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Caprylic acid (C8:0) promotes bone metastasis of prostate cancer by dysregulated adipo-osteogenic balance in bone marrow

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Abstract

Prostate cancer (PCa) continues to be the most common, noncutaneous cancer in men. Bone is the most frequent site of PCa metastases, and up to 90% of patients with advanced PCa develop bone metastases. An altered bone marrow microenvironment, induced by obesity, is a significant mediator for the bone tropism of PCa. However, the specific molecular mechanisms by which obesity causes changes in the bone marrow microenvironment, leading to PCa bone metastasis, are not fully understood. Our results demonstrate that a high-fat diet (HFD) leads to dyslipidemia and changes in bone marrow of nude mice: an increase in the area and number of adipocytes and a reduction in the area and number of osteoblasts. Moreover, a HFD promoted cyclooxygenase 2 (COX2) expression and inhibited osteoprotegerin (OPG) expression in the bone microenvironment. Additionally, the total level of free fatty acids (FFAs) and caprylic acid (C8:0) was significantly higher in PCa patients with bone metastases. In vitro, caprylic acid (C8:0) promoted bone mesenchymal stem cell (MSC)-derived adipocytic differentiation, COX2 expression, and prostaglandin E2 (PGE2) secretion, whereas osteoblastic differentiation and OPG expression were reduced. Furthermore, caprylic acid (C8:0)-treated adipocytes promoted the invasion and migration of PCa cells. Taken together, our findings suggest caprylic acid (C8:0) promotes bone metastasis of PCa by dysregulated adipo-osteogenic balance of bone marrow.

KEYWORDS

adipo-osteogenic balance, bone marrow microenvironment, caprylic acid (C8:0), obesity, prostate cancer bone metastasis

Cuizhe Wang, Jingzhou Wang and Keru Chen contributed equally to this work.

1 | INTRODUCTION

Prostate cancer (PCa) continues to be the most common noncutaneous cancer, and the third leading cause of cancer-related deaths, among men in the United States.[1] Moreover, while the incidence is lower, PCa cases have been steadily increasing in Asian countries for the past few decades.[1] Growing evidence suggests that the bone marrow microenvironment is a significant contributor to the bone tropism of PCa.[2] Bone is the most frequent site of PCa metastases, and up to 90% of patients with advanced PCa disease have bone metastases.[2]

Obesity is a highly significant risk factor for PCa progression and aggressiveness.[3] Previous findings from our laboratory, and other reports in the literature, have demonstrated that obesity can alter the bone marrow microenvironment and a high-fat diet (HFD) can lead to increased fat levels in bone marrow. In turn, this contributes to reduced trabecular bone volume and bone mineral density.[4,5] Mesenchymal stem cells (MSCs) have the capacity to differentiate to osteoblasts and adipocytes.[6] Numerous in vitro investigations have demonstrated that fat induction factors inhibit osteogenesis, and, conversely, bone induction factors hinder adipogenesis.[7] However, the contribution of obesity-induced dysregulation of the adipo-osteogenic balance to the growth and metastasis of PCa has not been fully elucidated.

Prostaglandin E2 (PGE2) is one of the most abundant lipid mediators in the human body.[8] It is constitutively produced in nearly all tissues by the coordinate enzymatic activities of cyclooxygenases (COX) and terminal PGE synthases. PGE2 acts on four types of prostaglandin E receptor (EP) subtypes (EP1-EP4) to exert its physiologic functions.[8,9] It has been reported that elevated expression of COX2 and concomitant production of PGE2 by adipocytes in bone marrow may contribute to the prosurvival signaling in PCa cells.[10] Osteoprotegerin (OPG), secreted by osteoblasts and bone marrow stromal cells, competitively binds to receptor activator of nuclear factor-κB ligand (RANKL). By inhibiting the interaction between RANKL and receptor activator of nuclear factor-κB (RANK), OPG regulates osteoclast differentiation, activation, and function.[11] Moreover, high levels of OPG inhibit PCa bone metastasis by blocking RANKL activity.[12] Thus, increased expression of COX2 and PGE2 from adipocytes and decreased OPG from osteoblasts may be linked to growth and metastasis of PCa.

However, the specific mechanisms underlying obesity-induced dysregulation of adipo-osteogenic balance are not fully understood. It has been reported that obesity can cause an increase in plasma free fatty acid (FFA) levels, eventually leading to lipid metabolism disorders.[13] FFAs exhibit considerable heterogeneity with respect to the length and spatial structure of the carbon chains; the many FFA subtypes play diverse roles in the human body.[14,15] Notably, increased FFA levels correlate with the occurrence and progression of PCa.[16] Our previous findings have shown that a mixture of oleic acid and palmitic acid promoted the development of PCa by upregulating peroxisome proliferator-activated receptor gamma (PPAR-γ).[17] Previous data from MD Brown demonstrated arachidonic acid is a potent inducer of adipocytic differentiation, whereas linoleic acid, α-linolenic acid, eicosapentaenoic acid, and oleic acid are not.[18] More importantly, arachidonic acid can promote PCa invasion by inducing bone marrow adipocyte formation.[18] It remains unknown if any other types of FFA are involved in obesity-related PCa bone metastasis.

In the present study, we first demonstrated that the level of caprylic acid (C8:0) was significantly higher in patients with PCa than normal individuals. In nude mice, HFD caused an increase in the area and number of adipocytes and a reduction in the area and number of osteoblasts. Moreover, HFD promoted COX2 expression and inhibited OPG expression in the bone microenvironment. In vitro, caprylic acid (C8:0) promoted adipocytic differentiation of MSCs, COX2 expression, and PGE2 secretion, whereas it inhibited osteoblastic differentiation of MSCs and OPG expression. Caprylic acid (C8:0)-induced adipocytes promoted the invasion and migration of PCa cells. Taken together, our findings suggest caprylic acid (C8:0) promotes growth and metastasis of PCa by dysregulated adipo-osteogenic balance of bone marrow.

2 | MATERIALS AND METHODS

2.1 | Animals

Eight 4-week-old male nude mice (BALB/c-nu) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. After adaptive feeding for 1 week, four mice were fed with normal diet (ND) containing 10% fat, and four mice were fed with HFD containing 60% fat (Medicience Ltd). Lee's index was used to evaluate the obese degree of mice (Lee's index = $[(\text{body weight (g)} \times 10^3)/\text{body length (cm)}]^{1/3}$). After HFD feeding for 9 weeks, the mice were euthanized and tissue samples were collected. All experiments involving mice were performed in accordance with the protocol approved by the Medical Ethics Committee at the First Affiliated Hospital, Shihezi University School of Medicine (reference number: A2017-115-01).

2.2 | Immunohistochemistry

Femur specimens were set in 10% neutral formaldehyde and embedded in 4 μm thick paraffin. After dewaxing, the sections were prepared with sodium citrate buffer, washed in 1X phosphate-buffered saline (PBS), and incubated overnight at 4°C with anti-COX2 antibody (Abcam) or anti-OPG antibody (Abcam) diluted in PBS containing 3% BSA. Anti-mouse HRP-conjugated secondary antibody (DAKO) was added, and the specimens were incubated at 37°C for 30 minutes. The scoring criteria are as follows: percentage of positive cells (0%-5%, 0; 6%-25%, 1; 26%-50%, 2; 51%-75%, 3; 76%-100%, 4), positive staining intensity (negative, 0; yellow, 1; brownish yellow, 2; brown, 3), and the final total score is equal to the percentage of positive cells multiplied by the positive staining intensity.

