IKKβ Enforces a LIN28B/TCF7L2 Positive Feedback Loop That Promotes Cancer Cell Stemness and Metastasis

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Abstract

Considerable evidence suggests that proinflammatory pathways drive self-renewal of cancer stem-like cells (CSC), but the underlying mechanisms remain mainly undefined. Here we report that the let7 repressor LIN28B and its regulator IKBKB (IKKβ) sustain cancer cell stemness by interacting with the Wnt/TCF7L2 (TCF4) signaling pathway to promote cancer progression. We found that LIN28B expression correlated with clinical progression and stemness marker expression in breast cancer patients. Functional studies demonstrated that the stemness properties of LIN28B-expressing human breast and lung cancer cells were enhanced by IKKβ, whereas loss of LIN28B abolished stemness properties in these settings. These phenomena were driven through interactions with TCF7L2, which enhanced LIN28B expression by direct binding to intron 1 of the LIN28B gene, which in turn promoted TCF7L2 mRNA translation through a positive feedback loop. Notably, RNAi-mediated silencing of LIN28B or pharmacologic inhibition of IKKβ was sufficient to suppress primary and metastatic tumor growth in vivo. Together, our results establish the LIN28B/TCF7L2 interaction loop as a central mediator of cancer stemness driven by proinflammatory processes during progression and metastasis, possibly offering a new therapeutic target for generalized interventions in advanced cancers. Cancer Res; 75(8); 1725–35. ©2015 AACR.

Introduction

Tumor-promoting inflammation has been recognized as one of the hallmarks of cancer (1). NF-κB activation is one of the pillars of inflammation that provides a mechanistic link between inflammation and cancer (2, 3). In fact, it is known that the IKKβ-dependent NF-κB activation pathway provides a critical molecular link between inflammation and colon cancer growth in mouse models (4, 5) and that the specific inhibitor for IKKβ shows promising antineoplastic effects (6–8). Cancer stem cells (CSC) are highly tumorigenic, relatively resistant to conventional chemotherapy and radiotherapy, and involved in both tumor initiation and metastasis (9, 10). It has been proposed that the development of more effective cancer therapies may require specific targeting of the CSCs population. Several studies have also revealed that the NF-κB pathway plays critical roles in sustaining the stemness of CSCs (11, 12) and that targeting this pathway impairs self-renewal of CSCs (8); however, the molecular mechanisms by which NF-κB regulates CSCs are not fully understood. Wnt/β-catenin signaling controls virtually every aspect of embryonic development and mediates homeostatic self-renewal in adult tissues (13, 14). Recent studies have demonstrated the essential role of transcriptional factor TCF7L2 in adult intestinal homeostatic self-renewal and oncogenic program (15–17). It is worth noticing that a recent study has revealed that interaction between NF-κB and Wnt signaling promotes tumor-initiating cell traits during intestinal tumorigenesis (18).

Mammalian homologs of LIN28, such as LIN28A and LIN28B, bind to terminal loops of the precursors of let-7 family miRNAs and block their processing into mature miRNAs (19, 20). Thus, repression of let-7 is important in establishing the pluripotent state of embryonic stem cells. Several reports also indicate that LIN28 can affect protein levels by regulating mRNA stability and modulate cell proliferation by enhancing translation of various cell-cycle regulators in murine embryonic stem cells (21, 22). Therefore, LIN28 may promote reprogramming through both miRNA-dependent and independent pathways. In fact, it has been reported that LIN28A/LIN28B can promote transformation by repressing let-7 miRNAs, and that activation of LIN28A/LIN28B occurs in many different human tumors with a frequency of approximately 15% (23, 24). Recent studies have also suggested that LIN28 plays an important role in the maintenance of CSCs (25, 26) and Wnt signaling could directly activate LIN28 to augment breast cancer stem cell expansion (27).

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This study aims to obtain evidences that LIN28B and TCF7L2 are the key molecular players that mediate the function of NF-kB signaling in CSCs and to identify a novel molecular mechanism by which the LIN28B/TCF7L2 interaction loop, when activated by IKKβ, sustains the stemness of CSCs to promote cancer progression and metastasis.

