Supplementary material for

Inhibition of KPNA4 Attenuates Prostate Cancer Metastasis

Supplemental Materials and Methods

MTT assay.

Cells were seeded in 96-well plate (1×10^4 cells/well) and cultured with the 1.1 mM MTT reagent (Thermo Scientific, Waltham, MA, USA) at at 37 ℃ for 4 hours. Remove all but 25 μl of the medium from the wells, add 50 μl DMSO and incubate the mixture at 37 ℃ for 10 minutes. Cell viability was determined by reading the absorbance at 540 nm.

q-PCR

Total RNA was purified using Trizol reagent (Invitrogen). We synthesized cDNA using a Taqman reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA). Taqman reverse transcription kit (Applied Biosystems) was applied for cDNA synthesis. SYBR green super mix (Applied Biosystems) was used for real-time PCR in a CFX384 Touch qPCR System (Bio-Rad, Hercules, CA, USA). For microRNA detection, TaqMan microRNA reverse transcription kit and TaqMan microRNA assay kit (Applied Biosystems) were applied for cDNA synthesis and qPCR respectively.

For TNF-α or β detection, NF-κB specific inhibitor 4-N-[2-(4-phenoxyphenyl) ethyl] quinazoline-4, 6-diamine (QNZ) was purchased from Selleck Chemicals (Houston, TX, USA). PC3 cells (2.5×10^5 cells/well) were seeded in 12-well plate and transfected with

KPNA4 overexpression plasmid (Addgene, Cambridge, MA, USA). 24 hours later, the cells were cultured with QNZ (10 μM) for another 48 hours before RNA purification.

X-ray imaging and bioluminescence detection

Hind limbs of the mice were placed on the top of a film and exposed to X-ray for 15 seconds to obtain the images.

Luciferase-labeled PC3 cells were seeded in 6-well plate (2×10^5 cells/well). D-luciferin (Rgis Technologies, Morton Grove, IL, USA) was diluted to 150 μg/mL in the culture medium and cultured with the Luciferase-labeled PC3 cells at 37 ℃ for 10 minutes prior to imaging using the IVIS Lumina XR system (Caliper Life Sciences, Waltham, MA, USA).

qPCR array and cytokines array

PC3-shKPNA4 or scramble control cells were seeded in 6-well plate (2×10^5 cells/well) for 48 hours to collect the conditioned medium (2ml/well).

Raw264.7 cells were cultured using a 1:1 mixture of RPMI 1640 medium and conditioned medium for 24 hours. Total RNA was isolated for qPCR array using an RT² Profiler™ PCR Array Mouse Inflammatory Cytokines and Receptors kit (Qiagen, Venlo, The Netherlands). Conditioned medium was also collected for human cytokines array assays (Abcam, Cambridge, United Kingdom) according to the guide of manufacturer.

Osteoclastic culture and TRAP staining

RAW264.7 cells were seeded in 96-well plate (5000 cells/well) and cultured in an osteoclast differentiation medium (50 ng/mL RANKL). 5ng/mL human recombinant TNF-α or –β, or 10% conditioned medium was supplemented to the osteoclast differentiation medium as indicated. After 4 days, the RAW264.7 cells were fixed in 10% formaldehyde for 10 minutes and washed with PBS. Cells that were TRAP-positive were detected using an Acid Phosphatase kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. To prepare the paraffin bone tissue slides, the samples underwent deparaffinization in xylene, 100% ethanol, 95% ethanol and deionized H_2O; they were then fixed in 10% formaldehyde for 30 seconds and thoroughly rinsed with deionized water, and followed by the TRAP staining. The osteoclastogenesis were then microscopically evaluated.

Supplementary figure legends

S. Figure 1. KPNA4 knockdown inhibits the migration of PCa cells. Cell migration of (**a**) C4-2B and (**c**) LNCaP with indicated transfection were determined by wound healing assay. Representative images at 0 and 48 hours after wounding are show on the left. Relative migration was quantitated by the filled wound area. Cell invasion of (**b**) C4-2B and (**d**) LNCaP with indicated transfection were determined by transwell assay. Invasive cells were quantitated by crystal violet assay to determine the relative invasion. Unpaired t test, *p<0.05, **p<0.01.

