Cancer-associated fibroblasts-derived exosomes suppresses immune cell function in breast cancer via miR-92/PD-L1 pathway

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Research

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Abstract

Background

Cancer associated fibroblasts (CAF) are important component in tumor microenvironment and has been reported contributes to tumor progression through many mechanisms, however, the detailed mechanism underling immune-suppression effect are not clearly defined.

Methods

In this study, human breast cancer-derived cancer associated fibroblasts was cultured, and CAF-derived exosomes in culture medium was isolated. Cancer cell migration was evaluated by transwell and wound healing assay, miR-92 binding to the LATS2 3’ untranslated region was validated by luciferase report assay, and underlying mechanism was investigated by chromatin immunoprecipitation and Immunoprecipitation.

Results

After treatment by CAF-derived exosomes, breast cancer cells express higher PD-L1, accompanied with increased miR-92 expression. Increased PD-L1 expression which induced by CAF- derived exosomes significantly promotes apoptosis and impaired proliferation of T cell. proliferation and migration of breast cancer cells was increased after transfection of miR-92, LATS2 was recognized as target gene of miR-92, which was proved by luciferase assay. Immunoprecipitation (IP) shown that LATS2 can interact with YAP1, after nuclear translocation, YAP1 could binds to enhancer region of PD-L1 to promotes transcription activity, which was confirmed by chromatin immunoprecipitation (ChIP). Furthermore, animal study confirmed that cancer associated fibroblasts significantly promotes tumor progression and impaired function of tumor infiltrated immune cells in vivo.

Conclusion

Our data revealed a novel mechanism which can induce immune suppression in tumor microenvironment.

Background

Breast cancer is the second most common cancer worldwide, the fifth most common cause of cancer death, and the leading cause of cancer death in women(1, 2).

And high expression of programmed cell death receptor ligand 1 (PDL1) was associated with poor prognosis in breast cancer (3, 4). Cancer associated fibroblasts (CAFs) is one of most important components in tumor microenvironment of breast cancer, it has reported that CAFs can support progression of breast cancer through many mechanisms, and promotes migration, proliferation of cancer cells(5–7). recently, several studies shown that CAFs also can suppress immune response in tumor

microenvironment, by recruit M2 macrophages or directly suppress function of immune cells(8–10). CAFs can affect other cells within tumor microenvironment by secreting growth factors, cytokines, and exosomes, and it is well documents that CAFs can interact with tumor cell by exosomes(11). Exosomes are small vesicle with a diameter ranging from 40 to 100 nm. The exosomes derived from endocytic compartments can contain RNA, protein, DNA, and microRNA(12).

MicroRNAs are evolutionarily conserved, class of 22-nucleotide non-coding RNAs. microRNA can negatively regulate target gene expression in a sequence-specific manner and has been reported play an important role in metabolism, migration and apoptosis of cancer cells(13–16).

To date, effect of breast cancer CAFs derived exosome on immune cells has not been studied. In this study, we found that CAFs derived exosomes can significantly promotes PD-L1 expression in breast cancer cells, and subsequently induce apoptosis of T cells, and impair NK cells function. Mechanically, it is observed that after treatment of CAFs derived exosomes, miR-92 expression in breast cancer cells was increased significantly, as target gene of miR-92, LATS2 was down-regulated, which subsequently enhance nuclear translocation of YAP1.YAP1 binds to enhancer region of PD-L1, and enhance PD-L1 transcription activity. Further, immune suppression effect was also confirmed in vivo, which shown that CAFs can suppress immune cell function.

Overall, this study revealed a novel mechanism which can induce immune suppression in tumor microenvironment.

Materials And Methods

Ethics statement

Breast cancer tissues were obtained from patients at the The First Affiliated Hospital of Zhengzhou University, and informed consent was obtained from each subject. Animal experiments were conformed to animal study guidelines of Zhengzhou University.

Antibodies And Reagents And Cell Lines

Anti-LATS2, YAP1, laminB, PPD-L1, and GAPDH rabbit polyclonal (Abcam, Cambridge, UK) and anti-actin polyclonal (Santa Cruz Biotechnology) antibodies were used at 1:1,000 dilution for western blotting. Anti-rabbit polyclonal secondary horseradish peroxidase- conjugated antibodies was used for detection (diluted; 1:2,000). Paclitaxel (TX) was purchased from Sigma (St. Louis, MO). The working stock was diluted in the media at a final concentration of 4 μM and further diluted when needed. The human breast cancer cell line, MCF7 cells were obtained from ATCC (Manassas, VA, USA), These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS) at 5% CO2 and 37 °C.

Detection Of NK Cells Killing Activity

NK-92 cells were added into 1 × 105 cancer cells which labeled with CFSE and incubated at 37 °C for 5 h. After the incubation, all cells were stained with 7-AAD (BD Bioscience). Cells were subsequently analyzed

by flow cytometry.

Flow Cytometry Analysis

Cells were digested with trypsin and then washed by PBS. Annexin V-FITC Apoptosis Detection Kit (Beyotime) was applied to practice cell apoptosis in line with the manufacturer's instructions. The apoptotic cells were dual-stained with PI and AnnexinV-FITC, using Annexin V/FITC kit (Thermo Scientific, Shanghai, China). Analysis was carried out via BDTM LSRII flow cytometer (BD Biosciences). Afterwards, Data was measured with the Cell Quest (BD Bioscience, San Jose, CA, USA) software

Luciferase Reporter Assay

Luciferase reporter assay was used to test the miR-92/LATS2 relationship. The firefly luciferase reporter gene expression vector, controlled with SV40 enhancer, was purchased from GeneCopoeia. The wild-type or mutant LATS2 3'- UTR sequence (LATS2; NM_014572; HmiT007288- MT06) was inserted downstream of the luciferase gene, whereas no oligonucleotides were inserted in the control vector. Renilla luciferase was used as a tracking indicator for transfection efficiency. The luciferase activity was measured using Light Switch Assay Reagent according to the manufacturer’s instructions (Switch Gear Genomics).