Materials and Methods

Cell lines and reagents

The human breast cancer cell lines MDA-MB-231 and MCF-7, NSCLC cell line H1299, human monocytic cell line THP1, and embryonic kidney cell line HEK293T cells were obtained from ATCC (October 2011) and cultured according to guidelines. All the cell lines were recently authenticated by cellular morphology and the short tandem repeat analysis at Microread Inc. (May 2014) according to the guidelines from ATCC (28). IMD-0354 was purchased from Sigma Aldrich and Taxol was obtained from Tocris.

Immunohistochemistry

The tissue microarray used for analysis of the correlation of LIN28B expression with stemness markers and clinical stages of breast cancer was purchased from Alenabio Inc. The tissue microarray used for analysis of the correlation of LIN28B expression with survival of patients with breast or lung cancer was purchased from Shanghai Outdo Biotech. The pictures of immunohistochemistry were captured by microscope (Leica). The expression level of LIN28B was based on the proportion of positive cancer cells of 5 random fields in the tissues and the cutoff defined as low (0%–30%), moderate (30%–60%), or strong (60%–100%) expression of LIN28B. Tyramide Signal Amplification (PerkinElmer, Inc.) was used for tissue immunofluorescence. Cy3, Cy5, and FITC-conjugated second antibody (Sigma) was used to visualize LIN28B, ALDH1, and OCT4, respectively, in immunofluorescence assay. The pictures of tissue immunofluorescence were captured by confocal microscopy (Olympus).

Flow cytometry

The ALDEFLUOR assay (Stemcell Technologies) was performed according to the manufacturer’s guidelines to identify cells with high ALDEFLUOR activity. Cells were incubated for 40 minutes at 37°C and the ALDH1 level was measured by flow cytometry (Olympus). All the cells were washed and incubated with appropriate secondary antibodies (Santa Cruz Biotechnology) and detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce). The band intensities in Western blotting were quantified with the LAS4000 Image Analyzer.

Luciferase assay

The Cignal Finder Reporter Array (Qiagen) was used to identify the signaling pathways that were regulated by IMD-0354. To generate the luciferase reporter vectors, the LIN28B gene fragments were amplified from human genomic DNA and cloned into the firefly luciferase plasmid pGL3-basic-IRE. For reporter assays, above distinct constructs with pRL-TK plasmid (reference) were cotransfected into MDA-MB-231 or H1299 cells. To analyze the activity of Wnt/β-catenin signaling, Top-flash or Fop-flash (Millipore) with pRL-TK plasmid (reference) were cotransfected into MDA-MB-321 or H1299 cells.

Animal study

Female NOD/SCID mice, 6 to 8 weeks of age, were purchased from Vital River Laboratories. Approximately 1 × 10^6 of MDA-MB-231 cells, mixed with Matrigel (1:1), were transplanted into 4th mammary gland fat pad of NOD/SCID mice. After 10 days, the NOD/SCID mice were treated with IMD-0354 or Taxol. Mice were randomly divided into experimental groups with 5 mice in each group. Primary and metastatic tumors in mice were detected by Bioluminescence (NIGHTOWL LB983, Berthold Technologies) or PET scan (MicroPET Focus 120, Siemens). Tissues of tumor and lung were fixed in formalin and embedded in paraffin for histologic analysis.

Statistical analysis

All results were presented as the SEM. The significance of correlation between LIN28B level and clinical stages in human breast cancer patients was analyzed by using the Spearman rank correlation test. The significance of correlation between LIN28B and ALDH1, SOX2, or TCF7L2 was determined by using the χ² test. Patient survival curves were plotted using Kaplan–Meier analysis, and the statistical parameters were calculated by log-rank (Mantel–Cox) test using GraphPad Prism. Other experiments were analyzed with the Student t test. P < 0.05 was considered as the criterion for
statistical significance (*, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \)).

