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## Supplemental information

# Exosomal and intracellular miR-320b promotes lymphatic metastasis in esophageal squamous cell carcinoma

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# Supplementary Materials and Methods

## Antibodies and reagents

The following antibodies were used: anti-LYVE-1, Abcam (ab14917), for IHC; anti-firefly luciferase antibody, Abcam (ab185924), for IHC; anti-PDCD4, Cell Signaling Technology (#9535), for IHC and immunoblot; anti-DGCR8, Abcam (ab191875), for immunoblot, immunoprecipitation and RIP; anti- N6-methyladenosine (m6A), Abcam (ab208577), for immunoblot; anti-METTL3, Abcam (ab195352), for immunoblot and immunoprecipitation; anti-E-cadherin, Cell Signaling Technology, #73195, for immunoblot; anti-Vimentin, Cell Signaling Technology, #5741, for immunoblot; anti-GAPDH, Cell Signaling Technology, #5174, for immunoblot; Control mouse IgG, control rabbit IgG, and anti-m6A were provided in the EZ-Magna RIP kit or Magna MeRIP™ m6A Kit (Millipore). DAPI (Thermo Scientific, 62247) and PKH67 Green Fluorescent Cell Linker Mini Kit (Sigma-Aldrich, MINI67) were also used.

## Lentiviral, plasmid and miRNA mimics package and cell transfection

Lentiviral plasmids encoding miR-320b overexpression, sponge and negative control were designed and produced by Wigene (Shandong, China). KYSE150 and EC9706 cells were transfected with lentivirus at a multiplicity of infection (MOI) of 20 and 20, respectively. The cells were then selected with 1μg/ml (KYSE150) or 2 μg/ml (EC9706) puromycin for 3 days. p-ENTER plasmid containing PDCD4 and negative control were purchased from Wigene. miR-320b mimics and control were produced by Biosune (Shanghai, China). Plasmid, mimics, inhibitor and negative control were transfected using (Invitrogen, California, USA) according to the manufacturer's instructions of Lipofectamine2000. The total RNA and protein were extracted after 48 hours for subsequent experiments.

## Exosome purification and identification

Serum exosome extraction and cell culture medium exosome were extracted using SBI regent or ultracentrifugation as previously described [1-3]. The morphology, quantification and size distribution were analyzed using transmission electron microscopy (TEM, JEM-1-11 microscope, Japan) and ZetaView

instrument and software (Particle Metrix Ltd. Germany), respectively.

### Immunohistochemistry

Immunohistochemistry was done as previously reported [3]. Positive cells were counted in five random fields per slide. Interpretation of staining intensity of anti-LYVE-1, anti-Luciferase and anti-PDCD4 was made independently by two specialists, as no staining=0, weak staining=1 (1-25%), moderate staining=2 (26-50%), and strong staining=3 (51-100%).

### Luciferase reporter assay

Cells were co-transfected with Dual-Luciferase reporter system using pmiR-REPORT$^{TM}$ luciferase vectors containing wild-type or mutant 3`-UTR of PDCD4 and miR-320b mimics or control using Lipofectamine 2000 (Invitrogen, California, USA). Luciferase activity was measured by Dual-Luciferase Reporter Assay System (Beyotime Biotechnology, Shanghai, China) 48h after transfection. Each group was run triplicate in 96-well plates.

### Quantitative real-time PCR

Total RNAs were extracted from cell and exosome by the miRNeasy Micro Kit (QIAGEN, Valencia, CA, USA). RNA was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Takara, Dalian, China). Quantitative real-time PCR was performed using Power TB Green (Takara, Dalian, China) on a CFX96 Real-Time PCR Detection System (Bio-Rad, USA). Data was collected and normalized to U6 (for cellular miR-320b), GAPDH (for cellular PDCD4 mRNA) or miR-16 (for exosomal miR-320b). MicroRNA and mRNA primers were designed and synthesized by Biosune (Shanghai, China). mRNA primers are listed in Supplementary table 2.

