Supplementary Materials for
Enhanced Cholesterol biosynthesis promotes breast cancer metastasis via modulating
CCDC25 expression and neutrophil extracellular traps formation
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Supplementary Materials and Methods

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using RNeasy Mini kit (Qiagen) and reverse transcription was performed using a reverse transcription kit (Vazyme, Nanjing, China). Complementary DNA (cDNA) was synthesized using HiScript II Q RT SuperMix (R223-01, Vazyme). Real-time PCR was completed using SYBR Green PCR Master Mix (Takara, Otsu, Japan) on QuantStudioTM 7 Flex (Thermo Fisher Scientific). All target genes were normalized to cyclophilin B. Gene expression was quantified using the delta Ct method.

Primer sequences were as follows:

**human HMGCR**: forward, TGATTGACCTTCCAGAGCAAG  
reverse, CTAAAATTGCCATTTCCAGGAGC

**human ASPP2**: forward, AGATAGTGATGGGATGGACGCC  
reverse, CCATCTTCTCCTGAACGCA

**mouse ASPP2**: forward, 5′-AGATAGTGATGGGATGGACGCC-3′  
reverse, 5′-CTGGGTCATCTTTTCACGGT-3′

**mouse HMGCR**: forward, 5′-CGATCCTTCCTTATTGGCGG-3′  
reverse, 5′-CGGATCTCAATGGAGGCCA-3′

**mouse CD133**: forward, 5′-CTGGACCAGGAGGGATG-3′  
reverse, 5′-AGCCATAGTTGGCCCTGA′

**mouse EpCAM**: forward, 5′-ATCGCCTGTCAATTGTGGTGTG-3′  
reverse, 5′-ACCACATCTCCCTCTATCTCAGCC-3′

**mouse OCT-4**: forward, 5′-CAGTGGGGCGCTTCTTTGAGT-3′
reverse, 5′-GGCTGAACACCTTTCCAAAGAGA-3′

mouse CD44 forward, 5′-CACCTTGGCCACCCTCCTAAT-3′

reverse, 5′-GATGGTTGGTGTGGGGCGAA-3′

**Lentivirus generation and infection**

Lentivirus was purchased from Hanheng Biological Technology Co., Ltd (Hanbio, Shanghai, China). The virus titers were all $\geq 10^9$ TU/ml. We used the 1/2 small volume infection method to explore the most suitable MOI for follow-up experiments. 4T1 cells were cultured in a 12-well plate. When the cell confluence rate reached 40%, 500μl medium was added to each well after washing with PBS, and then 2 μg/ml polybrene has been adjuncted before 10, 15, and 20μl of virus were added respectively. After 72 hours of culture, cellular RNA was extracted for RT-PCR detection to analyze infection efficiency and optimal MOI value. All lentiviruses were designed with three siRNA sequences and synthesized three shRNAs. The most suitable sequences were verified and selected for subsequent experiments. In the subsequent culture and experiments of lentivirus-infected cells, 2μg/ml puromycin was added from time to time to construct a stable lentivirus strain.

**Isolation of neutrophils**

Mouse neutrophils were collected from female BALB/c mice. Neutrophil isolation kit (2013NM, TBD, Tianjin, China) was used to extract and isolate neutrophils from mouse femur and tibia. Use homogenate to rinse out the lumen. After blowing and beating into a single cell suspension, filter with a 70um filter. Centrifuge at 500g for 10min, resuspend the cells with erythrocyte sedimentation solution, and add it to the separation
solution of the concentration gradient. 800g, centrifuge for 30min. Take the middle cell layer and add 3 times its volume of washing solution to resuspend. Centrifuge at 400g for min. Add erythrocyte lysis solution to remove red blood cells. After centrifugation, add washing solution to wash and centrifuge again to obtain neutrophil pellet. Add appropriate amount of DMEM medium to resuspend, and follow-up experiments can be carried out.

**Wound-Healing assay**

Tumor cells were culture in 6-well plant, until cell density reached to 90%. 10μl pipette tip was used to scar the cells. Cell wound healing ability was calculated using a Leica microscope at 0h, 12h and 24h. All assays were conducted three times.

**Immunohistochemical staining**

Briefly, the slide is dewaxed, hydrated, quenched endogenous peroxidase activity, recovered antigen, blocked and combined with anti-HMGCR (1:100, #271595, Santa cruz), anti-CCDC25 (1:100, #515201, Cell Signaling Technology), anti-MPO (1:200, #AF3667, R&D), anti-H3cit (1:300, #4499, Cell Signaling Technology), or anti-Caveolin-1 antibody (1:100, A4480, Cell Signaling Technology) overnight at 4°C. Then rinsed the sections, added separated secondary antibodies labeled with horseradish peroxidase, and incubate at 37°C for 1 h. After washing 3 times, use the diaminobenzidine colorimetric reagent solution from Dako (Carpinteria, CA). Subsequently, the slides were counterstained with hematoxylin and dehydrated in graded alcohol and mounted. The expression of CCDC25, Caveolin-1, HMGCR, MPO and H3cit was scored according to the signal intensity and distribution. We used a two-
level scoring method, staining degree 0-3 points (negative, light yellow, light brown, tan), and positive range 1-4 points (0-25%, 26%-50%, 51%-75%, 76%-100 %). The staining degree points were multiplied with positive range points to produce a final score for each case. Tissues with immunohistochemical scoring 2 or less were considered as low, 3 to 12 as high. The immunostaining evaluation was performed independently by two experienced pathologists.

