

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

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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.

## 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

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**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

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<sub>2</sub> 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<sup>-ΔΔC<sub>q</sub></sup> method (19). Primer (10 μM for each gene) sequences for each gene were as follows: HCAR1 forward, 5'-GCCCAGCAC TGTTCACCTTTTC-3' and reverse, 5'-CCCCAAAAGCCC AGTGTCTAC-3'; phosphofructokinase muscle type (PFKM) forward, 5'-GAGTGACTTGTGTGAGTGACCTCCAGAAA-3' and reverse, 5'-CACAATGTTTCAGGTAGCTGGACTTCG-3'; PFK liver type (PFKL) forward, 5'-GGCATTTATGTGGGT GCCAAAGTC-3' and reverse, 5'-CAGTTGGCCTGCTTG ATGTTCTCA-3'; hexokinase (HK)2 forward, 5'-GGGCAT CTTGAACAAG-3' and reverse, 5'-GGTCTCAAGCCC TAAAG-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<sub>3</sub>VO<sub>4</sub>, 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; cat. 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

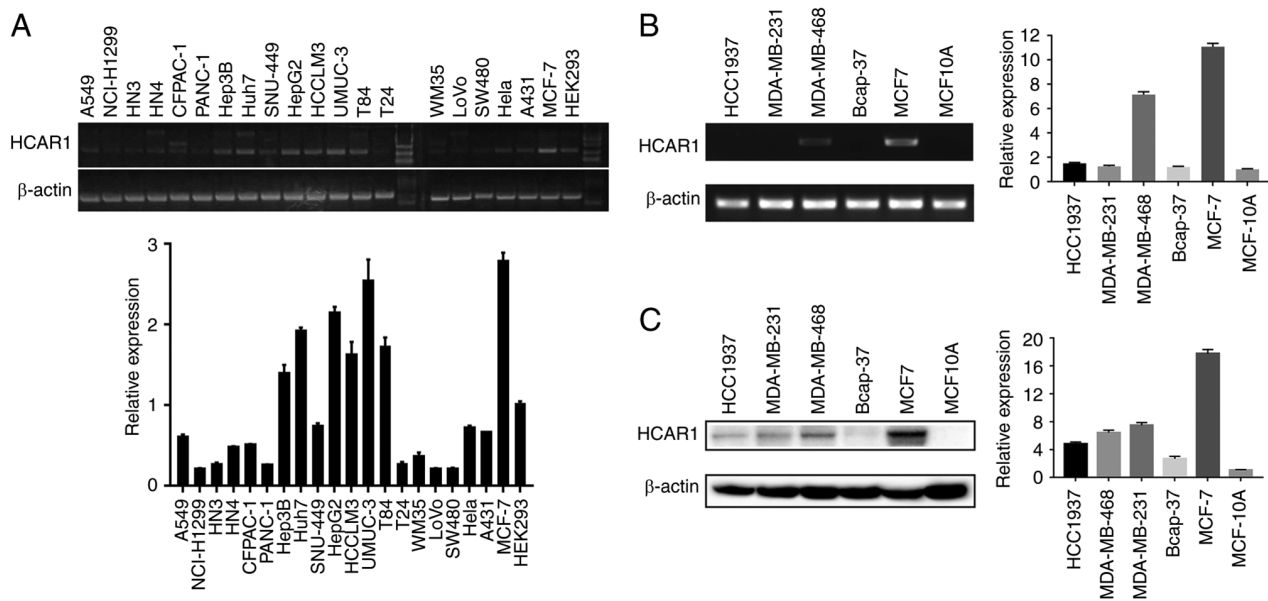


Figure 1. HCARI is highly expressed in human breast cancer cell lines. (A) The expression of HCARI in different cancer cell lines was assessed by semi-quantitative PCR. Data are presented as the mean  $\pm$  SD ( $n \geq 3$ ). (B) Relative mRNA expression of HCARI in 5 breast cancer cell lines and the 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 HCARI 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. HCARI, hydroxyl carboxylic acid receptor 1.

96 h) at 37°C. CCK-8 (10  $\mu$ l) 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  $1 \times 10^4$  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 HCARI-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  $\mu$ g/ml PI, 250  $\mu$ g/ml RNase A in PBS) for 15 min at 37°C in the dark. The level of PI incorporation was analyzed by FACSscan (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  $1 \times 10^5$  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  $\mu$ m). 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,  $\times 200$ ).

