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RESEARCH ARTICLE

Low-folate stress reprograms cancer stem cell-like potentials and bioenergetics metabolism through activation of mTOR signaling pathway to promote *in vitro* invasion and *in vivo* tumorigenicity of lung cancers

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Abstract

Low-folate (LF) nutritional status is associated with increased risks of lung cancer. It has unexplored effects on lung cancer malignancy, a cancer stem cell (CSC) disease. We hypothesized that LF may reprogram CSC-like potential and bioenergetics metabolism to increase metastasis potential of lung cancers. Cultivation of human non-small-cell lung cancer cells (H23) in an LF medium enhanced CSC-like properties signified by increased expressions of the CSC surface marker CD44 and pluripotency markers Sox2, Oct4 and ALDH1A1, and promoted self-renewal ability of anchorage-independent oncospheroid formation. The CSC-like phenotype of LF-treated H23 cells coupled with the metabolic reprogramming to aerobic glycolysis evident by elevated lactate release and medium acidification suppressed expressions of pyruvate dehydrogenase E1-α, and elevated redox status of the NADH/NAD+ and NADPH/NADP+ ratios. The LF-induced metabostemness phenotype of H23 cells was modified by DNA methylation inhibitor 5-AdC and histone acetylation inhibitor EX. Treatment of H23 cells with mTOR siRNA or the mTOR inhibitor rapamycin abrogated LF-activated Akt-mTOR-Hif1-Foxo signaling and stemness-associated sonic hedgehog pathway, reversed Warburg metabolic switch and diminished invasion of H23 cells. Intrapleural injection of LF-induced lung oncospheres into the LF recipient mice, but not the control recipient mice, caused metastasis xenograft lung tumors. The *in vitro* and *in vivo* data corroboratively demonstrate that LF stress reprograms metabostemness signatures through activated mTOR signaling pathway to promote metastasis tumorigenicity of lung cancers.

Keywords: Low folate; Lung cancer stem cell; Metabolic signaling; bioenergetics; Tumorigenesis

1. Introduction

Lung cancer is the leading cause of cancer deaths. The overall prognosis of lung cancers is poor with low 5-year survival rates [1]. The etiology of malignancy lung cancer development remains obscure. It has been proposed that malignancy cancers are the cancer stem cell (CSC) diseases [2]. The stemness potentials of CSC with epithelial–mesenchymal (EMT) transdifferentiation ensure their invasion and disseminate to distant organs for metastasis. The self-renewal capacity of CSC to form nonadherent oncospheroid mediated intrinsic drug resistance to cytotoxicity therapy and promoted aggressive relapse tumor [3]. A subpopulation of CD44+ lung cancer cells that expressed the pluripotent stemness genes OCT4, NANOG and SOX2 was capable of oncospheroid formation and *in vivo* tumor initiation [4]. Lung tumor cells with overexpressed aldehyde dehydrogenase 1 (ALDH1) exhibited enhanced self-renewal capacity, spheroid formation, tumorigenicity and metastatic activity [5,6]. Activation of embryonic developmental pathway, sonic hedgehog (Shh) signaling, is required to maintain this CSC-like stemness signature in non-small-cell lung cancer (NSCLC) and small-cell lung cancers (SCLC) [7–10]. Blockage of Shh signaling in NSCLC abolished EMT-induced SOX2 and NANOG, and subsequently abolished cancer metastasis [11]. Identification of CSC-like features and activated stemness signaling in heterogeneous tumor population may serve as the diagnostic markers for the early detection of malignancy cancer.

Recent advances have suggested that metabolic reprogramming on CSC bioenergetics plays a key role in the stemness development [12]. Malignancy cells uptake glucose for fermented glycolysis to produce lactate. Lactate release will result in acidified microenvironment which will subsequently trigger the metabolic stress signaling to drive pentose-phosphate pathway for generation of reducing agent and synthesis of fatty acid to support self-renewal capacity of cancer cells [13,14].

