Lactate receptor HCAR1 regulates cell growth, metastasis and maintenance of cancer-specific energy metabolism in breast cancer cells

LILI JIN[1], YANAN GUO[2], JIAWEN CHEN[2], ZHENZHEN WEN[2], YIBIN JIANG[2] and JING QIAN[2,3]

[1]Huzhou Key Laboratory of Molecular Medicine, Huzhou Central Hospital, Huzhou Hospital Affiliated with Zhejiang University; [2]Huzhou University Schools of Nursing and Medicine; [3]Key Laboratory of Vector Biology and Pathogen Control of Zhejiang, Huzhou University, Huzhou, Zhejiang 313000, P.R. China

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Abstract. Under aerobic conditions, the preferential use of anaerobic glycolysis by tumour cells leads to a high level of lactate accumulation in tumour microenvironment. Lactate acts not only as a cellular energy source but also as a signalling molecule that regulates cancer cell growth, metastasis and metabolism. It has been reported that a G-protein-coupled receptor for lactate named hydroxycarboxylic acid receptor 1 (HCAR1) is highly expressed in numerous types of cancer, but the detailed mechanism remains unclear. In the present study, it was reported that HCAR1 is highly expressed in breast cancer cells. Genetic deletion of *HCAR1* in MCF7 cells leads to reduced cell proliferation and migration. Moreover, it was observed that knockout (KO) of *HCAR1* attenuated the expression and activity of phosphofructokinase and hexokinase, key rate-limiting enzymes in glycolysis. Using an extracellular flux analyzer, it was showed that KO of *HCAR1* promoted a metabolic shift towards a decreased glycolysis state, as evidenced by a decreased extracellular acidification rate and increased oxygen consumption rate in MCF7 cells. Taken together, our results suggested that lactate acts through HCAR1 as a metabolic regulator in breast cancer cells that may be therapeutically exploited.

Correspondence to: Dr Jing Qian, Huzhou University Schools of Nursing and Medicine, Huzhou University, 1 Bachelor Road, Wuxing, Huzhou, Zhejiang 313000, P.R. China
E-mail: 02300@zjhu.edu.cn

Abbreviations: HCAR1, hydroxyl carboxylic acid receptor 1; PFK, phosphofructokinase; PK, pyruvate kinase; HK, hexokinase; ECAR, extracellular acidification rate; OCR, oxygen consumption rate; WT, wild-type; KO, knockout; NC, negative control; CCK-8, Cell Counting Kit-8; EdU, 5-ethynyl-2'-deoxyuridine; PTX, pertussis toxin

Key words: HCAR1, breast cancer, metastases, energy metabolism

Introduction

According to the Warburg effect, tumour cells obtain energy from the glycolysis pathway, which includes active glucose uptake and excessive lactate production (1). A growing body of studies on cancer metabolism (2) sparked our interest in the new relationship between lactate and tumour biology. In the glycolytic tumour microenvironment, a high level of lactate accumulation is associated with a poor survival rate and a higher incidence of cell metastasis (3-6). Numerous experimental studies have proposed that lactate may be not just a byproduct of metabolic reprogramming. Indeed, as a signalling molecule, lactate plays a crucial role in almost every step of tumourigenesis and development, such as angiogenesis, migration, metastasis, immune evasion and cancer stem cell formation (7-9). However, the mechanism regulating this process remains unclear.

Hydroxycarboxylic acid receptor 1 (HCAR1; also known as GPR81) is a member of the GPCR family, and lactate has been identified as its endogenous ligand (10). HCAR1 is mainly expressed in adipocytes and was originally reported to be associated with lipolysis inhibition in adipose tissue (11,12). Studies have shown that HCAR1 expression is strikingly high in several solid tumours, such as breast, cervical and pancreatic cancers (13,14). HCAR1 is closely associated with tumour growth and metastasis. In breast cancer, a high level of HCAR1 expression promoted cell proliferation and angiogenesis through a PI3K/Akt-dependent pathway (14). HCAR1 and HCAR3 are essential for breast cancer cells to control their lipid/fatty acid metabolism (15,16). Roland *et al* (13) revealed that HCAR1 expression was positively associated with pancreatic cancer progression. Moreover, the interference of HCAR1 expression dramatically prevented tumour proliferation and metastasis *in vitro* and *in vivo*. Surprisingly, lactate-induced PD-L1 expression in tumour cells is mediated by its receptor HCAR1, thus providing an effective means for tumour cells to evade the immune system through an autocrine mechanism (8). Lactate also controls immune evasion through activation of HCAR1 on stromal dendritic cells in a paracrine manner (17,18). Collectively, these findings suggested that HCAR1 engagement stimulates intracellular signalling

pathways that facilitate tumour growth, immune evasion and metastasis.

In the present study, it was found that breast cancer cell lines displayed high expression of HCAR1, which is involved in cell survival and migration. Furthermore, new insight was provided into the role of the lactate-HCAR1 pathway in maintaining energy metabolism balance. These findings suggested that autocrine activation of HCAR1 by lactate plays a key role in reprogramming cancer cell metabolism to meet the high requirements for rapid cell growth and migration. The discovery of HCAR1 provides novel ideas for the research and development of new antitumour drugs.