**Measurement of enzyme activities.** MCF7 cells were seeded at a density of  $2.0 \times 10^5$ /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

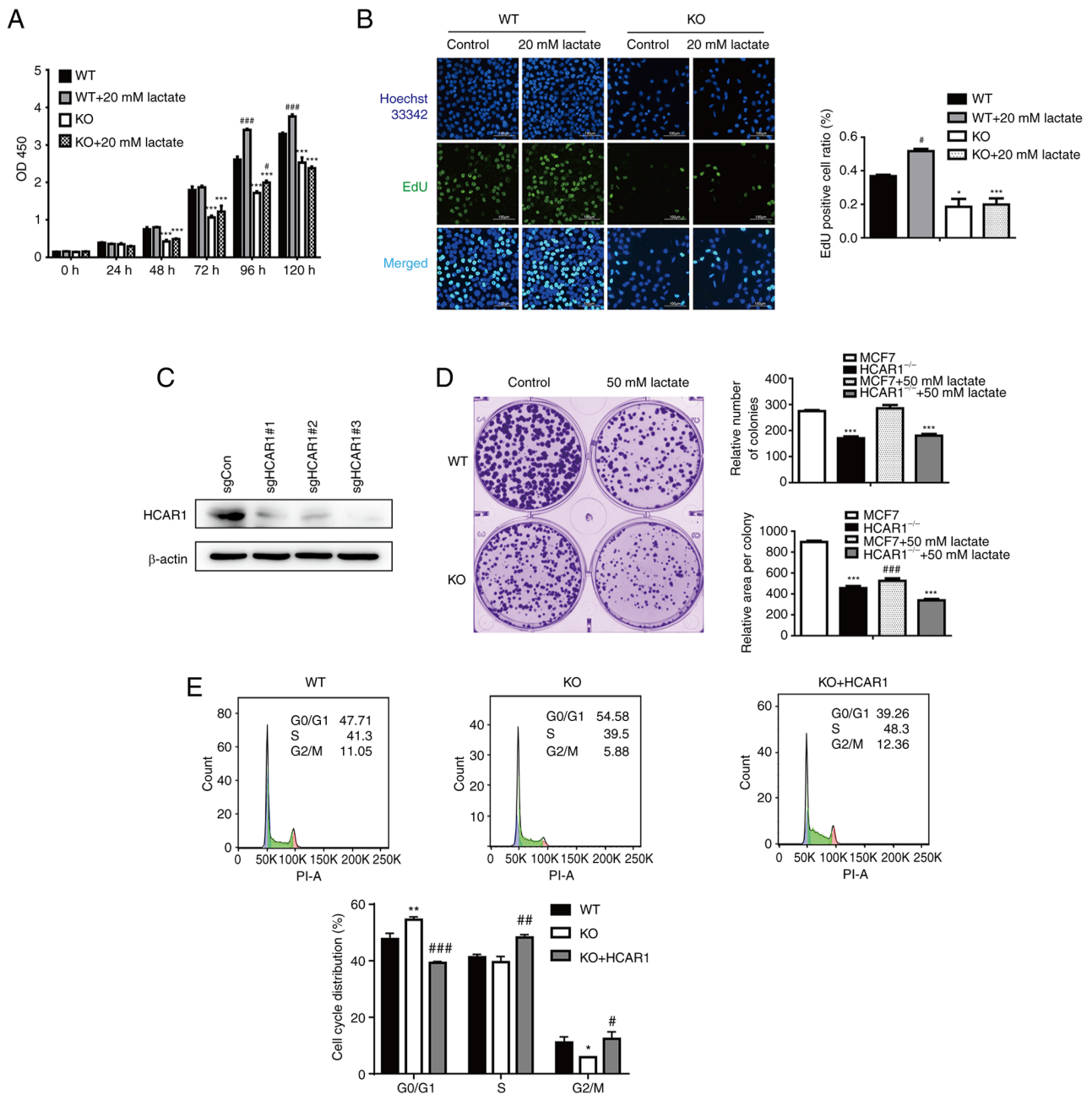


Figure 2. HCAR1 is an important regulator of MCF7 cell proliferation and survival. (A) After lactate treatment of WT and *HCAR1*-KO cell lines, cell proliferation was assessed using a Cell Counting Kit-8 (CCK-8). Experiments were repeated at least three times, and all data are presented as the mean  $\pm$  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,  $\times 200$ ). Data are presented as the mean  $\pm$  SD ( $n \geq 3$ ). (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  $\pm$  SD ( $n \geq 3$ ). (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  $1 \times 10^4$  cells/well into 96-well Seahorse microplates for 24 h, and the calibration plate was equilibrated overnight in a non- $\text{CO}_2$  incubator. Cells were washed twice with assay running media and equilibrated in a non- $\text{CO}_2$  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 ( $1 \mu\text{M}$ ), carbonyl