ChIP Assay

Fragmented chromatin from cancer cells was incubated with anti- IgG (negative control), anti-YAP. Recruited DNA was subjected to PCR using the primers for distal enhancer regions of PD-L1, and PCR products were electrophoresed in agarose gel. The ChIP assay was conducted using the Chromatin Immunoprecipitation (ChIP) Assay Kit (Millipore Corporation). Polyclonal antibodies for YAP (Cell Signaling Technology) and control rabbit antibody for IgG (Cell Signaling Technology) were used for ChIP. primers were used for RT-PCR to amplify the PD-L1gene.

Cell Viability Assay

Cell viability was determined by CCK8 assays. Briefly, cancer cells were seeded in 96-well plates (5 × 103 cells/well) and treated with corresponding processes. CCK8 was added into the wells for 3 h at indicated times. The absorbance in each well at wavelength of 450 nm (A450) was measured with a Thermomax microplate reader.

Western Blot Analysis And Immunoprecipitation (IP)

Cancer cells were collected, washed twice with cold PBS and lysed in NP-40 lysis buffer for 30 min at 4 °C. Protein concentration was measured using bicinchoninic acid assay kit (Thermo). Protein extracts were separated by electrophoresis in an 8 ~ 12% premade sodium dodecyl sulfate-polyacrylamide minigel (Tris-HCL SDS-PAGE) and transferred to a PVDF membrane. The membrane was incubated with indicated antibodies and detected by using a chemiluminescence method. For immunoprecipitation, total cell lysates were incubated with appropriate antibodies overnight and subsequently rotated with protein A/G

beads for 2 ~ 4 h at 4 °C. Beads were washed three times using NP-40 lysis buffer, mixed with 2 × SDS sample buffer and boiled for 5 ~ 10 min. The co-precipitates were analyzed by western blot analysis.

Animal Experiment

Animal assays were performed according to Experimental Animal Care Guidelines. 6–7 weeks old BALB/c mice were bred under specific pathogen-free (SPF) conditions. The mice were divided into four randomized groups (n = 6 per group), 1 × 105 MA782-mCherry with or without BALB/c derived BM-MSC were subcutaneously injected into flank of each mouse. The tumor size was measured using digital Vernier calipers every 3 days, the tumor volume was calculated as following formula: volume = 1/2 × (width2 × length). All mice were sacrificed after 18 days, and the tumors were collected and visually examined. Then the tumor was cut into sections and subjected to collagenase IV (Invitrogen, CA, USA) digestion for 3 h at 37 °C. After digestion, it was passed through a 70 μm mesh ((Miltenyi Biotech, Germany)) and analyzed by flow cytometry.

Statistical analysis

All the data were presented as the mean ± SEM. One-way analysis of variance (ANOVA) was adopted to analyze the differences among groups by using SPSS 13.0 (SPSS Inc., Chicago, Illinois, USA). Pair-wise comparisons were also made between groups using the Student–Newman–Kuels (SNK) test. P value less than 0.05 was considered as statistical significance.

Results

1.cancer associated fibroblasts-derived exosomes promotes miR-92 and PD-L1 expression in breast cancer.

Breast cancer associated fibroblasts (CAFs) was isolated and cultured, as shown in Fig. 1.a, immunofluorescence show that CAFs was positive for FSP and vimentin, the further confirm phenotype, western blot was performed. As shown in Fig. 1.b, CAFs express higher FSP, vimentin and α-SMA compared with normal fibroblasts (NFs) which isolated from para-cancer normal tissue. Those data indicated we have isolated breast cancer derived CAFs successfully. then CAFs derived exosomes was isolated, and as shown in Fig. 1.c, purified exosomes from condition medium of CAFs display typical morphology. To investigated function of CAFs derived exosomes on breast cancer cells, real-time PCR was performed, as shown in Fig. 1d, in breast cancer cells, there are no significant changes of selected microRNA expression after being treated by CAFs derived exosomes, except for miR-92, which significantly up-regulated after cancer cells was cultured with exosomes. Moreover, we also found PD-L1 expression in cancer cells was increased after administration of CAFs-derived exosomes (Fig. 1.e).

2.miR-92 promotes migration and proliferation of breast cancer cells.

Based on previously results, we are curious about the role played by miR-92 in breast cancer. As shown in Fig. 2.a, clinical sample shown that breast cancer tissue expresses higher miR-92 compared with normal

tissue, indicated that possibly, miR-92 can promotes tumor progression. Breast cancer cells MCF7 was transfected with mimic and inhibitor of miR-92, the efficiency of transfection was confirmed by real-time PCR (Fig. 2b), CCK-8 and colony genesis experiment shown that after up-regulation of miR-92, MCF7 presents higher proliferation rate compared with negative control (Fig. 2c,d), Wound healing assay shown that miR-92 promotes migration, and down-regulation of miR-92 inhibited migration of breast cancer cells, this effect was further confirmed by transwell assay (Fig. 2e,f). Taken together, our results indicated that miR-92 promotes migration and proliferation of breast cancer cells.

3. MiR-92 targeting LATS2 and enhance nuclear translocation of YAP1

To find out target gene for miR-92, bioinformation screening using the TargetScan database was performed. Based on the database, LATS2 may serve as a target protein of miR-92. luciferase report assay was used to confirm if LATS2 is direct target for miR-92, as shown in Fig. 1.a, Wild type LATS2 3'-UTR and mutant LATS2 3'-UTR with nucleotide substitution in the putative binding site was subcloned into luciferase report vector, after co-transfection of miR-92 mimic, luciferase activity was suppressed, whereas such effect did not observed in luciferase with mutant UTR. Those data shown that LATS2 is direct target of miR-92 in breast cancer cells. to figure out underlying role played by LATS2 in breast cancer, we investigated LATS2 expression in TCGA dataset, as shown in Fig. 3.b, LATS2 was upregulated in normal tissue compared with tumor tissue, indicated that LATS2 might be a tumor suppression gene. As shown in Fig. 3.c, based on gene analysis of STRING, LATS2 is possibly interact with YAP1 which is an important gene in Hippo pathway. To test this possibility, western blot was performed. As shown in Fig. 3.d, after LATS2 was down-regulated by miR-92 transfection, phosphating of YAP1 was decreased, and nuclear translocation of YAP1 was increased subsequently, moreover, after LATS2 was up-regulated by transfection of miR-92 inhibitor, nuclear translocation of YAP1 was decreased. To further confirm relationship between YAP1 and LATS2, co-IP assay was performed. As shown in Fig. 3e, it is observed that YAP1 could precipitate with LATS2, whose data confirmed that LATS2 can interact with YAP1 directly, promotes phosphating of YAP1 and reduce its nuclear translocation.

