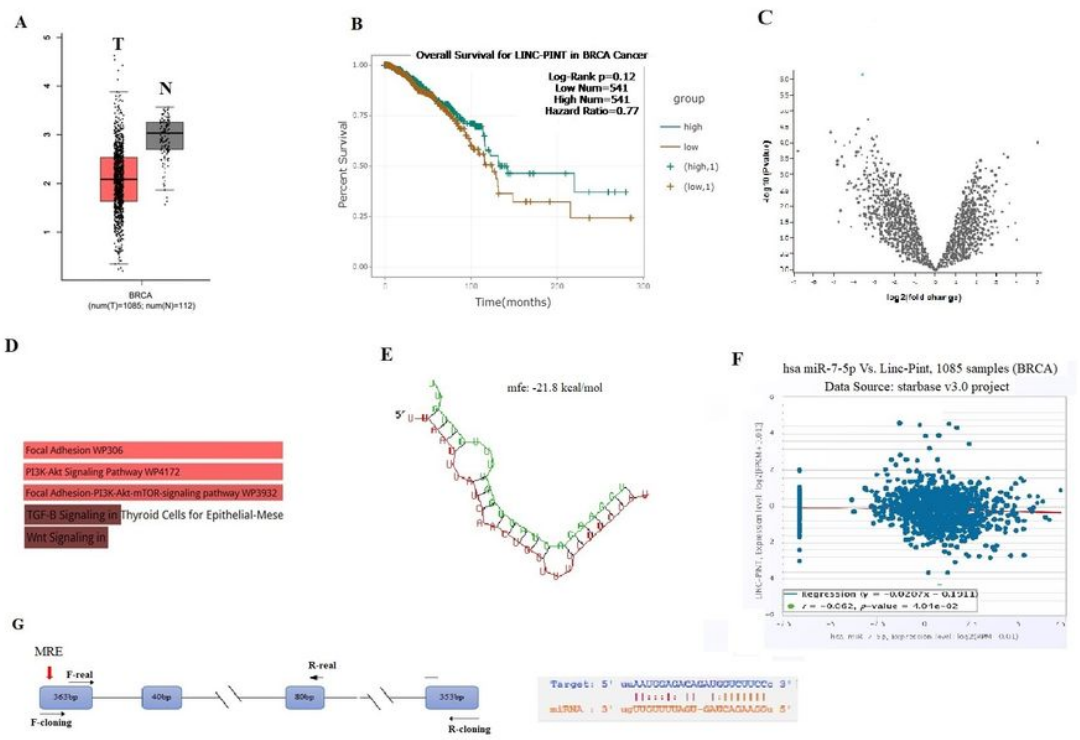

Figure 1

Bioinformatics evidences for Linc-PINT differential expression in breast Normal/ Tumor tissues and its relation to miR-7. (A) RNAseq data analysis indicated reduced Linc-Pint expression in breast cancer tumors compared with normal tissues. (B) Kaplan–Meier survival analysis of breast cancer patients from TCGA dataset with different Linc-Pint expression levels. (C) The volcano plot shows that the distribution of the stimulated genes is equal to the down regulated ones, indicating a correct analysis of the RNA-seq data. (D) The KEGG pathway analysis of Linc-PINT correlated genes in TCGA data. “Focal Adhesion” and “WNT signaling pathway” are prominent pathways for negatively correlated genes (Adjusted P-value = 2.48E-0.08). (E) RNAhybrid software predicted a relatively strong direct interaction between Linc-Pint and miR-7. (F) Shows a negative correlation of expression between Linc-Pint and miR-7 in breast cancer tissue adopted from TCGA database. (G) Clip-seq data analysis supported a direct interaction between Linc-Pint and miR-7 in breast cancer specimens.