cyanide-p-trifluoromethoxyphenylhydrazine ( $1 \mu\text{M}$ ), rotenone ( $1 \mu\text{M}$ ) and antimycin A ( $1 \mu\text{M}$ ), 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



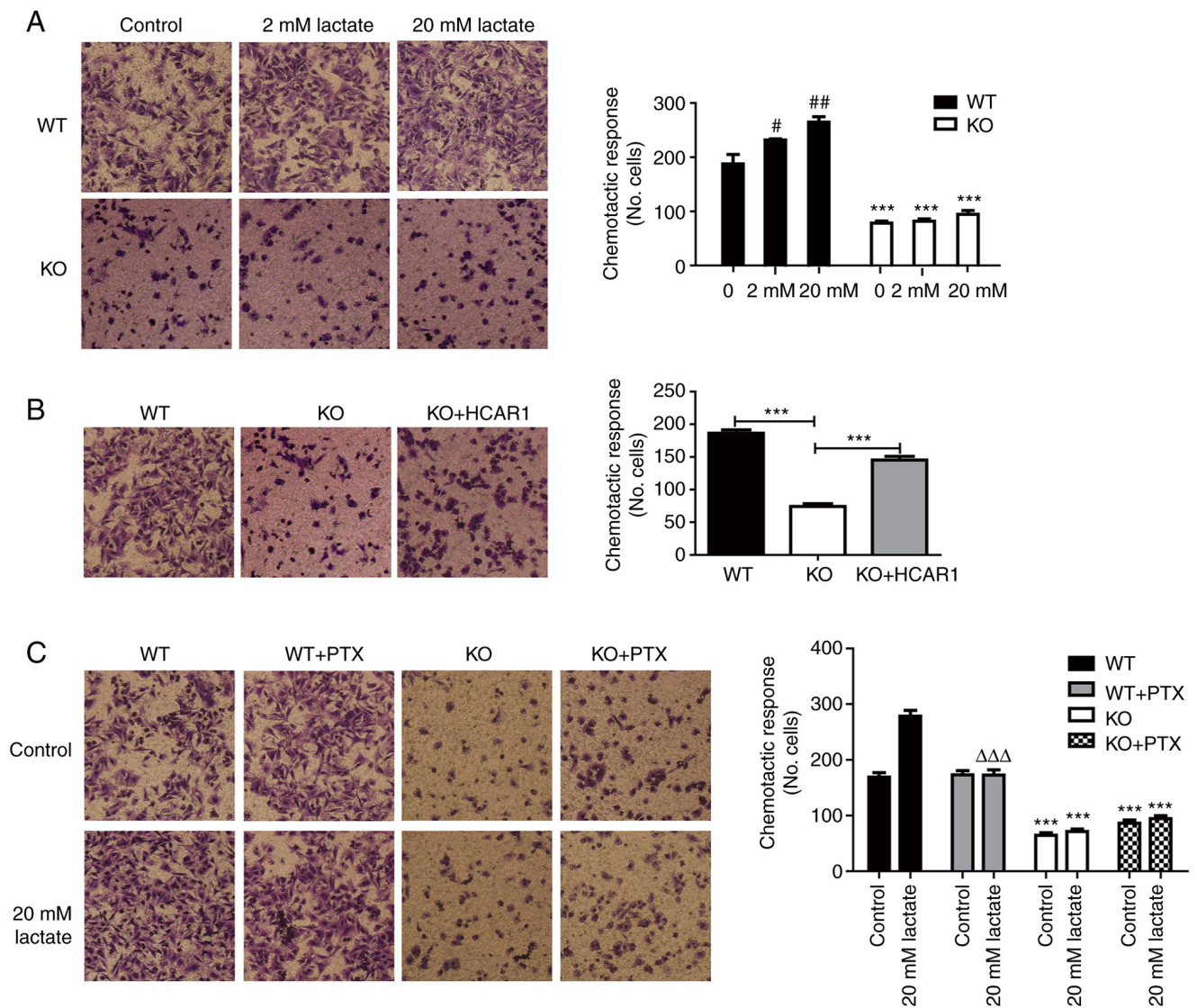


Figure 3. Effect of *HCAR1* KO MCF7 cell migration. (A) Complete medium containing 10% FBS with 2 or 20 mM lactate was added to the lower chambers to act as a chemotactic factor for 24 h, and the migratory cells were stained with 0.1% crystal violet. Pictures of the cell layers were obtained using a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