**Measurement of serum MPO-DNA level**

Anti-MPO monoclonal antibody (5μg/ml, R&D) was coated on a 96-well plate overnight at 4℃. Blocked with 1% BSA for 1h at room temperature then added 100μl serum diluted at 1:5 with normal saline, incubate at room temperature for 2h. After washed with PBST for five times, PicoGreen® dsDNA Quantitation Reagent (YEASEN, Shanghai, China) was used to measure MPO-DNA content based on the manufacturer’s protocol. Data were normalized to healthy mouse serum for each test. All assays were conducted at least 3 times.
Fig. S1  

**a** 4T1 and MDA-MB-231 cells cultured in 6-well plate to verify the efficiency of lentiviral interference with ASPP2 and HMGCR.  
**b** 4T1 cell treated with 2μM Simvastatin, 10μM Berberine, 2μM Simvastatin and 10μM Berberine or DMSO in 12 and 24 hours for viability assay.  
**c-d** LV-shHMGCR (c) and LV-shASPP2 (d) lentiviral sequence screening and optimal MOI. Finally, we selected the LV-shHMGCR lentivirus sequence number 56 or LV-shASPP2 lentiviral sequence 38 to infect 4T1 cells. When infecting in a six-well plate, add 20μl lentivirus solution per well. **P < 0.01; ***P < 0.001.**
Fig. S2 Analysis of ASPP2 and HMGCR mRNA expression by qRT-PCR in 4T1 cells by different treatment.
Fig. S3  

**a** Wound healing assay (top) and migration areas (down) of treated 4T1 cells. **b** 4T1 cells cultured with Matrigel in the upper chamber of the Transwells. 10μM BBR, 2μM simvastatin or DMSO was added to shASPP2 4T1 cells. 20% serum containing medium were add to the bottom wells. After cultured 22-26h, counted invading cells by a light Microscope. Invasion was calculated by compared with invading cells in shNon group. Scale bar: 200μm.
**Fig. S4** NETs formation was analyzed after adding MVA pathway additives MVAPP to BBR and simvastatin treated 4T1 cells to recover cholesterol metabolism levels. Scale bar: 50µm. *P < 0.05; ** P < 0.01; *** P < 0.001.
Supplementary Tables

Supplementary Table 1

The clinicopathologic characteristics of 60 breast cancer patients

| Variables                      | No. of patients(%) |
|-------------------------------|--------------------|
| Gender                        |                    |
| Male                          | 0 (0)              |
| Female                        | 60 (100)           |
| Age (year)                    |                    |
| <50                           | 16 (27)            |
| ≥50                           | 44 (73)            |
| AJCC stage                    |                    |
| I - II                        | 17 (28)            |
| III - IV                      | 27 (45)            |
| Tissue classification         |                    |
| Normal or paracancerous tissue| 11 (18)            |
| Primary breast cancer         | 27 (45)            |
| Metastatic tissue             | 17 (28)            |
| Tumor-free lymph nodes        | 5 (8)              |
| Primary tumor with metastasis |                    |
| Yes                           | 24 (87)            |
| No                            | 3 (13)             |
| Metastatic organ              |                    |
| Liver                         | 3 (27)             |
| Breast                        | 5 (45)             |
| other                         | 3 (27)             |
Supplementary Table 2

The HMGCR, CCDC25 and H3cit expression with clinicopathologic characteristics in 27 breast cancer patients

|                      | Whole study(n=27) | Whole study(n=27) | Whole study(n=27) |
|----------------------|-------------------|-------------------|-------------------|
|                      | HMGCR expression  | P                 | CCDC25 expression | P        | H3cit expression | P       |
|                      | negative (n=9)    | positive (n=18)   | negative (n=7)    | positive (n=20) | negative (n=6) | positive (n=21) |
| Age(years)           |                   |                   |                   |           |                   |         |
| <50                  | 3                 | 6                 | 1.00              | 4         | 5                 | 0.175   |
| ≥50                  | 6                 | 12                | 3                 | 15        | 3                 | 15      |
| Lymph node metastasis number |         |                   |                   |           |                   |         |
| <5                   | 6                 | 8                 | 0.42              | 4         | 10                | 1.00    |
| ≥5                   | 3                 | 10                | 3                 | 10        | 2                 | 11      |
| TNM Stage            |                   |                   |                   |           |                   |         |
| I                    | 3                 | 4                 | 0.387             | 1         | 6                 | 0.544   |
| II                   | 3                 | 7                 | 4                 | 6         | 1                 | 9       |
| III                  | 3                 | 4                 | 1                 | 6         | 1                 | 6       |
| IV                   | 0                 | 3                 | 1                 | 2         | 1                 | 2       |

