Tyrosine kinase SRC-mediated YAP1-KLF5 module regulates cancer stemness and metastasis in TNBC

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Tyrosine kinase SRC-mediated YAP1-KLF5 module regulates cancer stemness and metastasis in TNBC

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Abstract

SRC was the first identified oncogene, and its aberrant activation has been implicated as a driving event in tumor initiation and progression. However, its role in cancer stemness regulation and the underlying regulatory mechanism are still elusive. Here, we identified a YAP1 tyrosine phosphorylation-dependent YAP1-KLF5 oncogenic module, as the key downstream mediator of SRC kinase regulating cancer stemness and metastasis in triple negative breast cancer (TNBC). SRC was overexpressed in TNBC patient tissues and its expression level was highly correlated with the tumor malignancy. SRC activation induced, while inhibition of SRC kinase reduced the cancer stemness, tumor cell growth and metastasis in vitro and in vivo. Transcriptomic and proteomic analysis revealed that SRC-mediated YAP1 tyrosine phosphorylation induced its interaction with Kruppel-like factor 5 (KLF5) to form a YAP1/TEAD-KLF5 complex in TNBC cells. YAP1-KLF5 association further promoted TEAD-mediated transcriptional program independently of canonical Hippo kinases (MST-LATS), which eventually gave rise to the enhanced cancer stemness and metastasis. Disruption of YAP1-KLF5 module in TNBC cells dramatically attenuated the SRC-induced cancer stemness and metastasis in vitro and in vivo. Accordingly, co-upregulations of SRC and YAP1-KLF5 module in TNBC tissues were significantly positively correlated with the tumor malignance. Altogether, our work presents a novel tyrosine phosphorylation-dependent YAP1-KLF5 oncogenic module governing SRC-induced cancer stemness and metastasis in TNBC. Therefore, targeting of YAP1/KLF5-mediated transcription represents a promising strategy for TNBC treatment with SRC aberrantly activation.

Keywords: Triple negative breast cancer, Cancer stem cell, SRC kinase, YAP1-KLF5 module.

1. Introduction

BC is the leading cause of cancer-related deaths in women all around the world [1]. Among which, TNBC is the most aggressive BC subtype [2]. Moreover, due to the tumor heterogeneity of TNBC and lack of expressions of ER/PR and HER2, targeted therapies are very limited for this subgroup of BC patients. Despite these patients respond to conventional chemotherapy, most of the patients commonly develop chemo-resistance [3, 4]. In addition, the recently-reported immunotherapy by targeting of PD-1/PD-L1 module, produced very limited improvement in overall survival of TNBC patients [5]. Thus, finding new therapeutic strategies are very urgent for curing TNBC. Accumulating studies have revealed that BCSCs are primarily existed in TNBCs and these cell populations are positively correlated with the 'triple-negative' state and unfavorable prognosis of BC patients [6, 7]. Targeting of BCSC regulation thus serves as a very promising strategy for TNBC treatment.

c-SRC is the first identified proto-oncogene, and it encodes a non-receptor protein-tyrosine kinase [8, 9]. This kinase is usually activated by the receptor tyrosine kinases, such as EGFR and PDGFR, and then transduces the extracellular stimuli into intracellular signals [10]. Structurally, SRC protein consists of four SRC-homology (SH1-SH4) domains and a C-terminal tail regulatory region. In addition, two tyrosine sites, including Tyr416 (an activating autophosphorylation site) and Tyr527 (an inhibitory phosphorylation site), have proved to be essential for regulating SRC kinase activity [11, 12]. SRC is inactive when Tyr527 residue is phosphorylated, owing that phosphorylated Tyr527 could bind to the SH2 domain and protect the catalytic pocket of Tyr416 in the kinase domain from inappropriate phosphorylation [13]. The pro-mitogenic growth factor treatments, such as EGF and PDGF, will induce the dephosphorylation of Y527, unlocking the catalytic pocket of Tyr416 and consequential de-inhibition of SRC kinase [14, 15]. Aberrant overexpression or activation of SRC kinase were frequently identified in various human malignant cancers, including BCs [16, 17]. Furthermore, SRC activation has discovered to be involved in multiple processes underlying tumor development and progression, including cell

proliferation, migration, invasion, and metastasis [10, 18]. Meanwhile, lots of the downstream components have been identified to responsible for SRC kinase-mediated functions in different subtypes of BCs, including FAK, SGK1, LATS and so on [19-21].

Using the TNBC mouse model, a recent identification of the selective SRC family kinase inhibitor eCF506 has showed a very high antitumor efficiency against both primary tumors and metastatic site tumors [22]. In TNBC patients, SRC pathway has also been identified to be one of the most commonly upregulated pathways [23, 24]. SRC kinase inhibitor Dasatinib alone, particularly combining with cisplatin has been shown great potential in treating metastatic TNBC [23, 25, 26]. Moreover, emerging preclinical evidences have demonstrated that TNBC cells showed higher sensitivity to the c-SRC inhibitor than do other cancer subgroups by targeting BCSCs [27-30], further highlighting the potential significance of targeting BCSCs through inhibiting SRC activity in TNBC treatment. However, the role of SRC kinase in cancer stemness regulation and the underlying regulatory mechanisms are still obscured.

In current study, we identify the YAP1-KLF5 oncogenic module as the key downstream target of SRC kinase to regulate the cancer stemness, cell proliferation and metastasis in TNBC cells, and which is independent of canonical Hippo kinases. The SRC gain- and loss-of-function (GOF and LOF) assays demonstrated that SRC kinase regulated the cancer stemness and metastasis of TNBC cells in vitro and in vivo. Disruption of YAP1-KLF5 attenuated SRC activation-induced cancer stemness and metastasis. Furthermore, co-upregulations of SRC and YAP1-KLF5 module in TNBC tissues were positively correlated with the tumor malignance. Collectively, all our findings unraveled a novel molecular mechanism by which the TNBC stemness and aggressive behaviors can be developed through the SRC-YAP1/KLF5 signaling axis, and independently of the canonical Hippo kinases. Based on our findings, we proposed that targeting of YAP1/KLF5 module may represent a rational therapeutic strategy for SRC aberrantly activated TNBCs.