Materials and methods

Cell culture. The lung cancer cell lines (A549 and NCI-H1299), hepatoma cell lines (Hep3B, Huh7, HepG2 and HCCLM3), bladder cancer cell lines (UMUC-3 and T24), colorectal cancer cell lines (T84, LoVo and SW480) and pancreatic cancer cell lines (CFPAC-1 and PANC-1) were purchased from Procell Life Science & Technology Co., Ltd. The tongue squamous cell carcinoma cell lines (HN3, HN4), hepatoma cell line (SNU-449), melanoma cell line (WM35), cervix carcinoma cell line (Hela), epithelial carcinoma cell line (A431), breast cancer cell line (MCF7) and HEK293 cell were maintained by our laboratory. Cell lines were authenticated using short tandem repeat (STR) profiling. MCF7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS) and 1X penicillin/streptomycin and all cells were incubated at 37˚C in a humidified 5% CO_2 incubator.

Reverse transcription-quantitative (RT-q)PCR and semi-quantitative PCR. The total RNA from all the aforementioned cancer cell lines was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and converted into cDNA by the PrimeScript™ RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol. For RT-qPCR, cDNA templates were amplified on the ABI7500 System (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the SYBR Green PCR Kit (Takara Bio, Inc.). The qPCR thermocycling conditions were as follows: 95˚C for 2 min, followed by 40 cycles of 94˚C for 20 sec, 58˚C for 20 sec and 72˚C for 30 sec. Relative levels of mRNA expression were calculated using the $2^{-\Delta\Delta Cq}$ method (19). Primer (10 μM for each gene) sequences for each gene were as follows: HCAR1 forward, 5'-GCCCAGCAC TGTTTACCTTTTC-3' and reverse, 5'-CCCCAAAAGCCC AGTGTCTAC-3'; phosphofructokinase muscle type (PFKM) forward, 5'-GAGTGACTTGTTGAGTGACCTCCAGAAA-3' and reverse, 5'-CACAATGTTCAGGTAGCTGGACTTCG-3'; PFK liver type (PFKL) forward, 5'-GGCATTTATGTGGGT GCCAAAGTC-3' and reverse, 5'-CAGTTGGCCTGCTTG ATGTTCTCA-3'; hexokinase (HK)2 forward, 5'-GGGCAT CTTGAAACAAG-3' and reverse, 5'-GGTCTCAAGCCC TAAG-3'; pyruvate kinase M2 (PKM2) forward, 5'-GTGGCT CTGGATACAAAGGG-3' and reverse, 5'-ACTTCTCCATGT AAGCGTTGTC-3'; and β-actin forward, 5'-ACAATGTGG CCGAGGACTTT-3' and reverse, 5'-TGGGGTGGCTTTTAG GATGG-3'. The β-actin gene was used as an internal control.

Semi-quantitative PCR was performed using Taq PCR mastermix (Tiangen Biotech, Co., Ltd.), The PCR was conducted using the following thermal cycles: Initial denaturation at 94˚C for 5 min, followed by 28 cycles of 98˚C for 10 sec, 55˚C for 15 sec and 72˚C for 1 min, and then a final 5-min extension at 72˚C. Primer (10 μM for each gene) sequences for semi-quantitative PCR were as follows: HCAR1 forward, 5'-GGAGCATCGTGTTCCTTAC-3' and reverse, 5'-TTCTTC ATCCGAGCCTGT-3', which amplify a 349-bp fragment; and β-actin forward, 5'-TCTACAATGAGCTGCGTGTG-3' and reverse, 5'-CAACTAAGTCATAGTCCGCC-3', which amplify a 878-bp fragment. After amplification, the PCR products were visualized on 1.2% agarose gels containing GelRed (US Everbright, Inc.). The DNA bands were quantitated using ImageJ 1.52a software (National Institutes of Health).

CRISPR/Cas9-mediated HCAR1 knockout (KO). To knock out *HCAR1* via CRISPR/Cas9, three sgRNAs were designed using the CRISPR Design Tool (http://crispr.mit.edu/): *HCAR1* sgRNA-1, targeting CAGCACGACCCGTTG TACA; sgRNA-2, targeting GGTCGTGCTGCCGCATC GA; and sgRNA-3, targeting CACACAGGACCCGCATCCT. Oligos were annealed at 37˚C for 30 min and 95˚C for 5 min, and then the temperature was ramped down to 25˚C at 5˚C/min. The annealed fragments were treated with *BpiI* endonuclease and incorporated into the pSpCas9(BB)-2A-Puro(pX459) plasmid (Addgene, Inc.). MCF7 cells were seeded in six-well plates and transfected with 1 μg of pX459-HCAR1 plasmid by FuGENE® HD transfection Reagent (Roche Diagnostics) according to the manufacturer's protocol. Following 3 days after transfection, cells were selected using 2 μg/ml puromycin for 2 weeks. The KO efficiency was validated by genomic sequencing and western blot analysis.

Immunoblot analysis. Breast cancer cells were lysed for 30 min on ice in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM Na_3VO_4, 10 mg/ml leupeptin and 10 mg/ml aprotinin). Protein content was calculated using BCA Protein Assay Kit (Beyotime Institute of Biotechnology). Whole-cell lysates containing 50 μg of proteins were separated on 10% gels using SDS-PAGE and transferred to PVDF transfer membrane (MilliporeSigma) using a semidry transfer system (Bio-Rad Laboratories, Inc.). After blocking in a 5% BSA (Biosharp Life Sciences) solution in 1X TBST for 1 h at room temperature, the membrane was probed with anti-HCAR1 (1:1,000; cat. no. SAB1300090; Sigma-Aldrich; Merck KGaA) or β-actin (1:1,000; ca. no. 4967; Cell Signalling Technology, Inc.) antibody for 1 h at room temperature and subsequently with HRP-conjugated secondary antibodies (1:2,000; cat. nos. 7076/7074; Cell Signalling Technology, Inc.) for 1 h at 37˚C. Immunoreactive bands were detected using an enhanced chemiluminescent (ECL) reagent (Thermo Fisher Scientific, Inc.) by using Azure Biosystem C600 (Azure Biosystems, Inc.). Western blots were quantitated using ImageJ 1.52a software (National Institutes of Health).