To further confirm function of YAP1 in miR-92 induced effect, YAP1 was knocked down by shRNA transfection. The effect of YAP1 down-regulation was confirmed by western blot (Fig. 3.f). after YAP1 was knocked down, increased migration capacity which induced by miR-92 was partly impaired, indicated pro-tumor effect of miR-92 is partly rely on LATS2/YAP1 pathway.

Collectively, those results shown that LATS2 is direct target of miR-92, LATS can interact with YAP1 and regulate nuclear translocation of YAP1 in breast cancer cells.

4.miR-92 promotes PD-L1 transcription activity by enhance nuclear translocation of YAP1.

Because our previously study shown that CAFs derived exosomes treatment enhance PD-L1 expression, we sought to confirm that if up-regulation of miR-92 can promotes PD-L1 expression, as shown in Fig. 4.a, western blot indicated that miR-92 overexpression result in increased YAP1 nuclear translocation, accompanied with up-regulation of PD-L1, after expression of LATS2 was enhanced by miR-92 down-

regulation ,decreased YAP1 nuclear translocation can be observed, accompanied with up-regulation of PD-L1. This effect was further confirmed by flow cytometry, as shown in Fig. 4. b.

YAP1 is a transcription co-activator, together with TEAD family protein, regulated many genes, to explain underlying mechanism causes increased PD-L1 expression after nuclear translocation of YAP1, we hypothesis YAP1 could promotes PD-L1 transcription. An TEAD-binding site was observed in 7911 bps upstream of PD-L1 transcription start site, Chromatin immunoprecipitation (ChIP) was performed to test this possibility. As shown in Fig. 4.c, YAP1 antibody causes precipitation of PD-L1 enhancer regions encompassing the putative TEAD binding site. But this was not observed in ChIP assay using Rabbit IgG, those result confirmed that YAP1 could occupied the enhancer region of PD-L1 directly.

To further confirm whether occupation of YAP1 with enhancer region of PD-L1 can result in increased transcription activity of PD-L1, YAP1 was knocked down by transfection of siRNA, as shown in Fig. 4.d, after YAP1 was knocked down, nuclear translocation of YAP1 was also decrease significantly, as our expected, YAP1 down regulation also diminished PD-L1 up-regulation which result from miR-92 overexpression.

Collectively, our result confirmed that miR-92 can promotes PD-L1 expression by enhance occupation between YAP1 and PD-L1 enhancer region.

5. exosomes induced PD-L1 up-regulation suppress immune cell function in breast cancer.

PD-L1 is a type 1 transmembrane surface glycoprotein encode by CD274 gene, it promotes T cell tolerance and escapes host immunity. It can bind to its ligand PD-1 which usually expressed in immune cells and suppress function of immune cells. expression of PD-1 can be induced after NK cells co-cultured with tumor cells (data not shown), we asked whether the function of NK cells which express PD-1 can be suppressed by exosomes induced PD-L1 overexpression. To answer this question, NK cell killing assay was performed, as shown in Fig. 5.a, PD-1-expression NK cells can lyse cancer cells significantly, but function of NK cells was impaired when tumor cells was pre-treated by exosomes. But function of NK cells can be partly rescue after administration of anti-PDL1 antibody.

PD-1 is classically expressed in T cells, as shown in Fig. 5.b, c, when T cells was co-cultured with cancer cells which pre-treated by exosomes, enhanced apoptosis rate and impaired proliferation rate can be observed. As our expected, those effect can partly block by administration of anti-PD-L1 antibody. Importantly, knock down of YAP1 markedly limited exosomes induced apoptosis of T cells, indicated that immune suppression effect of exosomes was rely on expression of YAP1 and PD-L1.

6. cancer associated fibroblasts promotes tumor progression and suppress immune cell function in vivo.

Animal experiment was performed to investigated role played by CAFs in vivo. Mesenchymal stem cells which is a precursor of CAFs was used in this xenograft model, MA-782-mCherry alone and MA-782-mCherry mixed with MSCs was injected into flanks of Balb/c mice. The mice were sacrificed after 3 weeks, as shown in Fig. 6.a, the tumor produced by MA-782-mCherry mixed with MSCs were significantly

larger than those produced by MA-782-mCherry alone. This result indicated that CAFs can promotes tumor progression in vivo.

To investigate underlying mechanism responsible for tumor volume, Sigle cell suspension of tumor was analyzed by flow cytometry. as shown in Fig. 6.b, cancer cells in tumor which expressed mCherry was studied, which show that CAFs promotes PD-L1 expression in vivo. To test whether difference of PD-L1 expression can suppress immune cell function in vivo, NK cells and cytotoxic T cells were gated as CD49b + CD335 + and CD8 + CD4+, respectively, CD107a (LAMP-1) may be a marker for degranulation of NK and activated CD8 + T cells was used to test function of immune cells. As shown in Fig. 6.c-d, addition of MSCs significantly reduced CD107a expression in NK cells and T cells, which indicated that CAFs can suppress function of immune cells in vivo.

Taken together, our result indicated that cancer associated fibroblasts promotes tumor progression and suppress immune cell function in vivo.

Discussion

Breast cancer remains a significant threat to the health and wellness of women in the United States, accounting for 30% of all new cancer diagnoses and almost 41,000 deaths annually(17–19). Immunotherapy is an effectively strategy for a variety of cancer and has been shown impressive survival benefits in patients(20–23), However, most breast cancers are resistant to monotherapy with checkpoint inhibitors(24). Cancer associated fibroblasts is important component of tumor microenvironment and has been reported suppress immune cell function in a variety of tumor, but its underlying mechanism remain unknown. Exosomes based miRNA delivery is one strategy of cancer associated fibroblasts to influence other cells presents in microenvironment. In this study, we reported a novel mechanism of immune suppression which based on exosomes delivery- induced PD-L1 up-regulation.