2. Materials and Methods

2.1 Cell lines and DNA plasmids.

Human MCF10A cell, 293T and the human BC cell lines used in this study, including MDA-MB-231 and CAL51 were purchased from American Type Culture Collection (ATCC). MCF10A cells were grown in DMEM/F12 (Gibco), provided with 5% horse serum (Solarbio), 10 μg/mL insulin (MCE), 20 ng/ml epidermal growth factor (EGF) (MCE), 250 ng/μl Hydrocortisone (MCE), 100 ng/ml Cholera toxin (MCE) and 100 U/mL penicillin/streptomycin (Gibco). 293T cell and all human BC cells were grown in DMEM (High glucose) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 2 mmol/L L-glutamine (Gibco) and 100 U/mL penicillin/streptomycin (Gibco). The gene overexpressing plasmids (pUbi-MCS-3xFlag) and knockdown plasmids (pLKO.1-Puro) used for subcloning human *SRC, CA-SRC(Y530F), KLF5, YAP1* or *YAP1S127A* were purchased from GeneChem and TranSheepBio company respectively. Cell transfection, lentiviral production and infection assays were performed as previously described [31].

2.2 Reverse-transcription, real-time PCR and western blot.

All these procedures were performed as previously described [31]. The oligo sequences, and the antibody information used in this study were listed in the Supplementary table 1 and table 2 respectively.

2.3 Co-IP, luciferase assay and flow cytometry analysis.

Co-IP: Flag-IP was performed as previously described using the Anti-Flag M2 magnetic beads (Sigma) according the instructions [32]. The YAP1 and KLF5 antibody reciprocal Co-IP assay were performed using the protein A/G magnetic beads (MCE) according the product instructions. In brief, the human cells, including 293T, MCF10A and all BC cells were lysed first in 50 mM Tris-HCl (pH=7.5), 100 mM NaCl, 1% Triton X-100, 0.1 mM EDTA, 0.5 mM $MgCl_2$, 10% glycerol, protease inhibitor cocktail (Epizyme), phosphatase inhibitor cocktail (Epizyme), and 10 μM pervanadate (NEB), and then incubated with antibody-bound beads overnight at 4 °C.

Finally, the antibody/protein complexs were washed with lysis buffer for five times, boiled with protein loading sample buffer, and subjected to western blot analysis.

Luciferase assay: luciferase assay was performed as previously described using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions [31, 32]. In detail, 8xGTIIC plasmid was used to detect the TEAD-associated transcriptional activity and Renilla plasmid was used as the internal transfection control, whereas the pGL3 empty vector was used as a negative control. The transfection procedure was performed using Lipofectamine2000 (Invitrogen) following the manufacturer's instructions.

Flow cytometry analysis: 2×10^5 BC cells were first trypsinized, harvested and washed with PBS buffer, and then single-cell suspensions were incubated in 400 μL running buffer (PBS+5%FBS) with anti-human CD44 (BD company) and anti-human CD24 (BD company) on ice for 30 min. Finally, the percentages of labeled cells were analyzed using the CytoFLEX LX flow cytometer (Beckman).

2.4 Immunohistochemistry (IHC) staining and in vitro proximity ligation assay.

The IHC staining was performed as previously described [31] and the primary antibody for IHC was listed in the Supplementary table 1. In vitro proximity ligation assay was performed with the Duolink In Situ Orange Starter Kit Mouse/Rabbit (Sigma) according to the manufacturer's instructions. Images were captured by Carl Zeiss confocal microscope (LSM 800) using a 40x objective and analyzed with the image analysis software ZEN.

2.5 CCK-8 cell proliferation, tumorsphere growth, cell migration and invasion assays.

CCK-8 assay: cell viability assay was performed by using Cell Counting Kit-8 (Abbkine). Briefly, cells stably expressing the corresponding plasmids were seeded in triplicate in 96-well plates for indicated time, and then the OD value at 450 nm was detected according to the manufacturer's instruction.

Tumorsphere growth, cell migration and invasion assays were conducted as previously described [31].

2.6 In vivo xenograft assays.

All the animal-related protocols in this study were approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University. All the animal experiments were conducted following the approved protocols as we previously described, including limiting dilution assay (subcutaneous injection) and lung metastasis model (tail vein injection) [31].

2.7 Human samples and tissue microarray

The human BC tissues used in this study for western blotting were collected at the time of surgical resection in the Seventh Affiliated Hospital of Sun Yat-sen University. The human BC tissue microarrays used in this study were purchased from Shanghai Outdo Biotech, China. All the BC patients involved in this study provided an informed consent, and this study was ethically approved by the Ethics Committee of the Seventh Affiliated Hospital of Sun Yat-sen University. The IHC scores were calculated based on the percentage of positively-stained cells and staining intensity, as previously described [31].