**Results**

The expression of LIN28B is positively correlated with stemness markers and clinical progress of cancer patients

To test the correlation between LIN28B expression and clinical progress of breast cancer patients, immunohistochemical (IHC) staining was used to detect the expression of the stemness gene LIN28B in 102 patients with different clinical stages, including stage 1 \( (n = 15) \), stage 2 \( (n = 60) \), and stage 3 \( (n = 27) \). It was found that LIN28B expression was strongly correlated with clinical stages in breast cancer patients (Fig. 1A; Supplementary Table S4). It was also found by IHC staining that LIN28B expression was associated with ALDH1 and SOX2, two stemness markers (Fig. 1B and Supplementary Fig. S1A; Supplementary Tables S5 and S6). These results were extended by immunofluorescence staining, which indicated that LIN28B expression was correlated with OCT4 and ALDH1 expression (Fig. 1C). Furthermore, statistical analysis revealed that higher LIN28B level was associated with shorter overall survival of patients with either breast cancer \( (n = 159) \) or lung cancer \( (n = 150) \); Supplementary Fig. S1B). Together with

![Figure 1.](image)

LIN28B expression correlated with clinical stages and stemness markers in breast cancer tissues. A, IHC analyses for LIN28B expression were classified as absent/weak, moderate, or strong in either human breast cancer tissues with different clinic stages or normal human breast tissues. Percentages of LIN28B expression at different levels in each stage are depicted on the right. The representative staining results are shown on the left. Scale bars, 200 \( \mu m \); 20 \( \mu m \) (insets). B, IHC analyses were used for LIN28B and ALDH1 expression in 102 human breast cancer specimens. Analysis is shown for percentages of specimens with either low or high LIN28B expression relative to ALDH1 level. The representative cases are shown on the left. Scale bars, 200 \( \mu m \); 20 \( \mu m \) (insets). C, immunofluorescence staining for OCT4, ALDH1, and LIN28B expression in human breast cancer tissues. Scale bar, 10 \( \mu m \). D, Kaplan–Meier analysis of LIN28B expression in survival of patients with breast cancer \( (n = 159) \). *, \( P < 0.05 \).
clinical data, these results suggest that the expression of LIN28B in tumor tissue correlates positively with their metastasis and stemness markers, suggesting that LIN28B might be an important player in driving cancer progress.

LIN28B is a key factor in maintaining stemness phenotypes of CSCs and promotes tumor cell metastasis

As reported previously, LIN28B was involved in inflammation leading to cell transformation (23). Here, we initially found highly metastatic human breast cancer cell line MDA-MB-231 cells and human non–small cell lung cancer cell line H1299 cells with high levels of LIN28B expression. On the basis of these findings, we established siRNA-LIN28B (siLIN28B) and two LIN28B knockdown to be stable pools in MDA-MB-231 cells and H1299 cells (shLIN28B-1, shLIN28B-2; Supplementary Fig. S5A). As expected, low expression of LIN28B reduced the expression of stemness-related genes, including SOX2, OCT4, and NANOG, in MDA-MB-231 and H1299 cells (Fig. 2A). Moreover, we also found that low expression of LIN28B reduced the proportion of ALDH+ cells (Fig. 2B and Supplementary Fig. S5B) and restrained the tumorsphere forming ability of MDA-MB-231 cells and H1299 cells (Fig. 2C and D and Supplementary Fig. S5C and S5D; Supplementary Tables S9 and S10). Inversely, the CSC population CD44+CD24−/CD0 cells in less malignant MCF-7 human breast cancer cells was increased and its tumorsphere forming ability was enhanced after overexpression of LIN28B (Supplementary Fig. S4B, C). Moreover, the invasive ability of MDA-MB-231 cells and H1299 cells were both significantly decreased after LIN28B knockdown (Fig. 2E and Supplementary Fig. S5E). Furthermore, to address these effects in vivo, it is important to note that tumor grew much slower in the shLIN28B-H1299 group compared with the control group (Supplementary Fig. S5G), and the survival time of tumor-bearing mice was significantly extended in shLIN28B groups (Supplementary Fig. S5H). A limiting dilution assay demonstrated that 1 of 5 of mice of the shLIN28B group formed tumors compared with 5 of 5 of the control group when H1299 cells were injected below one million (Supplementary Table S12). Moreover, pulmonary metastasis in groups of mice with reduced LIN28B was clearly suppressed as indicated by PET scan and hematoxylin and eosin (H&E) staining analysis (Fig. 2G and H and Supplementary Fig. S5F). Together with these data, it was apparent that LIN28B played an important role in maintaining stemness phenotypes of cancer cells.