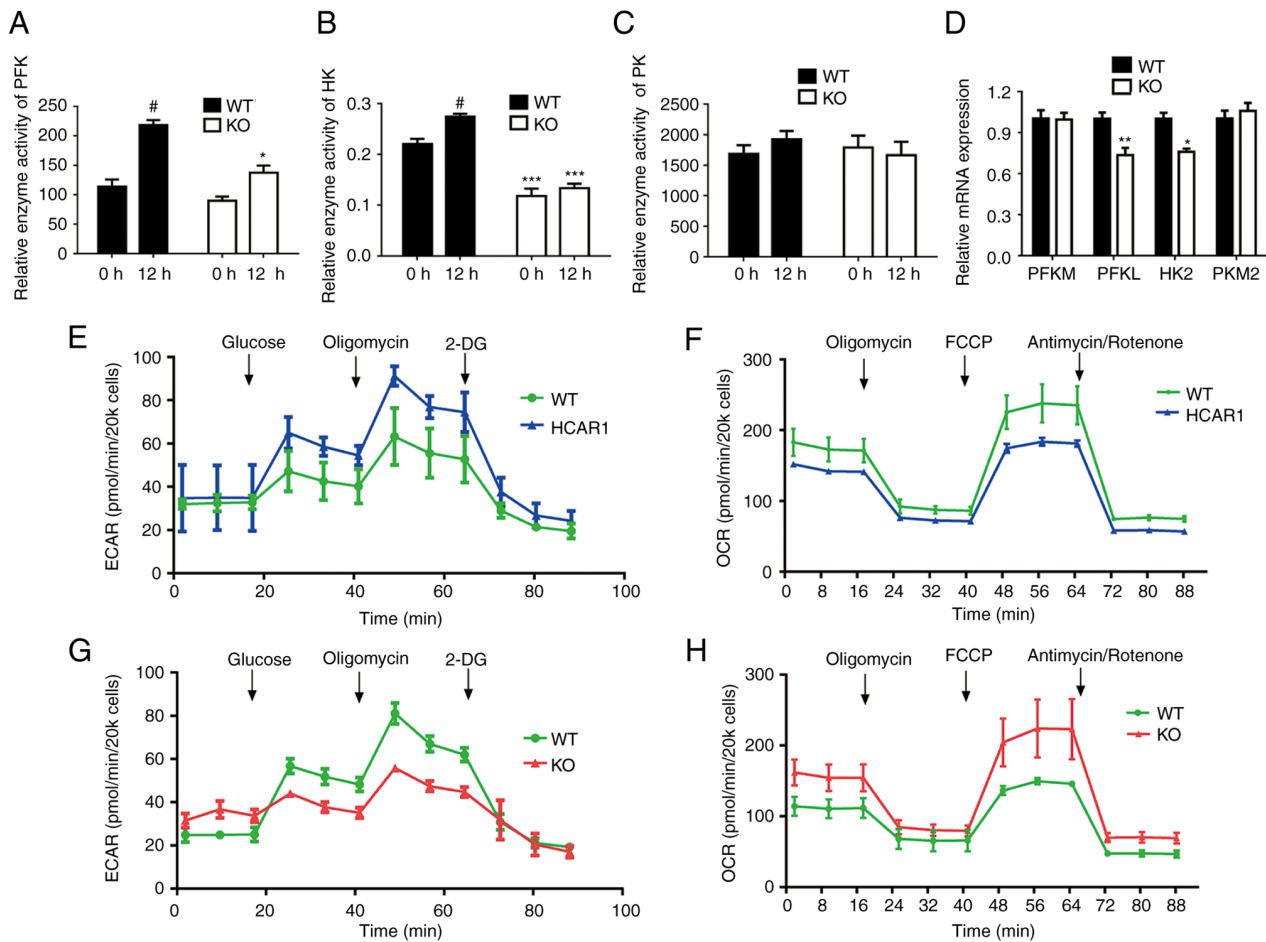


Figure 4. Effect of HCARI on MCF7 cell energy metabolism. (A-C) After 20 mM lactate treatment for 12 h, WT or KO MCF7 cell lysates were used to estimate the activities of the key enzymes for the glycolytic pathway. (A) PFK, (B) HK and (C) PK were assessed by respective enzyme activity kits according to the manufacturer's protocol. All data are presented as the mean  $\pm$  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  $\pm$  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 Biosciences XF96 analyzer in HCARI (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. <sup>#</sup> $P < 0.05$  compared with non-lactate treatment group; <sup>\*</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$  and <sup>\*\*\*</sup> $P < 0.001$  compared with WT group. HCARI, hydroxyl carboxylic acid receptor 1; WT, wild-type; KO, knockout; PFK, phosphofructokinase; HK, hexokinase; PK, pyruvate kinase.

(Fig. 2A). *HCARI* depletion potently inhibited the viability of MCF7 cells, and lactate incubation significantly promoted the proliferation of wild-type (WT) but not *HCARI*-KO cells. Consistent with these findings, colony formation and *EdU* assay results further demonstrated that *HCARI* 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 *HCARI*, 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 *HCARI*-KO cells (Fig. 2E). These results indicated that *HCARI* promoted MCF7 cell proliferation.

***HCARI* promotes breast cancer cell metastasis.** The effects of *HCARI* on the migratory ability of MCF7 cells were assessed by a Transwell migration assay. The depletion of *HCARI* 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 *HCARI* (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 *HCARI* 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 HCARI 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 *HCARI* 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 *HCARI*-KO cells, whereas a significant inhibition of the enzyme activity of PFK and HK was also observed in *HCARI*-KO cells.

***KO of HCARI reverses the Warburg effect in MCF7 cells.*** The glycolysis metabolism results suggested that *HCARI* 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<sup>+</sup> 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.