CSCs signal the master metabolic stress mediator, mammalian target of rapamycin (mTOR) pathways, to adapt fermented glycolysis for the maintenance of the CSC properties of self-renewal and pluripotency [15,16]. Blockage of mTOR signaling by rapamycin (Rap) mediates glycolysis metabolism to reduce cell viability and the colony-forming ability of NSCLC cells [17]. Treatment of lung CSC with

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Rap suppressed expressions of the stemness marker SOX2, the EMT phenotype and sphere formation [18]. Blockage of mTOR pathways reversed fermented glycolysis of CSC and cancer malignancy [19]. The metabostemness phenotype of cancer cells mediated by metabolic mTOR signaling was proposed as the new-dimensional hallmark of cancer stem cells.

The vitamin folate mediates cellular one-carbon metabolism and acts as a physiological precursor required for *de novo* purine and pyrimidine biosynthesis by both normal and tumor cells. The carcinogenic role of folate deficiency — because of dietary insufficiency, genetic defects and/or chemotherapeutic treatment — is well documented; it induces intracellular one-carbon deficits, aberrant DNA methylation and genetic abnormalities in cancer development [20,21]. Low levels of folate in the serum of cancer patients have been correlated with an elevated risk of developing metastatic tumors in hepatocellular carcinomas [22] and gastric cancer [23]. A recent study reported that continuous exposure of human NSCLC cell lines to pemetrexed, a multitarget antifolate chemotherapeutic drug used for the treatment of NSCLC, created a substantial drug-resistant subpopulation with a stem-cell-like phenotype characterized by an enriched stem cell gene signature, augmented ALDH activity and greater clonogenic potential [24]. It raises the possibility that a persistently low-folate (LF) microenvironment may interact with a developing lung tumor to promote malignancy transformation through induced metabostemness potentials before the cancer is diagnosed. In this study, we tested the hypothesis that an LF microenvironment promotes the *in vitro* and *in vivo* metastasis tumorigenicity of lung cancer cells and the molecular mechanisms associated with CSC signatures, metabolic phenotypes and signaling transduction pathways.

2. Materials and methods

2.1. Materials

Folate (pteroylmonoglutamic acid), cyclopamine (Cyc) and 5-aza-2′-deoxycytidine (5-AdC) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Invitrogen (Grand Island, NY, USA). Antibodies for ALDH1A1, Sox2, Oct4, forkhead box protein O 3a (FoxO3a), pyruvate dehydrogenase (PDHA) E1-α, Akt, mTOR, Hif1α, Gli1, shh-19 and shh-45 were purchased from Cell Signaling Technology (Boston, MA, USA), and vimentin was obtained from GeneTex (Irvine, CA, USA). The inhibitors Rap and EX-527 (EX) were from Cayman Chemical (Ann Arbor, MI, USA). Human recombinant basic fibroblast growth factor (hr-bFGF), recombinant epidermal growth factor (hr-EGF) and B-27 supplements without vitamin A were purchased from Gibco Laboratories (Grand Island, NY, USA).

2.2. Cell culture and treatments

The human NSCLC cell line NCI-H23 (ATCC CRL-5800) and human fetal lung fibroblast cell line MRC-5 (ATCC CCL-171) were purchased from the American Type Culture Collection. Each cell line was maintained as a monolayer culture in DMEM with 10% fetal bovine serum and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin) at 37°C in a humidified 5% CO_2 atmosphere. Folate-deprived cells were cultured in an LF DMEM (HiMedi ®AL007F+10 nM folic acid), whereas the control cells were cultivated in regular DMEM with a normal culture of folic acid at 2 nM. In the inhibitor experiments, cells were cultivated in various media for 2 days and then underwent an inhibitor treatment of Rap (10 μM), EX (10 μM), Cyc (10 μM) and 5-AdC (5 μM) in an LF-DMEM medium for 48 h. Cells were harvested for analysis at designated time points.