IKKβ activates LIN28B and sustains the cancer stemness in a human breast cancer model

During breast cancer progression, the NF-κB signaling pathway was found to be markedly activated by immune inflammatory cells and other factors in the tumor microenvironment (TME) such as tumor-associated macrophages (TAM; refs. 31–33). Here, we performed experiments to further explore the correlation

Figure 2. LIN28B maintains the stemness phenotype of CSCs and promotes tumor growth and metastasis in vivo. A, Western blot analysis for LIN28B, SOX2, OCT4, and NANOG in MDA-MB-231 cells and H1299 cells. shNC, shLIN28B, siLIN28B-1, and siLIN28B-2 are transfected with shLIN28B. B, percentage of ALDH+ cells of shLIN28B-MDA-MB-231 cells was detected by flow cytometry. C, Primary mammosphere frequencies of shLIN28B-MDA-MB-231 cells were calculated and representative single sphere images were visualized by microscope (bottom). D, secondary mammosphere frequency of shLIN28B-MDA-MB-231 cells was calculated and representative sphere images were visualized by microscope (bottom). Scale bar, 200 μm. E, Matrigel invasion assay for shLIN28B-MDA-MB-231 cells were detected by microscope and OD value. Scale bar, 100 μm. F, the curve of tumor growth in NOD/SCID mice after injection of shLIN28B-MDA-MB-231 cells (n = 5). G, the primary and metastasis tumor was monitored by PET scan. H, The pulmonary metastases in mice and lung metastatic foci were evaluated by H&E staining analyses (n = 5). Scale bars, 200 μm; 50 μm (insets). Error bars, mean ± SD/SEM from three independent experiments. **P < 0.01, ***P < 0.001.
between inflammatory signals induced by TME and the degree of stemness in CSCs. ALDH+ cells, isolated from MDA-MB-231 and H1299 cancer cells, were used as CSC model. We initially found ALDH+ cells to be strongly tumorigenic, as indicated by tumors growing more rapidly in NOD/SCID mice injected with ALDH+ cells (Supplementary Fig. S2D; Supplementary Table S8). In addition, mammosphere formation and expression of stemness-related genes, such as LIN28B, SOX2, OCT4, and NANOG, increased in ALDH+ cells (Supplementary Fig. S2A and S2B). We also used human macrophages THP1, which were treated with PMA and TH2 cytokines to induce M2 phenotype macrophages as TAMs (34) that were cocultured with MDA-MB-231 and MCF-7 cells (Supplementary Fig. S3A). It was found that this maneuver clearly increased the percentage of ALDH+ cells in MDA-MB-231 cells and the percentage of CD44+/CD24- cells in MCF-7 cells (Supplementary Fig. S3B). Similarly, tumoursphere formation of MDA-MB-231 cells was also increased in MDA-MB-231 cells that were cocultured with treated THP1 cells (Supplementary Fig. S3C). Results of Western blots indicated upregulated expressions of p-IKKβ, LIN28B in either MDA-MB-231 or MCF-7 cells that were cocultured with THP1-treated cells (Supplementary Fig. S3D). Moreover, cell surface marker CD44 was upregulated in MCF-7 cells when cocultured with THP1 cells by flow cytometry (Supplementary Fig. S3E). These results suggest that ALDH+ cells had CSCs’ properties, and that the inflammatory TME could activate IKKβ to enhance tumor stemness properties in cancer cells.

