Biochemical and Biophysical Research Communications 443 (2014) 239–245

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

EpCAM expression in the prostate cancer makes the difference in the response to growth factors

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ARTICLE INFO

Article history:
Received 18 November 2013
Available online 2 December 2013

Keywords:
EpCAM
Cancer stem-like cell
Prostate cancer
Heterogeneity
mTOR signaling
EGFR signaling

ABSTRACT

Introduction: Epithelial cell adhesion molecule (EpCAM) is expressed in tumors with an epithelial cell of origin, in a heterogeneous manner. Prostate cancer stem-like cells highly express EpCAM. However, little is known about how EpCAM is involved in the ability of cells to adapt to micro-environmental changes in available growth factors, which is one of the essential biological phenotypes of cancer stem-like cells (CSCs).

Methods: EpCAM-high and EpCAM-low subpopulations of cells were established from the prostate cancer cell line PC-3. Signal transductions in response to serum starvation, and on the exposure to EGF ligand or the specific inhibitor were analyzed in terms. Furthermore, we analyzed the expression level of amino acid transporters which contribute to the activation of mTOR signal between the two subgroups.

Results: EpCAM-high and EpCAM-low PC-3 subpopulations showed markedly different responses to serum starvation. EpCAM expression was positively correlated with activation of the mTOR and epithelial growth factor receptor (EGFR) signaling pathways. Furthermore, AMP-activated protein kinase (AMPK) was gradually de-activated in EpCAM-low PC-3 cells in the absence of serum.

Conclusions: EpCAM regulates the AMPK signaling pathway, essential for the response to growth factors characterized by EGF. LAT1, the amino acid transporter stabilized at the cellular membrane by EpCAM, is likely to be responsible for the difference in the susceptibility to EGF between EpCAM-high and EpCAM-low PC-3 cells.

1. Introduction

Prostate cancer is one of the most prevalent malignancies affecting men throughout the world [1]. Although screening for prostate-specific antigen (PSA) has dramatically increased early detection, many patients already have advanced disease at the time of initial diagnosis [2]. Given that androgen ligand is responsible for the deterioration of prostate tumor tissue, endocrine therapy to block this steroid hormone has been used effectively in prostate cancer patients [3]. However, this approach eventually fails due to the emergence of androgen-independent prostate cancer cells, which proliferate and metastasize to bones independently of androgen signals [4]. By contrast, many cases of prostate cancer are indolent and do not manifest clinically [5].

Recent studies have shown that intra-tumor heterogeneity is the main cause of therapeutic resistance in cancer [6]. Tumor tissue is composed of heterogeneous cellular populations with a hierarchical organization governed by stem/progenitor-like cells, also referred to as cancer stem-like cells (CSCs). Much the same as stem cells in normal tissue, CSCs have the potential for self-renewal and multi-lineage differentiation [7]. Furthermore, chronic inflammation and redox stress increases the plasticity between CSCs and non-CSCs via epigenetic regulation [8]. CSCs of prostate cancer (PCSCs) are also responsible for cellular diversity in terms of gene expression profiles and trans-differentiation patterns, which contributes to the heterogeneity of tumor tissue [9].

Epithelial cell adhesion molecule (EpCAM), also known as CD326, is over-expressed ubiquitously in CSCs that originate from epithelial lesions [10,11]. An antibody to EpCAM has been used clinically to detect circulating tumor cells (CTCs) in patients with cancer [11,12]. CTCs maintain epithelial characteristics even after dissociation and intravasation [13], which is apparently paradoxical to the conventional theory of epithelial–mesenchymal transition (EMT). Indeed, it has recently been reported that EMT is not required for tumor cells to dissociate from the primary site and enter blood vessels [14,15]. Over-expressed EpCAM in prostate CSCs is involved in the progression of prostate cancer and is a useful marker for the detection of minimum residual disease (MRD) after surgery, CTCs in the blood, and metastatic cancer cells in the bone, lymph node, and lung [10,11,16,17].

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http://dx.doi.org/10.1016/j.bbrc.2013.11.093

CD44, a widely recognized CSC maker, has been shown to be functionally involved in cancer biology. The CD44 variant including variable exon 6 (CD44v6) promotes invasion and metastasis in colon cancer, and CD44v8–10 induces resistance to redox stress [18].

By contrast, EpCAM has been widely used as a marker of CSCs, but the biological function EpCAM confers to CSCs remains elusive. We hypothesized that EpCAM expression in CSCs is involved in the maintenance of "stemness", the biological characteristics of CSCs.