## References

- Heiden MG, Cantley LC and Thompson CB: Understanding the Warburg effect: The metabolic requirements of cell proliferation. *Science* 324: 1029-1033, 2009.
- Cairns RA, Harris IS and Mak TW: Regulation of cancer cell metabolism. *Nat Rev Cancer* 11: 85-95, 2011.
- Brizel DM, Schroeder T, Scher RL, Walenta S, Clough RW, Dewhirst MW and Mueller-Klieser W: Elevated tumor lactate concentrations predict for an increased risk of metastases in head-and-neck cancer. *Int J Radiat Oncol Biol Phys* 51: 349-353, 2001.
- Walenta S and Mueller-Klieser WF: Lactate: Mirror and motor of tumor malignancy. *Semin Radiat Oncol* 14: 267-274, 2004.
- Walenta S, Schroeder T and Mueller-Klieser W: Lactate in solid malignant tumors: Potential basis of a metabolic classification in clinical oncology. *Curr Med Chem* 11: 2195-2204, 2004.
- Romero-Garcia S, Moreno-Altamirano MM, Prado-Garcia H and Sánchez-García FJ: Lactate contribution to the tumor microenvironment: mechanisms, effects on immune cells and therapeutic relevance. *Front Immunol* 7: 52, 2016.
- Iñigo SM and Carcinogenesis BGJ: Reexamining cancer metabolism: lactate production for carcinogenesis could be the purpose and explanation of the Warburg Effect. *Carcinogenesis* 119: 119-133, 2016.
- Feng J, Yang H, Zhang Y, Wei H, Zhu Z, Zhu B, Yang M, Cao W, Wang L and Wu Z: Tumor cell-derived lactate induces TAZ-dependent upregulation of PD-L1 through GPR81 in human lung cancer cells. *Oncogene* 36: 5829-5839, 2017.
- Dhup S, Dadhich RK, Porporato PE and Sonveaux P: Multiple biological activities of lactic acid in cancer: Influences on tumor growth, angiogenesis and metastasis. *Curr Pharm Des* 18: 1319-1330, 2012.
- Liu C, Wu J, Zhu J, Kuei C, Yu J, Shelton J, Sutton SW, Li X, Yun SJ, Mirzadegan T, *et al*: Lactate inhibits lipolysis in fat cells through activation of an orphan G-protein-coupled receptor, GPR81. *J Biol Chem* 284: 2811-2822, 2009.
- Liu C, Kuei C, Zhu J, Yu J, Zhang L, Shih A, Mirzadegan T, Shelton J, Sutton S, Connelly MA, *et al*: 3,5-Dihydroxybenzoic acid, a specific agonist for hydroxycarboxylic acid 1, inhibits lipolysis in adipocytes. *J Pharmacol Exp Ther* 341: 794-801, 2012.
- Ca TQ, Ren N, Jin L, Cheng K, Kash S, Chen R, Wright SD, Taggart AKP and Waters MG: Role of GPR81 in lactate-mediated reduction of adipose lipolysis. *Biochem Biophys Res Commun* 377: 987-991, 2008.
- Roland CL, Arumugam T, Deng D, Liu SH, Philip B, Gomez S, Burns WR, Ramachandran V, Wang H, Cruz-Monserrate Z and Logsdon CD: Cell surface lactate receptor GPR81 is crucial for cancer cell survival. *Cancer Res* 74: 5301-5310, 2014.
- Lee YJ, Shin KJ, Park SA, Park KS, Park S, Heo K, Seo YK, Noh DY, Ryu SH and Suh PG: G-protein-coupled receptor 81 promotes a malignant phenotype in breast cancer through angiogenic factor secretion. *Oncotarget* 7: 70898-70911, 2016.