MiR-92 has been reported play an oncogenic role in a variety of cancer(25). In this study, we observed that after treatment of CAF derived exosomes, the miR-92 expression was significantly increased, whereas other selected micro-RNA expression remains stable. We subsequently investigated role of miR-92 in breast cancer cells. as previously report, we found miR-92 up- regulation promotes migration and invasion in breast cancer cells.

PD-L1 is a type 1 transmembrane surface glycoprotein encode by CD274 gene, it promotes T cell tolerance and escapes host immunity, but the regulation of PD-L1 in tumor is still under investigation(26–28). In this study, we use luciferase activity assay confirmed that LATS2 which is an important component of Hippo pathway was direct target of miR-92, co-IP was performed to confirm LATS could directly interact with YAP1, promotes YAP1 phosphating and subsequently prevents YAP1 nuclear translocation. It has been reported that YAP activity can regulate PD-L1 expression in some type of cancer(29, 30), which similar with our result, in this study, Chromatin immunoprecipitation (ChIP) was used to confirmed YAP1 directly occupied the enhancer regions of PD-L1, and subsequently result in up-regulation of PD-L1.

We further confirm immune suppression effect of CAFs derived exosomes, and found that after cancer cells was treated by CAFs derived exosomes, co-culture with cancer cells results in increased apoptosis rate, and impaired immune cell function.

We further confirmed our proposed mechanism in vivo, based on flow cytometry, we confirmed that CAFs significantly suppress immune cell function in vivo, and promotes PD-L1 expression in breast cancer cells.

Collectively, this study revealed a novel mechanism which can induce immune suppression in tumor microenvironment.

Declarations

Ethics approval and consent for participation

Breast cancer tissues were obtained from patients at the The First Affiliated Hospital of Zhengzhou University, and informed consent was obtained from each subject. Animal experiments were conformed to animal study guidelines of Zhengzhou University.

Consent for publication

No applicable

Availability of data and material

The dataset generated and analyzed in the current study are available in TCGA dataset

Competing interest

The authors declare that they have no competing interests

Funding

No applicable

Authors' contributions

D.D. and X.W. conceived and designed the study. D.D. and X.TR did the main experiment. M.H. analyzed and interpreted the data. X.X. was responsible for reagents and materials. X.G. drafted the article. Y.G. and X.W. revised the article critically. All authors had final approval of the submitted versions.