### Immunoblotting assay

The cells were lysed using the protein extraction reagent RIPA buffer (Solarbio, China) supplemented with a protease inhibitor cocktail (Solarbio, China). Immunoblotting assay was performed as previously described [4]. Protein band was visualized using an ECL chemiluminescence kit and the intensity was quantified by

densitometry using Image Lab software (Bio-Rad, Hercules, CA, USA).

### CCK-8, colony formation and EdU assays

Cells viability was determined by Cell Counting Kit 8 (Dojindo, Japan) and the optical density (OD) value was measured at 450nm wavelength with the Thermo Scientific Multiskan FC (Thermo Fisher Scientific Corporation, USA).

For the colony formation assays, $1\times10^3$ ESCC cells were seeded into 6-well plates and cultured for 14 days. The colonies were stained with 0.1% crystal violet and visible colonies were counted.

For EdU assays, ESCC cells were seeded in 24-well plates and incubated for 24h. Cells were stained with EdU and DAPI according to the manufacturer's instructions of the EdU kit (RioboBio, Guangzhou, China). The images were obtained with Zeiss laser scanning microscope system (Zeiss, Germany).

### Cell migration and invasion assay

200μl cell suspension ($2.5\times10^5$ cells/ml) was inoculated in the apical chamber with or without the Matrigel membrane, and the culture medium containing 20% fetal bovine serum was added to the basolateral chamber. The cells passing through the membrane in each group was stained with 0.1% crystal violet and counted by inversion microscope (Zeiss, Germany).

### ELISA analysis

VEGF-C in the culture supernatants of KYSE150 cells was quantified using the Human VEGF-C Quantikine ELISA Kit (Elabscience, Wuhan, China) according to the manufacturer's protocol.

### Co-immunoprecipitations assay

Co-immunoprecipitation was performed in KYSE150 cells using Co-Immunoprecipitation Kit (ThermoFisher Scientific, USA) according to the manufacturer's protocol. Briefly, immunoprecipitations of METTL3 were performed using an anti-METTL3 antibody overnight at 4°C. After washing, the immunoprecipitated complex was treated with either RNase A or RNase inhibitor (Sigma Aldrich, USA) for 5 min at 37°C. Anti-DGCR8, anti-METTL3 or anti-m6A antibody was used for immunoblot analysis.