2.8 RNA-sequencing and mass spectrometry analysis

The methods for RNA-sequencing and analysis were followed as we previously described [31]. For the Flag-IP-MS assay, the normal epithelial cells stably expressing 3xFlag-WT-YAP1 or 3xFlag-mut-YAP1 (3YF mutant) were generated by retrovirus infection. Cells were cultured to 80% confluency and lysed with buffer containing protease/phosphatase inhibitor cocktails (Epizyme). Lysate was first incubated with anti-Flag antibody conjugated resin (Sigma) for 2 h at 4 °C and washed five times with lysis buffer. Bound proteins were then eluted with elution buffer containing 200 ng/μl 3xFlag peptide. The eluate was further incubated with streptavidin conjugated resin for 2 h at 4 °C. Resin was washed and then eluted with buffer containing 4 mM biotin. The purified YAP1 and associated proteins were trypsin digested for 16 hours at 37 °C. Trifluoroacetic acid was added to the digestion solution to a final concentration of 0.5%, incubated at 37 °C for 45 min, and centrifuged at 13,000 rpm for 10 min. The top 90% of the sample after centrifugation was desalted and concentrated using a Millipore microC18 ZipTip and dried and resuspended in 0.1% formic acid. The resulting sample was analyzed by LC/ESI MS/MS using an Eksigent

2D nanoLC coupled in-line with an LTQ-OrbiTrap mass spectrometer. The details to identify proteins from mass spectrometry data was conducted as previously described [33].

2.9 Statistical analysis

All the in vitro experiments in this study were repeated at least three times in independent experiments. The graphs represent mean ± standard deviation (SD). The statistical analysis for comparison between two groups was performed by an unpaired Student's t-test. For multiple groups, one-way ANOVA followed by Tukey's test was used as indicated in GraphPad Prism 5 software. P-values <0.05, or <0.01, or <0.001 were considered to be statistically significant and marked with *, ** and *** respectively. P<0.05 was considered to be non-significant and marked as NS.

3. Results

3.1 SRC expression is prominently upregulated in TNBC tissues and cells, and highly associated tumor malignancy.

To examine the associations of SRC kinase expression level with the molecular subtypes of BCs, we first evaluated *SRC* mRNA expression in breast tumors from the cancer genome atlas (TCGA)-BRCA datasets, including 114 normal tissues and 1097 primary BC tumor tissues. The results revealed that *SRC* mRNA level was significantly upregulated in primary tumor tissues compared in normal tissues, and the TNBC patients showed overall higher SRC expression than the other BC subtypes, including Luminal and HER2+ subtypes (Fig 1A-B). Further investigating the SRC protein expression by BC-TMAs (IHC score ≤6 was defined as low expression and a score >6 was considered to be high expression according to the SRC IHC signals) showed that SRC protein expression level was significantly higher in BC tissues compared in normal tissues (Fig S1A-B and 1C). According to the BC subtypes, we also observed that around 47% of the breast tissues had high levels of SRC expression, and its expression ratio with a IHC score>6 in TNBC was significantly higher compared to the other subtypes, such as HER2+ and Luminal subtypes (Fig 1D-F). In addition, we noticed that SRC expression level was highly related to breast tumor

node metastasis (TNM) stage, and the BC tissues at stage III usually had a more higher IHC score (Fig 1G-H). These data confirmed that SRC kinase expression was upregulated in TNBC tissues and its expression level was positively correlated with the tumor malignancy. Subsequently, we also conducted western blot analysis with BC patient tissue lysates, including tumor lesions and adjacent tumor-free tissues. Consistently, SRC overall showed a higher expression level in TNBC patients compared in non-TNBC patients, as well as in the adjacent normal tissues (Fig 1I-J and S8A-B). In addition, we detected SRC protein level in various types of BC cell lines, and found it was widely expressed in various BC cell lines; however, it also showed a relatively higher expression level in TNBC cell lines (Fig 1K and S8C). Altogether, our results showed that SRC was highly expressed in the TNBC tissues and cells, and its expression level was correlated with BC malignancy.

3.2 SRC-GOF enhances CSC-like properties, tumor growth and metastasis.

To examine the detailed functions of SRC kinase in TNBC, we conducted the GOF assays using lentiviruses to stably express constitutively activated SRC (CA-SRC: Y530F) in both CAL51 (without 3xFlag) and MDA-MB-231 (with 3xFlag) cells. Western blot analysis revealed that SRC activation in these cells dramatically induced the expression of cancer stemness-related markers, such as OCT4, SOX2, NANOG and C-MYC. Meanwhile, epithelial-mesenchymal transition (EMT)-associated markers, like E-cadherin was repressed, and Vimentin was enhanced in SRC-GOF TNBC cells (Fig 2A-B and S9A-B), revealing the potential roles for SRC kinase in cancer stemness and EMT regulations. We and others have previously proved that upregulation of stemness and EMT-related markers could confer tumor cells with CSC-like properties, and $CD44^{+}/CD24^{-/low}$ cells were widely used to characterize the BCSC populations or tumor-initiating cells [34]. Thus, to examine the potential effect of SRC activation on CSC-like properties, we conducted the flow cytometry analysis using CD44/24 antibodies. The result revealed that SRC activation significantly increased the percentage of $CD44^{+}/CD24^{-/low}$ population in TNBC cells (Fig S2A-B). In addition, tumorsphere formation assay was performed to assess the BCSC

self-renewal in vitro. We observed that SRC activation dramatically induced the tumorsphere growth derived from the TNBC cells (Fig S2C-D). All these data confirmed that SRC activation indeed enhanced the cancer stemness in TNBC cells. Owing that enhanced cancer stemness is closely correlated to the tumor cell growth and metastasis abilities, we thus performed the CCK-8 and Transwell assays to analyze the cell proliferation, migration and invasion abilities upon SRC kinase activation. As anticipated, SRC activation in TNBC cells dramatically induced the cell proliferation rate compared with the control cells (Fig S2E-F). Meanwhile, in vitro cell migration/invasion assays revealed that SRC activation significantly enhanced the migratory and invasive capabilities of these cells (Fig 2C-D). Next, we also did the in vivo xenograft assay to valid the in vitro phenotypes we have observed above in CA-SRC cells. Consistently, CAL51 cells with SRC activation remarkably induced the tumor cell growth and size, as evidenced by tumor weight and Ki67 IHC stain compared with the control group (Fig S2G-J). Utilizing a xenograft metastasis model via tail vein injection of MDA-MB-231 cells (Fig 2E), we discovered that SRC activating cells could form more pulmonary localizations, and larger lung metastasis nodules than the control cells in vivo (Fig 2F-I). Taken together, our results demonstrated that SRC-GOF enhanced cancer stemness, tumor cell growth and metastasis in TNBCs.

