IKKβ enforces a LIN28B/TCF7L2 positive feedback loop that promotes cancer cell stemness and metastasis

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Abstract

Considerable evidence suggests that pro-inflammatory 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 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 pharmacological 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 pro-inflammatory processes during progression and metastasis, possibly offering a new therapeutic target for generalized interventions in advanced cancers.
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 which 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 (CSCs) 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-kB and Wnt signaling promotes tumor-initiating cells 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 ~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).

This study aims to obtain evidences that LIN28B and TCF7L2 are the key molecular players that mediate the function of NF-κB 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. (Beijing, China; May 2014) according to the guideline 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 were purchased from Alenabio Inc. (Xi’an, China). 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 (Shanghai, China). The pictures of immunohistochemistry were captured by microscope (Leica, Germany). The expression level of LIN28B was based on the proportion of positive cancer cells of 5 random fields in the tissues and the cut off defined as low (0-30%), mediate (30-60%) or strong (60-100%) expression of LIN28B. Tyramide Signal Amplification (PerkinElmer, Inc. Waltham, MA) 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 immunofluorescence were captured by confocal (Olympus, Japan).

**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 in the presence or absence of the ALDH inhibitor.
diethylaminobenzaldehyde (DEAB). CD44 and CD24 primary antibodies were incubated with single cells in PBS/1% FBS for 30 minutes at 4°C. Cells were stained with 7-AAD to exclude non-viable cells.

**Tumorsphere assay**

Single cell suspensions were seeded in 6-well or 24-well ultra-low attachment plates (Corning) in sphere-culturing medium (Stemcell Technologies) for 7-14 days. For limited dilution assays, MDA-MB-231 cells were plated (after sorting) at 1, 10, 100, 1000 cells/well or H1299 cells were plated (after sorting) at 10, 100, 1000, 10000 cells/well in 24 wells (6 wells/condition). The number of wells with spheres was recorded on day 14 after culturing. Tumorsphere formation was monitored using an inverted Leica microscope fitted with a camera. Data analysis performed using the publically available ELDA software as detailed in reference (29). Secondary tumorspheres were obtained following the mechanical and enzymatic dissociation of primary tumorspheres as described previously (30).

**RNAi experiment**

RELA, RELB, IKKβ, LIN28B or TCF7L2 siRNA was transiently transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. LIN28B shRNA lentivirus vectors were obtained from GenePharma (Shanghai, China) and transfected into HEK293T cells (ATCC) to generate retroviral particles. MDA-MB-231 or H1299 cells were transduced with lentivirus-containing media and
selected with puromycin (2 μg/ml). After a week cell pools were obtained and expanded.

**Western blotting**

Cells were lysed in protein extraction reagent (Pierce) and loaded onto NuPAGE gels (Invitrogen). Blots were incubated with the respective primary antibody diluted in TBST (containing 0.1% Tween 20 and 5% BSA) overnight at 4 °C. Then, blots were washed and incubated with appropriate secondary antibodies (Santa cruz) 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 which 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-IRES. For reporter assays, above distinct constructs with pRL-TK plasmid (reference) were co-transfected 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 co-transfected into MDA-MB-321 or H1299 cells.

**Animal study**

Female NOD/SCID mice, 6- 8 weeks of age, were purchased from Vital River
Laboratories (Beijing, China). Approximately $1 \times 10^6$ 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, Germany) or PET scan (MicroPET Focus 120, Siemens, Germany). Tissues of tumor and lung were fixed in formalin and embedded in paraffin for histological analysis.