## Supplementary Table 1 Clinical characteristics

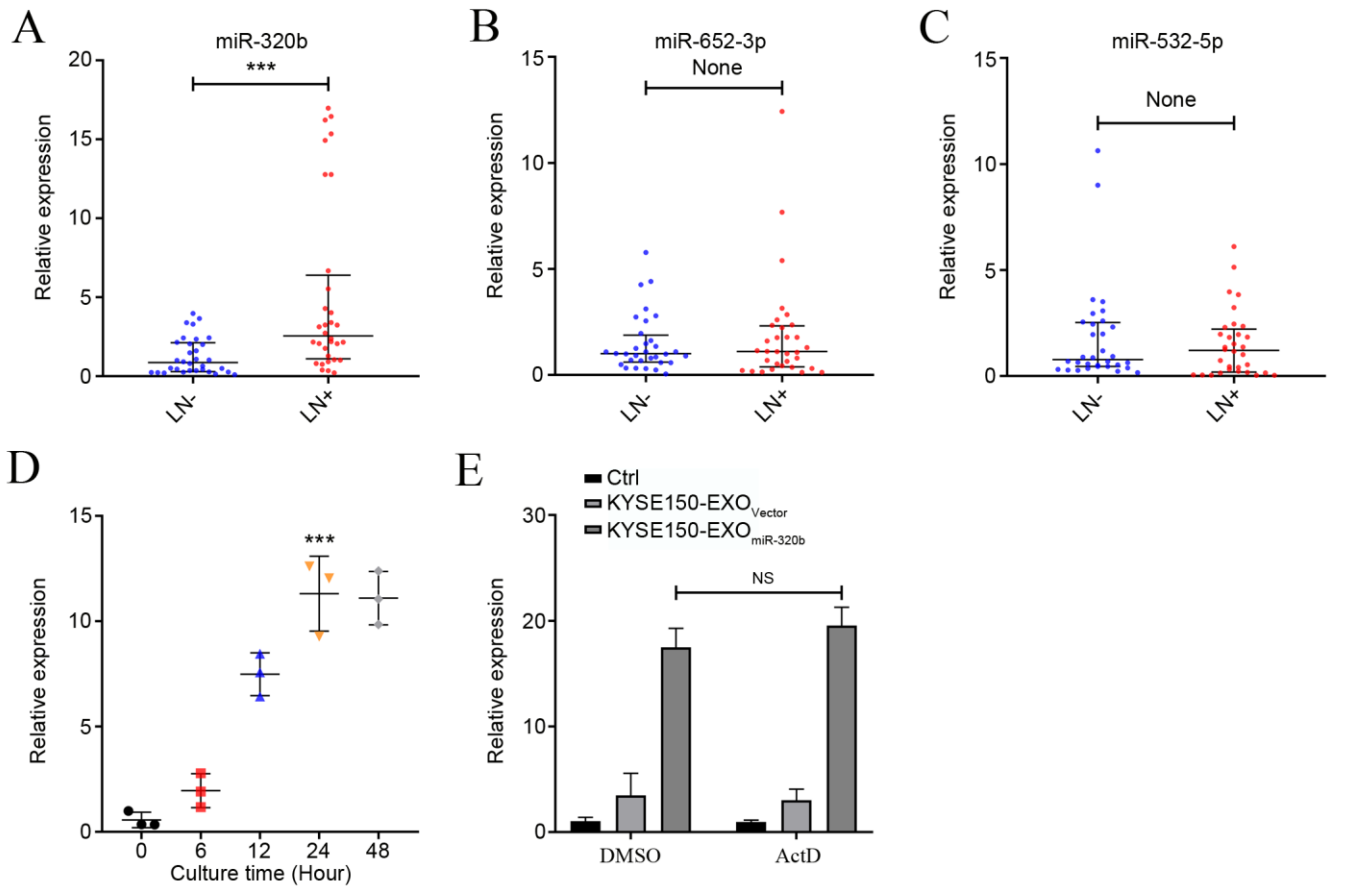
| Variable, N (%) | Cohort 1 | | | Cohort 2 | | |
|---|---|---|---|---|---|---|
| | Total | LNM (-) | LNM (+) | Total | LNM (-) | LNM (+) |
| **Sex** | | | | | | |
| Male | 128(71.9) | 68(71.6) | 60(72.3) | 137(72.9) | 61(67.0) | 76(78.4) |
| Female | 50(28.1) | 27(28.4) | 23(27.7) | 51(27.1) | 30(33.0) | 21(21.6) |
| **Age** [a] | | | | | | |
| <62 | 80(44.9) | 46(48.4) | 34(41.0) | 94(50.0) | 46(50.5) | 48(49.5) |
| ≥62 | 98(55.1) | 49(51.6) | 49(59.0) | 94(50.0) | 45(49.5) | 49(50.5) |
| **Tumor location** | | | | | | |
| Cervical-Middle thoracic | 102(57.3) | 49(51.6) | 53(63.9) | 111(59.0) | 55(60.4) | 56(57.7) |
| Low thoracic | 76(42.7) | 46(48.4) | 30(36.1) | 77(41.0) | 36(39.6) | 41(42.3) |
| **Differentiation** | | | | | | |
| Well-Moderate | 107(60.1) | 70(73.7) | 37(44.6) | 105(55.8) | 57(62.6) | 48(49.5) |
| Poor | 71(39.9) | 25(26.3) | 46(55.4) | 83(44.2) | 34(37.4) | 49(50.5) |
| **T stage** | | | | | | |
| T1-T2 | 85(47.8) | 55(57.9) | 30(36.1) | 86(45.7) | 51(56.0) | 35(36.1) |