NF-κB was shown to be important in TICs isolated from HER2+ breast cancer (35). On the basis of this fact, we investigated how NF-κB activation was involved in the maintenance of stemness in breast cancer cells. Initially, we found the expressions of p-IKKβ and LIN28B were much higher in ALDH+ of MDA-MB-231 and H1299 cells (Fig. 3A). We then defined the factor in the NF-κB signaling pathway that played a leading role in this process. To this end, the main molecules in the NF-κB signaling pathway were knocked down by siRNA, respectively, including siRELα, siRELβ, or siIKKβ. Only silence of IKKβ could reduce the expression of the stemness gene LIN28B and SOX2 in MDA-MB-231 and H1299 cells (Fig. 3B and Supplementary Fig. S6B). Furthermore, the above results were confirmed by an IKKβ-selective inhibitor (IMD-0354) to treat MDA-MB-231 or H1299 cells. As expected, it downregulated the expression of LIN28B and other stemness genes (SOX2, OCT4, NANOG) in a dose-dependent manner (Fig. 3F and Supplementary Fig. S6C). Moreover, treatment of MDA-MB-231 cells with 5 μmol/L IKKβ inhibitor (IMD-0354) resulted in a decreased mRNA expression of LIN28B, SOX2 (Fig. 3G), and other important stemness properties, including the percentage of ALDH+ cells (Fig. 3H), the frequency of tumoursphere (Fig. 3D; Supplementary Table S11), and invasion ability (Fig. 3E) among MDA-MB-231 cells. Similar results were obtained in H1299 cells treated with IMD-0354 (Supplementary Fig. S6C–S6F).

Furthermore, an in vivo experiment was performed to verify our in vitro results (Fig. 3H). The tumor volume was decreased in the IMD-0354 group at the end of 7 weeks (Supplementary Fig. S8A). However, there was no difference in body weight of mice after IMD-0354 treatment (Supplementary Fig. S8B). Pulmonary metastases in groups of mice treated with IMD-0354 were fewer than those in control groups (Fig. 3I). In fact, H&E staining of lung specimens indicated clearly that the number of metastatic foci in lung were much fewer in groups of mice subjected to IMD-0354 treatment (Fig. 3I). These results proved that IKKβ in the NF-κB signaling pathway, which was activated by the TME such as TAMs, played a critical role in up regulating LIN28B and in sustaining cancer stemness properties in a MDA-MB-231 breast cancer model.

**TCF7L2 is required for activation of LIN28B, which targets TCF7L2 directly via a positive feedback loop**

As described above, activation of LIN28B is required for maintenance of stemness and enhancement of cancer cell metastasis in MDA-MB-231 breast cancer cells and H1299 lung cancer cells. To unveil the molecular mechanism behind the process, we performed an experiment to test which signaling pathways were involved in regulation of IKKβ or LIN28B in MDA-MB-231 cells. A pathway reporter array including 10 selected cancer-related signaling pathways was used to detect whether it could be regulated by IMD-0354 on MDA-MB-231 cells. The result showed clearly that Wnt/β-catenin signaling pathway was significantly inhibited by IMD-0354 (Fig. 4A), suggesting a correlation between Wnt/β-catenin and NF-κB signaling. Western blot analyses further indicated that the expression of TCF7L2 (TCF4), a key transcription factor in the Wnt/β-catenin signaling pathway, was downregulated by either IKKβ inhibitor IMD-0354 or siIKKβ, but not by siRELα or siRELβ, in MDA-MB-231 and H1299 cells (Fig. 4B and Supplementary Fig. S6B and S6C). Furthermore, we investigated whether LIN28B could be regulated by TCF7L2. As expected, LIN28B expression was reduced by siTCF7L2 (Fig. 4D and Supplementary Fig. S7A) and LIN28B expression was correlated with TCF7L2 in breast cancer tissues (Fig. 5F; Supplementary Table S7). A further in depth investigation indicated the mechanism underlying the important interaction between TCF7L2 and LIN28B. In this regard, PROMO software analysis identified two putative binding sites of TCF7L2E (one isoform of TCF7L2) in intron 1 of the LIN28B gene, which were respectively named site 1 (AGCAAAAG) and site 2 (CTGTGAC; Fig. 4D). Chromatin immunoprecipitation (ChIP) found that only site 2 could be tested in the TCF7L2/DNA complex (Fig. 4E and Supplementary Fig. S7B).