S. Figure 2. KPNA4 knockdown inhibits the migration of PC3 cells. (**a**) KPNA4 knockdown efficiency PC3shKPNA4 cell line was determined by western blotting. α-tubulin and Histone H4 were used as loading control of cytoplasm and nuclear fracture respectively. (**b**) Cell migration of PC3shKPNA4 and scramble control cell lines were determined by wound healing assay. Relative migration was quantitated by the filled wound area. (**c**) Cell invasion of PC3shKPNA4 and scramble control cell lines was determined by transwell assay. Invasive cells were quantitated by crystal violet assay to determine the relative invasion. Unpaired t test, $*p<0.05$, $**p<0.01$.

S. Figure 3. KPNA4 knockdown does not affect PC3 cell proliferation and viability. (**a**) Luciferase-labeled PC3shKPNA4 and scramble control cells (2×10^5) were seeded in 6-well plate. Bioluminescence was detected daily for 3 days to generate the growth curve of indicated cell lines. (**b**) Cell viability of indicated cell lines seeded in 96-well plate (1×10^4) was determined by MTT assay.

S. Figure 4. RNA expression of KPNA4 is not up-regulated in prostate cancer versus normal control. Endogenous (**a**) KPNA4 mRNA and (**b**) miR-708 expression level of normal epithenial prostate RWPE-1 or indicated PCa cell lines were evaluated by real-time PCR. Unpaired t test, $***p<0.001$.

S. Figure 5 Absence of KPNA4 diminishes the inhibitory effect of miR-708 on PC3 invasion. Cell invasion of PC3shKPNA4 and scramble control cell lines transfected with miR-708 mimic or negative control mimic were determined by transwell assay. Invasive

cells were quantitated by crystal violet assay to determine the relative invasion. Unpaired t test, *p<0.05, **p<0.01.

S. Figure 6. Robust correlation between PC3 cell number and luciferase activity. *Ex vivo* imaging analysis was performed to determine the correlation between luciferase labeled PC3 cell number and the luciferase activity.

S. Figure 7. X-ray images of hind limbs from the metastatic mouse model. Hind limbs were collected from the intra-cardiac injected mice after 4 weeks post-injection. Both femurs and tibias were fixed and exposed to X-ray for imaging. The metastatic lesions were indicated by the yellow arrows.

S. Figure 8. KPNA4 regulates the production of TNF- α and β through the activation of NF-κB signaling in PC3 cells.

(**a**) Conditioned medium of PC3shKPNA4 or scramble control cell lines seeded in 6-well plate (2×10^5 per well) was collected after 48 hours of culture. Immunoblotting array was performed to detect the cytokines released in the conditioned medium. (**b**) TNF- α and β expression of PC3shKPNA4 or scramble control cell lines was determined by western blotting. β-actin was used as loading control. (**c**) PC3 was transfected with KPNA4 overexpression plasmid and treated with or without NF-κB specific inhibitor QNZ (10μM). RNA level of TNF- α and β was determined by real-time PCR. Non-treated PC3 cells were used as control. Unpaired t test, **p<0.01.

S. Figure 9. TNF-α and -β induction facilitates osteoclastogenesis.

RAW264.7 macrophages (**a-b**) were stimulated with TNF- α or TNF-β or combination (5ng/mL each); or (**c-d**) were cultured with the indicated conditioned medium (10%) with or without the supplement of TNF- α (5ng/mL). TRAP staining was performed to determine the osteoclast differentiation. (**e**) TRAP staining of the femur of mice with bone metastasis lesions. Unpaired t test, *p<0.05, **p<0.01.

S. Figure 10. KPNA4 knockdown induces the inhibition of inflammatory RAW264.7 macrophages. RAW264.7 cells were cultured with the indicated conditioned medium (10%) (**a**) Fold change of regulated genes of inflammatory cytokines&receptors was determined by qPCR array. (**b**) Scatter diagram shows the global tested genes of inflammatory cytokines&receptors.