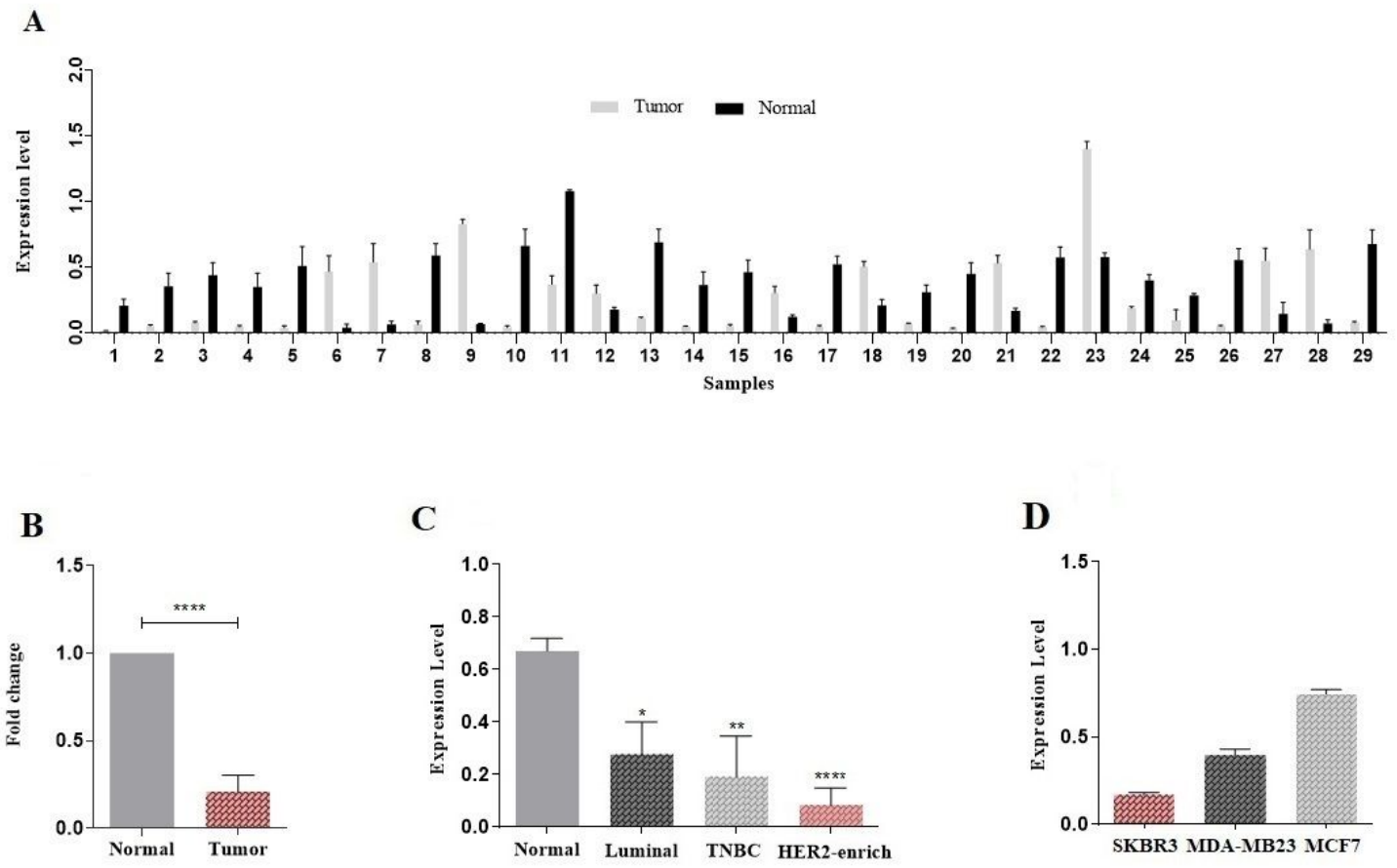

Figure 2

RT-qPCR results of Linc-Pint expression in breast cancer tissues and cell lines. (A) Shows the relative expression of Linc-Pint in 29 pairs of breast cancer tumor tissues and their normal pairs. (B) Average of Linc-Pint expression change in breast cancerous tissues compared to normal adjacent pairs shows a significant decrease. (C) Represents Linc-Pint expression status in 29 pairs of T/N breast specimens classified by subtype.(luminal=11, TNBC=10, HER2-enriched=8). (D) Shows the expression of Linc-Pint in different breast cancer cell lines.