| T3-T4 | 93(52.2) | 40(42.1) | 53(63.9) | 102(54.3) | 40(44.0) | 62(63.9) |
| **Tumor size** [b] | | | | | | |
| <4.0 | 94(52.8) | 64(67.4) | 30(36.1) | 95(50.5) | 58(63.7) | 37(38.1) |
| ≥4.0 | 84(47.2) | 31(32.6) | 53(63.9) | 93(49.5) | 33(36.3) | 60(61.9) |
| **SCC level** | | | | | | |
| Normal | 108(60.7) | 57(60.0) | 51(61.4) | 122(64.9) | 57(62.6) | 65(67.0) |
| Abnormal | 70(39.3) | 38(40.0) | 32(38.6) | 66(35.1) | 34(37.4) | 32(33.0) |
| **CEA level** | | | | | | |
| Normal | 113(63.5) | 63(66.3) | 50(60.2) | 126(67.0) | 63(69.2) | 63(64.9) |
| Abnormal | 65(36.5) | 32(33.7) | 33(39.8) | 62(33.0) | 28(30.8) | 34(35.1) |
| **CT reported LN status** | | | | | | |
| Negative | 107(60.1) | 70(73.7) | 37(44.6) | 105(55.8) | 67(73.6) | 38(39.2) |
| Positive | 71(39.9) | 25(26.3) | 46(55.4) | 83(44.2) | 24(26.4) | 59(60.8) |
| **Lymphovascular invasion** | | | | | | |
| Negative | 92(51.7) | 57(60.0) | 35(42.2) | 94(50.0) | 56(61.5) | 38(39.2) |
| Positive | 86(48.3) | 38(40.0) | 48(57.8) | 94(50.0) | 35(38.5) | 59(60.8) |