2. Materials and methods

2.1. Cell culture and treatment

The human prostate cancer cell lines PC-3 and DU-145 were obtained from ATCC and cultured under an atmosphere of 5% CO_2 at 37 °C in DMEM/F-12 (Dulbecco's Modified Eagle Medium/Ham's F-12) (Sigma–Aldrich) supplemented with 10% fetal bovine serum. The difference in EpCAM expression in sorted subpopulations of PC-3 cells was periodically examined by quantitative RT-PCR. The possibility that EpCAM-high and EpCAM-low subpopulations were contaminated with other cell lines was ruled out by STR multiplex system analysis (Promega), which revealed that both subpopulations originated from the parental PC-3 cell line. In serum depletion experiments, the cells were washed with PBS twice and then serum-free DMEM/F-12 was added. Human recombinant EGF ligand was obtained from Sigma–Aldrich (62253-63-8), reconstituted in PBS at a concentration of 100 μg/ml, and stored at −20 °C. Gefitinib, an EGFR inhibitor, was obtained from Funakoshi (184475-35-2), reconstituted in PBS at a concentration of 1 mM/ml, and stored at −20 °C.

2.2. Immunohistochemical staining

Formalin-fixed, paraffin-embedded prostate cancer tissue array (T195, Biomax) and PC-3-derived xenografts were depleted of paraffin by exposure to xylene for 15 min, rehydrated with a graded series of ethanol solutions (100–70%), and then heated in a microwave oven at 750 W for 10 min in citric acid buffer (0.01 M, pH 6.0) for antigen retrieval. The slides were then washed with PBS for 10 min and then treated for 5 min with PBS containing 3% hydrogen peroxide to block endogenous peroxidase activity. After an additional wash with PBS for 5 min, the slides were blocked with blocking buffer for 1 h at room temperature before incubation overnight at 4 °C with primary antibodies to EpCAM (1:100, NBP1-95700, Novus). Immune complexes were detected using the Vectastain ABC Kit (Vector) and DAB (3, 3-diaminobenzidine) (SK-4100, Vector). The slides were counterstained with hematoxylin and then examined with a BX51 microscope (OLYMPUS).

2.3. Animal experiments and ethical statement

Parental PC-3 cells (1×10^3 cells per animal) were subcutaneously implanted into 8-week-old male nude mice. After 6 weeks of transplantation, the animals were sacrificed and the xenografts were surgically resected for IHC. All these experiments were performed in accordance with the guidelines of Keio University. The ethics committee of Keio University specifically approved this study.

2.4. Quantitative RT-PCR

Total RNA was extracted from PC-3 cells with the RNeasy Micro Kit (QIAGEN). Extracted RNA was reverse-transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative RT-PCR analysis was performed with SYBR Premix Ex TaqII (TaKaRa Bio) and a Thermal Cycler Dice Real-Time System (TaKaRa Bio). The amplification protocol consisted of an initial incubation at 95 °C for 2 min followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. Dissociation curve analysis was performed to confirm specificity. Data were normalized against the corresponding amount of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA and are shown as the means ± SD of three independent experiments. Quantitative RT-PCR analysis was performed with the following primer sets (forward and reverse, respectively): EpCAM, 5′-CGCAGCTCAGGAAGAATGTG-3′ and 5′-TGAAGTACACTGCCATTGACG-3′; LAT1, 5′-CGGTGCGCAGAGCATGG-3′ and 5′-GCTTCAGTGCAAGTCTGTGG-3′; and GAPDH, 5′-GGTGGTCTCCTCTGACTTCAACA-3′ and 5′-GTTGCTGTAGCCAAATTCGTTGT-3′.

2.5. Flow cytometry

PC-3 cells ($1–5 \times 10^6$) were harvested with Enzyme-Free Cell Dissociation Buffer (Life Technologies), centrifuged, and re-suspended in 100 μl of PBS. Cells were incubated in a final volume of 100 μl for 15 min on ice with an allophycocyanin-conjugated antibody to human EpCAM (130-080-301, Miltenyi Biotec) [19]. As a negative control, cells were incubated with an isotype control antibody, APC-conjugated mouse IgG1 (SA1-12320, Thermo Scientific). The cells were then washed three times with PBS and stained with propidium iodide (PI) before analysis with a FACS Calibur flow cytometer (BD Technology). Data were analyzed using FlowJo software (Digital Biology).

2.6. Statistical analysis

All experiments were performed at least three times. Quantitative data were analyzed using Student's *t* test, with a *p*-value of <0.05 considered statistically significant.

3. Results

3.1. Prostate cancer shows heterogeneous expression of EpCAM both in vivo and in vitro

The expression level of EpCAM (CD326) in prostate cancer specimens (T195, Biomax) was examined by immunohistochemistry (IHC) on a tissue array, because over-expressed EpCAM in prostate cancer cells has been shown to contribute to malignant potential [16]. Well-differentiated prostate cancers highly expressed EpCAM on both on the lateral and basal membranes. There was a great deal of inter- and intra-tumoral heterogeneity with respect to EpCAM expression (Fig. 1A-1, 2, 3). Because stromal cells do not express EpCAM, scirrhous-type prostate tumors were negative for EpCAM expression (Fig. 1A-4). By contrast, budding tumor cells had a high level of EpCAM expression not only on the cellular membrane but also in the nucleus (Fig. 1A-5). Budding cells are thought to be responsible for invasion and metastasis [20]. It was also notable that even the prostate intraepithelial neoplasia (PIN), which is a counterpart of ductal adenocarcinoma *in situ* (DCIS) in the breast tissue, expressed high level of EpCAM (Fig. 1A-6).