2.3 | Hematoxylin and Eosin (H&E) staining

Femurs were immersed in 4% paraformaldehyde for 4 hours and then transferred to 70% ethanol. Femur biopsy was placed in processing cassettes, dehydrated through a serial alcohol gradient, and then embedded in paraffin wax blocks. Before immunostaining, the femur was dewaxed in xylene, rehydrated through decreasing concentrations of ethanol, and washed in PBS. Prepared specimens were then stained with hematoxylin and eosin (shxibio). After staining, the sections were dehydrated through increasing concentrations of ethanol and xylene.

2.4 | Human samples and measurement of biochemical indices

From September 2017 to March 2018, blood samples of 32 individuals were collected in the Department of Urology, First Affiliated Hospital of Shihezi University School of Medicine. The subjects were divided into three groups: normal individuals without PCa (n = 16), patients with PCa (n = 8), and patients with PCa bone metastasis (n = 8). General data including age, weight, and body mass index (BMI = body weight (kg)/height $(m)^2$) were collected. Total prostate-specific antigen (TPSA), FFA, triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and fasting plasma glucose (FPG) were measured.

For inclusion in the study, PCa patients had to meet specific criteria: TPSA > 4.57 ng/L, pathological diagnosis of PCa or PCa bone metastasis, and no prior history of diabetes mellitus (DM), other tumors, acute inflammation, or liver and kidney diseases. The consent form and ethical approval were provided by the Medical Ethics Committee at the First Affiliated Hospital, Shihezi University School of Medicine (reference number 2017-049-01).

2.5 | Preparation of caprylic acid(C8:0) solution

Caprylic acid (C8:0) (Sigma-Aldrich) was added to 3 mL 0.1 mol/L NaOH and then incubated at 75°C for 30 minutes, until the liquid became colorless and transparent. Immediately, the caprylic acid (C8:0) solution was quickly added to 3 mL 40% BSA solution. BSA (biofroxx) was not containing fatty acid (FA), and was dissolved in PBS to obtain BSA solution. The resultant solution was 40 mmol/L and stored at −20°C.

2.6 | Cell lines and culture conditions

Human MSCs (Cyagen) were cultured in human mesenchymal stem cell growth medium (hMSC-GM) supplemented with 10% fetal calf serum (Cyagen). PC3 and 22RV1 cells were purchased from the Chinese Academy of Sciences cell bank. PC3 cells were cultured in F12 supplemented with 10% FBS, and 22RV1 cells were cultured in RPMI 1640 supplemented with 10% FBS. All cells were maintained in a 37°C humidified incubator, ventilated with 5% CO_2.

For adipogenic differentiation, cells were grown in adipogenic medium (culture medium supplemented with 10 ng/mL insulin, 1 μmol/L dexamethasone, 60 nmol/L Rosiglitazone, and 0.5 mmol/L isobutylmethyl-xanthyne; Sigma-Aldrich). For adipocyte maintenance, the culture medium was supplemented with 10 ng/mL insulin. For osteogenic differentiation, cells were grown in osteogenic medium (culture medium supplemented with 50 nmol/L ascorbic acid, 10 mmol/L β-glycerol phosphate, and 100 nmol/L dexamethasone; Sigma-Aldrich). In caprylic acid (C8:0)-treated samples, caprylic acid (C8:0) was added to the medium at a final concentration of 100 μmol/L unless otherwise noted. After the adipogenic and osteogenic differentiation had been complete, the culture medium was harvested. The stored or fresh medium was then used as conditioned medium for assays with PC3 and 22RV1 cells.

2.7 | Quantitative Real-Time PCR

The reverse transcription (Eppendorf AG) program settings were 42°C for 60 minutes and 70°C for 15 minutes. The mRNA expression level was detected by using a qRT-PCR instrument Rotor-Gene Q (QIAGEN) with the following program settings: 95°C for 3 minutes, 45 cycles at 95°C for 10 seconds, and 60°C for 30 seconds. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Data were obtained as CT (cycle threshold) values, and the $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ method was used in the analysis. Table S1 shows the primer sequences.

2.8 | Western blot

The separated proteins were electroblotted onto PVDF membranes and blocked for 2 hours at room temperature with Tris-buffered saline containing 5% BSA. Nylon membranes were incubated at 4°C overnight with antibodies to GAPDH (36 kDa; Zhongshan Jinqiao), CCAAT-enhancer–binding protein-α (CEBPα; 43 kDa; Abcam), PPARγ (50 kDa; Abcam), COX2 (68 kDa; Abcam), adiponectin (APN; 30 kDa; Abcam), vimentin (57 kDa; Abcam), matrix metallopeptidase 2 (MMP2; 72 kDa; CST), OPG (46 kDa; Abcam), and Runt-related transcription factor 2 (RUNX2; 55 kDa; Abcam). The second antibody was incubated at room temperature for 2 hours at a working ratio of 1:10,000. The immune complexes were detected by chemiluminescence (Thermo Scientific).

2.9 | Cell Counting Kit-8 (CCK8) assay

MSCs in logarithmic growth phase were inoculated into 96-well plates (1×10^4 cells/well). After 24 hours of culture, the wells were divided into three groups: blank (unstimulated cells), cell-free (medium only), and caprylic acid (C8:0) stimulated. Each group was assessed in quadruplicate. The cells were stimulated for 24, 48, 72,

and 96 hours. To determine viability, 10 μL CCK8 solution was added to each well, and then the plate was incubated (37°C, 5% CO_2) for 2 hours. The absorbance value (CD value) at 450 nm was detected by a microplate reader (Bio-rad), and the growth curve of different groups of cells was plotted continuously for 5 days.

2.10 | Cell migration assay

PC3 and 22RV1 cells were transferred to the upper transwell chamber (3×10^4 cells/room), a polycarbonate filter membrane with a diameter of 6.5 mm and pore size of 8 μm with a serum-free cell suspension, and a different conditioned medium in the lower chamber. After 48 hours of incubation, the upper surface of the transwell membrane was gently wiped with a cotton swab to remove nonmigrating cells. Cells settled on the lower surface of the membrane and were stained using crystal violet for 7-8 minutes and counted microscopically. The mean average value was calculated based on nine different observation fields.

2.11 | Cell invasion assay

The upper transwell chamber was covered with matrigel and incubated for 48 hours in an incubator at 37°C. PC3 and 22RV1 cells were transferred to the upper transwell chamber (3×10^4 cells/room), and a different conditioned medium was used in the lower chamber. After 48 hours of culture, cells that moved to the lower surface of the membrane were stained using crystal violet for 7-8 minutes and counted microscopically. The mean cell count was calculated based on nine different observation fields.

2.12 | ELISA assay

OPG and PGE2 levels were detected using an ELISA Kit (Xitang Biotechnology).

2.13 | Calculation of the number and area of cells

The number and area of adipocytes and osteoblasts were calculated using cellSens Imaging Software (Olympus).[19] The number and area of cells were counted semi-automatically using a 200× objective; the field of view was 414 040 μm^2.

2.14 | Statistical analysis

SPSS (v. 17.0) software was used for all statistical analysis. Mean and standard deviation were used as the main parameters, and an unpaired two-sided *t* test was used to assess differences between control group and experimental group. Values of $P < .05$ were considered statistically significant.

3 | RESULTS

3.1 | HFD promoted adipocytes and inhibited osteocytes in bone marrow

First, we investigated whether HFD would have an impact on the bone marrow microenvironment. After 4 weeks of HFD feeding, the weight of the HFD group was significantly higher than the mice fed a ND ($P < .01$) (Figure 1A). Moreover, Lee's index was significantly higher in the HFD group than the ND group ($P < .05$) (Figure 1B). After 9 weeks, the weight and Lee's index of HFD mice remained significantly higher than those of ND mice ($P < .05$) (Figure 1A,B). More importantly, the levels of FFA, TG, and HDL were all significantly increased in HFD mice over ND mice ($P < .05$) (Figure 1C).