[a] The average age was 62.

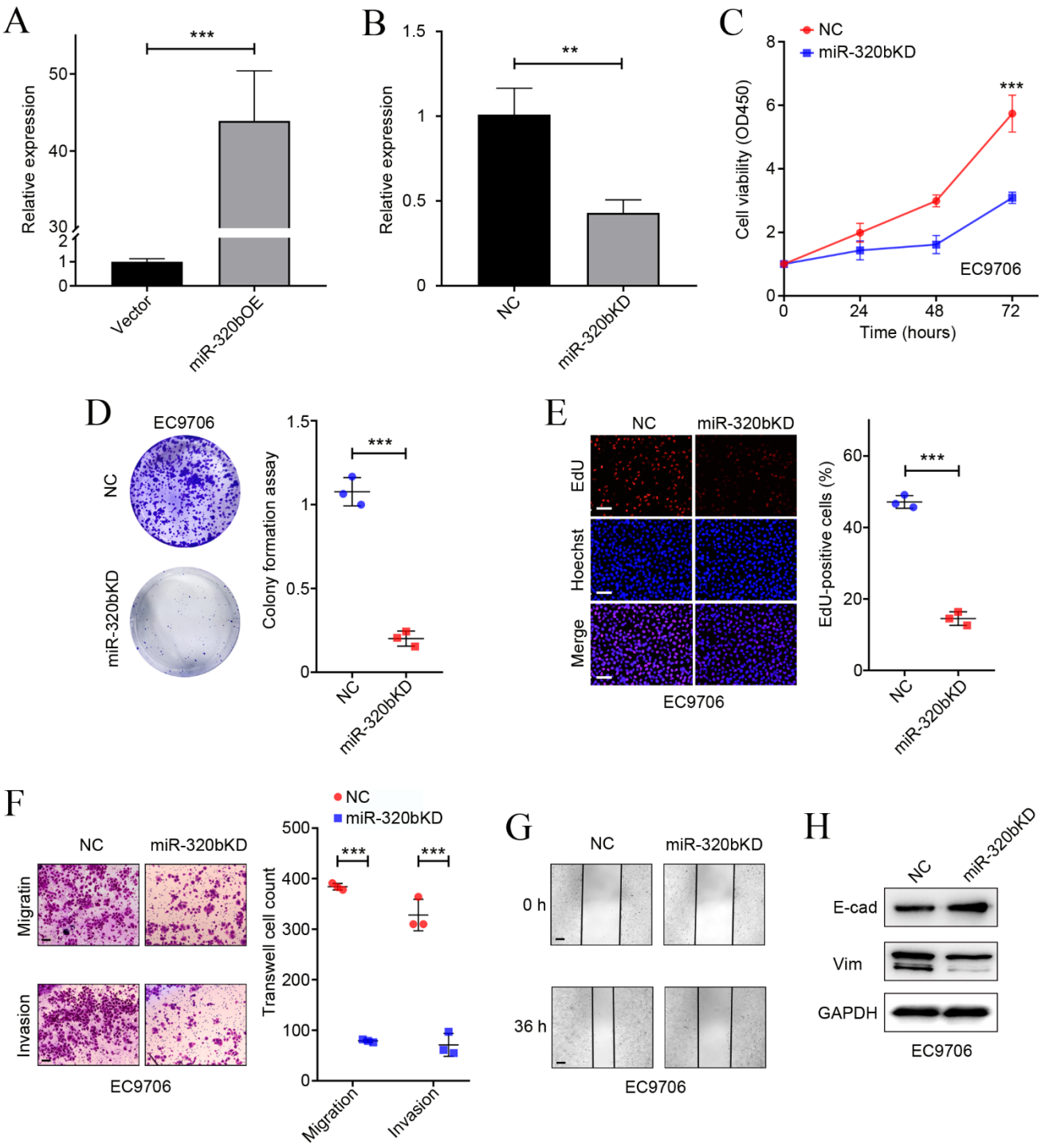
[b] Tumor size measured in longest diameter (cm), and the mean was 4.0cm.