Cell Counting Kit-8 (CCK-8) assay. Cell viability was assessed using a CCK-8 assay (Dojindo Laboratories, Inc.). Briefly, cells were seeded at 1,000 cells/well in 96-well plates and were incubated for different time periods (24, 48, 72 and

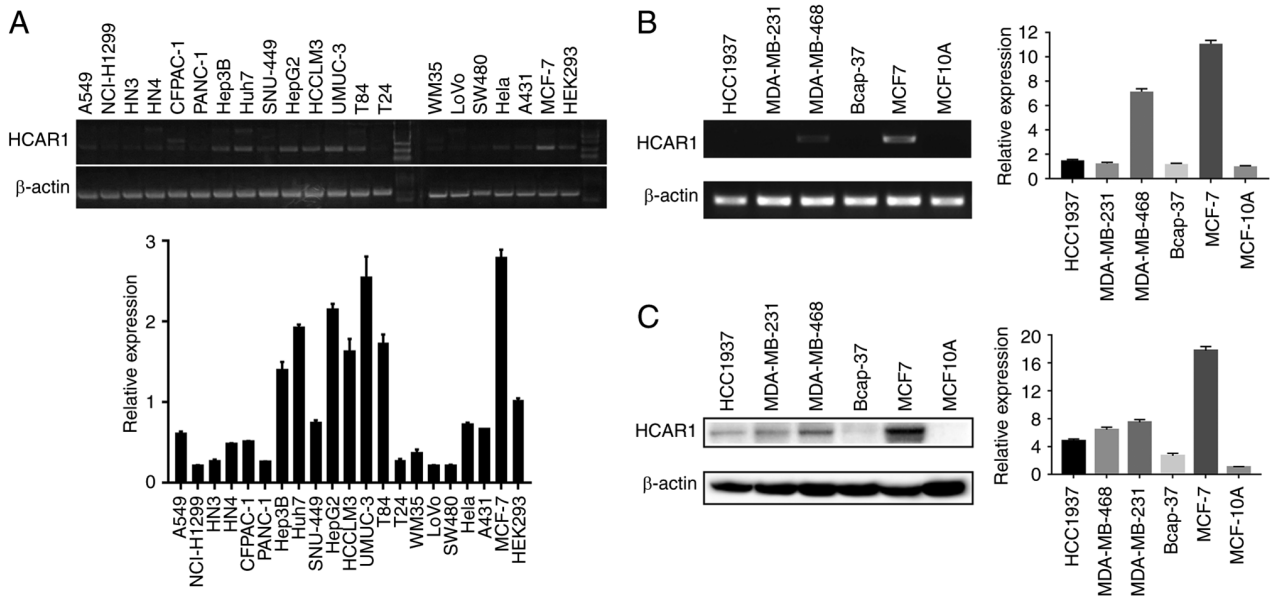

Figure 1. HCAR1 is highly expressed in human breast cancer cell lines. (A) The expression of HCAR1 in different cancer cell lines was assessed by semi-quantitative PCR. Data are presented as the mean ± SD (n≥3). (B) Relative mRNA expression of HCAR1 in 5 breast cancer cell lines and the immortalized breast epithelial cell line MCF10A was quantified by semiquantitative PCR. All determined by reverse transcription-quantitative PCR and expressed as relative values compared with MCF10A group. (C) Western blot analysis measurement of HCAR1 protein expression in 5 breast cancer cell lines and the immortalized breast epithelial cell line MCF10A. Data is expressed as fold change compared with MCF10A group. HCAR1, hydroxyl carboxylic acid receptor 1.

96 h) at 37˚C. CCK-8 (10 μl) solution was added to each well and then incubation followed for 2 h. The absorbance value (OD) of each well was measured at 450 nm with a microplate reader. Each sample was assayed in four replicates. Three independent experiments were performed.

EdU assay. The EdU incorporation assay was carried out by a Click-iT® EdU Alexa Fluor® 555 Imaging Kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, MCF7 cells were seeded into 24-well plates at $1x10^4$ cells/well and incubated at 37˚C overnight. After treatment with 20 mM lactate or DMEM for 24 h, the cells were treated with EdU (10 mmol/l) for 1 h. Then, the cells were fixed at room temperature for 15 min, permeabilized and incubated with the Click-iT reaction cocktail (Invitrogen; Thermo Fisher Scientific, Inc.), followed by Hoechst 33342 (Invitrogen; Thermo Fisher Scientific, Inc.) staining, and observed using a fluorescence microscope (Nikon Corporation). The number of EdU-positive cells and Hoechst 33342-positive cells were counted using ImageJ 1.52a software (National Institutes of Health).

Colony formation assay. The colony formation ability of *HCAR1*-KO and control MCF7 cells was estimated by a colony formation assay. Briefly, cells pretreated with lactate (50 mM, with a stronger effect) for 24 h were seeded in a six-well plate at a density of 300 cells/well, followed by ten days of culture. Then, the clones (a colony was defined as >50 cells) were stained with 0.1% crystal violet (Solai Bao Technology Co., Ltd.) for 30 min at room temperature and counted using ImageJ 1.52a software. The data shown represent the average of three independent experiments.

Cell cycle analysis. Cell cycle distribution was assessed with a Cell Cycle Analysis kit (BD Biosciences) by flow cytometry. Cells were harvested and washed with PBS containing 1% FBS. Then, the cells were fixed with 70% ethanol at 4˚C overnight, washed twice with PBS and incubated with propidium iodide (PI)/RNase A staining solution (5 μg/ml PI, 250 μg/ml RNase A in PBS) for 15 min at 37˚C in the dark. The level of PI incorporation was analyzed by FACScan (BD FACS Canto II). The percentage of cells in the respective cell cycle phase was determined using Modfit LT version 3.2 software (Verity Software House, Inc.).