3.3 SRC-LOF reduces CSC-like properties, tumor growth and metastasis.

To validate the phenotypes we have observed above in SRC-GOF TNBC cells, we further performed the SRC-LOF studies using short hairpin RNAs (ShRNAs)-mediated SRC knockdown technology. As anticipated, SRC knockdown obviously inhibited the expressions of CSC-associated transcription factors and the EMT program (Fig 2J-K and S9C-D). Flow cytometry analysis of the BCSC population and tumor sphere growth assay using the SRC-LOF cells also revealed that downregulation of SRC dramatically reduced the percentage of $CD44^{+}/CD24^{-/low}$ population, and the tumor cell self-renewal ability (Fig S3A-C). Moreover, we also performed the limiting dilution assay using the SRC stable knockdown CAL51 cells,

to evaluate the tumor-initiating frequency in vivo. The xenograft assay showed that decrease of SRC indeed was able to reduce tumor incidence (Fig S3D-F). Subsequently, we also did the CCK-8 and Transwell assays to evaluate the tumor cell proliferation, migration/invasion abilities upon SRC downregulation, and the results showed that SRC-LOF in TNBC cells significantly restrained these cell behaviors in vitro (Fig S3G-H and 2L-M). More importantly, in vivo xenograft experiments also displayed that the SRC knockdown TNBC cells significantly attenuated the tumor growth and size, as well as the pulmonary localization ability compared to the control cells (Fig S3I-J and 2N-Q). Taken together, all our results suggested that SRC regulated the cancer stemness, tumor cell growth and metastasis in TNBC cells.

3.4 SRC regulates YAP1 activation independently of the canonical Hippo kinases.

To clarify the molecular mechanism of SRC kinase-regulated phenotypes in TNBC cells, RNA-sequencing was conducted using both SRC-GOF and -LOF CAL51 cells. The analysis of differentially expressed genes (DEGs) in SRC activated cells showed that total 4378 genes were down- or upregulated with log2-fold change>2 and $p<0.05$, while 6990 common DEGs were identified in SRC knockdown cells with two different SRC ShRNAs (Fig S4A-D), indicating a fundamental role of SRC kinase in TNBC cells. Further characterization of these common DEGs revealed that CSC-associated markers, including *CD44*, *CD24*, *SOX2*, *KLF4*, *KLF9*, were regulated by SRC kinase activation, which was consistent with our data as previously described in Figure 2. More interestingly, we found that a substantial number of Hippo-YAP1 downstream target genes reported in BC cells [35-38], including *CYR61*, *CTGF*, *ETS1*, *FOSL1*, *JUNB*, were significantly increased upon SRC activation, while dramatically decreased in SRC knockdown cells (Fig 3A-B), which were further validated by qRT-PCR analysis (Fig 3C-D), indicating that YAP1-associated transcription was regulated by SRC kinase in these TNBC cells. In addition, luciferase assay using the 8XGTIIC reporter also demonstrated that SRC activation could promote the TEAD-associated transcriptional activity, resembling with the effect by

overexpression of YAP1 in these cells. Co-transfection of CA-SRC and YAP1 showed a synergistically effect on the luciferase activity, as evidenced by comparison with either CA-SRC or YAP1 single overexpression group (Fig S4E). Taken together, all these data indicated that SRC kinase directly regulated YAP1-TEAD-associated transcriptional outputs in TNBC cells. YAP1 was the downstream effector of Hippo kinases, and our previous study has revealed that YAP1 regulated cancer stemness and tumor progression in TNBC cells [31]. Meanwhile, another study also revealed that SRC activation was able to inhibit the Hippo pathway by tyrosine phosphorylation of LATS1, thereby leading to YAP1 activation [20]. Here, to shed light on whether SRC-mediated YAP1 activation was dependent on canonical Hippo kinases in TNBC cells, we further examined YAP1 phosphorylation status at S127 and S397 (the main catalytic sites by Hippo kinases) by western blot. The results showed that the phosphorylation levels of these two sites were not affected in TNBC cells expressing either CA-SRC or SRC ShRNAs, while the YAP1 downstream targets, like CYR61, were fluctuated resembling with SRC kinase, suggesting that SRC-mediated activation of YAP1 did not involve in the changes of canonical Hippo kinase cascade. On the contrary, the phosphorylation level of YAP1 at Y357, was dramatically induced in SRC-activated cells, while repressed in SRC knockdown TNBC cells (Fig 3E-F and S10A-B). Taken together, all these data demonstrated that SRC directly activated YAP1 through tyrosine phosphorylation in TNBC cells, rather than in a Hippo kinase-dependent manner.

3.5 YAP1 tyrosine phosphorylation enhances its interaction with transcription factor KLF5.

Tyrosine residue phosphorylation induced by SRC family kinase has been revealed to be an essential context for YAP1 interaction with its DNA-binding partners in multiple studies, such as the interactions between YAP1 and TBX3/5 and P73 [39-41]. Here, to identify novel YAP1 tyrosine phosphorylation-dependent interacting transcriptional factors, we first generated YAP1 knockout MCF10A cells, and then rescued these cells with either Flag-labeled WT-YAP1, or 3Y to F/E mutant YAP1