- Offermanns S: Hydroxy-carboxylic acid receptor actions in metabolism. *Trends Endocrinol Metab* 28: 227-236, 2017.
- Stäubert C, Broom OJ and Nordström A: Hydroxycarboxylic acid receptors are essential for breast cancer cells to control their lipid/fatty acid metabolism. *Oncotarget* 6: 19706-19720, 2015.
- Lundø K, Trauelsen M, Pedersen SF and Schwartz TW: Why warburg works: Lactate controls immune evasion through GPR81. *Cell Metab* 31: 666-668, 2020.
- Brown TP, Bhattacharjee P, Ramachandran S, Sivaprakasam S, Ristic B, Sikder MOF and Ganapathy V: The lactate receptor GPR81 promotes breast cancer growth via a paracrine mechanism involving antigen-presenting cells in the tumor microenvironment. *Oncogene* 39: 3292-3304, 2020.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- Jiang J, Huang D, Jiang Y, Hou J, Tian M, Li J, Sun L, Zhang Y, Zhang T, Li Z, *et al*: Lactate modulates cellular metabolism through histone lactylation-mediated gene expression in non-small cell lung cancer. *Front Oncol* 11: 647559, 2021.
- Salem A, Asselin MC, Reymen B, Jackson A, Lambin P, West CML, O'Connor JPB and Faivre-Finn C: Targeting hypoxia to improve non-small cell lung cancer outcome. *J Natl Cancer Inst* 110: doi: 10.1093/jnci/djx160, 2018.
- Yu G, Yu W, Jin G, Xu D, Chen Y, Xia T, Yu A, Fang W, Zhang X, Li Z and Xie K: PKM2 regulates neural invasion of and predicts poor prognosis for human hilar cholangiocarcinoma. *Mol Cancer* 14: 193, 2015.
- Li Y, Wang Y, Liu Z, Guo X, Miao Z and Ma S: Atractylenolide I induces apoptosis and suppresses glycolysis by blocking the JAK2/STAT3 signaling pathway in colorectal cancer cells. *Front Pharmacol* 11: 273, 2020.
- Goodwin ML, Jin H, Straessler K, Smith-Fry K, Zhu JF, Monument MJ, Grossmann A, Randall RL, Capecchi MR and Jones KB: Modeling alveolar soft part sarcomagenesis in the mouse: A role for lactate in the tumor microenvironment. *Cancer Cell* 26: 851-862, 2014.
- Sakurai T, Davenport R, Stafford S, Grosse J, Ogawa K, Cameron J, Parton L, Sykes A, Mack S, Bousba S, *et al*: Identification of a novel GPR81-selective agonist that suppresses lipolysis in mice without cutaneous flushing. *Eur J Pharmacol* 727: 1-7, 2014.
- Ward C, Langdon SP, Mullen P, Harris AL, Harrison DJ, Supuran CT and Kunkler IH: New strategies for targeting the hypoxic tumour microenvironment in breast cancer. *Cancer Treat Rev* 39: 171-179, 2013.
- Wagner W, Ciszewski WM and Kania KD: L- and D-lactate enhance DNA repair and modulate the resistance of cervical carcinoma cells to anticancer drugs via histone deacetylase inhibition and hydroxycarboxylic acid receptor 1 activation. *Cell Commun Signal* 13: 36, 2015.