Next, we examined the content of adipocytes and osteoblasts in bone marrow. Our results showed that the area and number of adipocytes were significantly higher in the HFD group compared with the ND group ($P < .001$) (Figure 1D-F, Figure S1). Not surprisingly, in contrast to adipocytes, HFD inhibited the area and number of osteoblasts ($P < .01$) (Figure 1G-I, Figure S2). This strongly indicated an association between HFD-induced dyslipidemia and imbalanced bone marrow adipocytes and osteoblasts.

3.2 | HFD promoted COX2 expression and inhibited OPG expression in the bone marrow microenvironment

To explore the expression of COX2 and OPG in the bone microenvironment, we performed immunohistochemical analyses of the bone marrow cavity from ND and HFD mice. ND mice exhibited very modest COX2 expression, whereas strong COX2 presence was detected in HFD mice ($P < .01$) (Figure 2A,B). In addition, COX2 mRNA levels in HFD mice were slightly higher than in ND mice ($P = .145$) (Figure 2C). In contrast to COX2 expression, immunohistochemical results showed significantly reduced expression of OPG in HFD mice than ND mice ($P < .05$) (Figure 2A,D). Moreover, OPG mRNA level was slightly lower in HFD mice than ND mice ($P = .398$) (Figure 2E).

3.3 | FFA levels were significantly higher in PCa bone metastasis patients than in PCa patients without bone metastasis or normal patients

The animal results suggest that HFD-induced dyslipidemia may be correlated with the change in the bone marrow microenvironment. Previous research suggests that the bone marrow microenvironment significantly contributes to the bone tropism of PCa.[2] Thus, we examined blood lipid levels among three groups of patients: normal patients, PCa patients, and patients with PCa bone metastasis. Table 1 presents the general information and various biochemical indicators of the subjects. We found no significant differences in age, weight, BMI, TC, TG, LDL-C, HDL-C, or FPG among the three

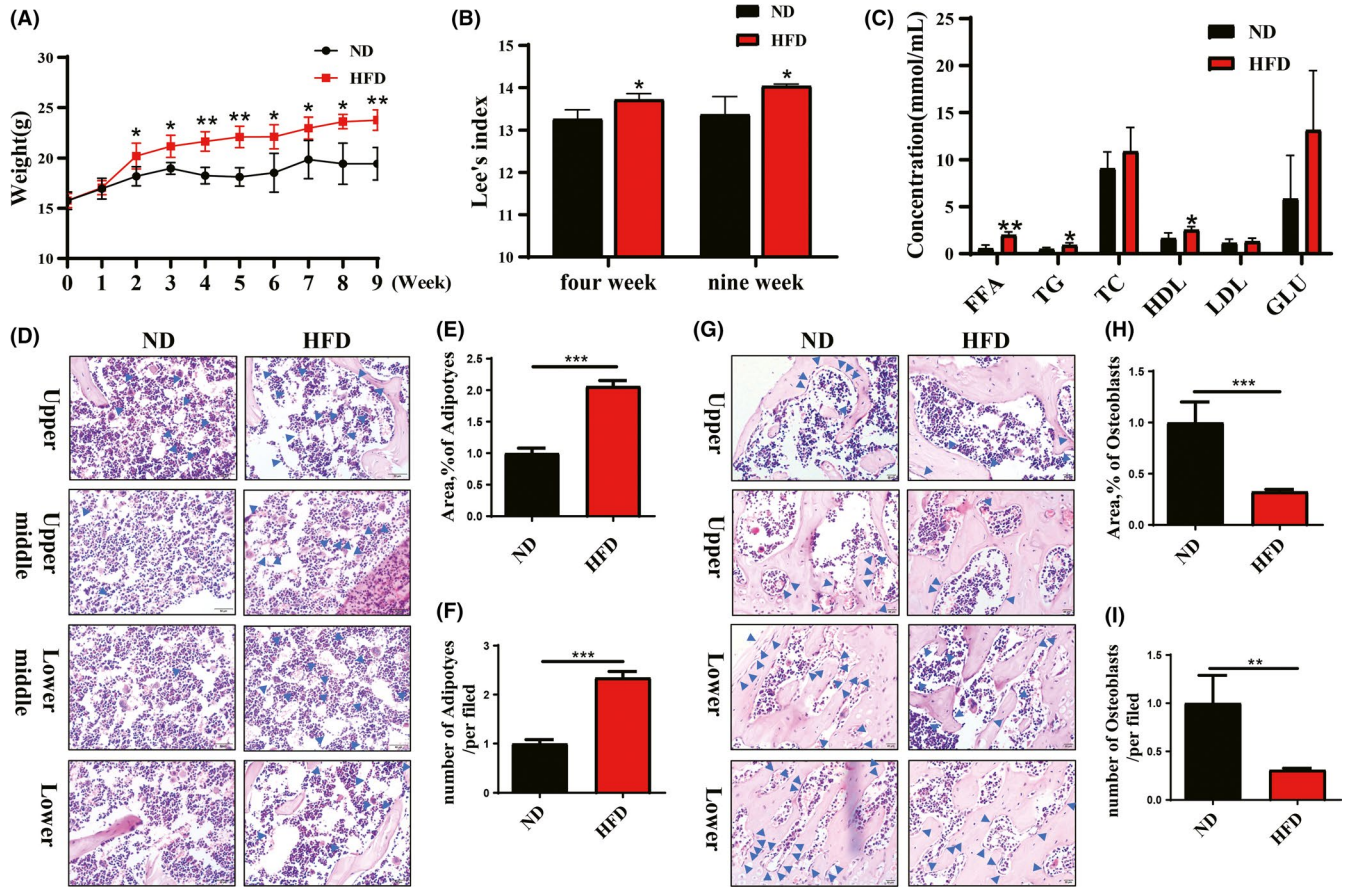

FIGURE 1 High-fat diet caused increased adipocytes and reduced osteoblasts in the bone marrow cavity. After 1 week of adaptive feeding, four mice were fed with normal diet (ND), and four mice were fed with high-fat diet (HFD). A, The body weight of the ND group (n = 4) and HFD group (n = 4). B, Lee's index of the two groups in the fourth and ninth week. C, The level of plasma FFA, TG, TC, HDL, LDL, and GLU in the ninth week. D, G, H&E-stained image of femur. Four different locations in the bone marrow cavity were chosen to present adipocytes, and two different locations rounded the bone cortex were chosen to present osteoblasts. 400 × magnification was used. E, H, Area of adipocytes and osteoblasts in the bone marrow cavity. F, I, Number of adipocytes and osteoblasts in the bone marrow cavity. FFA, free fatty acids; GLU, glucose; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TC, total cholesterol; TG, triglycerides. *t* test, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$; the difference was statistically significant

groups ($P > .05$). However, the level of TPSA, a marker of PCa, was significantly higher in both groups of PCa patients than in normal individuals ($P < .05$). Compared with normal individuals and patients with PCa, the level of FFA was significantly higher in patients with PCa bone metastasis ($P < .05$); notably, there was no significant difference in FFA levels between normal patients and patients with PCa. Therefore, an increased level of FFA could be associated with PCa bone metastasis.