We then constructed 4 plasmids containing both site 1/site 2, site 1 or site 2 separately. A Luciferase reporter assay revealed that only site 2 had transcriptional activity that was in accord with the result of our ChIP assay (Fig. 4F and Supplementary Fig. S7C). Moreover, the transcriptional activity of the plasmid containing both site 1 and site 2 was increased significantly when β-catenin was overexpressed (Fig. 4G and Supplementary Fig. S7D). These results suggest that Lin28B was a novel target gene of Wnt/β-catenin signaling pathway and TCF7L2 could promote LIN28B expression by directly binding to intron 1 of the LIN28B gene.

Unexpectedly, we observed an interesting fact that expression of TCF7L2, but not β-catenin, could be inhibited as well by shLin28B (Fig. 5A). It indicated that downregulation of LIN28B could reduce luciferase activity of TOP-flask (Fig. 5C and Supplementary Fig. S7E), as well as the expression of such TCF7L2 downstream genes as BCL2, CCND1, and MYC in MDA-MB-231 cells (Fig. 5D). Furthermore, RNA immunoprecipitation (RIP) experiments showed that LIN28B could directly bind to the mRNA of TCF7L2 in both of MDA-MB-231 and H1299 cells (Fig. 5E).
immuno-fluorescence (Fig. 5B). We therefore proposed that LIN28B protein binding with TCF7L2 mRNA occurred in the cytoplasm, a finding consistent with our prior data indicating that LIN28B protein appeared in both cytoplasm and nucleus in breast cancer tissues. Moreover, the expression of LIN28B and TCF7L2 was highly correlated in breast cancer tissues (Fig. 5F). It is evident that TCF7L2, sustained by IKKb, promoted LIN28B transcription by binding site 2 (CTGTACT) in intron 1 of the LIN28B gene and LIN28B targets with mRNA of TCF7L2 directly as a positive feedback loop. The important interaction of LIN28B and TCF7L2 represents a novel mechanism underlying the regulation of LIN28B to Wnt/β-catenin signaling pathway via the protein/RNA interaction.

IMD-0354 blocks the p-IKKb/LIN28B/TCF7L2 signaling pathway and suppresses stemness and lung metastasis of breast cancer cells.

Recent evidences suggested that breast CSCs were relatively resistant to chemotherapy and contributed to tumor recurrence after therapy (36). Previous reports indicated Taxol, one of

Figure 3.