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Figures

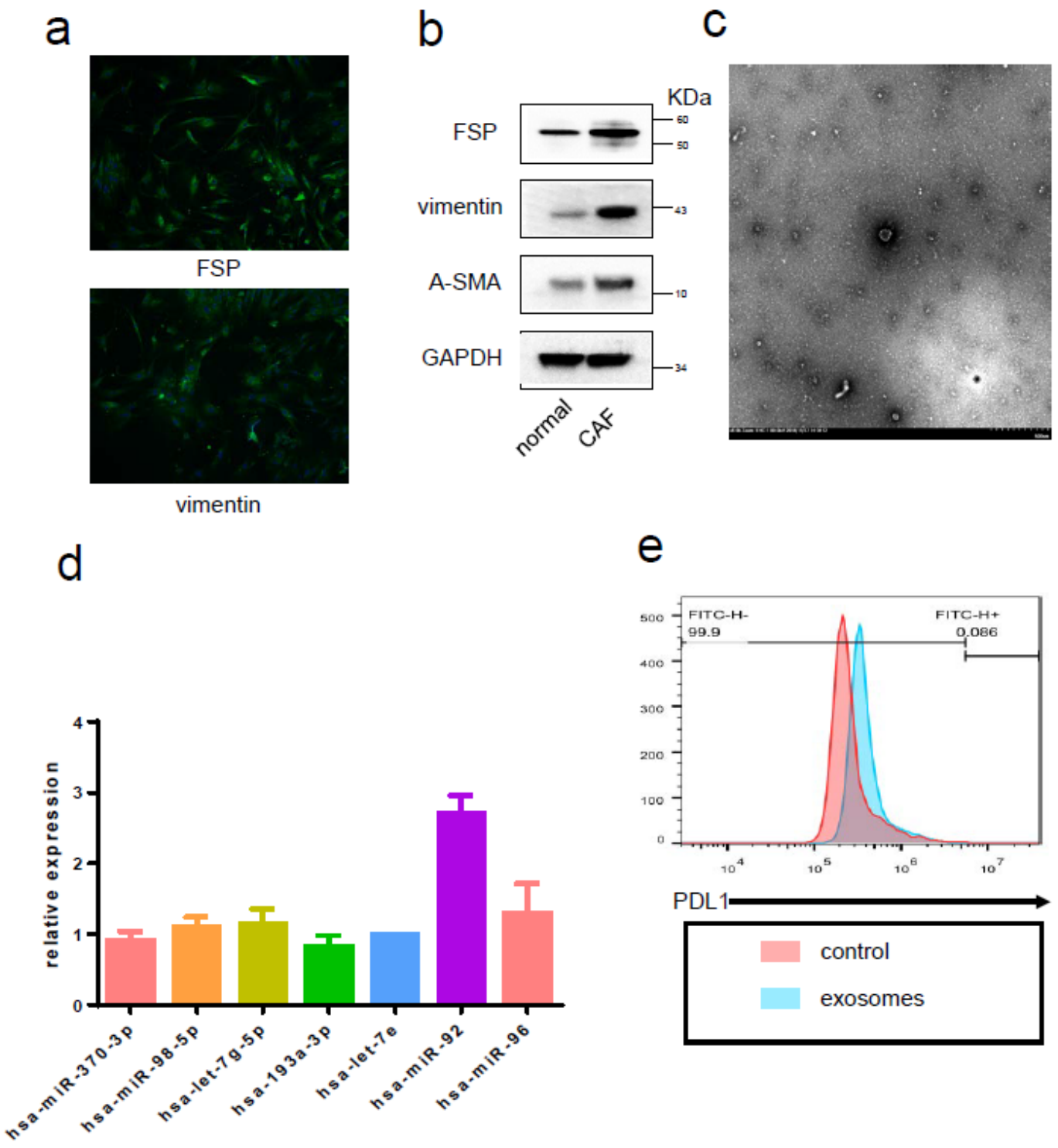

Figure 1

Cancer associated fibroblasts-derived exosomes promotes miR-92 and PD-L1 expression in breast cancer. a immunofluorescence image of FSP and vimentin in breast cancer derived cancer associated fibroblasts b western blot analysis of FSP, vimentin, α-SMA expression in cancer associated fibroblasts and normal fibroblasts. c transmission electron microscopy image of exosomes. d micro-RNA expression after

exosomes treatment e flow cytometry analysis of PD-L1 expression in breast cancer cells. Error bars represent mean ± s.d.; ****P < 0.0001; n.s. not significant; by paired two-sided Student's t-test.

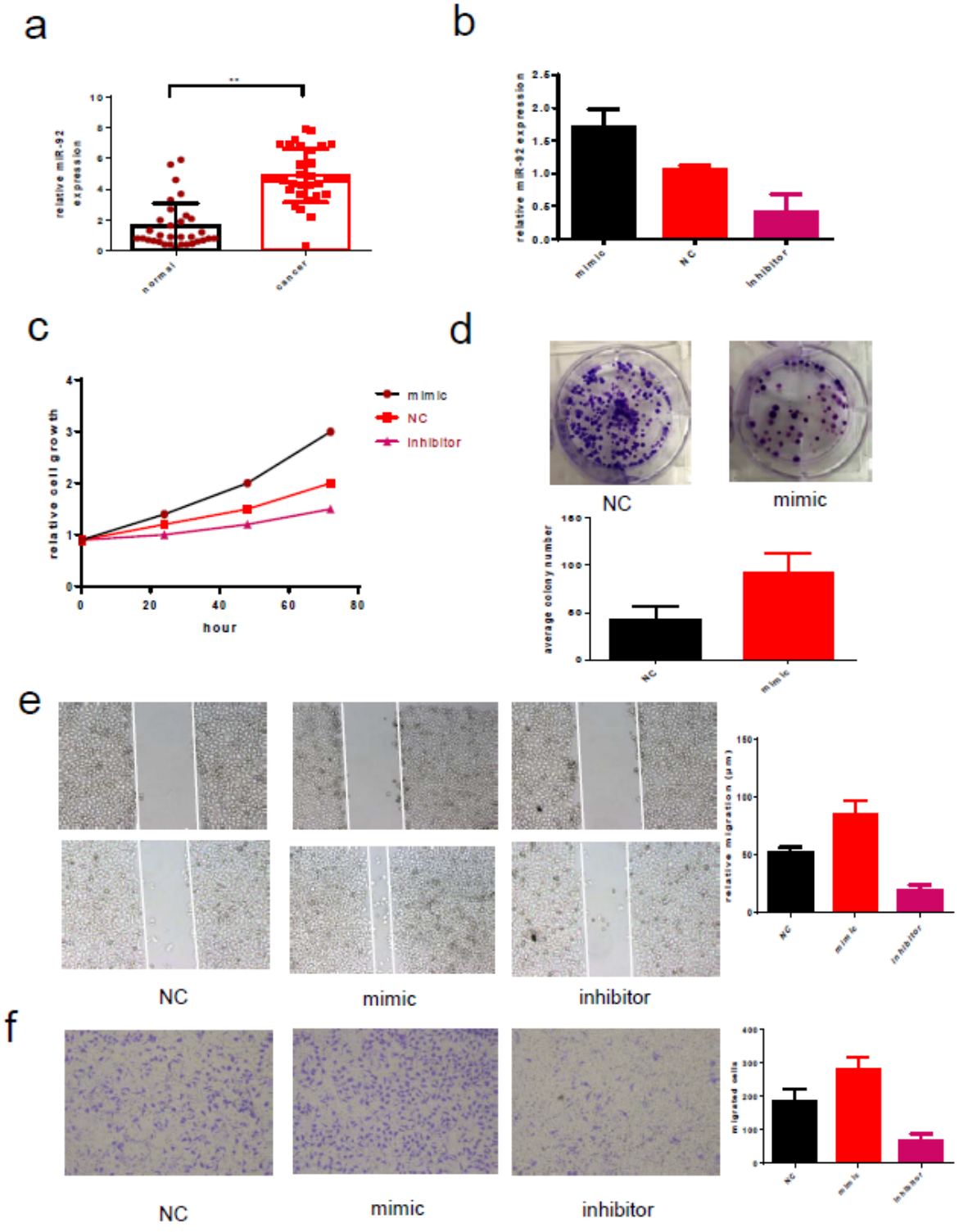

Figure 3

miR-92 promotes migration and proliferation of breast cancer cells. a the miR-92 expression was examined by qRT-PCR in human breast cancer tissue and normal tissue. In total, 32 cancer tissues and 34 normal tissue were analyzed b miR-92 expression after transfection of miR-92 mimic or inhibitor was

analyzed by qRT-PCR (n=3). c miR-92 overexpression promotes cell proliferation. cancer cells were transfected with miR-92 mimic or inhibitor and the absorption (A450 nm) was detected at 0, 24, 48 and 72 h, respectively. d. cancer cells transfected with miR-92 mimic and NC control were assayed for clonogenicity in adherent cultures. e Wound-healing closure assay for cancer cells which transfected with miR-92 mimic or inhibitor. (magnification,x50,scale bar: 500μm) f transwell assay for cancer cells which transfected with miR-92 mimic or inhibitor. (magnification, x100). Error bars represent mean ± s.d.; ****P < 0.0001; n.s. not significant; by paired two-sided Student's t-test.