3.4 | The caprylic acid (C8:0) levels were significantly higher in patients with PCa than in normal patients

To determine which FFAs play an important role in the process of PCa bone metastasis, patients (eight normal patients, four patients with PCa, and four patients with PCa bone metastasis) were selected for a detailed FFA profile analysis. The levels of 34 FFAs in serum samples were determined by ultrahigh-performance liquid chromatography (UHPLC) on the Tsinghua University metabolomics platform.[20] Among all the FFAs tested, only caprylic acid (C8:0) was found to be significantly higher in PCa patients and PCa bone metastasis patients than in the normal group ($P < .01$). The caprylic acid (C8:0) level was slightly higher in PCa bone metastasis patients in comparison with PCa patients, although the level of caprylic acid (C8:0) was lower than some of the other FFAs (Table 2). These data indicated that increased level of caprylic acid (C8:0) could be linked to the development of PCa bone metastasis.

3.5 | Caprylic acid (C8:0) promoted adipocytic differentiation and COX2/PGE2 level while antagonizing osteoblastic differentiation and OPG expression

We next evaluated the effects of caprylic acid (C8:0) on adipocytic and osteoblastic differentiation in MSCs. First, MSCs were treated with adipocytic medium or adipocytic maintenance medium

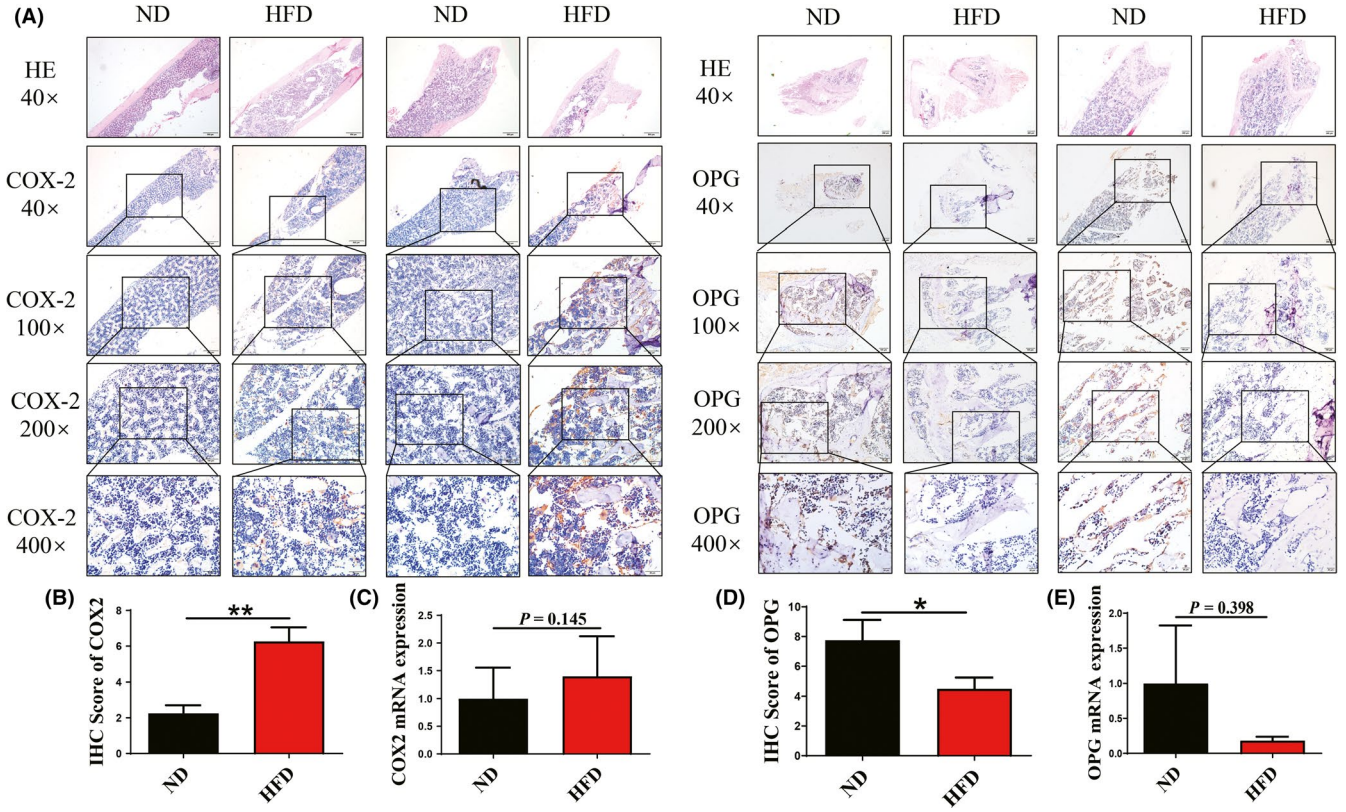

FIGURE 2 Detecting the expression levels of COX2 and OPG in the bone microenvironment. A, Immunohistochemical analyses to detect the protein expression of COX2 and OPG in the bone marrow cavity. Two different locations were presented and four different magnifications (40×, 100×, 200×, 400×) were used. B, D, The results of immunohistochemistry were scored. C, E, The mRNA expression of COX2 and OPG in the bone marrow cavity. COX2, cyclooxygenase 2; OPG, osteoprotegerin. *t* test, $^{*}P < .05$, $^{**}P < .01$; the difference was statistically significant

TABLE 1 General information and various biochemical indicators

Testing index	Normal individuals (n = 16)	Patients with prostate cancer (n = 8)	Patients with prostate cancer bone metastasis (n = 8)
Age (y)	75.05 ± 6.63	75.87 ± 6.40	75.62 ± 5.06
Weight (kg)	74.14 ± 8.43	80.0 ± 12.35	66.25 ± 15.40
BMI (kg/m^2)	25.86 ± 2.90	28.49 ± 4.41	23.80 ± 5.18
TC (mmol/L)	1.35 ± 0.44	1.29 ± 0.48	1.69 ± 1.08
TG (mmol/L)	3.83 ± 0.73	4.09 ± 0.82	3.72 ± 0.47
LDL-C (mmol/L)	1.59 ± 0.67	1.30 ± 0.31	1.29 ± 0.35
HDL-C (mmol/L)	1.46 ± 0.60	2.10 ± 0.78	1.74 ± 0.66
FPG(mmol/L)	5.15 ± 1.31	5.80 ± 2.00	4.99 ± 0.37
TPSA (ng/mL)	1.10 ± 0.82	55.62 ± 47.86**	65.80 ± 42.32$^{\#}$
FFA (μmol/L)	0.61 ± 0.21	0.55 ± 0.24	0.87 ± 0.38*$^{\#}$

Note: t test, $^{*}P < .05$, $^{**}P < .01$; compared with normal individuals, the difference is significant.
$^{\#}P < .05$; compared with patients with prostate cancer, the difference is significant.
Abbreviations: BMI, body mass index; FFA, free fatty acids; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides; TPSA, total prostate-specific antigen.

including caprylic acid (C8:0) at varying concentrations (25, 50, 100, and 125 μmol/L) to assess toxicity. Based on CCK8 assay, 100 μmol/L caprylic acid (C8:0) caused no toxicity or inhibition of cell

TABLE 2 Free fatty acid profiles in the serum of normal individuals, patients with prostate cancer, and patients with prostate cancer bone metastasis