**Statistical analysis**

All results were presented as the standard error of the mean (SEM). The significance of correlation between LIN28B level and clinical stages in human breast cancer patients was analyzed by using the Spearman’s rank correlation test. The significance of correlation between LIN28B and ALDH1, SOX2 or TCF7L2 was determined by using the $\chi^2$ 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’s 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 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 4). It was also found by IHC staining that LIN28B expression was associated with ALDH1 and SOX2, two stemness markers. (Fig. 1B, Supplementary Fig. 1A, Supplementary Table 5, 6). 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; Fig. 1D) or lung cancer (n=150; Supplementary Fig. 1B). Together with 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 cancer stem cells 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. Based on 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. 5A). 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; Supplementary Fig. 5B) and restrained the tumorsphere forming ability of MDA-MB-231 cells and H1299 cells (Fig. 2C, D; Supplementary Fig. 5C, D; Supplementary Table 9, 10). Inversely, the cancer stem cell population CD44$^-$CD24$^+$ 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. 4B, C). Moreover, the invasive ability of MDA-MB-231 cells and H1299 cells were both significantly decreased after LIN28B knockdown (Fig. 2E; Supplementary Fig. 5E). Furthermore, to address these effects in vivo, it is important to note that tumor grew distinctly slower in NOD/SCID mice injected with shLIN28B-MDA-MB-231 cells than with wild type MDA-MB-231 cells in control mice (Fig. 2F). Similarly, tumor grew much slower in shLIN28B-H1299 group compared to the control group (Supplementary Fig. 5G) and the survival time of tumor-bearing mice was significantly extended in shLIN28B groups (Supplementary Fig. 5H). Limiting dilution assay demonstrated that 1/5 of mice formed tumor compared with 5/5 of the control group when H1299 cells were injected below one million (Supplementary Table 12). Moreover, pulmonary metastasis in groups of mice with reduced LIN28B was clearly suppressed as indicated by PET scan and H&E staining analysis (Fig. 2G, H, Supplementary Fig. 5F). 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 (TAMs) (31-33). Here we performed experiments to further explore the correlation 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 cancer stem cell 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. 2D, Supplementary Table 8). In addition, mammosphere formation and expression of stemness related genes, such as LIN28B, SOX2, OCT4 and NANOG, increased in ALDH\(^+\) cells (Supplementary Fig. 2A, B). We also used human macrophages THP1, which were treated with PMA and TH2 cytokines to induce M2 phenotype macrophages as TAMs (34) that were co-cultured with MDA-MB-231 and MCF-7 cells (Supplementary Fig. 3A). 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. 3B). Similarly, tumorsphere formation of MDA-MB-231 cells was also increased in MDA-MB-231 cells that were co-cultured with treated THP1 cells.
Results of Western blots indicated up-regulated expressions of p-IKKβ, LIN28B in either MDA-MB-231 or MCF-7 cells that were co-cultured with THP1 treated cells (Supplementary Fig. 3D). Moreover, cell surface marker CD44 was up-regulated in MCF-7 cells when co-cultured with THP1 cells by Flow Cytometry (Supplementary Fig. 3E). These results suggest that ALDH+ cells had CSCs properties, and that the inflammatory tumor microenvironment 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). Based on 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 was knocked down by siRNA respectively, including siRELA, siRELB or siIKKβ. Only silence of IKKβ could reduce the expression of stemness gene LIN28B and SOX2 in MDA-MB-231 and H1299 cells (Fig. 3B; Supplementary Fig. 6B). Furthermore, the above results were confirmed by an IKKβ selective inhibitor (IMD-0354) to treat MDA-MB-231 or H1299 cells. As expected, it down regulated the expression of LIN28B and other stemness genes (SOX2, OCT4, NANOG) in a dose-dependent manner (Fig. 3F; Supplementary Fig. 6C). Moreover, treatment of MDA-MB-231 cells with 5 μM 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. 3C), the frequency of tumorsphere (Fig. 3D, Supplementary Table 11) and invasion ability (Fig. 3E) among MDA-MB-231 cells. Similar results were obtained in H1299 cells treated with IMD-0354 (Supplementary Fig. 6C-F).