Transwell migration assay. Transwell chambers (MilliporeSigma) were used to investigate cell migration ability. A total of $1x10^5$ cells were washed with serum-free medium and treated with 100 ng/ml pertussis toxin (PTX) for 6 h at 37˚C. Cells were plated in the upper chambers (8.0 μm). Medium containing 10% FBS with 2 or 20 mM lactate was added to the lower chambers to serve as a chemoattractant. After 24 h of incubation, migratory cells in the lower chambers were fixed, stained with 0.1% crystal violet solution (Solai Bao Technology Co., Ltd.) for 30 min and quantified using light microscopy. The cell numbers were counted in 5 different random fields (magnification, x200).

Measurement of enzyme activities. MCF7 cells were seeded at a density of $2.0x10^5$/well in six-well plates. The cells were then collected and the enzyme activity of PFK, HK and PK were determined using test kits from Nanjing Jiancheng Bioengineering Institute (catalog nos. A129-1-1, A077-3-1 and A076-1-1) according to the manufacturer's instructions.

Oxidative phosphorylation (OXPHOS) and glycolysis assay. The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of cells were evaluated using a Seahorse XF96 Extracellular Flux Analyzer (Seahorse

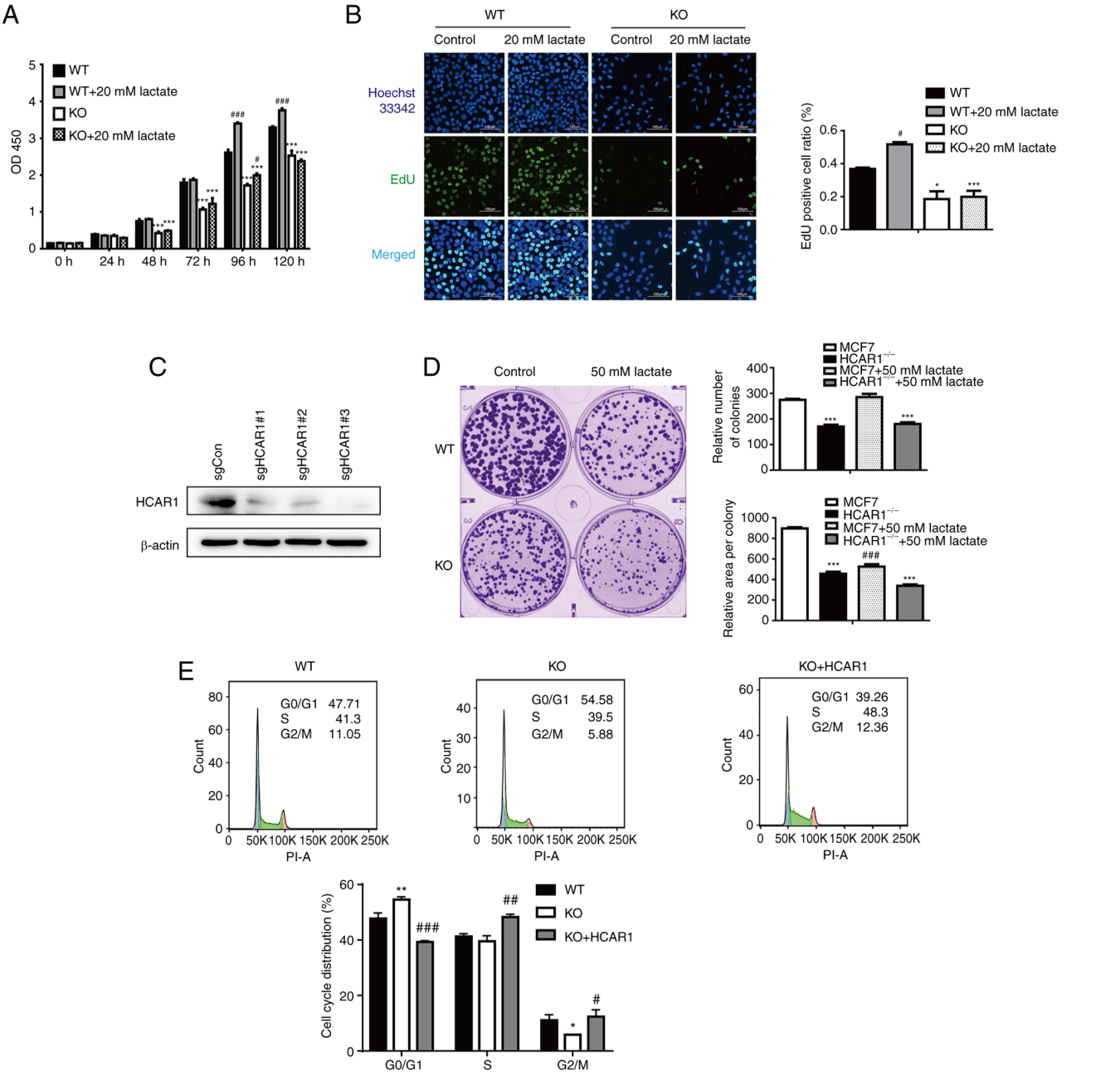

Figure 2. HCAR1 is an important regulator of MCF7 cell proliferation and survival. (A) After lactate treatment of WT and *HCAR1*-KO cell lines, cell proliferation was assessed using a Cell Counting Kit-8 (CCK-8). Experiments were repeated at least three times, and all data are presented as the mean ± SD. Statistical analyses of the parametric data were carried out using two-factor analysis of variance (ANOVA). (B) After Lactate treatment of WT and KO cell lines, cell proliferation was assessed using an EdU staining proliferation kit. Nuclei were stained using Hoechst 33342, and EdU was labeled with Alexa Fluor488 (magnification, x200). Data are presented as the mean ± SD (n≥3). (C) *HCAR1*-KO MCF7 cell clones were generated via CRISPR/Cas9 technology, and knockout efficiency was detected by western blot analysis. (D) The colony formation experiment was performed by staining each cell line with crystal violet and counting the number and size of effective clones. Data are presented as the mean ± SD (n≥3). (E) Flow cytometric analysis of the cell cycle in WT, KO and KO re-overexpressing HCAR1 cells. Data are representative results from experiments repeated at least three times. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with the non-lactate treatment group; *P<0.05, **P<0.01 and ***P<0.001 compared with the WT group. HCAR1, hydroxyl carboxylic acid receptor 1; WT, wild-type; KO, knockout.

Bioscience). Briefly, MCF7 cells were seeded at a density of 1x10^4 cells/well into 96-well Seahorse microplates for 24 h, and the calibration plate was equilibrated overnight in a non-CO_2 incubator. Cells were washed twice with assay running media and equilibrated in a non-CO_2 incubator before starting the test. Once the probe calibration was completed, the calibration plate was replaced with the cell plate to simultaneously measure the OCR and ECAR of the cells. After injection of oligomycin (1 μM), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (1 μM), rotenone (1 μM) and antimycin A (1 μM), OCR and the corresponding ECAR were determined. Once completed, the protein concentration was quantified (BCA Protein Assay Kit; Thermo Fisher Scientific, Inc.) to normalize the OCR and ECAR values.

Statistical analysis. All data were analyzed using SPSS pack 26.0 software (IBM Corp,), and the graphs were constructed