FFA (μmol/μL)	Normal individuals (n = 8)	Patients with prostate cancer (n = 4)	Patients with prostate cancer bone metastasis (n = 4)
C (7:0)	0.0011 ± 0.0008	0.0017 ± 0.0011	0.0020 ± 0.0019
C (8:0)	0.0225 ± 0.0048	0.0386 ± 0.0187**	0.0463 ± 0.0132**
C (9:0)	0.0161 ± 0.0052	0.0191 ± 0.0058	0.0214 ± 0.0091
C (10:0)	0.0140 ± 0.0036	0.0207 ± 0.0090	0.0349 ± 0.0343
C (11:0)	0.0021 ± 0.0006	0.0027 ± 0.0004	0.0025 ± 0.0005
C (12:0)	0.0366 ± 0.0086	0.0664 ± 0.0309	0.1130 ± 0.1195
C (13:0)	0.0036 ± 0.0010	0.0046 ± 0.0013	0.0060 ± 0.0026
C (14:0)	0.2639 ± 0.0702	0.3815 ± 0.1277*	0.5105 ± 0.4280
C (14:1)	0.0124 ± 0.0049	0.0187 ± 0.0103	0.0328 ± 0.0372
C (15:0)	0.0441 ± 0.0142	0.0142 ± 0.0232	0.0678 ± 0.04840
C (15:1)	0.0010 ± 0.0002	0.0011 ± 0.0003	0.0036 ± 0.0021
C (16:0)	1.3670 ± 0.3643	1.7110 ± 0.7327	1.8871 ± 0.9976
C (16:1)	0.4346 ± 0.1253	0.5678 ± 0.2838	0.8021 ± 0.8127
C (17:0)	0.3151 ± 0.0893	0.3679 ± 0.1277	0.4489 ± 0.2435
C (17:1)	0.0294 ± 0.0086	0.0359 ± 0.0128	0.0412 ± 0.02752
C (18:0)	1.3347 ± 0.3243	1.3074 ± 0.4847	1.9685 ± 1.3219
C (18:1)	4.0821 ± 0.8167	4.636 ± 1.9817	5.5383 ± 3.2666
C (18:2)	4.4120 ± 1.6866	5.2169 ± 2.2802	5.0541 ± 2.5797
C (18:3)	0.0716 ± 0.0275	0.0671 ± 0.0277	0.0656 ± 0.0392
C (19:0)	0.1249 ± 0.0359	0.1347 ± 0.0283	0.1587 ± 0.0951
C (20:0)	0.1163 ± 0.0807	0.1565 ± 0.0366	0.2845 ± 0.2531
C (20:1)	0.0319 ± 0.0107	0.0391 ± 0.0261	0.0692 ± 0.0633
C (20:2)	0.0717 ± 0.0368	0.0846 ± 0.04062	0.0981 ± 0.0751
C (20:3)	0.1017 ± 0.0291	0.0948 ± 0.0255	0.0801 ± 0.0220
C (20:4)	0.4376 ± 0.1240	0.3828 ± 0.1865	0.4179 ± 0.1830
C (20:5)	0.0412 ± 0.0393	0.0313 ± 0.0083	0.0284 ± 0.0104
C (21:0)	/	/	/
C (22:0)	0.3881 ± 0.1590	0.4233 ± 0.1978	0.4229 ± 0.2552
C (22:1)	0.0024 ± 0.0014	0.0066 ± 0.0078	0.0059 ± 0.0075
C (22:2)	0.0014 ± 0.0003	0.0017 ± 0.0010	0.0043 ± 0.0024
C (22:6)	0.3072 ± 0.1033	0.3650 ± 0.0971	0.3365 ± 0.1475
C (23:0)	0.5892 ± 0.2415	0.4919 ± 0.2546	1.2149 ± 0.7244
C (24:0)	2.0923 ± 0.4896	2.0883 ± 0.4432	2.0979 ± 0.6377
C (24:1)	0.1055 ± 0.0192	0.1092 ± 0.0107	0.1174 ± 0.0348

Note: t test, *$P < .05$, **$P < .01$; compared with normal individuals, the difference is significant.
Abbreviation: FFA, free fatty acid.

proliferation. Thus, we used 100 μmol/L caprylic acid (C8:0) to treat MSCs cells (Figure S3) in all subsequent experiments.

We found that treatment with caprylic acid (C8:0) increased the area and number of adipocytes (Figure 3A-C). Additionally, the protein expression levels of CEBPα and PPARγ were higher in the caprylic acid (C8:0)-treated group (Figure 3D), indicating that caprylic acid (C8:0) promoted adipocytic differentiation of MSCs. Interestingly, mRNA and protein expression levels of APN, specifically secreted by adipocytes, were significantly reduced in the caprylic acid (C8:0)-treated group although the content of adipocytes was increased (Figure 3E, G). Furthermore, COX2 protein expression was higher under caprylic acid (C8:0) treatment (Figure 3E,F). It has been reported that COX2 is a critical enzyme involved in the biosynthesis of prostaglandin E2 (PGE2), and PGE2 supplied by adipocytes is a potential regulator of prosurvival pathways in PCa.[10] Thus, we next investigated whether caprylic acid (C8:0) treatment promoted PGE2 secretion by adipocytes. Our results demonstrate that PGE2 secretion was slightly, but significantly,

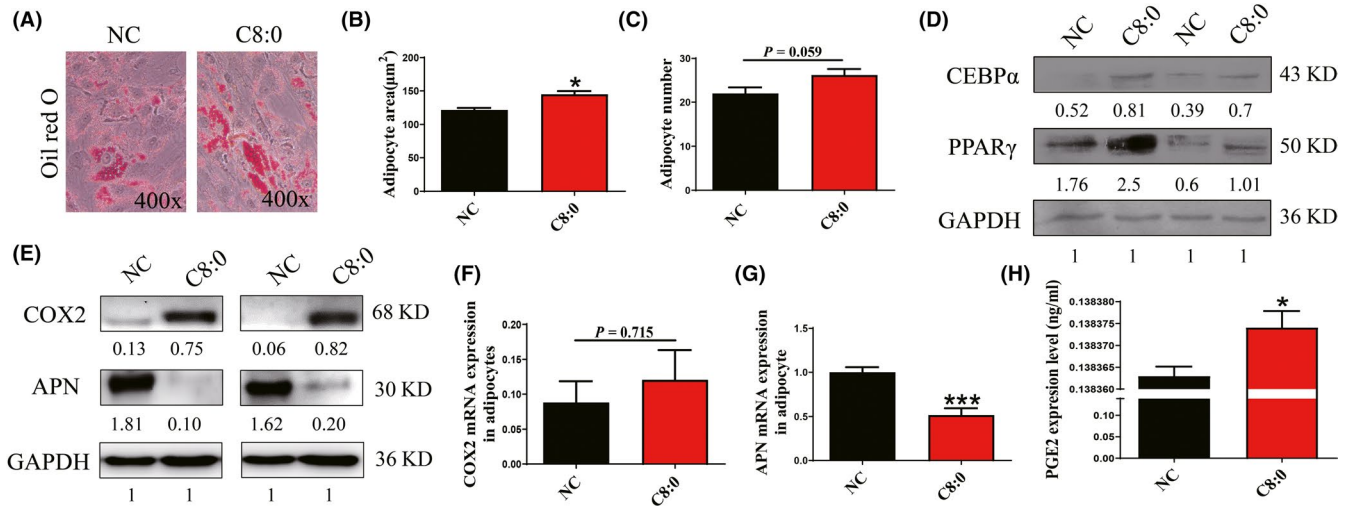

FIGURE 3 Caprylic acid (C8:0) promoted adipocytes differentiation and COX2/PGE2 level. MSCs were cultured with adipogenic medium or adipogenic maintenance medium, alone (Normal Control, NC) or in the presence of caprylic acid (C8:0). A, Adipogenic differentiation was evaluated with Oil-Red-O staining. B, The area of adipocytes was measured. C, The number of adipocytes was measured. D, The protein expression levels of CEBPα and PPARγ were detected. E, The protein expression levels of COX2 and APN were detected. F-G, The mRNA expression levels of COX2 and APN were detected. H, The secretion level of PEG2 was detected. APN, adiponectin; CEBPα, CCAAT-enhancer–binding protein-α; COX2, cyclooxygenase 2; GADPH, glyceraldehyde 3-phosphate dehydrogenase; HFD, high-fat diet; MSCs, mesenchymal stem cells; ND, normal diet; OPG, osteoprotegerin; PGE2, prostaglandin E2; PPAR-γ, peroxisome proliferator-activated receptor gamma. *t* test, $^{*}P < .05$, $^{***}P < .001$; the difference was statistically significant