There have been several reports that the intracellular domain of EpCAM (EpICD) activates canonical Wnt signaling in the nucleus [21,22]. It is noteworthy that both well-differentiated cancer cells and de-differentiated budding cells showed a significantly higher level of EpCAM expression than the normal prostate tissue (Fig. 1A-N). These seemingly contradictory findings are supported by the quantitative IHC data, which showed that the Gleason score did not correlate with the EpCAM expression level (Fig. 1B).

EpCAM expression was next analyzed in two prostate cancer cell lines, PC-3 and DU-145, to understand the contribution of

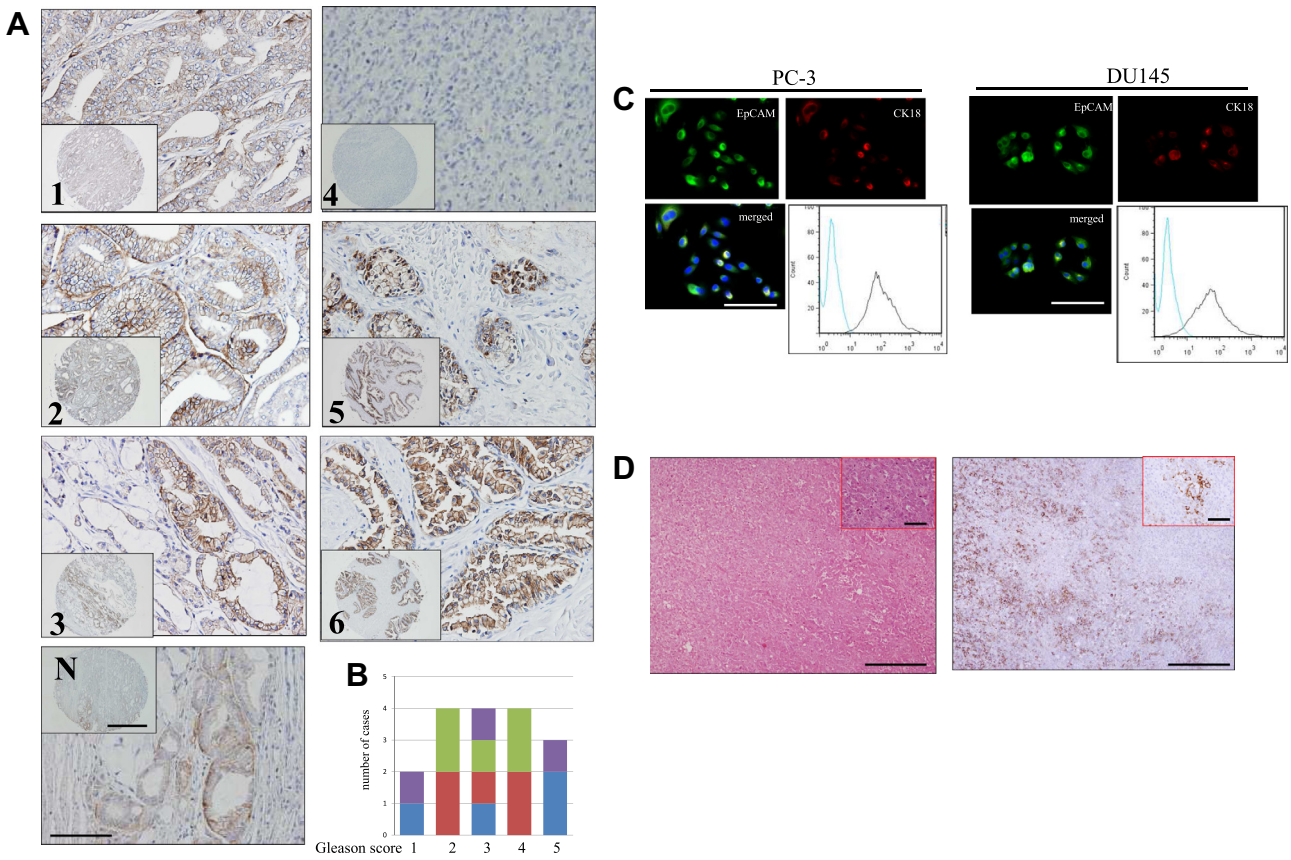


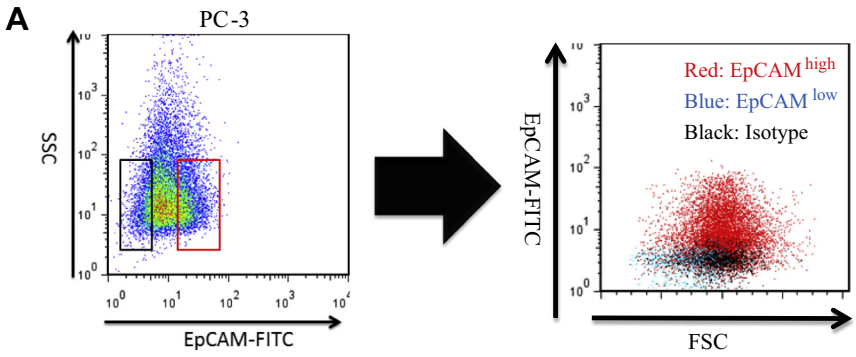
Fig. 1. Heterogeneity of EpCAM expression both *in vivo* and *in vitro*. (A) Immunohistochemistry (IHC) analysis of EpCAM expression of a prostate cancer tissue array. Six tumor specimens (1–6) and normal prostate tissue (N) samples are shown. Scale bar, 100 μm (inset, 500 μm). (B) Number of prostate cancer cases by EpCAM IHC score. A total of 17 cases were analyzed (Gleason score 1, 2 cases; Gleason score 2–4, 4 cases each; Gleason score 5, 3 cases). Blue: 0 (not stained), red: +1 (weakly positive), green: +2 (moderately positive), and purple: +3 (strongly positive). (C) Fluorescent immunostaining of PC-3 and DU-145 cells with EpCAM (green), CK18 (red), and DAPI (blue), and flow cytometry with a FITC-conjugated EpCAM antibody (isotype control, blue trace; EpCAM, black trace). Scale bar, 100 μm. (D) Xenografts derived from parental PC-3 cells stained with H&E and an EpCAM antibody. Scale bar, 500 μm (inset, 100 μm).