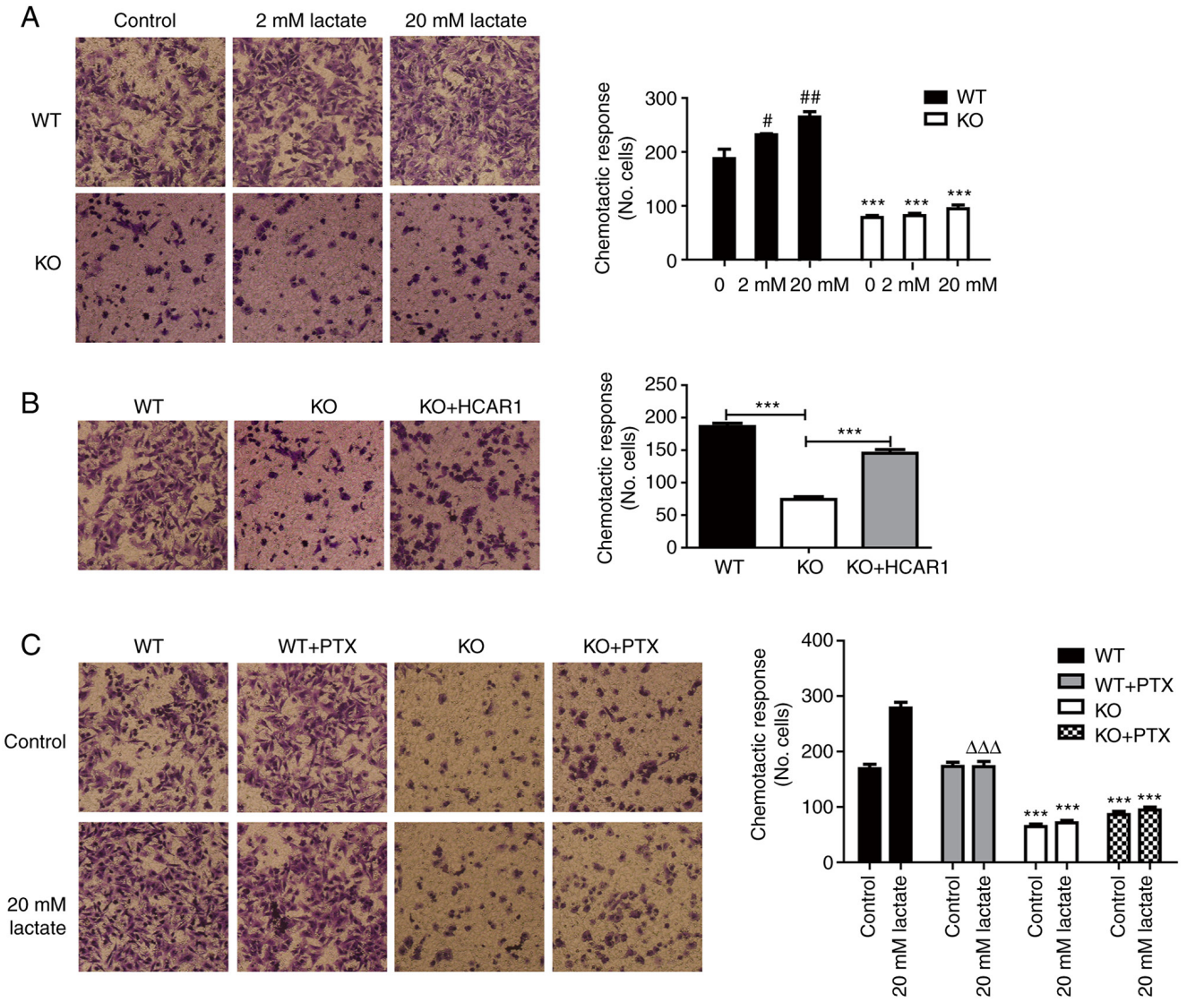

Figure 3. Effect of *HCAR1* KO MCF7 cell migration. (A) Complete medium containing 10% FBS with 2 or 20 mM lactate was added to the lower chambers to act as a chemotactic factor for 24 h, and the migratory cells were stained with 0.1% crystal violet. Pictures of the cell layers were obtained using a light microscope (x100 magnification). Data are presented as the mean ± SD (n≥3). (B) Cell migration of the WT, KO and re-overexpression groups were assessed by Transwell assays. Data are presented as the mean ± SD (n≥3). (C) After incubation with 100 ng/ml PTX (Gi inhibitor) for 6 h, the migration of WT and KO cells was accessed by Transwell assays. Data are presented as the mean ± SD (n≥3). $^{\#}$P<0.05 and $^{\#\#}$P<0.01 compared with non-lactate treatment group; ***P<0.001 compared with WT group; $^{\Delta\Delta\Delta}$P<0.001 compared with non-PTX group. HCAR1, hydroxyl carboxylic acid receptor 1; KO, knockout; WT, wild-type; PTX, pertussis toxin.

by GraphPad Prism 5.0 software (GraphPad Software, Inc.). Data are presented as the mean ± SD and represent at least three independent experiments. Unpaired Student's t-tests, or one-way ANOVA with Bonferroni's post hoc test was used as indicated. P<0.05 was considered to indicate a statistically significant difference.

Results

HCAR1 is highly expressed in breast cancer. To determine whether HCAR1 was expressed in cancer cells of solid tumours, whose interior are hypoxic and shows more lactate accumulation (20,21), HCAR1 mRNA levels were analyzed in various cancer cell lines. It was found that HCAR1 was expressed in tongue, lung, breast, bladder, pancreatic, hepatocellular, colorectal, cervical and epidermal carcinoma cells (Fig. 1A). Due to high HCAR1 expression, breast cancer cells were selected for further study. Compared with all kinds of breast cancer cell lines, a higher level of HCAR1 mRNA and protein expression was observed in MCF-7 cells (Fig. 1B and C). These results demonstrated that HCAR1 was expressed in numerous cancer cell types and in almost all human breast cancer cells.

KO of HCAR1 inhibits breast cancer cell survival and proliferation. To elucidate the potential effect of HCAR1 on cell proliferation, *HCAR1*-KO MCF7 cells were generated by the CRISPR/Cas9 system. A total of 3 lenti-CRISPR/Cas9-KO constructs containing nonoverlapping sgRNAs ('sgRNA1/2/3') were utilized to establish stable *HCAR1*-KO MCF7 cells (Figs. 2C, S1A and B). The survival of HCAR1-KO MCF7 cells treated with lactate was analyzed by CCK-8 assay