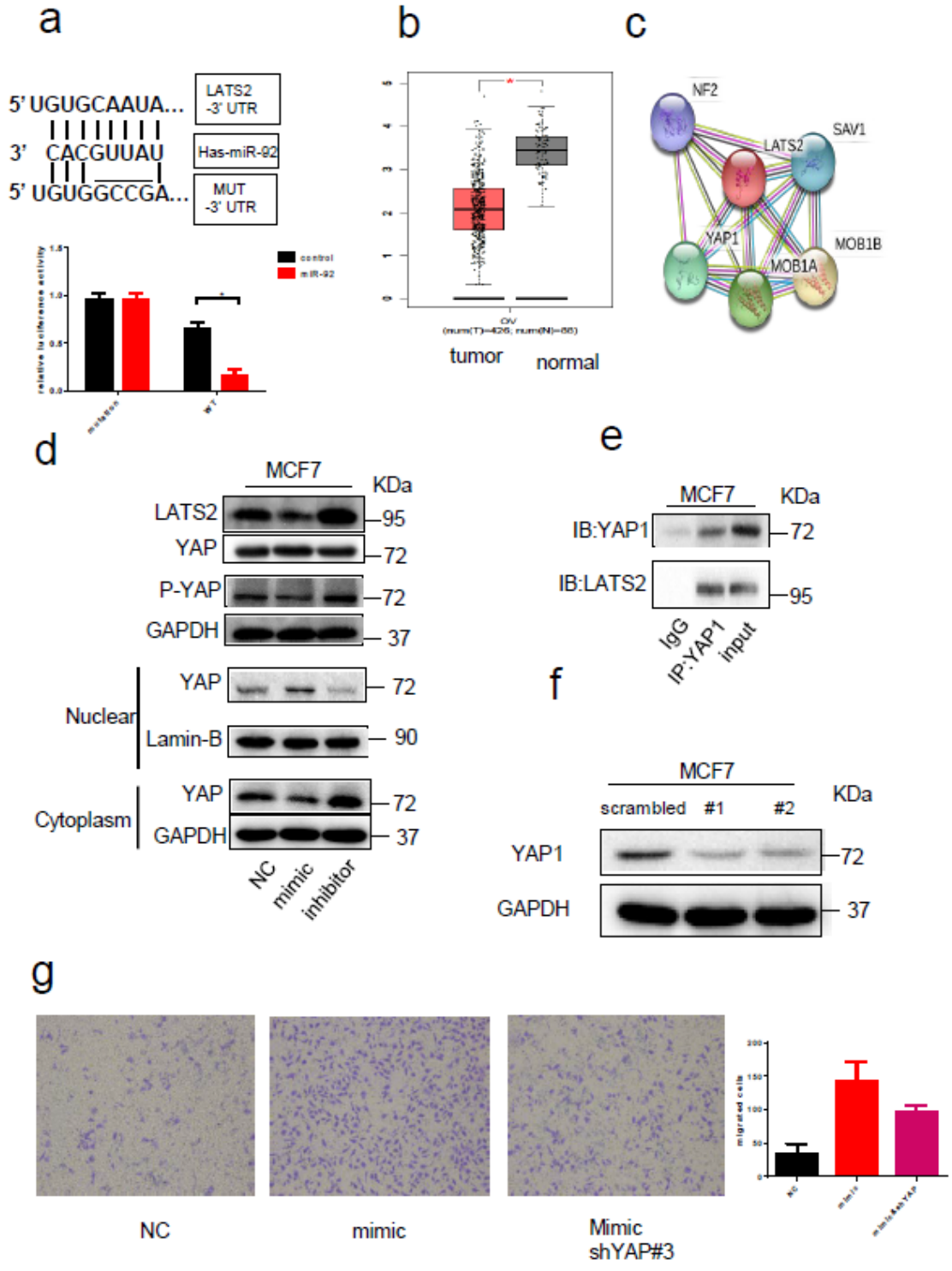

Figure 5

miR-92 targeting LATS2 and enhance nuclear translocation of YAP1. a Schematic representa¬tion of the miR 92 targeting sequences within the 3'UTR of LATS2. Luciferase reporter assay was conducted in MCF7 cells following transfection with miR 92 mimic or NC, and together with WT or Mut LATS2 3'UTR luciferase reporter plasmid. b LATS2 expression was examined in normal and breast cancer tissue from public dataset. c the protein-protein network view from STRING database showing the networks of LATS2. d western blot shown the YAP1, LATS2 expression in MCF7 which transfected with miR-92 mimic or inhibitor. e co-IP analysis with anti-YAP1 antibody or IgG in MCF7 f western blot analysis of YAP expression after two shRNA target YAP was transfected g transwell assay for breast cancer cells which transfected with miR-92 mimic and/or shRNA targeting YAP. (magnification, x100). Error bars represent mean ± s.d.; ****P < 0.0001; n.s. not significant; by paired two-sided Student’s t-test.