IKKb up-regulates expression of LIN28B and sustains cancer stemness in cancer cells. A, Western blot analysis for LIN28B, p-IKKb, and IKKb in either ALDH+/ or ALDH- cells of MDA-MB-231 and H1299 cells. B, Western blot analysis for IKKb, RELA, REBL, and LIN28B in MDA-MB-231 cells that were transfected with siRELA, siREBL, or siIKKb, respectively. C, percentage of ALDH+ cells of MDA-MB-231 cells that were treated with IMD-0354 (1 μmol/L) were detected by flow cytometry. D, mammosphere frequency of MDA-MB-231 cells that were treated with IMD-0354 (1 μmol/L) were calculated and representative images were visualized by microscope (below). Scale bar, 200 μm. E, Matrigel invasion assay for MDA-MB-231 cells that were treated with IMD-0354 (2 μmol/L) were detected by microscope and OD value. Scale bar, 100 μm. F, Western blots for p-IKKb, IKKb, p-RELA, RELA, LIN28B, SOX2, OCT4, and NANOG in MDA-MB-231 cells treated with IMD-0354 at doses of 1, 5, 10, and 50 μmol/L. G, qPCR for LIN28B, SOX2, OCT4, and NANOG in MDA-MB-231 cells that were treated with IMD-0354 (5 μmol/L). H, schedule of IMD-0354 treatment in vivo (n = 6). I, bioluminescent imaging was used to detect the pulmonary metastasis at week 7 (n = 6). J, lung metastatic foci were evaluated by H&E staining (n = 6). Scale bars, 200 μm; 50 μm (insets). Error bars, mean ± SD from three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
first-line clinical chemotherapy drug used in breast cancer treatment, could upregulate CSC populations in vitro (37) or in xenografts models (38, 39). To further investigate the therapeutic potential of IMD-0354, this study investigated the roles of the two drugs in CSCs and tumor progression. Results showed that Taxol could efficiently improve the proportion of ALDH+ cells and the frequency of tumorsphere in MDA-MB-231 cells (Fig. 6A and Supplementary Fig. S9A). On the basis of the inhibiting effect of IMD-0354 on CSCs phenotype and function in vitro, we designed a combination therapy strategy. To test the effect of this therapeutic strategy in vivo, MDA-MB-231 or 4T1 cells were transplanted into the 4th mammary fat pads of NOD/SCID mice or BALB/C mice and tumor growth and lung metastasis were monitored. The mice were treated with DMSO (control group), IMD-0354 alone, Taxol alone, or both IMD-0354 and Taxol at the time when the tumor volume reached approximately 100 mm3 (Fig. 6B and Supplementary Fig. S10A). Results showed that tumor volumes of the original site of the tumor in the combined treatment group were significantly smaller compared with those in the control group (Fig. 6C and Supplementary Fig. S10A). Results showed that tumor volumes of the original site of the tumor in the combined treatment group were significantly smaller compared with those in the control group (Fig. 6C and Supplementary Fig. S10A). Moreover, the degree of pulmonary metastasis in the IMD-0354--only group and the combined treatment group significantly reduced (Fig. 6D and E and Supplementary Figs. S9B, S9CC, and S10C). In the Taxol-only group, tumor volumes in situ of the MDA-MB-231 model were obviously suppressed (Fig. 6C), but no significant difference was found in the 4T1 model (Supplementary Fig. S10B); however, tumor metastases in the lungs of these mice in both MDA-MB-231 and 4T1 models were not decreased in the Taxol-only group (Fig. 6D and E and Supplementary Fig. S10C). The proportion of ALDH+ cancer stem cells in the IMD-0354--only group and the combined treatment group were reduced in both MDA-MB-231 and 4T1 models; however, it increased in the Taxol-only group in both two cell models (Fig. 6F and Supplementary Fig. S10D). Furthermore, histologic analyses revealed that this combination therapy strategy could suppress the expression of p-IKKb, TCF7L2, LIN28B, ALDH1, SOX2, and NANOG in tumor tissues, which supported our hypotheses (Fig. 6G). Taken together, these results suggest that blockade of IKKb activation with IMD-0354 may be able to inhibit TCF7L2/LIN28B interaction, suppress cancer stemness properties, and reduce tumor metastasis.

Discussion

Our experiment showed that LIN28B was upregulated by inflammatory factor IKKb and together with TCF7L2 sustained the stemness of cancer cells. Marotta and colleagues have demonstrated previously that the inflammatory transcription factor STAT3 was highly activated in the CD44+CD24~ population (40). Recent reports analogously indicated that STAT3 phosphorylation

Figure 4. TCF7L2 is required for IKKb-mediated activation of LIN28B. A, signaling pathway reporter array was used for seeking the signaling pathway associated with IKKb on MDA-MB-231 cells treated with IMD-0354 (5 μmol/L). B, Western blot analysis for TCF7L2 and β-catenin in MDA-MB-231 cells that were treated with IMD-0354 or siRNAs. C, software PROMO was used to identify two putative binding sites of TCF7L2 in intron 1 of the LIN28B gene. D, Western blots for TCF7L2 and LIN28B in MDA-MB-231 cells that were treated with siTCF7L2. E, ChIP was performed to verify TCF7L2 binding with the LIN28B gene in MDA-MB-231 cells. F, luciferase reporter system was used to identify the TCF7L2 binding site of the LIN28B gene in MDA-MB-231 cells that were transfected with plasmids containing both site 1/site 2, site 1, site 2, or mutated site 2 separately. G, reporter plasmid containing both site 1/site 2 and a β-catenin or control plasmid were cotransfected into MDA-MB-231 cells, and luciferase activity was detected. Error bars, mean ± SD from three independent experiments. ***, P < 0.001.
was positively correlated with ALDH1 expression in cancer cell lines and human breast cancer samples (41). However, this study demonstrated a novel mechanism, indicating that activation of IKKβ, an inflammatory kinase in the NF-κB pathway, was strongly correlated with the expression of stemness genes, especially LIN28B in human breast cancer cell lines or tissues. Previous studies also showed that LIN28B promoted tumor cell transformation (23, 24) and had a functional role in the maintenance of CSCs (25, 26). Consistent with these findings, our experiment results showed that expression of LIN28B was highly positively correlated with tumor progress in human breast cancer patients, indicating that there were high levels of LIN28B in stage 3 patients with lymphatic metastasis of breast cancer. LIN28B was shown to be a powerful predictor of poor clinical outcome. Besides, LIN28B was also shown highly positively correlated with stemness markers (ALDH1, SOX2, and OCT4) in breast cancer tissues. As far as mechanisms are concerned, we determined that selected blockade of IKKβ phosphorylation reduced the CSC population and LIN28B expression by using either silKKβ or IKKβ inhibitor IMD-0354. In this regard, Iliopoulos and colleagues found that LIN28B was activated by RELA to promote cell transformation (23). In contrast, results from our study indicated that RELA was not the only player in regulating LIN28B expression; instead, IKKβ did play a key role in our human breast and lung cancer model.