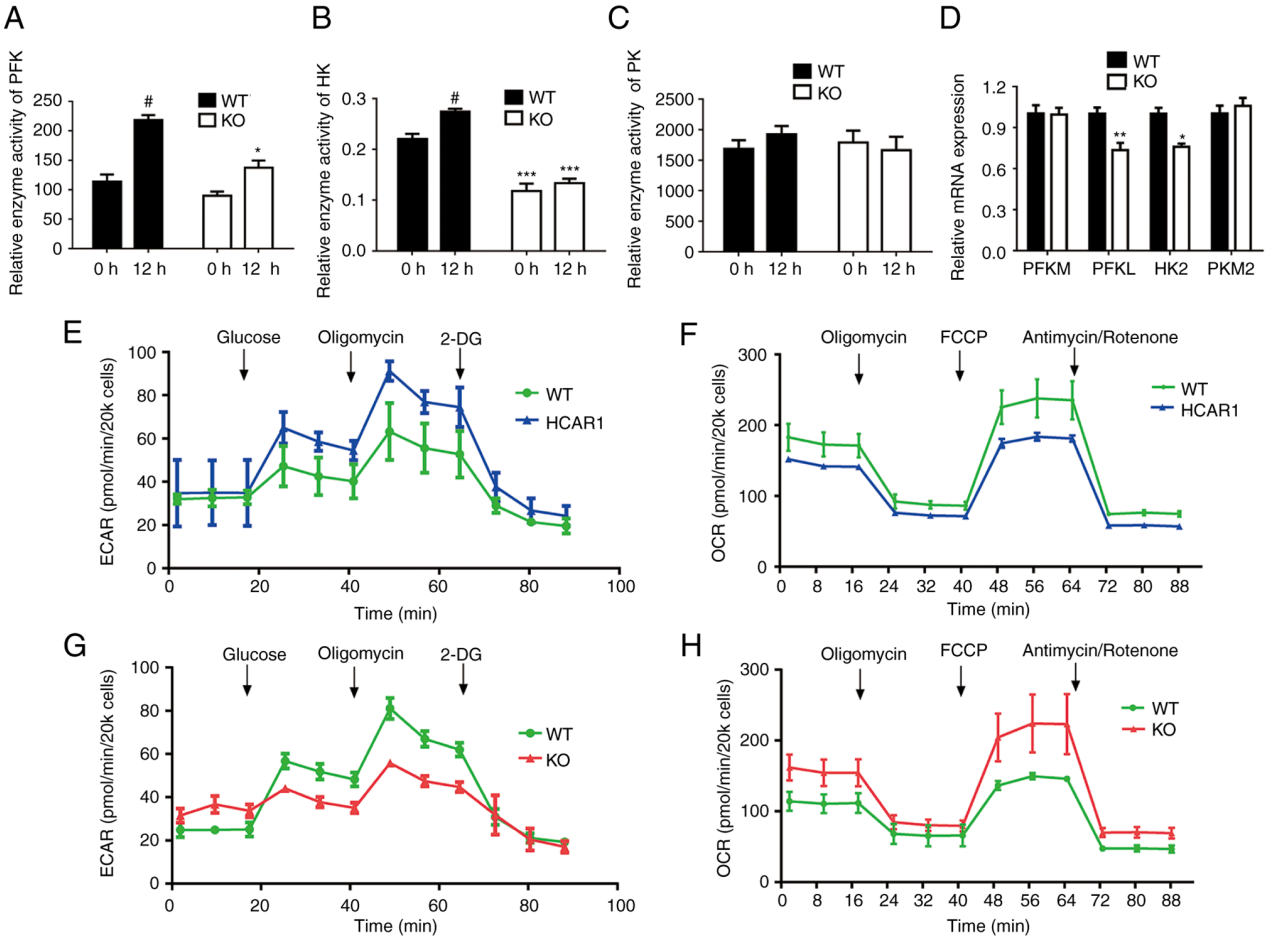

Figure 4. Effect of HCAR1 on MCF7 cell energy metabolism. (A-C) After 20 mM lactate treatment for 12 h, WT or KO MCF7 cell lysates were used to estimate the activities of the key enzymes for the glycolytic pathway. (A) PFK, (B) HK and (C) PK were assessed by respective enzyme activity kits according to the manufacturer's protocol. All data are presented as the mean ± SD from three replicates. (D) mRNA expression levels of key glycolytic enzymes were determined by reverse transcription-quantitative PCR. All data are presented as the mean ± SD from three replicates. (E-H) The (E and G) ECAR and (F and H) OCR were determined by extracellular flow analysis using a Seahorse Bioscienses XF96 analyzer in HCAR1 (E and F) overexpressing or (G and H) KO MCF7 cells. Experiments were repeated three times and six parallel wells were set in each experiment. $^{\#}$P<0.05 compared with non-lactate treatment group; *P<0.05, **P<0.01 and ***P<0.001 compared with WT group. HCAR1, hydroxyl carboxylic acid receptor 1; WT, wild-type; KO, knockout; PFK, phosphofructokinase; HK, hexokinase; PK, pyruvate kinase.

(Fig. 2A). *HCAR1* depletion potently inhibited the viability of MCF7 cells, and lactate incubation significantly promoted the proliferation of wild-type (WT) but not *HCAR1*-KO cells. Consistent with these findings, colony formation and *EdU* assay results further demonstrated that *HCAR1* KO inhibited MCF7 cell proliferation, and lactate treatment did not have a proliferation-promoting effect compared with WT MCF7 cells (Fig. 2B and D). As proliferation-suppression phenotypes were observed after depletion of *HCAR1*, cell cycle distribution was further detected by PI staining and flow cytometry. Consistently, a significant increase in the G1 phase and a decrease in the G2 phase were observed in MCF7 *HCAR1*-KO cells (Fig. 2E). These results indicated that HCAR1 promoted MCF7 cell proliferation.

HCAR1 promotes breast cancer cell metastasis. The effects of HCAR1 on the migratory ability of MCF7 cells were assessed by a Transwell migration assay. The depletion of *HCAR1* in MCF7 cells resulted in a significant reduction in cell migration (Fig. 3A). However, the inhibition of cell migration was attenuated after re-overexpression of HCAR1 (Figs. 3B and S2). In addition, lactate promoted WT MCF7 cell migration in a lactate concentration-dependent manner, which was inhibited after Gi inhibitor-PTX treatment (Fig. 3C). These data indicated that HCAR1 played a vital role in breast cancer cell migration and that the Gi protein may participate in the regulation of MCF7 cell migration.