Furthermore, an in vivo experiment was performed to verify our in vitro results (Fig. 3H). The tumor volume was decreased in IMD-0354 group at the end of 7 weeks (Supplementary Fig. 8A). However, there was no difference in body weight of mice after IMD-0354 treatment (Supplementary Fig. 8B). 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. 3J). 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 down-regulated by either IKKβ inhibitor IMD-0354 or siIKKβ, but not by siRELA or siRELB, in MDA-MB-231 and H1299 cells (Fig. 4B; Supplementary Fig. 6B, C). Furthermore, we investigated whether LIN28B could be regulated by TCF7L2. As expected, LIN28B expression was reduced by siTCF7L2 (Fig. 4D, Supplementary Fig. 7A) and LIN28B expression was correlated with TCF7L2 in breast cancer tissues (Fig. 5F, Supplementary Table 7). 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 LIN28B gene which were respectively named site 1 (AGCAAAAG) and site 2 (CTGTACT) (Fig. 4D). Chromatin immunoprecipitation (ChIP) found that only site 2 could be tested in the TCF7L2/DNA complex (Fig. 4E; Supplementary Fig. 7B). We then constructed 4 plasmids containing both site 1/site 2, site 1, site 2 or mutant site 2 separately. A Luciferase reporter assay revealed that only site 2 had transcriptional activity which was in accord with the result of our ChIP assay (Fig. 4F; Supplementary Fig. 7C). Moreover, the transcriptional activity of the plasmid containing both site 1 and site 2 was increased significantly when β-catenin was over expressed (Fig. 4G; Supplementary Fig. 7D). 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 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 down-regulation of LIN28B could reduce luciferase activity of TOP-flash (Fig. 5C; Supplementary Fig. 7E), 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). The down expression of LIN28B decreased the expression of TCF7L2 in MDA-MB-231 cells that was also confirmed by immunofluorescence (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 IKKβ, promoted LIN28B transcription by binding site 2 (CTGTACT) in intron 1 of 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-IKKβ/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 first line clinical chemotherapy drug used in breast cancer treatment, could up-regulate CSC populations in vitro (37) or in xenografts models (38, 39). To further investigate the therapeutic potential of IMD-0354, this study investigated their 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; Supplementary Fig. 9A). Based on 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 mm^3^ (Fig. 6B; Supplementary Fig. 10A). Results showed that tumor volumes of the original site of the tumor in combined treatment group were significantly smaller compared to those in the control group (Fig. 6C; Supplementary Fig. 10B). Moreover, the degree of pulmonary metastasis in the IMD-0354 only group and the combined treatment group significantly reduced (Fig. 6D, E, Supplementary Fig. 9B, C; Supplementary Fig. 10C). In the Taxol only group, tumor volumes in situ of MDA-MB-231 model were obviously suppressed (Fig. 6C), but no significant difference
was found in 4T1 model (Supplementary Fig. 10B); 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-E; Supplementary Fig. 10C). 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; Supplementary Fig. 10D). Furthermore, histological analyses revealed that this combination therapy strategy could suppress the expression of p-IKKβ, TCF7L2, LIN28B, ALDH1, SOX2 and NANOG in tumor tissues, which supported our hypotheses (Fig. 6G). Taken together, these results suggest that blockade of IKKβ 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 up-regulated by inflammatory factor IKKβ 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 CD44$^+$CD24$^-$ population (40). Recent reports analogously indicated that STAT3 phosphorylation 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 CSCs maintenance (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 mechanism are conserved, we determined that selected blockade of IKKβ phosphorylation reduced the CSC population and LIN28B expression by using either siIKKβ 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 up regulated 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 reports 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 analogous biological 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 CSCs population was significantly reduced by either IMD-0354 treatment alone or in combined therapy with Taxol. However, the CSCs 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. 8B). Our results suggested that blocking
inflammatory activation may represent a novel and effective strategy to target CSCs.

Based on two cancers model (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 which targets CSC and suppresses tumor metastasis (Fig. 7).

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Development of methodology: Chong Chen, Fengqi Cao and Lipeng Bai

Acquisition of data (provided animals, acquired and managed patients, provided
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Chong Chen, Yan Liu, Junling Xie and Wei Wang

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Study supervision: Chong Chen and Yunping Luo

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Figure legends

Figure 1

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 tissue. 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 μm; 20 μm (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 μm; 20 μm (insets). (C) Immunofluorescence staining for OCT4, ALDH1 and LIN28B expression in human breast cancer tissues. Scale bar: 10 μm. (D) Kaplan-Meier analysis of LIN28B expression in survival of patients with breast cancer (n=159). *P< 0.05.

Figure 2

LIN28B maintains the stemness phenotype of cancer stem cells and promotes tumor growth and metastasis in vivo.
Figure 3

**IKKβ up regulates expression of LIN28B and sustains cancer stemness in cancer cells.**

(A) Western blot for LIN28B, SOX2, OCT4 and NANOG in MDA-MB-231 cells that were transfected with shLIN28B. (B) Percentage of ALDH+ cells of shLIN28B-MDA-MB-231 cells was detected by Flow Cytometry. (C) Primary mammosphere frequency of shLIN28B-MDA-MB-231 cells were calculated and representative single sphere images were visualized by microscope (below). (D) Secondary mammosphere frequency of shLIN28B-MDA-MB-231 cells was calculated and representative sphere images were visualized by microscope (below). 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 injected with 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 represent mean ± SD/SEM from 3 independent experiments. **P<0.01, ***P<0.001.