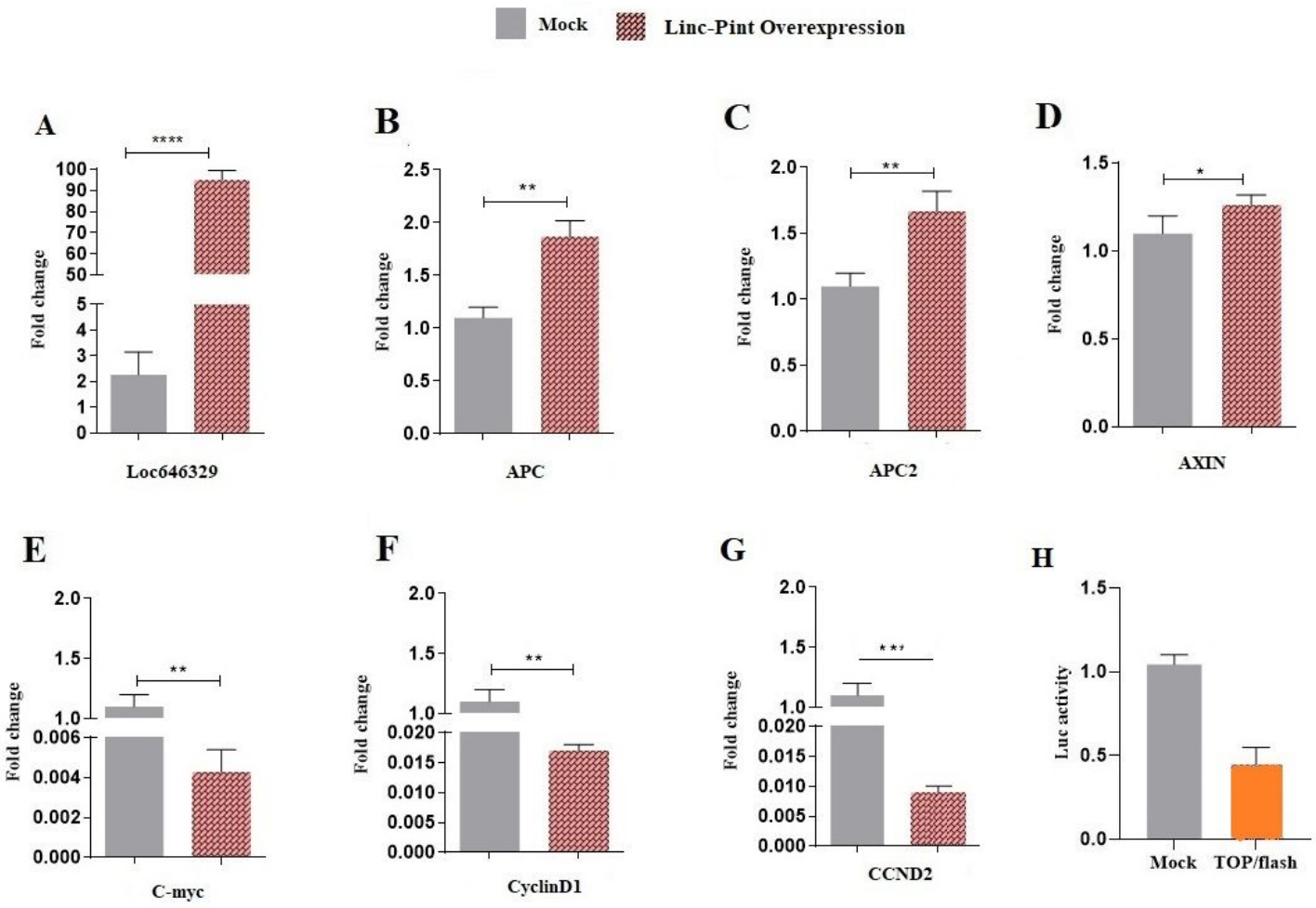

Figure 3

Experimental evidences for the effect of Linc-Pint on Wnt signaling in BC A) RT-qPCR results show that Linc-Pint was successfully overexpressed in transfected cell lines in comparison with cells transfected with mock vector. B) and C) and D) Up-regulation of APC, APC2, AXIN genes in response to Linc-Pint overexpression. E) and F) and G) shows downregulation of C-myc, CCND1 , and CCND2 following the Linc-Pint overexpression, detected by RT-qPCR. H) Represents the results