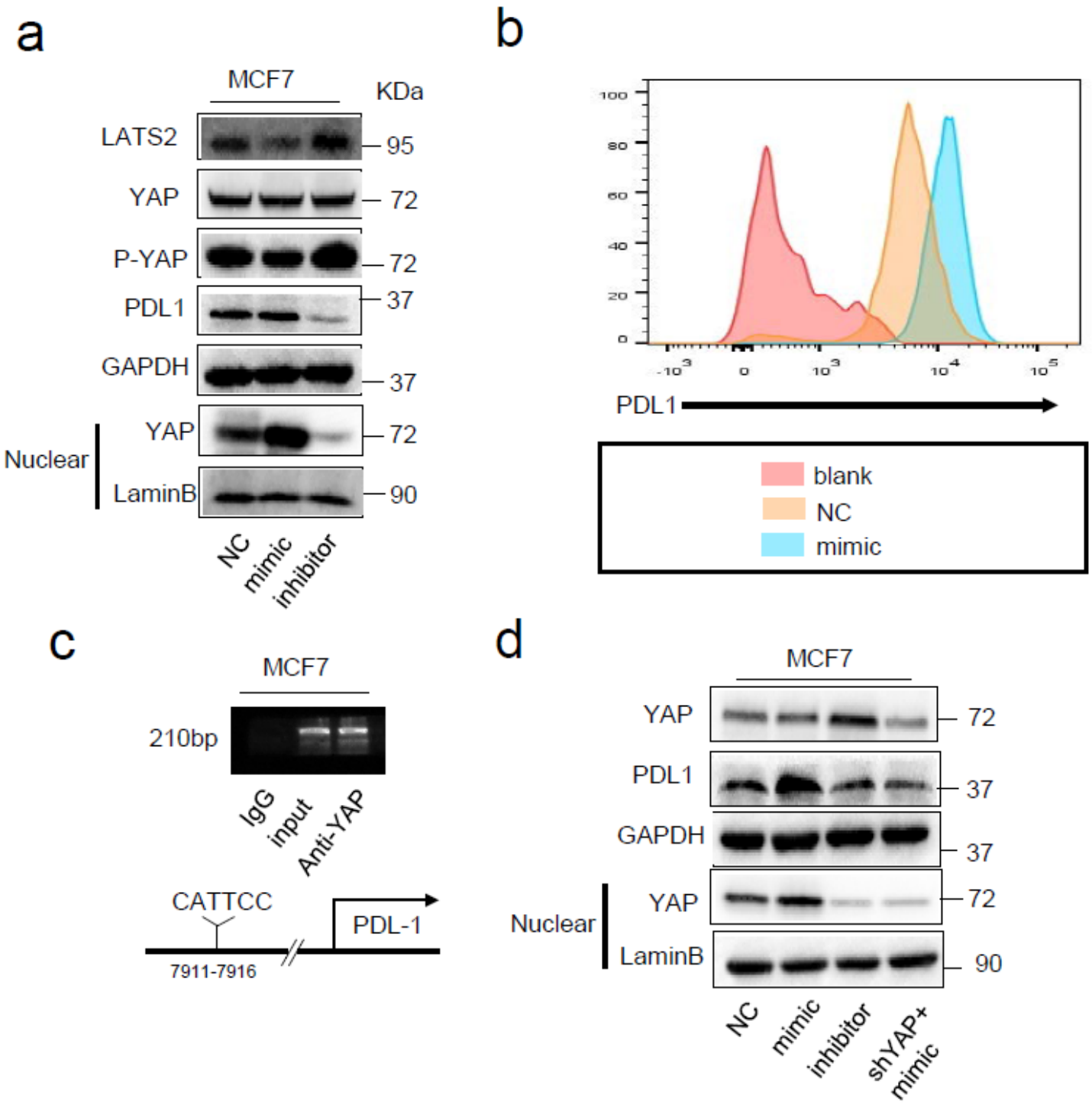

Figure 7

MiR-92 targeting LATS2 and enhance nuclear translocation of YAP1. a western blot shown the YAP1, LATS2. PD-L1 expression in MCF7 which transfected with miR-92 mimic or inhibitor. b flow-cytometry analysis for PD-L1 expression in MCF7 which transfected with NC or miR-92. c ChIP assay was performed with MCF7.which are shown by gel bands of RT-PCR products with 30 cycle. d western blot

shown the YAP1. PD-L1 expression in MCF7 which transfected with miR-92 mimic or inhibitor or/and shRNA targeting YAP.