(Y341/357/394F and Y341/357/394E) to analyze the cell transformation abilities (Fig S5A and S11A). The knockdown of endogenous YAP1 dramatically decreased MCF10A cell proliferation without EGF growth factor, and then overexpression of sgRNA-resistant human WT-YAP1 could enhance the cell growth; however, overexpression of the 3YF mutant-YAP1 failed to show above phenotypes, while the 3YE mutant-YAP1 showed a very similar efficiency as the WT-YAP1 (Fig S5B). Subsequently, we performed a proteomic screen by mass spectrometry using Flag-labeled WT-YAP1 or 3YF mutant-YAP1 immunoprecipitates from SRC-activated MCF10A cells (Fig S5C). We screened the Flag-immunoprecipitates for candidates and found that Kruppel family member KLF5 was one of the most potential candidate transcription factors specifically interacting with WT YAP1 based on the binding score with 1% FDR and q-value validation (<0.05) (Fig 4A and S5D). In our data, we also observed that YAP1-P73 interacting efficiency was decreased in mutant-YAP1 immunoprecipitates compared with in WT-YAP1 immunoprecipitates, while the YAP1 and C-JUN interaction was similar between WT and mutant YAP1 immunoprecipitates (Fig S5D). Further validation of the mass spectrometry data by Flag-IP and western blot assays revealed that mutations of YAP1 tyrosine residues targeted by SRC kinase prominently reduced YAP1-KLF5 interaction efficiency (Fig 4B and S11B). To further confirm this association, we conducted the in situ proximity ligation assays in CA-SRC MCF10A cells and found that YAP1 in close proximity with KLF5 in the cell nuclear, resembling with YAP1-TEAD4 interacting module in these cells (Fig 4C). Subsequently, the Co-IP assays also showed that SRC kinase activation could robustly enhance both the exogenous YAP1-KLF5 interactions in 293T cells, and also their endogenous interactions in TNBC cells (Fig 4D-F and S11C-E). Further mapping of the YAP1-KLF5 interacting motifs in 293T cells revealed that both of the SH3 domain (for binding SRC family kinases) and transactivation domain (containing the 3 tyrosine residues) of YAP1 were indispensable for their interactions (Fig 4G-H and S11F). Then to investigate the outcomes of SRC-mediated YAP1-KLF5 associations, we further conducted luciferase assay using the 8XGTIIC reporter. The results showed that KLF5

overexpression was able to promote the luciferase activity, and which was similar as YAP1 overexpressing in these cells (Fig 4I). Whereas knockdown of KLF5 led to reduced TEAD-dependent transcriptional activity, which was similar with the effect of downregulating of YAP1 (Fig 4J). Moreover, co-overexpression of WT-YAP1, rather than Y-F mutant YAP1, together with KLF5 synergistically boosted TEAD-mediated transcriptional activity (Fig 4K). In addition, the DEG analyzing in KLF5 and YAP1 knockdown cells obtained from RNA-sequencing data showed that 6885 genes were down- or upregulated with more than 2-fold changes in KLF5 knockdown cells, while only 1936 DEGs were identified in YAP1 knockdown cells (Fig S5E-F). Further characterization of these common DEGs in both KLF5 and YAP1 knockdown cells revealed that 258 and 786 genes respectively were up or downregulated (Fig 4L-M). We also have done the qRT-PCR assay to validate some of the differentiated genes commonly regulated by both YAP1 and KLF5 (Fig 4N and S5G). All these findings demonstrated that KLF5 could interact with YAP1 to further enhance the YAP-TEAD-dependent transcriptional outputs.

KLF5 has demonstrated to be highly expressed in basal-type poorly differentiated BCs and its high expression is an unfavorable prognostic marker of BC patients [42, 43]. Moreover, previous studies showed that YAP1 promoted BC cell growth by stabilizing the KLF5 protein [44, 45]. We previously have found that YAP1 regulated the cancer stemness and metastasis in TNBC cells [31]. Here we further generated the YAP1 and KLF5-LOF TNBC cells and performed the in vivo xenograft experiments via tail vein injection of these cells. The results showed that KLF5 knockdown dramatically reduced the TNBC cell localizations and growth in lung, resembling with the phenotypes observed in YAP1-LOF TNBC cells (Fig S5H-M). These data suggested that YAP1 and KLF5 indeed performed very similar functions in TNBC cells.

3.6 YAP1-KLF5 oncogenic module is responsible for SRC activation-induced CSC-like properties, tumor growth and metastasis.

To clarify whether activation of the YAP1-KLF5 oncogenic module is responsible for the phenotypes we observed in SRC-GOF cells, we generated the CA-SRC TNBC cells, and followed by either stable knockdown of YAP1 or KLF5 using the ShRNAs, to assess their effects on cancer stemness, tumor growth and metastasis abilities. Firstly, we confirmed that decrease of YAP1 or KLF5 expression in SRC-activated TNBC cells could attenuate the expressions of YAP1 downstream targets, including CYR61 and CTGF (Fig 5A and D, and S12A-B). Further analyzing the percentages of $CD44^{+}/CD24^{-/low}$ population and the tumorsphere growth ability revealed that knockdown of either YAP1 or KLF5 in CA-SRC TNBC cells was able to rescue SRC-enhanced CSC-like properties (Fig S6A-D). We further investigated the effect of YAP1-KLF5 module disruption on SRC-induced cell growth, cell migration/invasion behaviors in vitro and in vivo. CCK-8 and xenograft assay results showed that disruption of this module in SRC-activated TNBC cells could potentially inhibit the hyperproliferation in vitro (Fig S6E-F) and the tumor overgrowth in vivo (Fig 5G-J). Besides, the Transwell assay and xenograft metastasis assay also demonstrated that attenuation of YAP1-KLF5 association in SRC-activated TNBC cells dramatically repressed their migration and invasion capacities in vitro (Fig 5B-F and S6G-H), as well as their pulmonary localization abilities in vivo (Fig 5K-N). Taken together, all above findings revealed that YAP1-KLF5 module functions as the key downstream regulator of SRC-mediated BCSC stemness regulation, and the enhanced tumorigenesis and metastasis in TNBC cells.