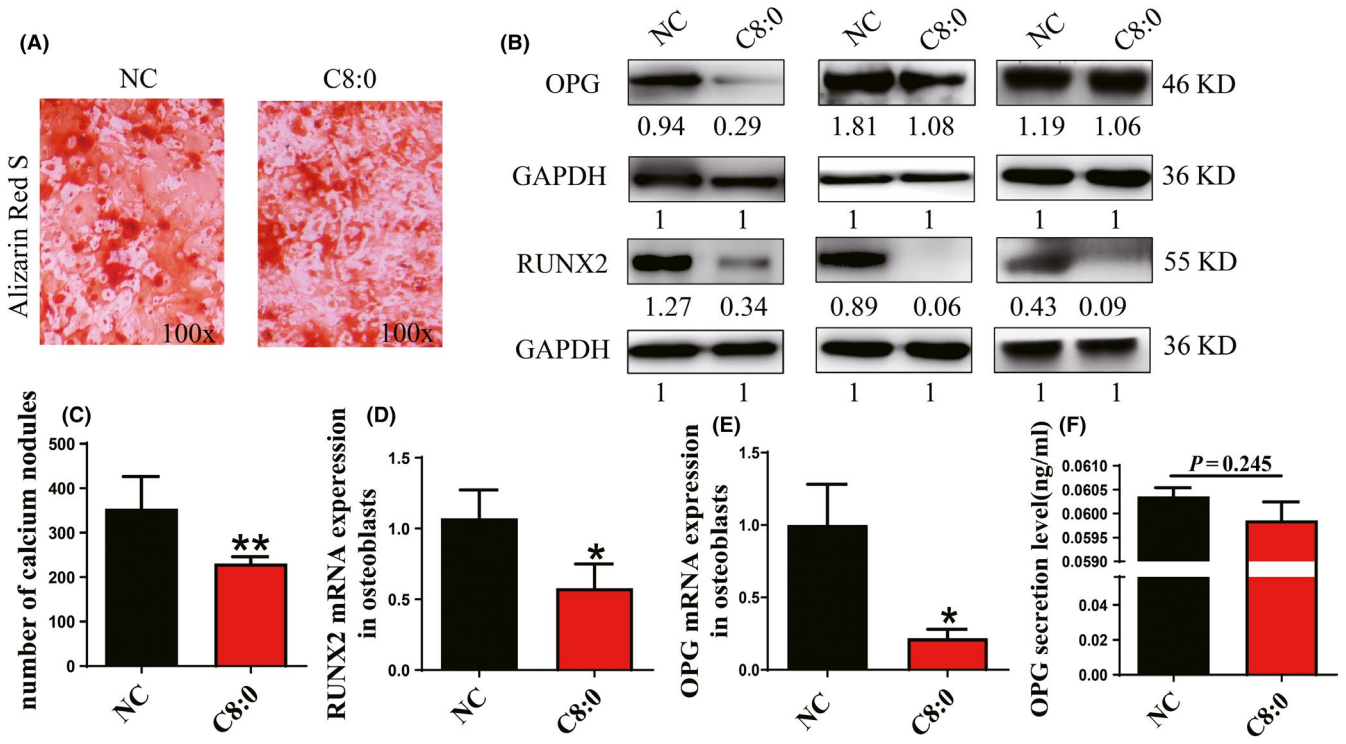

FIGURE 4 Caprylic acid (C8:0) reduced osteoblastic differentiation and OPG expression. MSCs were cultured with osteogenic medium alone (NC) or in the presence of caprylic acid (C8:0). A, Osteogenic differentiation was evaluated with alizarin red staining. B, The protein expression levels of OPG and RUNX2 were detected. C, The number of calcium nodules was measured. D, E, The mRNA expression levels of RUNX2 and OPG were detected. F, The secretion level of OPG was detected. GADPH, glyceraldehyde 3-phosphate dehydrogenase; MSCs, mesenchymal stem cells; OPG, osteoprotegerin; RUNX2, Runt-related transcription factor 2. *t* test, $^{*}P < .05$, $^{**}P < .01$; the difference was statistically significant