of Top/flash assay at the condition of Linc-Pint overexpression in MCF7 cell. Pointing to the attenuation of Wnt signaling following the Linc-Pint overexpression.

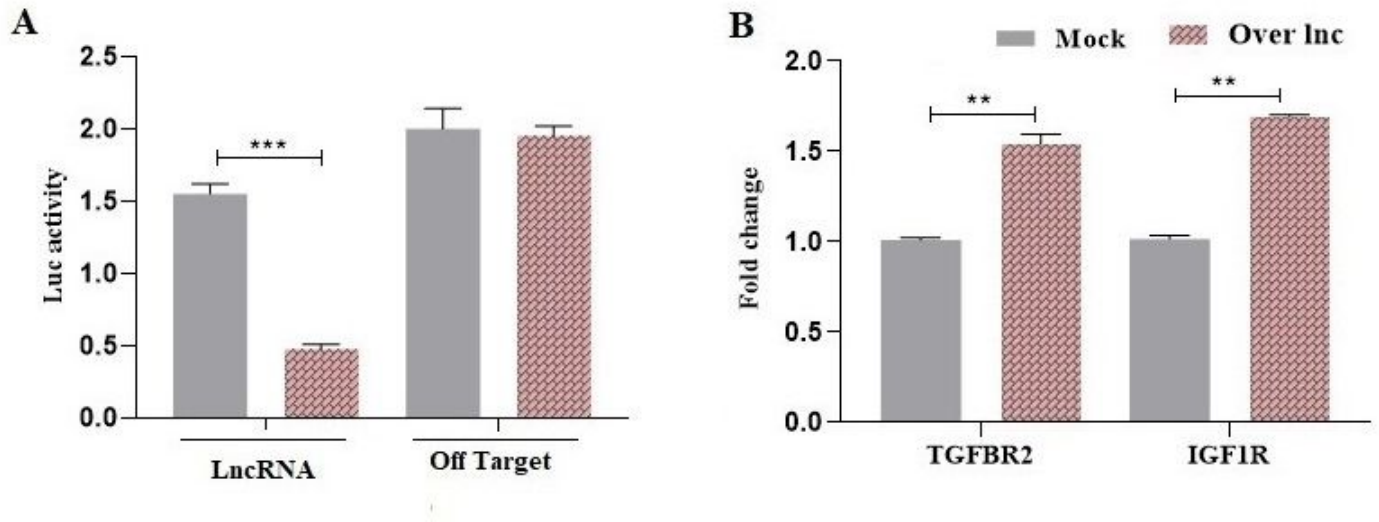

Figure 4

Experimental evidences showing interaction between Linc-Pint and miR-7 in MCF7 cells. A) Dual luciferase assay supported a direct interaction between miR-7 and Linc-Pint transcripts. B) TGBR2 and IGFIR target genes of miR-7 had a significant increase in expression following the overexpression of Linc-Pint.