**Supplementary Table 2 Primer sequences used in this manuscript.**

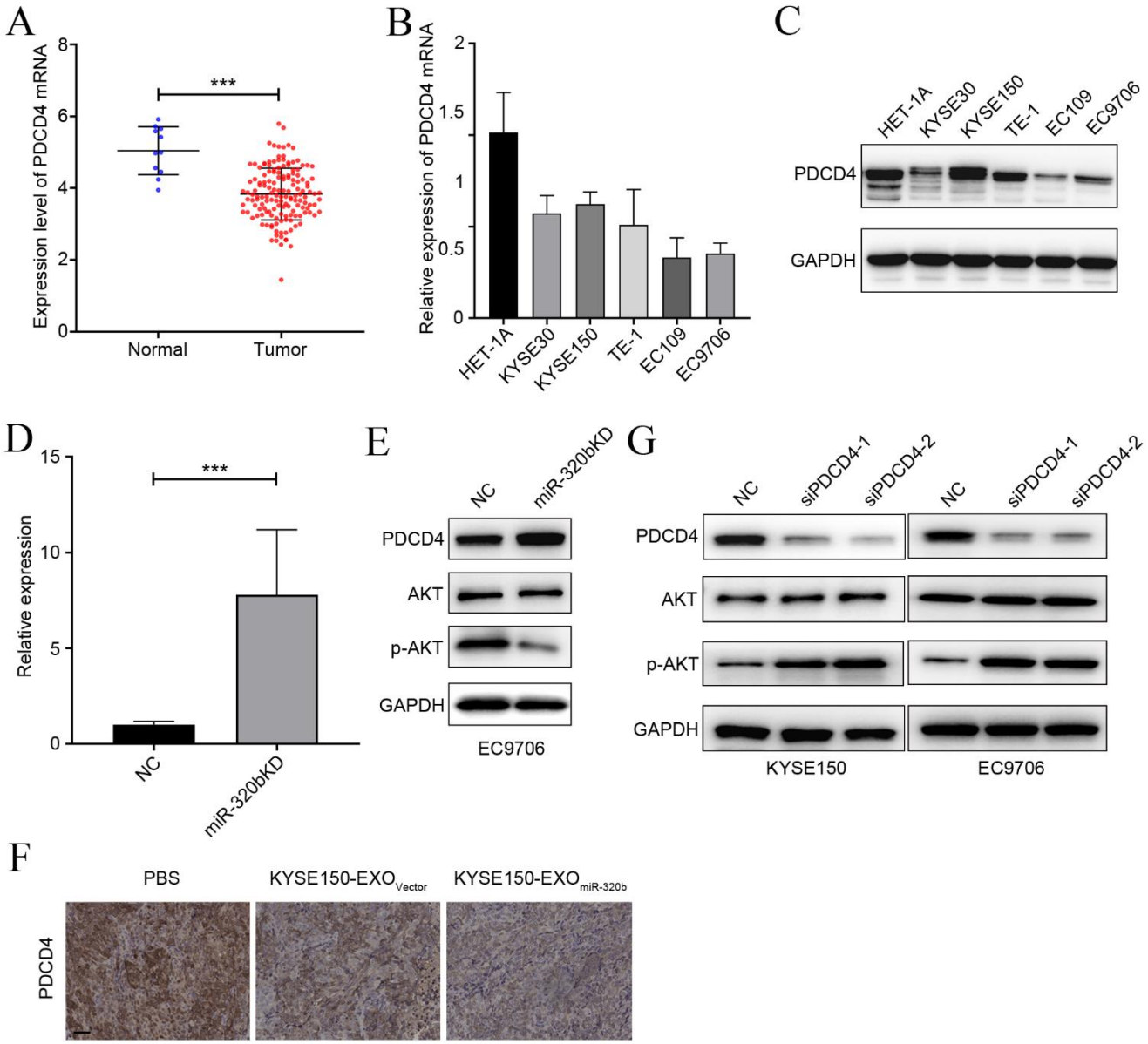
| Primer name | Forward primer sequence | Reverse primer sequence | Application |
|---|---|---|---|
| PDCD4 | AGGCAAAAAGGCGACTAAGGA | TCCAGCAACCTTCCCTTTGG | qRT-PCR |
| METTL3 | GTGTCAGGGCTGGGAGACTA | TAGATCCAAGTGCCCCGAGT | qRT-PCR |
| GAPDH | GCACCGTCAAGGCTGAGAAC | TGGTGAAGACGCCAGTGGA | qRT-PCR |
| Pri-miR-320b | AGCTGTTAGGCAATCTCTCCTTA | CAGCAATCCTTGCTCTGAGG | qRT-PCR |
| Pre-miR-320b | GTCTCTTAGGCTTTCTCTTCCCAG | TTTTTCCTTTTGCCCTCTCAAC | qRT-PCR |
| pENTER | CGCAAATGGGCGGTAGGCGTG | CCTCTACAAATGTGGTATGGC | plasmid |
| PDCD4 | CGCAAATGGGCGGTAGGCGTG | CCTCTACAAATGTGGTATGGC | plasmid |
| METTL3 | CGCAAATGGGCGGTAGGCGTG | CCTCTACAAATGTGGTATGGC | plasmid |
| siPDCD4-1 | CUGGGACUGAGGAAAUAAATT | UUUAUUUCCUCAGUCCCAGTT | siRNA |
| siPDCD4-2 | GCCCUUAGAAGUGGAUUAATT | UUAAUCCACUUCUAAGGGCTT | siRNA |
| siMETTL3-1 | CCUGCAAGUAUGUUCACUATT | UAGUGAACAUACUUGCAGGTT | siRNA |
| siMETTL3-2 | GCUACCUGGACGUCAGUAUTT | AUACUGACGUCCAGGUAGCTT | siRNA |
| miR-320b mimics | AAAAGCUGGGUUGAGAGGGCAA | GCCCUCUCAACCCAGCUUUUUU | mimics |