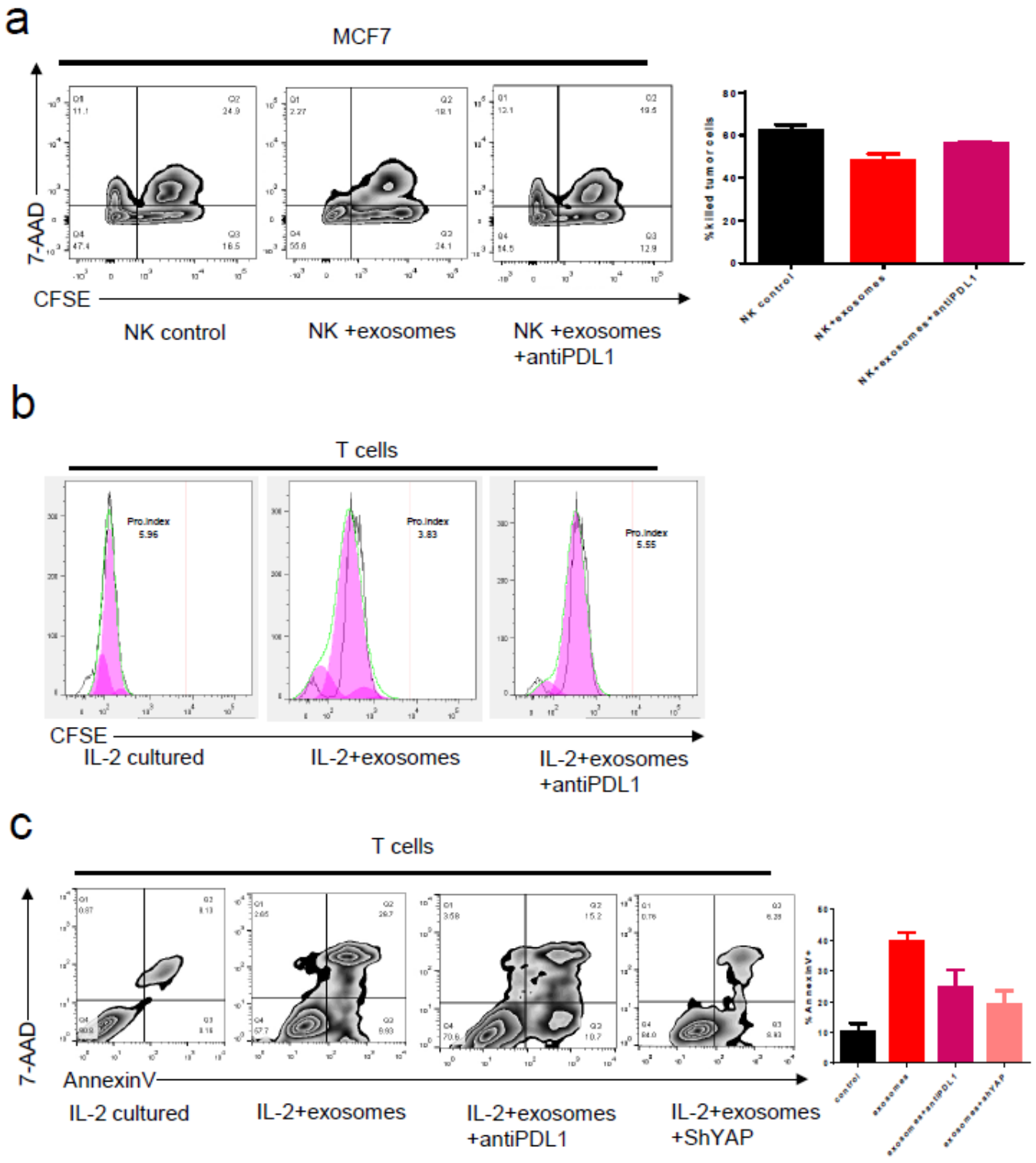

Figure 10

exosomes induced PD-L1 up-regulation suppress immune cell function in breast cancer. a NK cell cytotoxicity assay was performed, MCF7 treated by exosomes or not was used as target cells. quantification of cytotoxicity of NK cells was shown in right panel b CFSE was used to evaluate

proliferation of T cells. T cells was cultured directly with MCF7 treated by exosomes or not for 48h c T cells was cultured directly with MCF7 treated by exosomes or not for 48h, apoptosis rate was evaluated by Annexin V stain. Error bars represent mean ± s.d.; ****P < 0.0001; n.s. not significant; by paired two-sided Student's t-test.

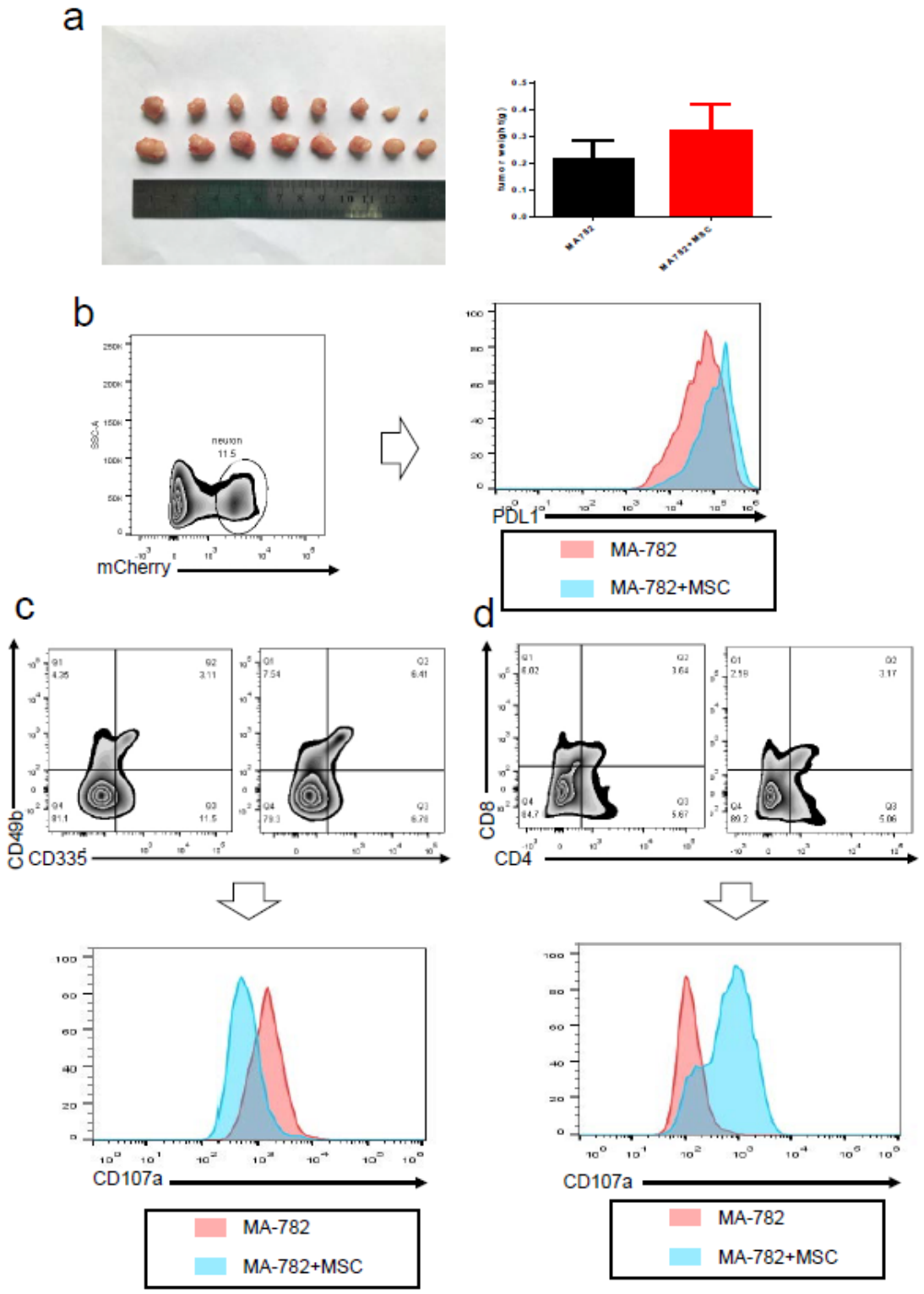

Figure 12

Cancer associated fibroblasts promotes tumor progression and suppress immune cell function in vivo. a the weight of tumors isolated from the mice with MA-782 cells co-injected with or without MSC (n = 8). b PD-L1 expression of MA-782 which gated as mCherry+ c,d CD107a expression of cytotoxicity T cells and NK cells Error bars represent mean ± s.d.; ****P < 0.0001; n.s. not significant; by paired two-sided Student's t-test.