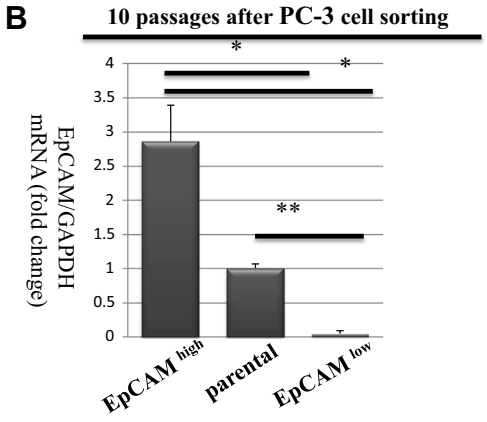
EpCAM to the acquisition of stemness and malignant potential. The PC-3 cell line was established from a bone metastatic lesion [23], while DU-145 was established from a brain metastatic lesion [24]. Both cancer cell lines are insensitive to androgen. Fluorescent immunohistochemistry (F-IHC) showed that cytokeratin-18 (CK18) co-localized with EpCAM in both cell lines (Fig. 1C). CK18 has attracted much attention as a prognostic marker for patients with advanced prostate cancer [25,26].

To demonstrate EpCAM expression *in vivo*, 1×10^3 PC-3 cells were subcutaneously injected into mice and tumors were harvested for analysis after 6 weeks. IHC analysis of xenograft tumors derived from PC-3 cells showed a heterogeneous pattern of EpCAM expression, although the xenografts were composed entirely of PC-3 tumor cells (Fig. 1D). Similarly to the *in vitro* analysis (Fig. 1C), there was a pronounced heterogeneity in terms of EpCAM expression in the xenograft tumors (Fig. 1D). This is consistent with a hierarchical model in which CSCs highly positive for EpCAM give rise to both CSCs and non-CSCs that are weakly positive or negative for EpCAM. It is important to note that the plasticity of EpCAM expression depends on the microenvironment.