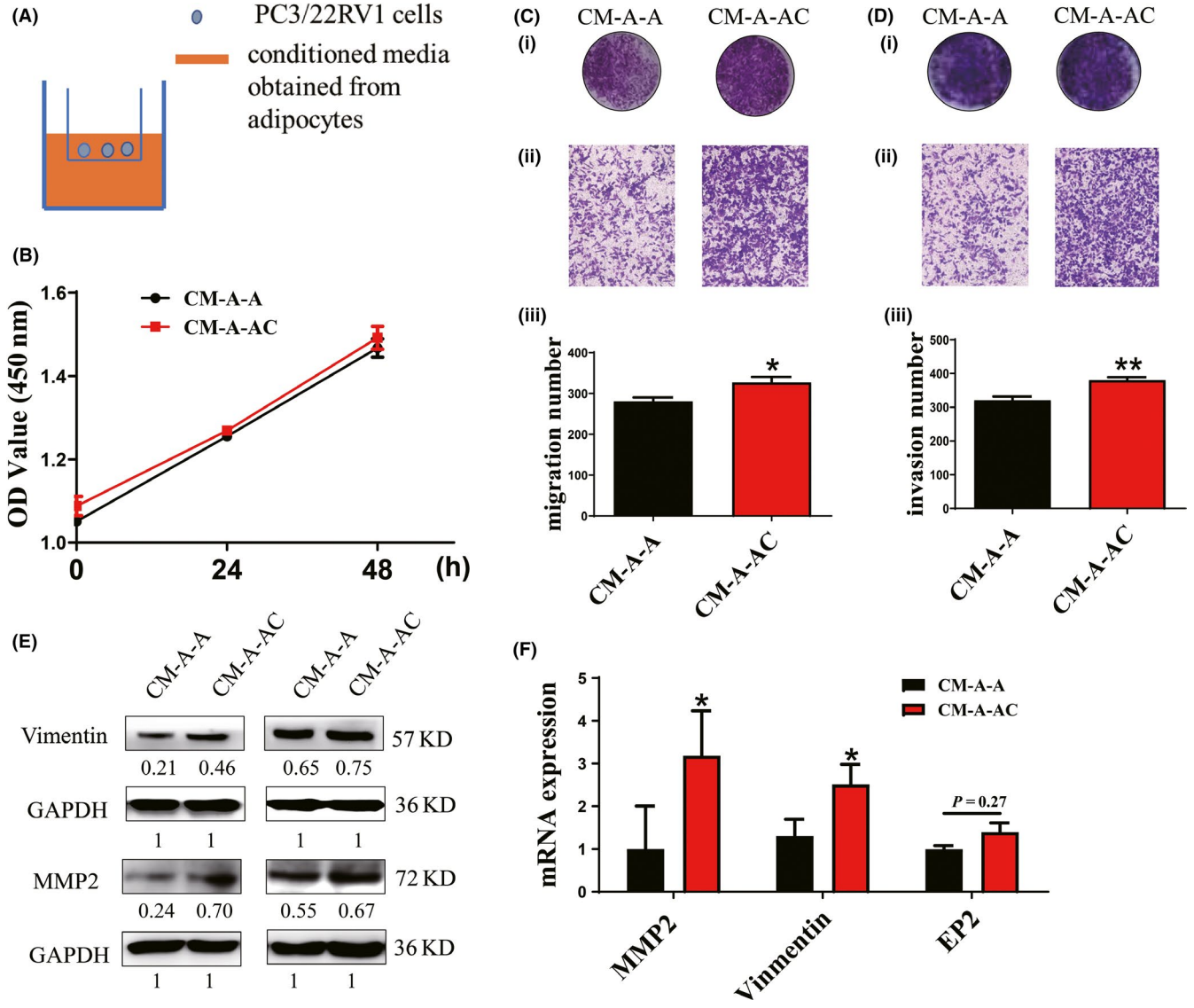

FIGURE 5 Caprylic acid (C8:0) regulated the crosstalk between adipocytes and PC3 cells. A, The conditioned medium obtained from adipocytes treated with caprylic acid (CM-A-AC) or the conditioned medium obtained from adipocytes without caprylic acid (CM-A-A) were used to treat the PCa cells, respectively. B, Proliferation bioassay of PC3 cells was detected. According to the optical density (OD) value, to determine the number of living cells. C, D, Transwell migration and invasion assays of PC3 cells were detected. (i) Whole visual field, (ii) local visual field observed by microscope, and (iii) quantified invasion number. (E) The protein expression levels of vimentin and MMP2 were detected. F, The mRNA expression levels of MMP2, vimentin, and EP2 were detected. EP2, E receptor 2; GADPH, glyceraldehyde 3-phosphate dehydrogenase; MMP2, matrix metallopeptidase 2; PCa, prostate cancer. *t* test, $^{*}P < .05$, $^{**}P < .01$; the difference was statistically significant

elevated in the caprylic acid (C8:0)-treated cells compared with the untreated cell ($P < .05$) (Figure 3H).

In contrast to the stimulatory effects of caprylic acid (C8:0) effects on adipocytes differentiation, we found that treatment with caprylic acid (C8:0) reduced the number of osteoblasts ($P < .01$) (Figure 4A,C), indicating an inhibition of osteoblastic differentiation. RUNX2, an osteogenesis-related gene, was significantly downregulated under caprylic acid (C8:0) treatment ($P < .05$) (Figure 4B,D). Similarly, caprylic acid (C8:0) treatment also significantly reduced the expression of OPG ($P < .05$) (Figure 4B,E). However, the level of OPG secretion was not significantly different between the two groups ($P = .245$) (Figure 4F).

3.6 | Caprylic acid (C8:0) regulates the crosstalk between adipocytes and PCa cells

To identify and characterize any potential crosstalk between PCa cells and adipocytes, conditioned medium obtained from adipocytes grown with (CM-A-AC) or without (CM-A-A) caprylic acid (C8:0) was used to treat PCa cell lines. PC3 cells (fully able to migrate) and 22RV1 cells (limited ability to migrate) (Figure 5A) were treated with conditioned medium and then monitored for proliferation, migration, and invasion. While we observed no significant differences in PC3 cell proliferation between the CM-A-A– and

CM-A-AC–treated groups (Figure 5B), PC3 cell migration and invasion were significantly higher in the CM-A-AC–treated group than in the CM-A-A–treated group ($P < .05$) (Figure 5C,D). Moreover, in the CM-A-AC group, mRNA and protein expression levels of the metastasis-associated factors vimentin and MMP2 were significantly higher than that in the CM-A-A group ($P < .05$) (Figure 5E,F). However, the mRNA expression level of EP2, a PGE2 receptor, had no significant difference between the two treatment groups ($P = .27$) (Figure 5F). In 22RV1 cells, we observed no significant differences in proliferation, migration, and invasion between the CM-A-A and CM-A-AC group; however, the mRNA expression of vimentin and MMP2 was significantly higher in the CM-A-AC group ($P < .05$) (Figure 6A-D).

We also performed analogous experiments to investigate the effects of caprylic acid (C8:0) on the crosstalk between osteoblasts and PCa-derived cells. Conditioned medium obtained from osteoblasts treated with caprylic acid (C8:0) (CM-O-AC), or without (CM-O-A), were used to treat PC3 and 22RV1 cells. However, we observed no significant differences between the treatment groups (Figure S4).

Collectively, we observed that caprylic acid (C8:0) primarily regulated crosstalk between osteolytic PCa cells (PC3 cell line) and adipocytes rather than osteoblasts.

4 | DISCUSSION

PCa is the most frequent noncutaneous cancer occurring in men.[21] On average, men with localized PCa have a high, 10-year survival rate, and many can be cured.[21] However, men with metastatic castrate-resistant PCa have incurable disease with poor survival despite intensive therapy.[21] Bone is the most frequent site of PCa