**Figure 3**

**IKKβ up regulates expression of LIN28B and sustains cancer stemness in cancer cells.**

(A) Western blot for LIN28B, p-IKKβ and IKKβ in either ALDH+ or ALDH- cells of MDA-MB-231 and H1299 cells. (B) Western blot for IKKβ, RELA, RELB and LIN28B in MDA-MB-231 cells that transfected with siRELA, siRELB or siIKKβ, respectively. (C) Percentage of ALDH+ cells of MDA-MB-231 cells that were treated with IMD-0354 (2
μM) was detected by Flow Cytometry. (D) Mammosphere frequency of MDA-MB-231 cells that were treated with IMD-0354 (1 μM) 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 μM) were detected by microscope and OD value. Scale bar: 100 μm. (F) Western blots for p-IKKβ, IKKβ, p-RELA, RELA, LIN28B, SOX2, OCT4 and NANOG in MDA-MB-231 cells treated by IMD-0354 at doses of 1, 5, 10 and 50 μM. (G) qPCR for LIN28B, SOX2, OCT4 and NANOG in MDA-MB-231 cells that were treated with IMD-0354 (5 μM). (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 represent mean ± SD from 3 independent experiments. *P<0.05, **P< 0.01, ***P< 0.001.

**Figure 4**

**TCF7L2 is required for IKKβ mediated activation of LIN28B.**

(A) Signaling pathway reporter array was used for seeking the signaling pathway associated with IKKβ on MDA-MB-231 cells treated with IMD-0354 (5 μM). (B) Western blot 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 TCF7L2E 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) Chromatin immunoprecipitation (ChIP) was performed to verify TCF7L2 binding with LIN28B
gene in MDA-MB-231 cells. (F) Luciferase reporter system was used to identify the TCF7L2 binding site of LIN28B gene in MDA-MB-231 cells which were transfected with plasmids containing both site 1/site 2, site 1, site 2 or mutated site 2 separately. (G) Reporter plasmid containing both site1/site2 and a β-catenin or control plasmid were co-transfected into MDA-MB-231 cells, and luciferase activity was detected. Error bars represent mean ± SD from 3 independent experiments. ***p<0.001.

Figure 5

LIN28B regulates Wnt/TCF7L2 signaling via direct binding to the mRNA of TCF7L2.

(A) Western blot for LIN28B, β-catenin and TCF7L2 in MDA-MB-231 cells that were transfected with shLIN28B. (B) Immunofluorescence staining for LIN28B and TCF7L2 in MDA-MB-231 cells that were treated with shLIN28B. Scale bar: 100 μm. (C) TOP-flash/FOP-flash and Renilla pRL-TK plasmid were co-transfected into shLIN28B-MDA-MB-231 cells, and luciferase reporter activity was detected. (D) Western blot for LIN28B, BCL2, CCND1 and MYC in MDA-MB-231 cells that were treated with shLIN28B. (E) RNA binding protein Immunoprecipitation (RIP) was performed and TCF7L2 mRNA was detected by RT-PCR. (F) LIN28B expression was associated with TCF7L2 expression in 102 clinical breast cancer specimens. Shown are visualizations of 2 representative cases (left) and percentages of specimens showing low or high LIN28B expression relative to TCF7L2 level (right). Scale bars: 200 μm; 20 μm (insets). Error bars represent mean ± SD from 3 independent experiments. **P<0.01,
***P< 0.001.

Figure 6

(A) Mamosphere frequency of MDA-MB-231 cells that were treated with DMSO, IMD-0354 (2 μM), Taxol (5 nM), Taxol+IMD-0354 were calculated and representative images were visualized by microscope (below). Scale bar: 200 μm. (B) Schedule of IMD-0354 or Taxol treatment in vivo (n=5). (C) Progression of tumor volumes during days 7–42 in mice treated with IMD-0354 or Taxol (n=5). (D) Bioluminescent imaging was used to detect the pulmonary metastasis at week 8 (n=5). (E) Lung metastatic foci were evaluated by H&E staining (n=5). Scale bars: 200 μm; 50 μm (insets). (F) Single cell suspensions derived from MDA-MB-231 xenografts harvested on day 56 were analyzed for ALDH activity using the ALDEFLUOR assay. (G) MDA-MB-231 xenografts from each group were collected, and immunohistochemistry staining was done to detect the expression of p-IKKβ, TCF7L2, LIN28B, ALDH1, SOX2 and NANOG. Scale bar: 20 μm. Error bars represent mean ± SD/SEM from 3 independent experiments. *P<0.05, **P<0.01, ***P<0.001.