3.2. Sorted PC-3 subpopulations maintain differences in EpCAM expression after several passages

Parental cancer cells were next separated into EpCAM-high and EpCAM-low/negative fractions from bulk populations using a cell sorter. MDA-M-231 (breast cancer cell line), MKN-74 (gastric cancer cell line), PC-3 (Fig. 2A), DU-145 (prostate cancer cell line), and ASPC-1 (pancreatic cancer cell line) cells were analyzed. Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis of *EpCAM* mRNA demonstrated that of the five cancer cell lines, only the PC-3 cell sublines maintained their differences in EpCAM expression ten passages after the initial separation (Fig. 2B). Furthermore, the EpCAM expression patterns of the parental, EpCAM-high, and EpCAM-low/negative PC-3 cells were compared by flow cytometry 15 passages after cell sorting. Approximately 30% of the EpCAM-high population showed the same EpCAM expression as the parental PC-3 cells, which suggests that EpCAM-low/negative cells were derived from EpCAM-high stem-like cancer cells. On the other hand, there was no emergence of an EpCAM-high subpopulation from within the EpCAM-low population (Fig. 2C). The possibility that the EpCAM-high and -low subpopulations were contaminated with other cell lines was ruled out by short tandem repeat (STR) analysis, by which it was confirmed that both subpopulations originated from the parental PC-3 cell line (Supplementary File 1). We therefore have established subpopulations of PC-3 cell lines that stably differ in the expression level of EpCAM. Given that EpCAM is a cancer stem cell marker [10,11], we hypothesized that EpCAM-high PC-3 cells are stem-like cancer cells, while EpCAM-low PC-3 cells are transit-amplifying (TA) cells, which are relatively differentiated cells derived from EpCAM-high cells.





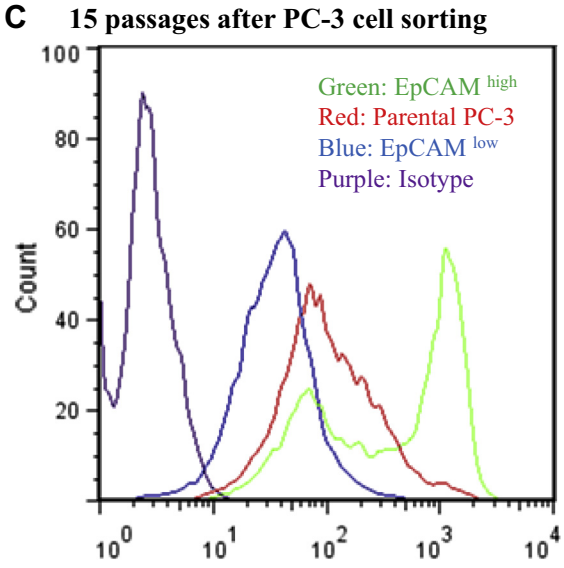


Fig. 2. Maintenance of EpCAM expression in PC-3 cells after passage. (A) Parental PC-3 cells were sorted into EpCAM-high (about 10,000 cells) and EpCAM-low (about 4000 cells) subpopulations. (B) Quantitative RT-PCR for EpCAM 10 passages after PC-3 cell sorting. $^{*}p < 0.05$, $^{**}p < 0.001$ (Student's *t* test). (C) Flow cytometry analysis of EpCAM expression 15 passages after PC-3 cell sorting.

3.3. EpCAM allows cells to adapt to a low-nutrient microenvironment via AMPK signaling

AMP-activated protein kinase (AMPK) is a signal transduction molecule that is involved in the maintenance of cellular energy homeostasis by controlling the AMP:ATP ratio. AMPK is a heterotrimeric protein activated by phosphorylation at thereonine-172 (Thr-172) [27,28]. The function of AMPK in cancer metabolism is still controversial; AMPK-mediated phosphorylation of the cyclin-dependent kinase (CDK) inhibitor p27 stabilizes AMPK and induces autophagy when cells are exposed to nutrient-deficient conditions [29]. Furthermore, AMPK not only promotes mitochondrial oxidative metabolism, but also suppresses excessive glycolysis in cancer cells with the declined ATP:AMP ratio [27,30].

To investigate differences in the response to lack of growth factors between stem-like cancer cells and daughter cells, amount of

ATP synthesized in response to short-term serum starvation were evaluated in EpCAM-high and EpCAM-low PC-3 cells. MTT assays 6 and 12 h after serum depletion revealed that only EpCAM-high PC-3 cells significantly decreased ATP synthesis in the absence of starvation, while EpCAM-low PC-3 cells were insensitive to serum starvation (Fig. 3A), which strongly suggested that EpCAM expression makes PC-3 cells more susceptible to the induction of dormant status under the serum depletion. To be sure, serum starvation was likely to induce apoptosis or autophagy, but the division of total ATP amount by live cell count makes synthesized ATP titer per cell, excluding the existence of apoptotic cells in the absence of growth factors. (Fig. 3A).

Quantitative RT-PCR was conducted to evaluate differences in the expression of amino acid transporters, including ASCT1/2 (small neutral amino acid transporter including alanine, serine, and cysteine), L-type amino acid transporter-1 (LAT1), and xCT (cysteine/glutamine exchange transporter) [31], between EpCAM-high and -low PC-3 cells. These amino acid transporters contribute to the uptake of metabolites and affect mTOR signaling activity, depending on nutrient availability. The LAT1 transporter was significantly more highly expressed in EpCAM-high PC-3 cells than in EpCAM-low cells (Fig. 3B). The Spearman's rank correlation between EpCAM and LAT1 in parental, EpCAM-high, and EpCAM-low PC-3 cells was very high (0.859), which implies that the expression of EpCAM correlates with that of LAT1. LAT1 promotes the intake of leucine, the amino acid that activates mTOR signaling [31], suggesting that mTOR signaling may be differentially activated in the EpCAM-high and EpCAM-low subpopulations.