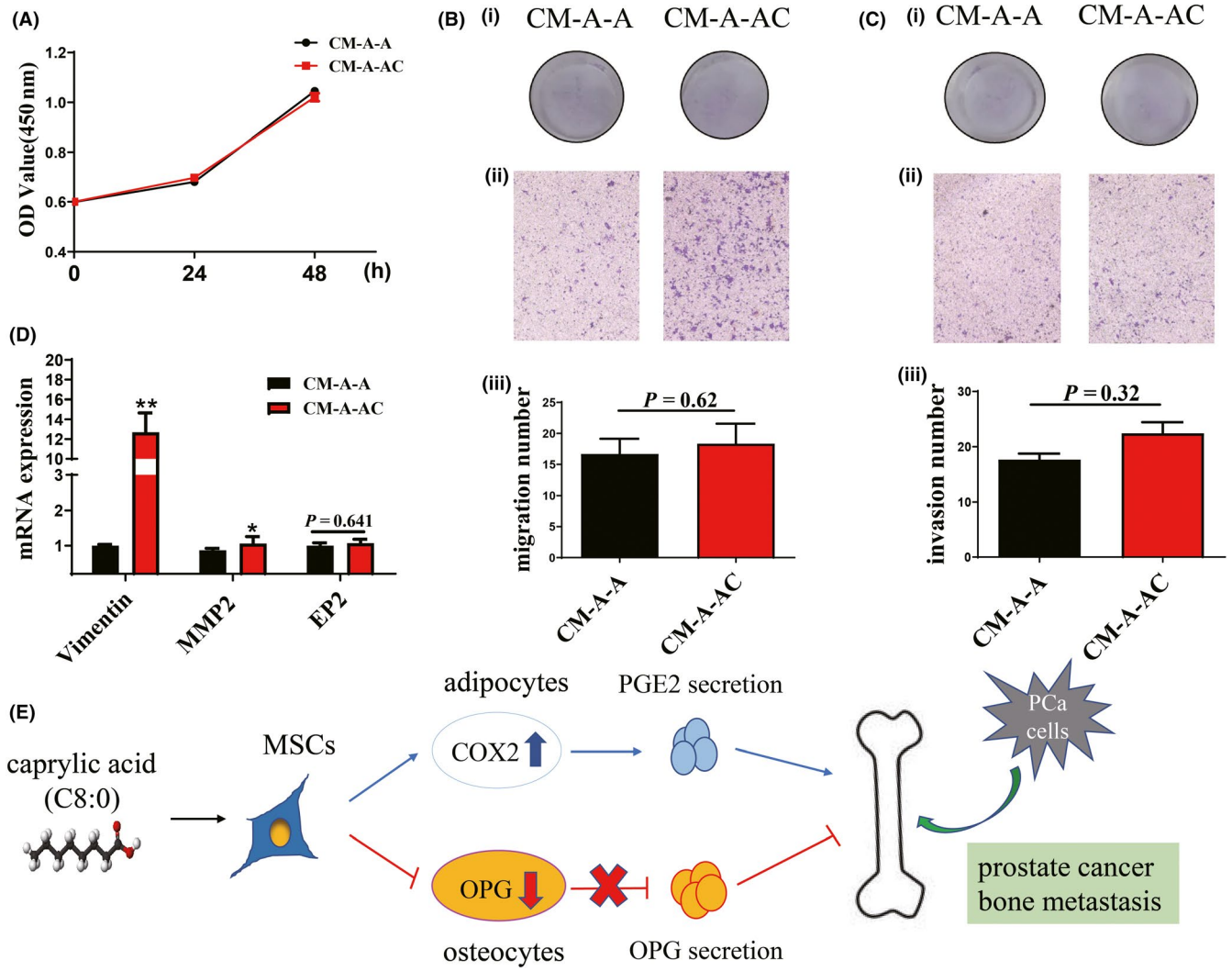

FIGURE 6 Caprylic acid (C8:0) had no effect on the proliferation, migration, and invasion of 22RV1 cells. A, Proliferation bioassay of 22RV1 cells was detected. B, C, Transwell migration and invasion assays of 22RV1 cells were detected. (i) Whole visual field, (ii) local visual field observed by microscope, and (iii) quantified invasion number. D, The mRNA expression levels of vimentin, MMP2, and EP2 were detected. E, Proposed mechanism of caprylic acid (C8:0) promoting bone metastasis of prostate cancer by dysregulated adipo-osteogenic balance in bone marrow. COX2, cyclooxygenase 2; EP2, E receptor 2; MMP2, matrix metallopeptidase 2; MSCs, mesenchymal stem cells; PCa, prostate cancer; OPG, osteoprotegerin. *t* test, $^*P < .05$, $^{**}P < .01$; the difference was statistically significant

metastases, though the exact mechanisms that favor bone metastasis versus other sites remain to be elucidated.[2]

Obesity, an increasingly prevalent global issue, is associated with an increased risk of developing several types of cancer, including PCa.[1,2,22] This is consistent with our data, summarized in Table 1. More importantly, clinical studies have linked obesity with PCa aggressiveness, biochemical recurrence, and incidence of metastases.[23-27] A higher BMI has been reported to be associated with several risks: more than threefold increase in the progression to castration-resistant PCa, higher trend of developing metastases, and a trend toward worsening PCa-specific mortality.[27] Taken together, this indicates that obesity is associated with aggressive PCa. In our present study, body weight is actually lower in patients with bone metastasis. This is likely due to the fact that bone metastases can have a substantial detrimental effect on patients' quality of life, as well as significant morbidity due to complications collectively known as skeletal-related events.[28] Moreover, PCa cells are characterized by an uptake of FAs,[29] which suggests that the accelerated decomposition of TG and increased FA content may occur in patients with PCa bone metastases. Therefore, although we observed patients with bone metastasis have a lower BMI, this does not exclude obesity as a potential risk factor for PCa bone metastases.

Previous research, and data from our laboratory, has demonstrated that obesity could influence the bone marrow microenvironment and promote adipogenesis in bone marrow MSCs.[5,30,31] The bone microenvironment may regulate metastatic PCa cells between dormant and proliferative states.[2] Our results also showed that a HFD increased the area and number of adipocytes while reducing the area and number of osteocytes in bone marrow. Correspondingly, the adipocytokine COX2 was increased, and the osteoblastic cytokine OPG was decreased. The results indicated that a HFD dysregulated the adipo-osteogenic balance in the bone marrow microenvironment.

Obesity-induced increases of circulating FFAs correlated with the occurrence and progression of PCa.[18] This supports our data that show a HFD could also increase the level of FFAs in mice. Further, the level of FFAs was significantly higher in patients with PCa bone metastasis compared with normal individuals and PCa patients without metastasis. However, the precise mechanisms underlying the promotion of PCa bone metastasis by increased FFAs have not been clarified. It was reported that arachidonic acid prepared the "soil," making it more supportive for propagation and implantation of the migrating metastatic PCa cells.[18] We recently observed that the serum level of caprylic acid (C8:0) was significantly higher in the human obesity group compared with the normal-weight group.[20] It has been reported that decreasing dietary caprylic acid (C8:0) and ghrelin O-acyltransferase activity can resist obesity by modulating octanoylated ghrelin functions.[32] These studies suggest that the serum level of caprylic acid (C8:0) is much higher in obesity state. Narayanan et al[33] observed that caprylic acid (C8:0) significantly inhibited the proliferation of colorectal cancer cells, skin cancer cells, and breast cancer cells in vitro. Yamasaki et al[34] found that caprylic acid (C8:0) inhibited the proliferation of bladder cancer cells, while inconsistent studies have shown that colorectal cancer patients with lower serum caprylic acid (C8:0) had better prognosis than those with higher serum caprylic acid.[35] Notably, the relationship between caprylic acid (C8:0) and the occurrence and development of PCa has not been defined. In the current study, our results showed that only caprylic acid (C8:0) was significantly higher in PCa patients (with and without bone metastasis) than in normal individuals. Further, caprylic acid (C8:0) levels were slightly higher in PCa patients with bone metastasis compared with PCa patients without bone metastasis, suggesting caprylic acid (C8:0) could be correlated with bone metastasis.

In this study, we found that caprylic acid (C8:0) promoted the differentiation of MSCs into adipocyte in vitro, whereas it inhibited the differentiation of MSCs into osteoblasts. Significantly, adipogenic markers C/EBP-α and PPAR-γ were increased and the osteogenesis-related genes RUNX2 and OPG were inhibited under caprylic acid (C8:0) treatment. These results suggested that caprylic acid (C8:0) may regulate adipo-osteogenic balance in bone marrow. Interestingly, the expression level of APN, specifically secreted by adipocytes, was significantly inhibited although the number of adipocytes was increased. We previously reported that APN expression was lower in adipose tissue of obese individuals, and palmitic acid inhibits APN expression in 3T3-L1 adipocytes.[36] Reports in the literature have described how FAs, in addition to their role as bioenergetic sources, can also act as extracellular signaling molecules: FAs may affect different signaling pathways crucial to the biological functions of tumor cells, such as proliferation and invasion.[37] Thus, the reduced expression level of APN of adipocyte may be caused by higher levels of caprylic acid (C8:0), and further studies should be carried out to investigate this hypothesis.

Additionally, our conditioned medium experiments suggest that caprylic acid (C8:0) promotes the migration and invasion of PCa cells by regulating crosstalk between adipocytes and osteolytic PCa cells. A recent report demonstrated that PGE2-EP2–dependent intracrine mechanisms are involved in the pathogenesis of PCa.[38,39] Moreover, PGE2 has been shown to significantly upregulate the mRNA and protein expression levels of the MMP-2 and matrix metalloproteinases 9 (MMP-9); PGE2 was also found to promote PCa cell proliferation and invasion via the cAMP-PKA/PI3K-Akt signaling pathway.[40] It has been reported that adipocytes highly expressed COX2, a vital enzyme, involved in the biosynthesis of PGE2.[10] Our results demonstrate that caprylic acid (C8:0) significantly enhances both COX2 protein expression and PGE2 secretion by adipocytes, thus promoting the migration and invasion of PCa cells.

Above all, obesity-induced high FFA levels, in particular caprylic acid (C8:0), could change the bone marrow microenvironment, providing "fertile soil" for PCa bone metastasis. We present for the first time that an increase in caprylic acid (C8:0) could promote conditions which lead to PCa bone metastasis: growth and proliferation of adipocytes and enhanced COX2 and PGE2 expression in the bone marrow cavity (Figure 6E). Our data provide a theoretical basis for elucidating the specific molecular mechanism

of obesity-induced PCa bone metastasis and present possible molecular targets for the prevention and treatment of PCa bone metastasis.

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DISCLOSURE

The content has not been published or submitted for publication elsewhere. All authors listed agree to publish the manuscript with no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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