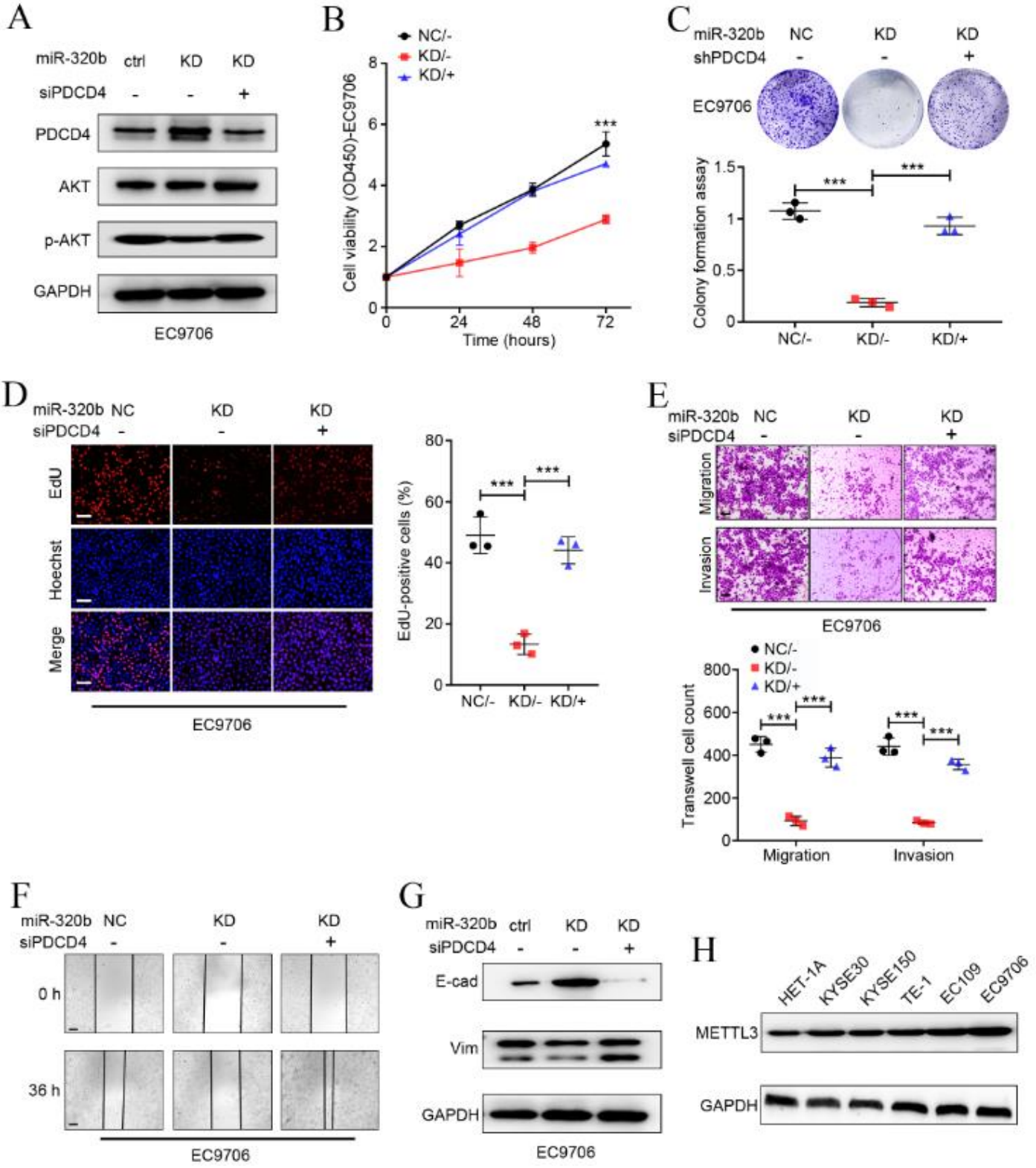

**Supplementary Figure 1. Upregulation of exosomal miR-320b is associated with LN metastasis**




**Supplementary Figure 2. miR-320b overexpression promote ESCC malignant phenotypes.**




**Supplementary Figure 3. Knockdown PDCD4 evokes oncogenic of ESCC cells.**




**Supplementary Figure 4. miR-320b promotes ESCC cell malignant phenotypes by targeting PDCD4.**

# REFERENCE

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