We next evaluated the activation of mTOR signaling, characterized by p70S6K phosphorylation, in serum-starved PC-3 levels with varying levels of EpCAM. AMPK was gradually activated in EpCAM-high PC-3 cells in response to serum starvation, whereas AMPK activity in EpCAM-low PC-3 cells decreased (Fig. 3C), probably due to the significant decrease of growth factor stimuli from such as insulin growth factor (IGF) and epithelial growth factor (EGF). Phosphorylated p70S6 kinase (p-p70S6K) and p-AMPK (the

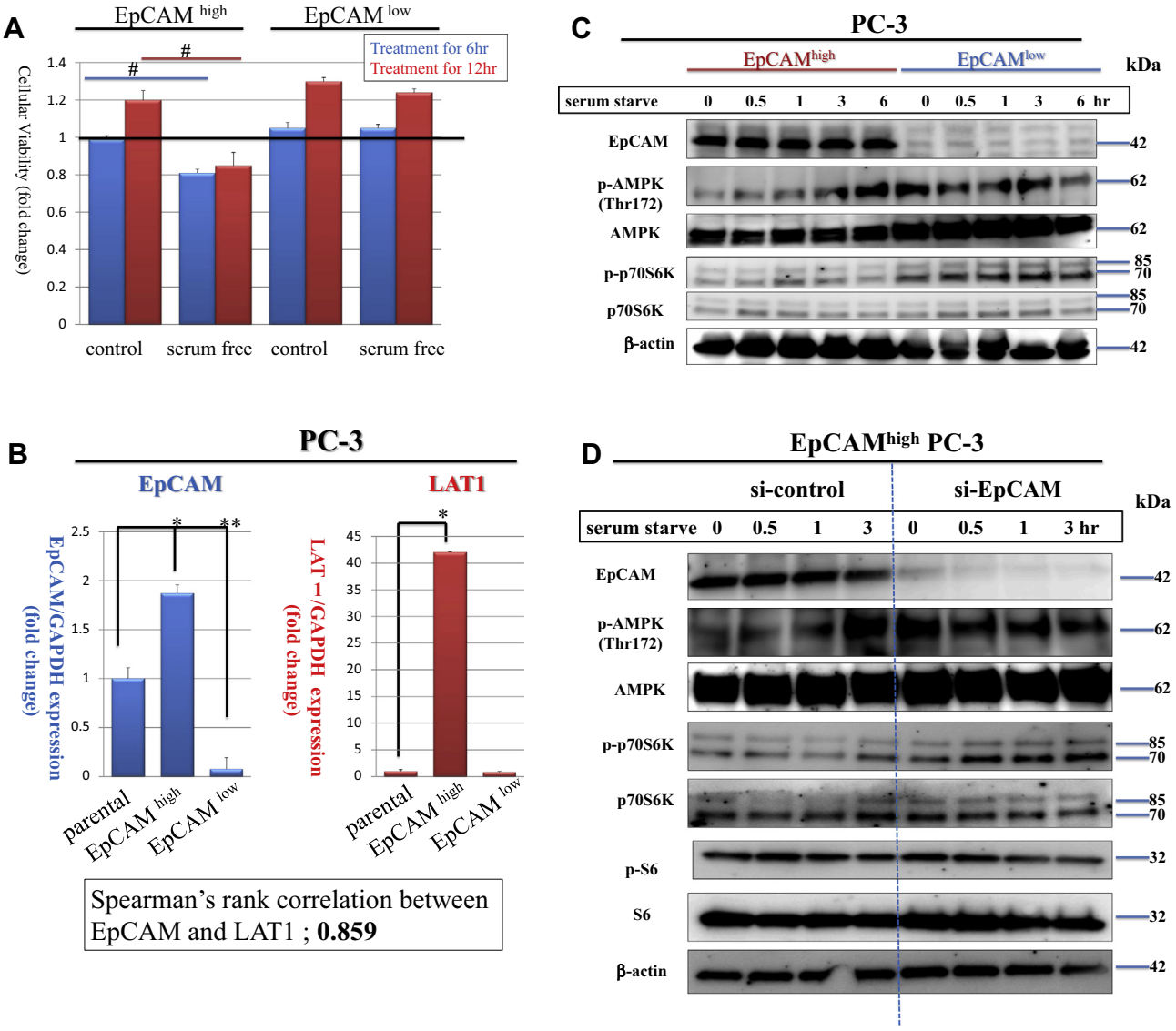


Fig. 3. Serum-starvation-induced AMPK activation is responsible for EpCAM expression. (A) MTT assay to detect cellular viability 6 and 12 h after serum starvation in EpCAM-high and -low cells. The cell count before serum depletion was normalized to 1.0. $^{\#}P < 0.001$ (Student's t test). (B) Quantitative RT-PCR analysis of LAT1 and EpCAM 15 passages after PC-3 cell sorting. The Spearman's rank correlation between EpCAM and LAT1 was approximately 0.86. $^{*}P < 0.05$, $^{**}p < 0.01$ (Student's t test). (C) Immunoblot analysis to detect chronological changes in AMPK and mTOR signaling under serum starvation (after 0, 0.5, 1, 3, and 6 h). (D) Immunoblot analysis to detect chronological changes in AMPK and mTOR signaling under serum starvation (after 0, 0.5, 1, and 3 h) after siRNA-mediated depletion of EpCAM in EpCAM-high PC-3 cells.

active form of AMPK) negatively regulate each other, but there was no significant change in p70S6K activity in the EpCAM-high subpopulation. By contrast, p70S6K activity slightly increased in the EpCAM-low subpopulation. Altogether, EpCAM-low PC-3 cells showed an atypical response to serum depletion – simultaneous activation of p70S6K and AMPK (Fig. 3C).

To determine whether EpCAM directly contributes to differences in mTOR signaling in the absence of serum, transient depletion of EpCAM was performed using small-interfering RNA (siRNA) (Fig. 3D). EpCAM-high PC-3 cells subjected to siRNA-mediated EpCAM depletion failed to activate AMPK in response to serum starvation, and responded similarly to EpCAM-low PC-3 cells under serum starvation with respect to the activation of mTOR signaling (Fig. 3D). Taken together, these data suggest that EpCAM plays a role in the response to growth factor depletion.

3.4. EGF-induced signaling under serum starvation is affected by EpCAM expression