28. Xu B, Wilson BA and Lu L: Induction of human myeloblastic ML-1 cell G1 arrest by suppression of K<sup>+</sup> channel activity. *Am J Physiol* 271: C2037-C2044, 1996.
29. Marakhova I, Domnina A, Shatrova A, Borodkina A, Burova E, Pugovkina N, Zemelko V and Nikolsky N: Proliferation-related changes in K(+) content in human mesenchymal stem cells. *Sci Rep* 9: 346, 2019.
30. Tateyama M and Kubo Y: Gi/o-coupled muscarinic receptors co-localize with GIRK channel for efficient channel activation. *PLoS One* 13: e0204447, 2018.
31. Days E, Kaufmann K, Romaine I, Niswender C, Lewis M, Utley T, Du Y, Sliwoski G, Morrison R, Dawson ES, *et al*: Discovery and Characterization of a Selective Activator of the G-Protein Activated Inward-Rectifying Potassium (GIRK) Channel. In: *Probe Reports from the NIH Molecular Libraries Program* [Internet]. National Center for Biotechnology Information (US), Bethesda, MD, 2010.
32. Dascal N and Kahanovitch U: The roles of G $\beta\gamma$  and G $\alpha$  in gating and regulation of GIRK channels. *Int Rev Neurobiol* 123: 27-85, 2015.
33. Park HJ, Lyons JC, Ohtsubo T and Song CW: Acidic environment causes apoptosis by increasing caspase activity. *Br J Cancer* 80: 1892-1897, 1999.
34. Porporato PE, Dhup S, Dadhich RK, Copetti T and Sonveaux P: Anticancer targets in the glycolytic metabolism of tumors: A comprehensive review. *Front Pharmacol* 2: 49, 2011.
35. Shi Q, Abbruzzese JL, Huang SY, Fidler IJ, Xiong Q and Xie K: Constitutive and inducible interleukin 8 expression by hypoxia and acidosis renders human pancreatic cancer cells more tumorigenic and metastatic. *Clin Cancer Res* 5: 3711-3721, 1999.
36. Shi Q, Le X, Wang B, Abbruzzese JL, Xiong Q, He Y and Xie K: Regulation of vascular endothelial growth factor expression by acidosis in human cancer cells. *Oncogene* 20: 3751-3756, 2001.
37. Rozhin J, Sameni M, Ziegler GH and Sloane BF: Pericellular pH affects distribution and secretion of cathepsin B in malignant cells. *Cancer Res* 54: 6517-6525, 1994.
38. Xie Q, Zhu Z, He Y, Zhang Z, Zhang Y, Wang Y, Luo J, Peng T, Cheng F, Gao J, *et al*: A lactate-induced Snail/STAT3 pathway drives GPR81 expression in lung cancer cells. *Biochim Biophys Acta Mol Basis Dis* 1866: 165576, 2020.
39. Goetze K, Walenta S, Ksiazkiewicz M, Kunz-Schughart LA and Mueller-Klieser W: Lactate enhances motility of tumor cells and inhibits monocyte migration and cytokine release. *Int J Oncol* 39: 453-463, 2011.
40. Dusaban SS, Purcell NH, Rockenstein E, Masliah E, Cho MK, Smrcka AV and Brown JH: Phospholipase C $\epsilon$  links G protein-coupled receptor activation to inflammatory astrocytic responses. *Proc Natl Acad Sci USA* 110: 3609-3614, 2013.
41. Wang Y, Liao R, Chen X, Ying X, Chen G, Li M and Dong C: Twist-mediated PAR1 induction is required for breast cancer progression and metastasis by inhibiting Hippo pathway. *Cell Death Dis* 11: 520, 2020.
42. Liberti MV and Locasale JW: The warburg effect: How does it benefit cancer cells? *Trends Biochem Sci* 41: 211-218, 2016.
43. Warburg O: On the origin of cancer cells. *Science* 123: 309-314, 1956.



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