3.7 SRC-YAP1-KLF5 signaling axis prognosticates the malignance of TNBC patients.

To assess the potential relevance of SRC-YAP1-KLF5 signaling axis with the BC patients, we further performed the TMA analysis using the YAP1 and KLF5 antibodies as we previously have done for analyzing SRC expression in patient BC tissues (Fig S1A). As we observed like SRC IHC staining, YAP1 and KLF5 also showed significantly higher expression levels in BC tissues based on the YAP1/KLF5 IHC scores (Fig 6A and S7A). Meanwhile, both of them showed prominently higher

expression level in TNBC tissues compared in other BC subtypes, including luminal and HER2+ BCs (Fig 6B-C and S7B-C). In addition, both YAP1 and KLF5 protein expression levels were significantly related to the BC TNM stage and showed relatively higher IHC scores and ratios in BC tissues at Stage III (Fig 6D and S7D). Furthermore, the expression of SRC in these tissues was positively associated with YAP1-KLF5 module, and co-expressions of SRC and YAP1-KLF5 module were significantly correlated to the TNM stage and tumor malignancy in BCs (Fig 6E-H). Consistent with these findings, we found that BC patients with SRC or KLF5 high expression suffered shorter overall survival based on the TCGA-BRCA datasets (Fig 6I-J). Taken together, all our findings indicated the clinical relevance of SRC-YAP1-KLF5 signaling axis in BC patients. Therefore, targeting of YAP1-KLF5 module-dependent transcriptions may represent a rational therapeutic strategy for SRC aberrantly activated TNBCs.

4. Discussion

In present study, we demonstrate the tyrosine kinase SRC directly regulated a YAP1-KLF5 oncogenic module to enhance the cancer stemness, tumorigenesis and metastasis in TNBC cells through YAP1 tyrosine residue phosphorylation, and independently of the canonical Hippo kinases (Fig 6K). These results therefore revealed a novel SRC-YAP1-KLF5 regulatory axis to induce the cancer stemness and metastasis.

The SRC kinase is a well-known therapeutic target for various types of cancer including BCs. However, the impact of SRC inhibition appears different among subtypes of BC cells in multiple of preclinical studies. For instance, inhibition of SRC kinase activity with Dasatinib decreases the growth of TNBC cells without clear effect on other BC subtypes [27]. Furthermore, Dasatinib sensitises TNBC cells to chemotherapy by targeting BCSCs [28], and its antitumor effect is highly associated with a decreased proportion of aldehyde dehydrogenase 1-positive cells [29]. All these studies indicated the essential role of SRC kinase in BCSCs. However, the role of

SRC kinase in BCSC stemness regulation and the underling mechanism are rarely reported. TNBCs are enriched in BCSCs that are characterized by expression of cell surface marker $CD44^{high}CD24^{low}$, and which may contribute to the recurrence and metastasis of TNBC patients [6]. Here we have demonstrated that SRC kinase was prominently expressed in TNBC patients and it was a BCSC stemness regulator in TNBC cells, which may address the issue that TNBCs are more sensitive to the SRC inhibitor than the other BC subgroups. We further identified the YAP1-KLF5 oncogenic module was responsible for SRC-induced cancer stemness in TNBC cells, targeting of YAP1-KLF5 module may represent a more precise therapeutic strategy for treating SRC activation-associated TNBC.

Hippo signaling plays essential roles in cell growth and differentiation, thereby regulating organ size and tumorigenesis [46, 47]. The canonical Hippo signaling consists of MST1/2 kinases that directly phosphorylate LATS1/2. LATS1/2 then phosphorylate YAP1 to sequester it into the cytoplasm, where it could be firstly marked by 14-3-3 protein, and then degraded by ubiquitination-mediated pathway [33]. Inhibition of this pathway will allow YAP1 shuttle to the nucleus where it forms a transcriptional complex with TEAD family members to regulate the downstream genes [48, 49]. Despite that SRC plays a central role by modulating several important signaling pathways, such as PI3K-AKT, RAS-MAPK, STAT3 and Hippo signaling [50]. Here, we identified YAP1 was the key downstream substrate of SRC-induced cancer stemness and metastasis in TNBC cells. Furthermore, SRC-mediated YAP1 regulation was not associated with the canonical Hippo kinases. YAP1 was firstly identified as Yes-associated protein [51], and later it was shown to be tyrosine-phosphorylated by SRC family kinases, including YES, ABL and SRC [52, 53]. In line with our findings, SRC-mediated YAP1 activation through direct phosphorylation has implicated in many cancer progressions and tumor microenvironment regulation [32]. For example, phosphorylation of YAP1 by the YES is necessary for survival and tumorigenesis β-catenin-driven cancers, which are sensitive to the SRC inhibitor Dasatinib [40]. Similarly, tyrosine phosphorylation of

YAP1 by SRC is also required for RASSF1C overexpression-induced cell motility, invasion, and tumorigenesis [41]. In addition, SRC-dependent YAP1 activation has demonstrated to be critical for the establishment and maintenance of cancer-associated fibroblasts [54]. Therefore, YAP1 tyrosine phosphorylation represents a potential mechanism for SRC activation-associated human cancers.

Due to lack of a DNA-binding domain, TEAD family transcriptional factors are required to mediate YAP1-associated transcriptional output [47, 55]. However, they are not the sole members, and not sufficient to execute all transcriptional programs in different contexts. Accumulating studies have demonstrated that cellular context has a pivotal role in the choice of the YAP/TAZ partners and consequently on the final transcriptional outputs [56]. YAP1 tyrosine phosphorylation mediated by SRC family kinase has demonstrated to be a critical context for the recruitment of YAP1-interacting proteins, like RUNX2, P73 and TBX3/5 [39-41, 53]. In our study, we have also identified KLF5 as a YAP1 tyrosine phosphorylation-dependent interacting transcriptional factor to mediate SRC activation-enhanced cancer stemness and metastasis in TNBCs. Therefore, targeting YAP1-KLF5-mediated transcriptional output may represent a more precise strategy for TNBC treatment induced by SRC aberrant activation.