Importantly, we noted that suppression of IKKβ can inhibit the activity of Wnt/β-catenin signaling and reduced the expression of TCF7L2, which was a Wnt/β-catenin effector. We identified for the first time that TCF7L2 directly bound to intron 1 of LIN28B and upregulated LIN28B expression. This finding suggests that LIN28B a novel direct downstream target gene of the Wnt/β-catenin pathway. Interestingly, a recent research revealed that LEF1, another effector of Wnt/β-catenin pathway, could activate LIN28A via direct binding to the LIN28A promoter (27). This report provided compelling evidence that both LIN28A and LIN28B were direct target genes of the Wnt/β-catenin pathway. Hence, our results suggest that IKKβ regulated LIN28B expression with TCF7L2 assistance. However, we unexpectedly discovered that LIN28B bound directly to mRNA of TCF7L2, thereby activating the Wnt/β-catenin signaling pathway. In this regard, it is well known that LIN28B plays analogic biologic function due to their parallel structural domains (42). In fact, they bind to the terminal loops of precursors of let-7 family miRNAs and block their processing into mature miRNAs. Furthermore, another study reported that let-7-dependent effects of LIN28B may supersede let-7–independent effects on intestinal tissue growth (43). Therefore, our study revealed a novel let-7–independent mechanism of LIN28B in regulating the function of CSC.

The development of strategies to effectively target the CSC population needs to be extended to improve outcome of cancer therapy. Here, we proposed such an approach, based on blocking the IKKβ kinase activation to eliminate CSCs. Meanwhile, we also designed a combination therapeutic...
strategy of IKKβ inhibitor IMD-0354 and the cytotoxic agent Taxol. We tested the effects of this treatment in NOD/SCID and BALB/c mice and found that IMD-0354 treatment inhibited tumor metastasis to lung. In fact, the CSC population was significantly reduced by either IMD-0354 treatment alone or in combined therapy with Taxol. However, the CSC population increased after Taxol treatment alone, which was analogous with other studies (36, 37). Moreover, we found that expression of TCF7L2, LIN28B, SOX2, and NANOG were all decreased in tumor tissues following the combination therapy. In fact, IMD-0354 did not result in any side effect in mice (Supplementary Fig. S8B). Our results suggested that blocking
inflammatory activation may represent a novel and effective strategy to target CSCs.

Based on two cancer models (breast cancer and lung cancer), this study proposes a novel mechanism of the inflammatory gene IKKβ regulating stemness and identifying the TCF7L2/LIN28B loop as the pivotal molecular bridge connecting inflammation and stemness through their interaction with CSCs. This mechanism suggests a novel therapeutic strategy with clinical potential, i.e., blocking activation of inflammation, that targets CSC and suppresses tumor metastasis (Fig. 7).

**References**


**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

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IKKβ Activates LIN28B/TCF7L2 Loop to Maintain Cancer Stemness


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