A phosphorylated tyrosine kinase (RTK) array was performed to determine which RTKs were activated or inactivated when the two subpopulations of PC-3 cells were subjected to serum starvation for 6 h. The RTK assay revealed that the activity of EGF receptor (EGFR) was significantly regulated by serum concentration (data not shown). Cellular viability assays were then performed in which the cell count in the absence of serum was normalized to 1.0. Cell viability following treatment with EGF ligand (10 ng/ml) in the absence of serum was higher in EpCAM-high PC-3 cells than in EpCAM-low cells (Fig. 4A). Furthermore, treatment with the EGFR tyrosine kinase inhibitor gefitinib (0.5 μM for 12 h) decreased ATP synthesis and reduced viability in both EpCAM-high and EpCAM-low PC-3 subpopulations (Fig. 4B), which suggests that EpCAM-low cells also depend on EGF-EGFR signaling, with much higher sensitivity to gefitinib. Thus, EpCAM-high PC-3 cells tended to show the prolonged activation of EGFR signal pathway as compared with EpCAM-low cells.

Endogenous EGFR activity (determined by phosphorylation at the residue Tyr1068) and susceptibility to EGF ligand was higher in EpCAM-high PC-3 cells than in EpCAM-low cells in the presence of serum. After serum starvation for 1 h, treatment with gefitinib suppressed AMPK activity in EpCAM-high PC-3 cells but not in EpCAM-low cells. In all the samples, mTOR signaling activity was not influenced by this short-term serum depletion (Fig. 4C). Taken together, there is a significant difference in the response to EGF ligand and the drug gefitinib between the two subpopulations of PC-3 cells. EpCAM-high PC-3 cells were more susceptible to EGF stimulation than EpCAM-low PC-3 cells and sustained EGFR activation in the presence of gefitinib, as demonstrated also in the previous experiments (Fig. 4A and B).

4. Discussion

EGFR signaling pathway is known to be an upstream component of the phosphatidyl-inositol-3-kinase (PI3K)/Akt/mTOR axis and is constitutively activated in many cancers [32,33]. LAT1 plays a crucial role in the maintenance of malignant neoplasms. It has recently been revealed that LAT-1 is highly expressed in a subset of esophageal adenocarinomas derived from Barrett's esophagus precursor lesions [33], and is a poor prognostic marker for malignant pleural mesothelioma [32]. Given that LAT1 expression was shown to be positively correlated with the expression of EpCAM in this study, this CSC marker is highly likely to function as a sensor for