5. Abbreviations

BC: breast cancer; BCSC: breast cancer stem cell; Co-IP: coimmunoprecipitation; DEG: differentially expressed genes; EMT: epithelial-mesenchymal transition; GOF: gain-of-function; IHC: immunohistochemistry; KLF5: kruppel like factor 5; LOF: loss-of-function; SD: standard deviation; SH: SRC-homology; TCGA: the cancer genome atlas; TMAs: tissue microarrays; TNBC: triple negative breast cancer; TNM: tumor node metastasis.

6. Declarations

Ethical Approval and Consent to participate

Not applicable

Consent for publication

All authors read and approved the submission and final publication.

Availability of supporting data

The datasets used and analyzed in this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflicts of interest.

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Authors' contributions

PL conceived and designed the study, HZ and JL performed the major experiments and analyzed the data, JL and PL wrote and revised the manuscript.

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Not applicable

Authors' information

Not applicable

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Figure legends

Figure 1. SRC expression in human BC tissues and cells.

(A-B). Expressions of *SRC* mRNA in TCGA BC RNA-seq dataset including 114 normal and 1097 tumor tissues.

(C-D). Expressions of SRC protein in 6 normal and 74 BC tissues based on SRC IHC scores.

(E-F). Quantitation of SRC expression level in 74 BC tissues with different subtypes according to the IHC score. Representative images indicated the expression of SRC in different BC subtypes. Scale bars=100 μm.

(G-H). Quantitation of SRC expression level in 74 BC tissues with different TNM stages from I to III according to the IHC score. Representative images indicated the expression of SRC in different TNM stages of BCs. Scale bars=100 μm.

(I-K). Western blot analyses of total proteins from BC patient tumor tissues or cell lines with different subtypes using the indicated antibodies.

Figure 2. SRC regulates CSC-like properties, cell growth and migration/invasion behaviors.

(A-B). Western blot analyses of total proteins from the CAL51 or MDA-MB-231 cells stably expressing vector control (Ctrl) or CA-SRC using the indicated antibodies.

(C-D). In vitro cell migration/invasion ability was measured in Ctrl or CA-SRC TNBC cells using the Transwell chamber or Transwell chamber containing the Matrigel as barrier. Representative images of migrated/invaded cells were shown. Scale bars=100 μm. The quantitation data represent means ± SD with 3 biological replicates.

(E). Schematic for tail vein injections of MDA-MB-231 cells, luciferase-labeled cells were captured and analyzed every two-weeks after the injection for 10 weeks. Mice were terminated and the lungs were collected for HE staining at the tenth week.

(F-G). Bioluminescence images of lung-colonized tumor cells injected through the tail vein using NOD/SCID mice at the tenth week (n=6 per group), the quantification data was based on the bioluminescence signal intensities and represent means ± SD.

(H-I). HE staining of sections from lung nodules and the quantification data represent the relative area of lung nodules. n=6. Scale bar=100 μm.

(J-K). Western blot analyses of total proteins from the CAL51 or MDA-MB-231 cells stably expressing ShRNA vector control (ShCtrl) or ShSRC using the indicated antibodies.

(L-M). In vitro cell migration/invasion ability was measured in ShCtrl or ShSRC TNBC cells using the Transwell chamber or Transwell chamber containing the Matrigel as barrier. Representative images of migrated/invaded cells were shown. Scale bars=100 μm. The quantitation data represent means ± SD with 3 biological replicates.

(N-O). Bioluminescence images of lung-colonized tumor cells injected through the tail vein using NOD/SCID mice at the tenth week (n=6 per group), the quantification data was based on the bioluminescence signal intensities and represent means ± SD.

(P-Q). HE staining of sections from lung nodules and the quantification data represent the relative area of lung nodules. n=6. Scale bar=100 μm.

Figure 3. SRC directly activates YAP1 independently of canonical Hippo kinases.

(A-B). The heatmap indicated the YAP1 target gene expression changes induced by overexpression of CA-SRC or SRC knockdown in CAL51 cells. Red color indicated the known YAP1 target genes in BC cells.

(C-D). Quantitative real-time PCR to examine the mRNA level of the indicated gene expression in CA-SRC or SRC knockdown CAL51 cells. The data are shown as the mean ± SD (n=3). Statistically significant differences were indicated.

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Figure 4. Identification of a SRC kinase-dependent YAP1-KLF5 interacting module.

(A). The unique KLF5 peptides identified by LC-MS in Flag-WT-YAP1 immunoprecipitates.

(B). Western blot analyses of input or Flag-IP proteins from CA-SRC cells stably expressing Ctrl, Flag-WT or mut-YAP1 using the indicated antibodies.

(C). In situ proximity ligation assay showing an interaction between YAP1 and KLF5

in the nucleus of MCF10A cells indicated by red fluorescent spots. YAP1 or KLF5 antibody alone was used as negative control, and YAP1-TEAD4 interaction signals were used as the positive control. Scale bar=100 μm.

(D-F). Co-IP showing the interaction between exogenous YAP1 and KLF5 in 293T cells, or endogenous YAP1 and KLF5 in CAL51 and MDA-MB-231 cells stably expressing Ctrl or CA-SRC plasmids.

(H). Identification of the molecular motifs on YPA1 required for its interactions with KLF5 through Flag/Myc-IP and western blot analysis with the indicated antibodies in 293T cells.

(I). Luciferase assay using 8XGTIIC reporter in 293T cells transiently transduced with different concentrations of YAP1/KLF5 overexpressing plasmids respectively. The data are shown as the mean ± SD (n=3).