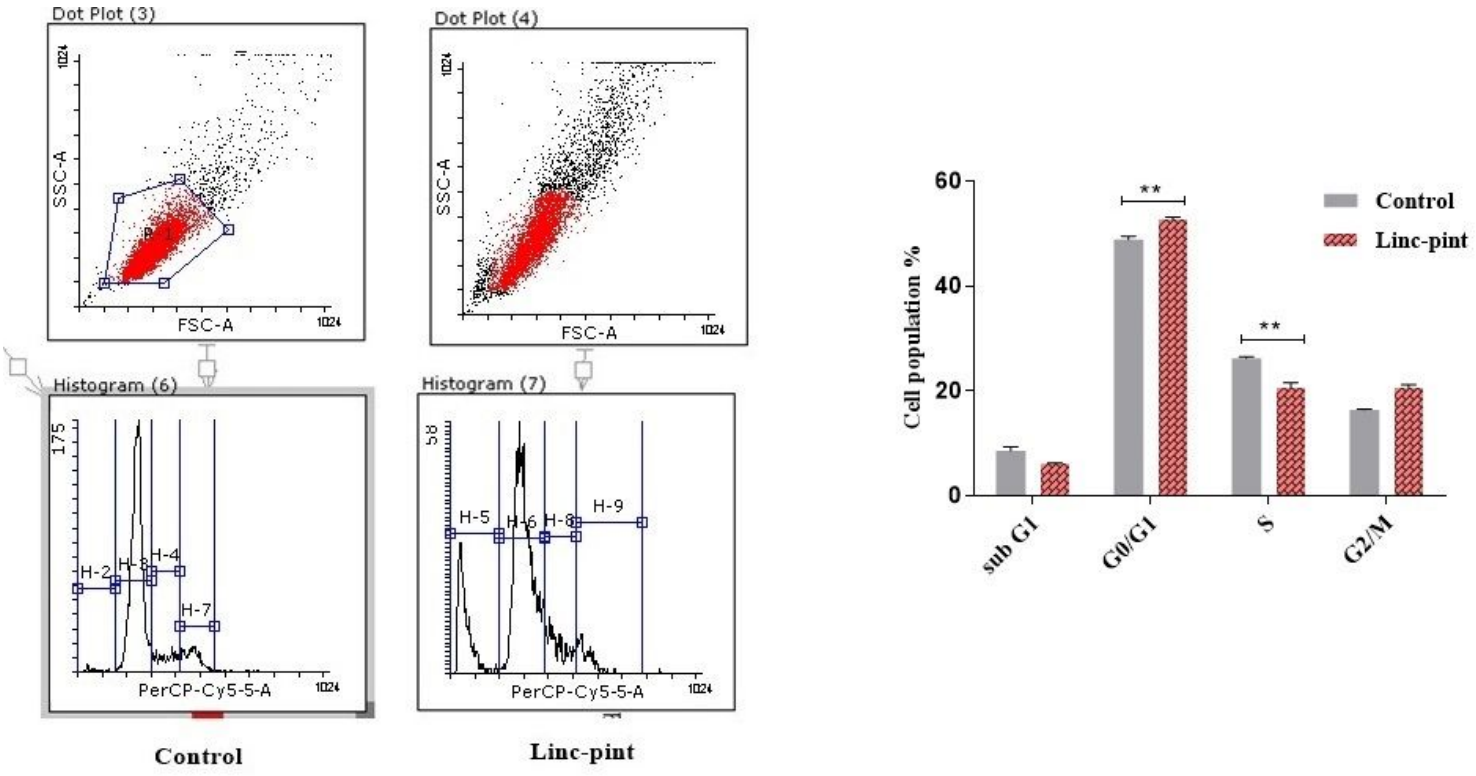

Figure 5

Linc-Pint overexpression effect on cell cycle status of MCF7 cells Represents flow cytometry results of Linc-Pint overexpression in MCF7 cells, in which significant G1 arrest and reduction in S phase cell

proportion is evident.

