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ECM1 promotes the Warburg effect through EGF-mediated activation of PKM2

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ABSTRACT

The Warburg effect is an oncogenic metabolic switch that allows cancer cells to take up more glucose than normal cells and favors anaerobic glycolysis. Extracellular matrix protein 1 (ECM1) is a secreted glycoprotein that is overexpressed in various types of carcinoma. Using two-dimensional digest-liquid chromatography–mass spectrometry (LC–MS)/MS, we showed that the expression of proteins associated with the Warburg effect was upregulated in trastuzumab-resistant BT-474 cells that overexpressed ECM1 compared to control cells. We further demonstrated that ECM1 induced the expression of genes that promote the Warburg effect, such as glucose transporter 1 (*GLUT1*), lactate dehydrogenase A (*LDHA*), and hypoxia-inducible factor 1 α (*HIF-1*α). The phosphorylation status of pyruvate kinase M2 (PKM-2) at Ser37, which is responsible for the expression of genes that promote the Warburg effect, was affected by the modulation of ECM1 expression. Moreover, EGF-dependent ERK activation that was regulated by ECM1 induced not only PKM2 phosphorylation but also gene expression of *GLUT1* and *LDHA*. These findings provide evidence that ECM1 plays an important role in promoting the Warburg effect mediated by PKM2.

1. Introduction

Proliferating cancer cells generate the energy required to support rapid cell division. Various cancer cells exhibit different metabolic phenotypes, such as increased glucose uptake and lactate production [1]. The Warburg effect is the most commonly observed metabolic phenotype in cancer cells and involves a shift from ATP production through oxidative phosphorylation to ATP production through glycolysis, converting most of the incoming glucose to lactate rather than metabolizing it in the mitochondria through oxidative phosphorylation regardless of oxygen supply [2,3]. This metabolic switch means that the rapidly proliferating cancer cells are provided with more energy and materials needed for biosynthesis [1]. It has previously been reported that several regulators participate in the modulation of this metabolic switch. Hypoxia-inducible factor 1 (HIF1) causes a metabolic shift from mitochondria-dependent oxidative phosphorylation to an increase in glycolysis [4]. HIF1 regulates the expression of various functional genes associated with glucose metabolism, such as glucose transporter 1 (GLUT1) [5] and lactate dehydrogenase A (LDHA) [6,7]. It is also known that inhibition of HIF1 is an efficient approach to block metabolic effects in cancer cells [8]. A high expression level of the M2 isoform of pyruvate kinase (PKM2) has been detected in many types of cancer cell [9], suggesting its involvement in mediating the Warburg effect. It is well-known that PKM2 catalyzes the generation of pyruvate from phosphoenolpyruvate (PEP) in the glycolytic pathway [10]. PKM2 also plays critical roles in tumorigenesis [10].

Extracellular matrix protein 1 (ECM1) is a secreted glycoprotein [11] and is considered a prognostic marker in various types of cancer [12,13]. Moreover, a high level of ECM1 is correlated with metastatic progression in breast cancer [14], cholangiocarcinoma [15], and laryngeal carcinoma [16]. Although it is well-established that ECM1 plays a role in tumor development and metastasis, the exact function of ECM1 is unclear.

In this report, we demonstrate that ECM1 potentiates a metabolic shift promoting the Warburg effect through modulating the gene expression of *GLUT1*, *HIF-1α*, and *LDHA*. We also show that expression of *GLUT* and *LDHA*, but not HIF-1α, is mediated by ERK-dependent activation and nuclear translocation of PKM2.

2. Materials and methods

2.1. Cell lines, antibodies, reagents, and plasmids

The human breast carcinoma cell lines BT-474 and MDA-MB-231 were obtained from American Type Culture Collection (ATCC). BT-474 and MDA-MB-231 cells were maintained in DMEM (Thermo Scientific Hyclone, Rockford, IL, USA) supplemented with 10% fetal bovine

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Table 1
LC/MS–MS identification of differentially expressed proteins between BT-474 WT and BT-474 TR cells.

Uniprot accession number	Protein name	Gene name	SI_{WT}[a]	SI_{TR}	Log_2 (SI_{TR}/SI_{WT})
P05091	Aldehyde dehydrogenase, mitochondrial	*ALDH2*	4.0301	20.7205	2.9090
P04075	Fructose-bisphosphate aldolase A	*ALDOA*	272.7292	475.6882	1.3494
P48735	Isocitrate dehydrogenase [NADP], mitochondrial	*IDH2*	25.2579	3.6217	−2.2551
P00338	L-lactate dehydrogenase A chain	*LDHA*	1.4633	26.5479	4.7282
P37837	Transaldolase	*TALDO1*	7.6586	11.7740	1.1673
P31040	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	*SDHA*	2.6194	0.0508	−5.1428

[a] SI = spectral index.

serum (FBS, Thermo), 1% penicillin/streptomycin (Thermo), and 1% antimycotic solution (Thermo). These cells were incubated at 37 °C in 5% CO_2 and 95% humidified air. Overexpression of ECM1 in BT-474 wild type (WT) cells was achieved by transfection with pBABE-puro-ECM1 vector using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. ECM1 silenced cells were generated by transfection with ECM1 shRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using Lipofectamine 2000. Trastuzumab-resistant (TR) BT-474 cells were established through tumor xenografts as described in our previous report [17]. Antibodies against the following proteins were used in this study: ECM1, LDHA, GLUT1, Actin, Egr-1, p-ERK, and ERK (Santa Cruz Biotechnology); EGFR (Epitomics, Burlingame, CA, USA); PKM2 and c-Jun (Cell Signaling Technology, Danvers, MA, USA); and p-PKM2 antibody (Signalway Antibody, College Park, MD, USA). The MEK inhibitor U0126 was obtained from Calbiochem (Darmstadt, Germany).