(J-K). Luciferase assay using 8XGTIIC reporter in CAL51 cells transiently transduced with YAP1 or KLF5 ShRNAs respectively, or WT-YAP1/mut-YAP1+KLF5 overexpressing plasmids. The data are shown as the mean ± SD (n=3).

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Figure 5. YAP1-KLF5 oncogenic module is responsible for SRC-enhanced CSC-like properties, cell growth and migration/invasion behaviors.

(A). Western blot analyses of total proteins from MDA-MB-231 cells stably expressing Ctrl, CA-SRC or CA-SRC+ShYAP1 using the indicated antibodies.

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(G-J). Xenograft tumor formation assays in NOD-SCID mice using CAL51 cells stably expressing Ctrl, CA-SRC or CA-SRC+ShYAP1 or ShKLF5. Quantitation of tumor weight represent means ± SD with n=8. Representative images for Ki67 IHC and quantitation data were shown with means ± SD. Scale bars=100 μm.

(K-N). Bioluminescence images of lung-colonized tumor cells injected through the tail vein using NOD/SCID mice at the tenth week (n=6 per group), the quantification data was based on the bioluminescence signal intensities and represent means ± SD. HE staining of sections from lung nodules and the quantification data represent the relative area of lung nodules. n=6. Scale bar=100 μm.

Figure 6. Clinical relevance of SRC-YAP1-KLF5 signaling axis with human BCs.

(A-B). KLF5 protein expression levels in 6 normal and 74 BC tissues were detected by IHC and analyzed by IHC scores.

(C-D). Quantitation of KLF5 expression level in 74 BC tissues with different subtypes or TNM stages according to the IHC scores.

(E-F). Representative images indicated the expression level of SRC, YAP1 or KLF5 in BC tissues or with different TNM stage from I to III according to the IHC scores. Scale bars=100 μm.

(G-H). Positive correlation of SRC with YAP1-KLF5 module was assessed using Pearson correlation coefficient analysis according to the corresponding IHC scores.

(I-J). Kaplan-Meier analysis of overall survival curves for BC patients grouped according to SRC or KLF5 expression level.

(K). Schematic of the proposed model to illustrate that SRC-YAP1-KLF5 regulatory

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Figures

Figure 1

SRC expression in human BC tissues and cells. (A-B). Expressions of SRC mRNA in TCGA BC RNA-seq dataset including 114 normal and 1097 tumor tissues. (C-D). Expressions of SRC protein in 6 normal and 74 BC tissues based on SRC IHC scores. (E-F). Quantitation of SRC expression level in 74 BC tissues with different subtypes according to the IHC score. Representative images indicated the expression of SRC in different BC subtypes. Scale bars=100 μm. (G-H). Quantitation of SRC expression level in 74 BC tissues with different TNM stages from I to III according to the IHC score. Representative images indicated the expression of SRC in different TNM stages of BCs. Scale bars=100 μm. (I-K). Western blot analyses of total proteins from BC patient tumor tissues or cell lines with different subtypes using the indicated antibodies.

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expressing Ctrl, CA-SRC or CA-SRC+ShYAP1 using the indicated antibodies. (B-C). In vitro cell migration/invasion ability was measured in MDA-MB-231 cells stably expressing Ctrl, CA-SRC or CA-SRC+ShYAP1 using the Transwell chamber or Transwell chamber containing the Matrigel as barrier. Representative images of migrated cells were shown. Scale bars=100 μm. The quantitation data represent means ± SD with 3 biological replicates. (D). Western blot analyses of total proteins from MDA-MB-231 cells stably expressing Ctrl, CA-SRC or CA-SRC+ShKLF5 using the indicated antibodies. (E-F). In vitro cell migration/invasion ability was measured in MDA-MB-231 cells stably expressing Ctrl, CA-SRC or CA-SRC+ShKLF5 using the Transwell chamber or Transwell chamber containing the Matrigel as barrier. Representative images of migrated cells were shown. Scale bars=100 μm. The quantitation data represent means ± SD with 3 biological replicates. (G-J). Xenograft tumor formation assays in NOD-SCID mice using CAL51 cells stably expressing Ctrl, CA-SRC or CA-SRC+ShYAP1 or ShKLF5. Quantitation of tumor weight represent means ± SD with n=8. Representative images for Ki67 IHC and quantitation data were shown with means ± SD. Scale bars=100 μm. (K-N). Bioluminescence images of lung-colonized tumor cells injected through the tail vein using NOD/SCID mice at the tenth week (n=6 per group), the quantification data was based on the bioluminescence signal intensities and represent means ± SD. HE staining of sections from lung nodules and the quantification data represent the relative area of lung nodules. n=6. Scale bar=100 μm.

Figure 6

Clinical relevance of SRC-YAP1-KLF5 signaling axis with human BCs. (A-B). KLF5 protein expression levels in 6 normal and 74 BC tissues were detected by IHC and analyzed by IHC scores. (C-D). Quantitation of KLF5 expression level in 74 BC tissues with different subtypes or TNM stages according to the IHC scores. (E-F). Representative images indicated the expression level of SRC, YAP1 or KLF5 in BC tissues or with different TNM stage from I to III according to the IHC scores. Scale bars=100 μm. (G-H). Positive correlation of SRC with YAP1-KLF5 module was assessed using Pearson correlation coefficient analysis according to the corresponding IHC scores. (I-J). Kaplan-Meier analysis of overall survival curves for BC patients grouped according to SRC or KLF5 expression level. (K). Schematic of the proposed model to illustrate that SRC-YAP1-KLF5 regulatory axis induces the CSC properties, tumor growth and metastasis in TNBC independently of canonical Hippo kinases.

Supplementary Files

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- Supplementaryfigurelegends.pdf
- Supplementaryfigures.pdf
- Supplementarytable1.pdf

- Supplementarytable2.pdf