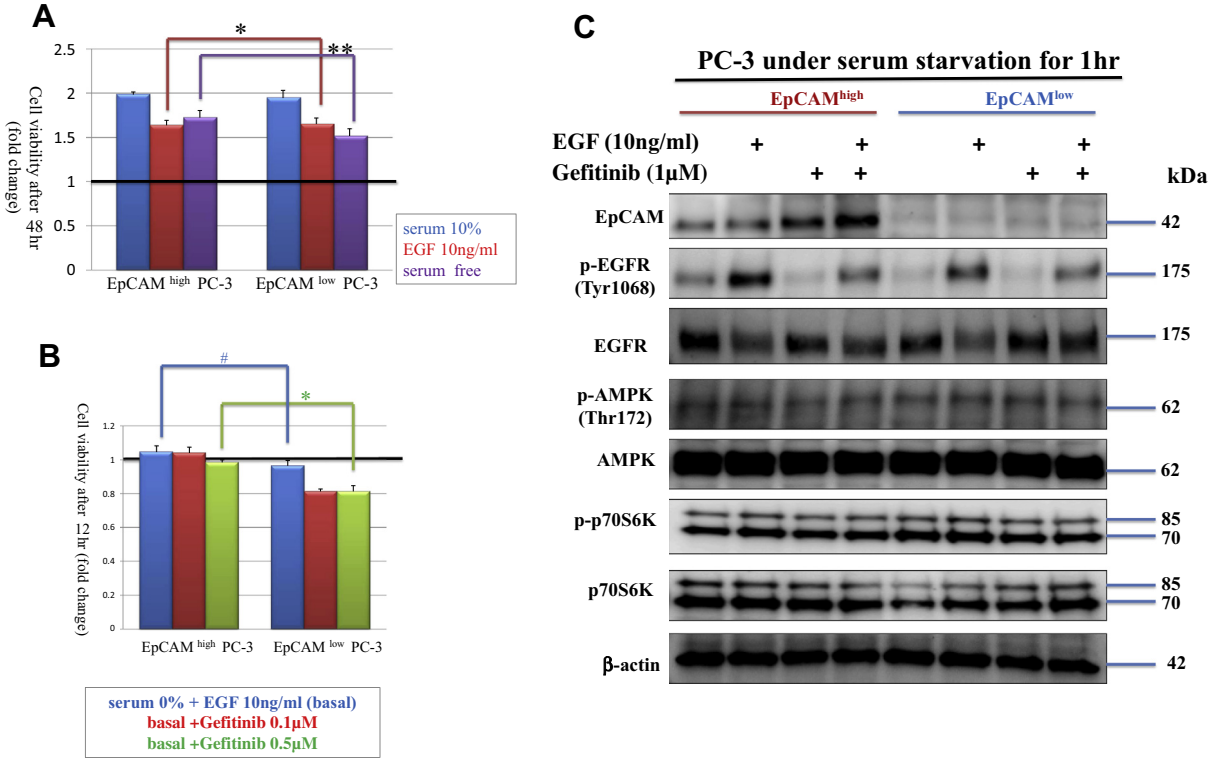


Fig. 4. EGF differentially affects the response to serum starvation. (A) MTT assay to detect cellular viability 48 h after the treatment with serum starvation or simultaneous treatment with serum depletion and EGF ligand (10 ng/ml) in EpCAM-high and -low cells. The cell count before serum depletion was normalized to 1.0. $^{*}P < 0.05$, $^{**}p < 0.005$ (Student's *t* test). (B) MTT assay 12 h after serum starvation and EGF ligand (10 ng/ml) stimulation in EpCAM-high and -low cells. Concentration-dependent effect of gefitinib effect on cell proliferation. The cell count before serum depletion was normalized to 1.0. $^{*}P < 0.05$, $^{\#}p < 0.0005$ (Student's *t* test). (C) Immunoblot analysis of EpCAM-high and -low PC-3 cells under serum starvation for 1 h, in response to stimulation with EGF ligand (10 ng/ml) and/or gefitinib (1 μM).

growth factors in the microenvironment via LAT1 and PI3K/Akt signaling, which regulates mTOR signaling. LAT1 has been regarded as a promising therapeutic target molecule [32,33], but the specific inhibition of LAT1 might be a novel therapeutic strategy to deplete CSCs.

The phenotypes induced by EpCAM expression are mainly responsible for the persistence of minimal residual disease and the latent relapse of prostate cancers. Therefore, EpCAM may be a promising therapeutic target to block the ability of prostate cancer CSCs to adapt to the lack of growth factors in the tumor microenvironment.

5. Conclusions

CSCs have widely been accepted as responsible for therapeutic resistance, invasion, and metastasis, which result in a poor prognosis for many patients. This study demonstrates that EpCAM is not only useful to detect CSCs as a surface marker, but is also functionally involved in the ability of CSCs to sense and adapt to changes in available growth factors in the tumor microenvironment. It is important to develop new therapeutic strategies focusing on the as-yet-unknown functions of CSC markers.

Competing interests

The authors have no competing interests to declare.

Author contributions

GJY conceived, designed, and performed experiments, analyzed data, and wrote the paper. HS conceived experiments and wrote the paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.093.

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