Figure 7
The work model of interaction of LIN28B/TCF7L2 loop induced by IKKβ promotes cancer stemness and metastasis.
The tumor inflammatory environment activates the NF-κB signaling pathway in CSC. Transcription factor TCF7L2 which was regulated by p-IKKβ transferred into nucleus from cytoplasm to active LIN28B expression by directly binding to intron 1 of LIN28B DNA. The up-regulated LIN28B protein directly binds to the mRNA of TCF7L2 in cytoplasm and promotes TCF7L2 mRNA translation. This interaction constitutes a positive feedback loop to maintain the stemness of CSC. Inhibition of inflammation (p-IKKβ) could block this loop and impair the function of CSC thereby reduce the latent metastasis.
Figure 1

A

Absent/Weak  Moderate  Strong

LINC28B expression (%)  
- Normal (n=12)  
- Stage I (n=15)  
- Stage II (n=60)  
- Stage III (n=27)  

r=0.488  P<0.0001

B

Case 1  Case 2

High ALDH1 expression  Low ALDH1 expression

% of specimens

C

DAPI  OCT4  ALDH1  LINC28B  Merge

D

LIN28B low expression (n=62)  LIN28B high expression (n=97)

Cumulative survival

Survival time (mo)

p=0.0042
Figure 2

A

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<td>LIN28B</td>
<td>SOX2</td>
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B

![Bar chart showing ALDH^+ cells](chart)

C

![Line graph showing primary spheres/500 cells](chart)

D

![Graph showing secondary spheres/500 cells](chart)

E

![Bar chart showing A570](chart)

F

![Line graph showing tumor volume (mm^3) over days after injection](chart)

G

![Images showing shNC and shLIN28B](chart)

H

![Images showing shNC and shLIN28D](chart)
Figure 3

**A**

<table>
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**B**

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**C**

ALDH^+ cells (%)

**D**

Spheres/500 cells

**E**

A570

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**F**

IMD-C354 (µM) | 1 | 5 | 10 | 50

| p-IKKα/β | IKKβ | p-RELA | RELA | LIN28B | SOX2 | OCT4 | NANOG | α-tubulin |

**G**

mRNA relative expression

**H**

Tumor cells injection | IMD-0354 treatment | Tumor harvest

0 | 10 | 49 | Days

**I**

Hematoxylin and eosin-stained images of tumor xenografts

**J**

Metastasis number

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Figure 4

A

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Relative Luciferase activity

B

- IMD-0354 (µM): 1, 5, 10, 50
- siNC, siRELA, siRELβ, siKKβ

TCF7L2
β-catenin
β-actin

C

+1

AGCAAG
CTTTGCT

D

siNC, siTCF7L2
TCF7L2, LIN28B, β-actin

E

IP

Input

G

Luciferase activity

-73 +2493 -1064 +1569

***

F

-78 +2493
-1064 +1569
+1848 +2160
+1848 +2160

Luciferase activity

0.0 0.1 0.2 0.3 0.4 0.5
Figure 5

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B

shNC | shLIN28B

 TCFL72/Nucleus: LIN28B/Nucleus

C

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F

Case 1: High TCF7L2 expression - LIN28B Low, TCF7L2 High
Case 2: Low TCF7L2 expression - LIN28B High, TCF7L2 Low

% of specimens

Low LIN28B expression

High LIN28B expression
Figure 6

A

B

C

D

E

F

G

H

I

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K

L

M

N

O

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Q

R

S

T

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V

W

X

Y

Z

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Figure 7
IKKβ enforces a LIN28B/TCF7L2 positive feedback loop that promotes cancer cell stemness and metastasis

Chong Chen, Fengqi Cao, Lipeng Bai, et al.

Cancer Res Published OnlineFirst March 5, 2015.