Effects of HCAR1 on breast cancer cell glycolysis metabolism. The speed and direction of the metabolic reaction are associated with rate-limiting enzymes such as PFK, HK and PK (22,23). Therefore, it was investigated whether HCAR1 regulated the expression of those key rate-limiting enzymes in glucose catabolism by RT-qPCR. As revealed in Fig. 4A-D, there was a significant decrease in the mRNA expression of PFKL and HK2 in MCF7 *HCAR1*-KO cells, whereas a significant inhibition of the enzyme activity of PFK and HK was also observed in *HCAR1*-KO cells.

KO of HCAR1 reverses the Warburg effect in MCF7 cells. The glycolysis metabolism results suggested that HCAR1 could regulate the expression and activity of glycolytic enzymes.

To evaluate whether KO of *HCAR1* reversed metabolic reprogramming consistent with the Warburg effect, cell energy phenotype assays were conducted using the XFp seahorse bioanalyzer system. This assay delineated the metabolic phenotypes of MCF7 cells under baseline, KO and overexpression conditions. These results indicated that *HCAR1* KO reduced the ECAR of MCF7 cells and augmented the OCR compared with control cells, which shifted their energy derivation towards OXPHOS (Fig. 4G and H). Moreover, HCAR1 overexpressing cells had an increased ECAR and a reduced OCR compared with WT cells (Fig. 4E and F). These results indicated that HCAR1 could metabolically control breast cancer by favoring glycolysis over OXPHOS.

Discussion

Since the identification of HCAR1 as a lactate receptor, studies have revealed that, similar to the other members of the HCA receptor family, HCAR1 is predominantly expressed in adipose tissue and suppresses lipolysis by reducing cAMP levels via a Gi protein-coupled pathway (10,24). However, in contrast to GPR109A (HCA2), HCAR1 could inhibit lipolysis without provoking skin flushing (14,25). Therefore, HCAR1 has promising potential in dyslipidemia. Cancer cells use anaerobic glycolysis for energy intake, even in normoxic conditions, causing increased production of lactate (26). Insufficient tumour blood supply leads to the accumulation of lactic acid (20-40 mM) (6) in the tumour microenvironment, which may be sufficient to activate HCAR1. Altogether, the role of HCAR1 in tumours has markedly attracted attention.