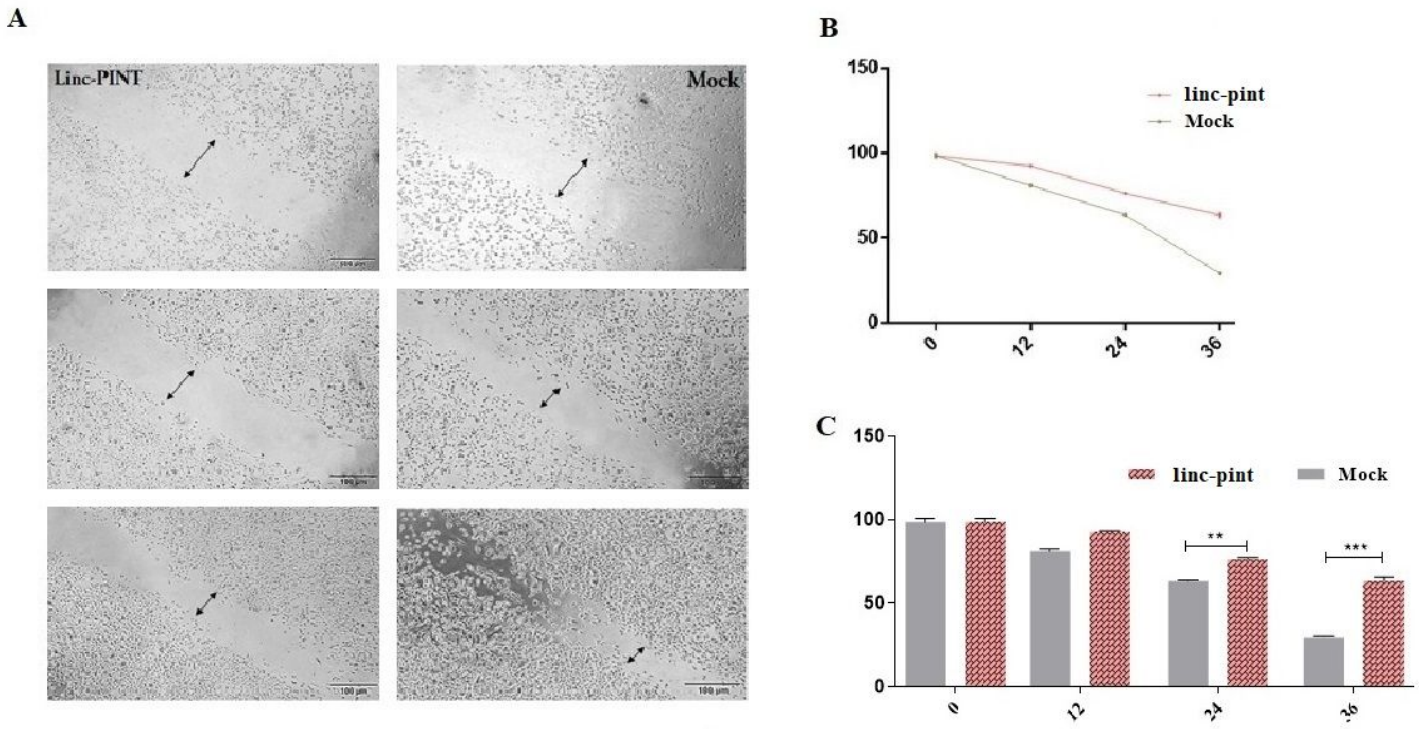

Figure 6

Linc-Pint overexpression effect on cell migration of transfected MCF7 cells Wound healing assay indicates the significant suppressive effect of Linc-Pint on cell migration and invasion in MCF7, after 24 and 36 hrs of transfection.