2.2. Two dimensional digest (ChemDigestTM/Trypsin)-LC–MS/MS and data analyses

The total proteome and secretome from TR BT-474 and BT-474 WT cells were analyzed exactly as described previously [17].

2.3. Cell-based assay for glucose uptake

The levels of glucose uptake were measured with a Glucose Uptake Cell-Based Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). Cells were seeded in 96-well plates at a density of 1×10^4 cells/well. After 24 h, glucose uptake assays were performed according to the manufacturer's protocol. Relative fluorescence units were determined at 485–535 nm using a VARIOSKAN FLASH (Thermo).

2.4. Lactate assay

The levels of lactate production were examined with a Lactate Assay Kit (Biovision, Milpitas, CA, USA). Cells were plated in 100-mm culture dishes at a density of 1×10^6 cells/plate. After incubation for 24 h the culture medium was replaced with FBS-free DMEM. After a further 8 h, lactate assays were performed with culture media collected from each sample according to the manufacturer's protocol and the optical density was measured at 570 nm using a Multiskan EX (Thermo).

2.5. Quantification of ATP levels

Cellular levels of ATP were examined with a CellTiter-Glo Luminescent Cell Viability Assay (Promega, San Luis Obispo, CA, USA). Cells were seeded in 96-well plates at a density of 5×10^4 cells/well. After 24 h the levels of ATP were measured according to the manufacturer's protocol. The luminescence was measured using a VARIOSKAN FLASH.

2.6. Western blot analysis

Harvested cells were lysed with lysis buffer containing 50 mM of Tris/HCl; pH 8.0, 150 mM of NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM of NaF, 1 mM of Na_3VO_4, and protease inhibitor (Roche, Indianapolis, IN, USA). Protein concentrations in the cell lysates were quantified using the BCA Protein Assay kit (Thermo Scientific). Proteins were separated on SDS-PAGE gels and transferred to nitrocellulose membrane (Whatman, Maidstone, UK). After blocking with 5% skimmed milk in TBS containing 0.05% Tween-20, the membranes were incubated in 5% skimmed milk containing the appropriate primary antibodies overnight, followed by 2-h incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. The protein bands were visualized by reaction with WEST ZOL plus (iNtRON, Seongman, Korea) or Lumigen™ TMA-6 (GE Healthcare).

2.7. Real-time quantitative PCR (RT-qPCR)

cDNA was synthesized from 5 μg of total RNA using the Gene Amp RNA PCR Core kit (Applied Biosystems, Carlsbad, CA, USA). cDNA was amplified with the KAPA SYBR Fast universal qPCR kit (KAPA BIOSYSTEMS, Cape Town, South Africa) using the following primers: human GLUT1, Fwd 5′- AAG CTG ACG GGT CGC CTC ATG -3′ and Rev 5′- CTC TCC CCA TAG CGG TGG ACC -3′; human LDHA, 5′- GTG GGT CCT TGG GGA ACA TGG AG -3′ and Rev 5′- GTC CAA TAG CCC AGG ATG TGT AGC C -3′; human HIF-1α, Fwd 5′- ACC ACC TAT GAC CTG CTT GGT GCT G -3′ and Rev 5′- CAT ATC CAG GCT GTG TCG ACT GAG G -3′; and human GAPDH, 5′-TCG ACA GTC AGC CGC ATC TTC TTT-3′ and Rev 5′-ACC AAA TCC GTT GAC TCC GAC CTT-3′. Gene expression levels were normalized to those of GAPDH. RT-qPCR was performed with a TP 850 Thermal Cycler Dice real time system (TAKARA BIO INC).

Table 2
LC/MS–MS identification of differentially secreted proteins between BT-474 WT and BT-474 TR cells.

Uniprot accession number	Protein name	Gene name	SI_{WT}[a]	SI_{TR}	Log_2 (SI_{TR}/SI_{WT})
P04075	Fructose-bisphosphate aldolase A	*ALDOA*	319.9010	887.5438	0.9212
P09972	Fructose-bisphosphate aldolase C	*ALDOC*	2.8674	53.4931	3.6782
P11413	Glucose-6-phosphate 1-dehydrogenase	*G6PD*	1.1699	25.3598	3.9067
P00338	L-lactate dehydrogenase A chain	*LDHA*	55.1457	171.2111	1.0651
P37837	Transaldolase	*TALDO1*	0.9971	12.5367	3.0830

[a] SI = spectral index.