HCAR1 has been identified to be expressed at high levels in a variety of tumour cells, where it is able to induce cancer cells to proliferate and differentiate constantly, thus enhancing tumour growth. A previous study has shown that HCAR1 is highly expressed in prostate cancer cells and may inhibit pancreatic cancer cell progression by regulating lactate transporter expression (13). Moreover, daily intraperitoneal injection with isotonic lactate or sodium lactate in a mouse xenograft model promoted the proliferation of tumour cells (24). Furthermore, Wanger *et al* (27) found that lactate present in the uterine cervix may participate in the modulation of cellular DNA damage repair processes and in the resistance of cervical carcinoma cells to anticancer therapy. In the present study, it was revealed that after KO of *HCAR1*, the proliferation, cell cycle distribution and migration of breast cancer cells were greatly affected. It is noteworthy that a significant increase in the G1 phase and a decrease in the G2 phase were observed in *HCAR1*-KO cells. K^+ channel activity has been reported to be crucial in cell progression through the G1 checkpoint of the cell cycle (28,29). There are studies regarding co-localization of GIRK channel and Gi coupled GPCR (muscarinic receptor) for efficient channel activation (30-32). However, there is no study about HCAR1 associated K-channel activity. Thus, whether the cell cycle changes in *HCAR1*-KO MCF7 cells associated with K-channel function need to be further explored. Collectively, these results demonstrated that activation of the HCAR1 receptor was important for maintaining breast cancer cell survival.

High lactate levels in the tumour microenvironment play a critical role in promoting cell migration and invasion. On the one hand, extracellular acidification activated p53-mediated apoptosis in a caspase-dependent manner in normal cells (33). Cancer cells can survive more easily in acidified microenvironment due to their high proton transport activity and low expression level of the P53 gene (34). On the other hand, acidification promoted tumour angiogenesis by activating VEGF and IL-8 release (35,36) and promoting degradation of the extracellular matrix by proteolytic enzymes to drive tumour metastasis and invasion (36,37). GPR132 functions as a key macrophage sensor of the rising lactate in the acidic tumour milieu to promote the alternatively activated macrophage (M2)-like phenotype, which facilitates cancer cell migration and invasion (38). High lactate content promoted tumour progression by contributing to the phenomenon of tumour immune escape and by enhancing the migratory potential of the malignant cell population (39). Less conclusive evidence has been reported concerning the role of the HCAR1 pathway in breast cancer cell migration. In the present study, it was found that KO of *HCAR1* resulted in a significant inhibition of MCF7 cell motility. After re-expressing HCAR1 in the KO cell line, the number of cells that passed through the Transwell chamber showed a significant improvement. The results suggested that HCAR1 played an important role in breast cancer migration. Quite a few G protein-coupled receptors (GPCRs), such as CXCR4, LPA and PAR1, participate in the regulation of tumour cell migration (40,41). In the present study, the Gi protein inhibitor PTX was used to determine whether Gi coupled with the HCAR1 signalling pathway mediates MCF7 cell migration.

The Warburg effect, a reprogrammed metabolic pathway that meets the rapidly proliferating tumour cell energy requirement, has been observed in a variety of malignant tumours (1,42,43). Enhanced glycolysis leads to increased glucose uptake and lactate production, which has a significant influence on the initiation, development and progression of cancer. In the present study, it was found that the expression and activity of key glycolytic enzymes were affected by HCAR1 in breast cancer. After *HCAR1* KO in MCF7 cells, the expression of enzymes, such as PFKL and HK decreased to varying degrees, and the enzyme activity of PFK and HK showed a significant reduction, indicating that the levels of glycolysis were to a moderate extent suppressed by *HCAR1* knockdown. Furthermore, evidence provided by cell energy phenotype assays suggested a similar result accomplished with the use of MCF7 cells. Initially, the WT and overexpression HCAR1 cell lines demonstrated a glycolytic phenotype consistent with the Warburg effect that would be expected in cancer. However, with CRISPR-Cas9 *HCAR1* gene KO, a more energetic phenotype was demonstrated by reduced ECAR and increased OCR. The results implied an impairment of cell glycolysis and showed an oncogenic role for HCAR1.

The limitation to the present study is the performance of experiments using only one cell line, as the proliferation, migration and energy metabolism phenotype can vary in different breast cancer cell types. In the present study, focused was addressed on MCF7 cells based on higher expression of HCAR1 in the aforementioned cell line. Further studies are required to investigate the aforementioned roles of HCAR1 in other breast cancer cells so as to reveal the relation between HCAR1 and breast cancer in an improved way.

In conclusion, it was revealed that HCAR1 was overexpressed in breast cancer cells, particularly in MCF7 cells. In addition, KO of *HCAR1* could substantially inhibit breast cancer cell proliferation, migration and glycolysis in an *in vitro* study. Collectively, the present findings indicated that HCAR1 may be a tumour promoting factor and that lactate activated this receptor and hence promoted tumour growth and metastasis by regulating cellular energy metabolism through glycolysis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JQ and LJ conceived and designed the study. LJ, YG, JC and ZW performed experiments. YJ helped with the collection and assembly of data. LJ, ZW and JQ analyzed the data and prepared the figures. LJ and JQ drafted and revised the manuscript. All authors read and approved the final manuscript. LJ and YJ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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