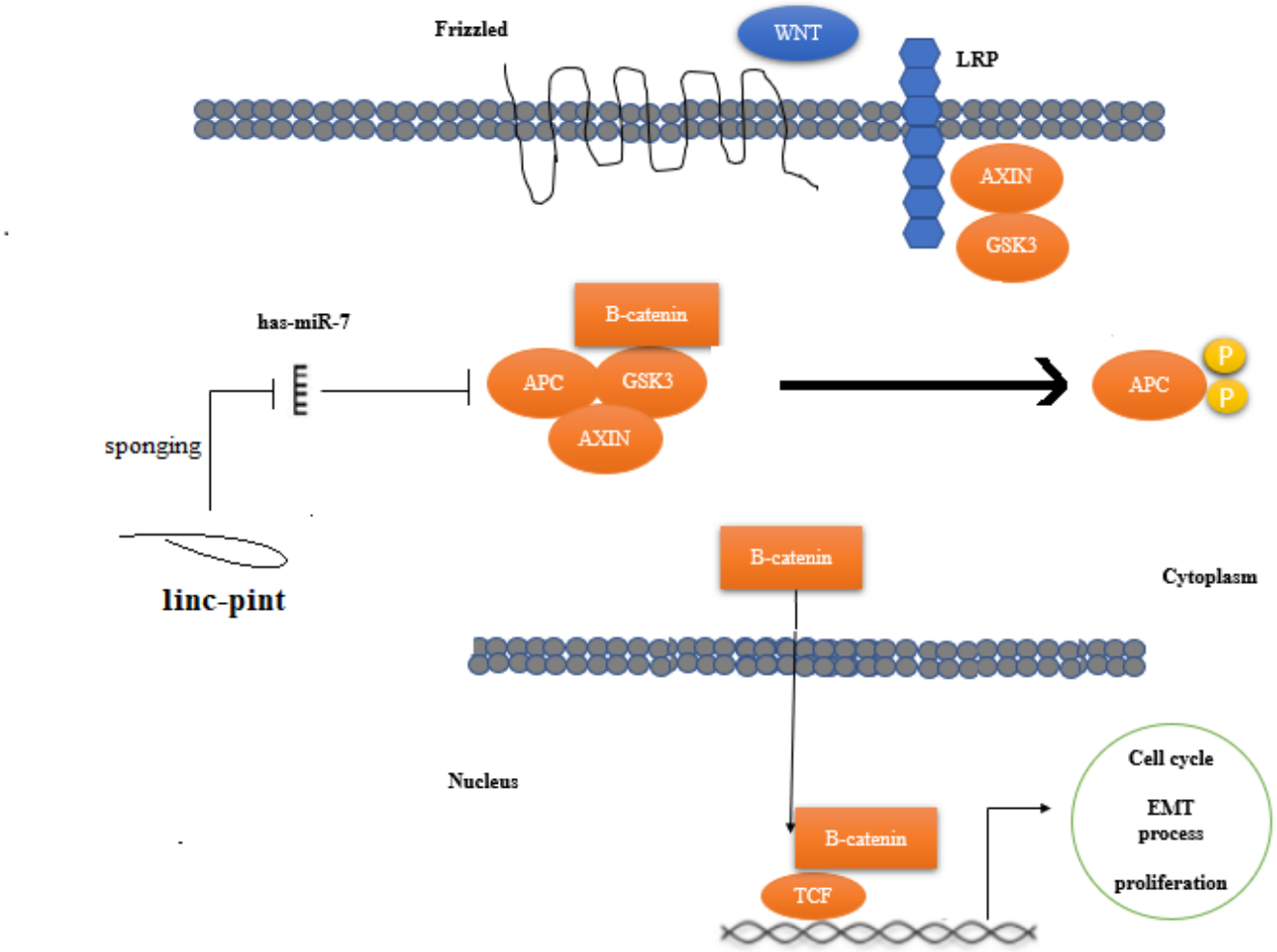

Figure 7

Schematic representation of a deduced regulatory network in which Linc-Pint regulates WNT signaling pathway through sponging miR-7.