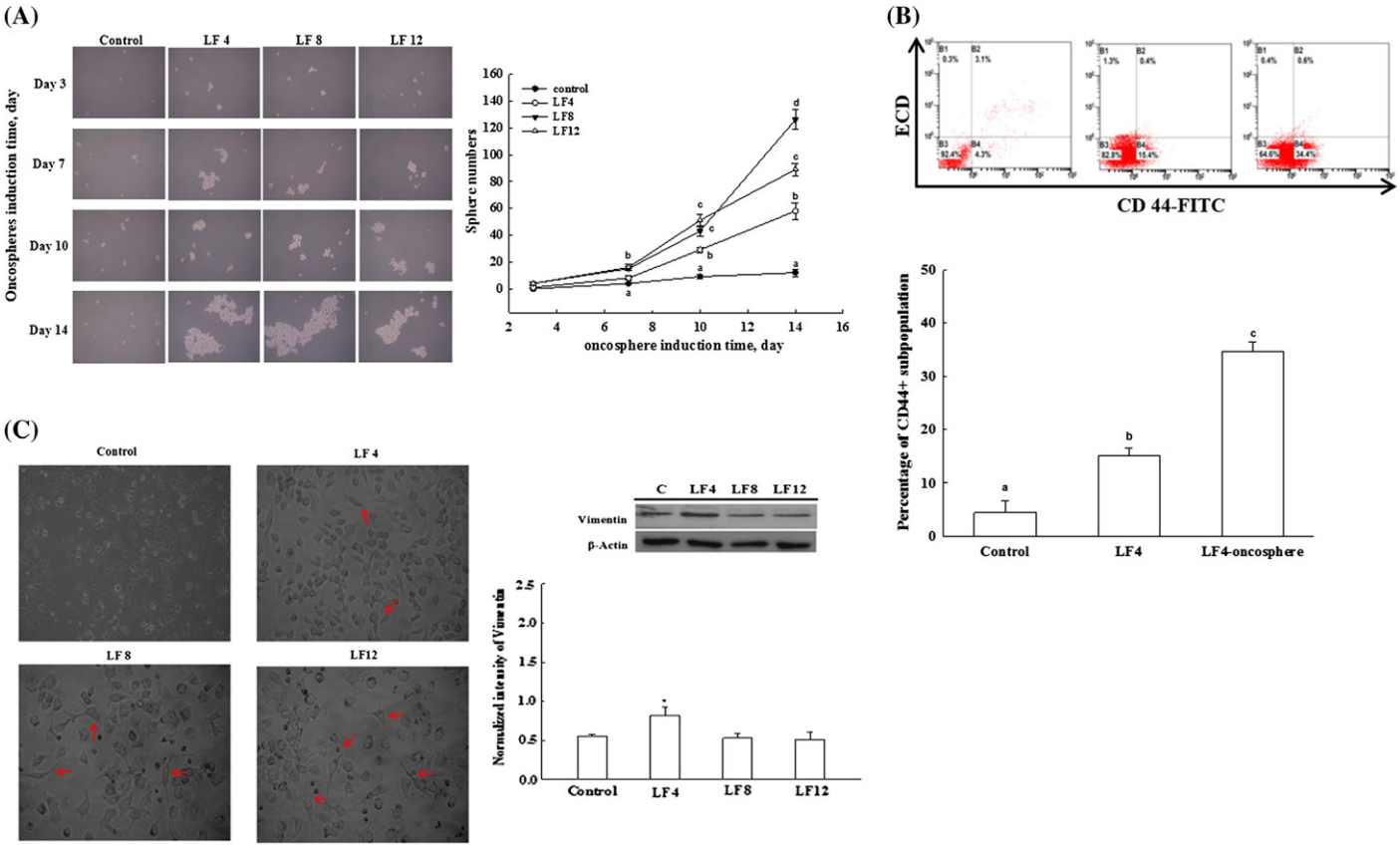

Fig. 1. *In vitro* stemness-associated tumorigenicity of H23 cells in the absence and presence of LF exposure. (A) Representative images of the density and morphology of oncospheroids at various times. Quantitative analysis of oncospheroids numbers upon the control and LF cultivations at the designated time points are shown on the right panel. Data from three individual experiments are shown as the mean±S.D. Values of various treatments after each incubation period with different letters differ significantly at $^{*}P<.05$. (B) Flow image and quantitative analysis of the $CD44^{+}$ subpopulations of the control, the 4-day LF-exposed H23 cells (LF4) and the LF-promoted 8-day oncospheroids (LF-sphere). Data from three independent samples are expressed as the mean±S.D. (C) Representative images of the mesenchymal morphology and immunoblot of the mesenchymal marker of vimentin. (D) Representative images and quantitative analysis of the invasiveness of H23 cells cultivated with the control, LF and oncospheroid media. The result is expressed as the number of invasive cells per microscope field. All data are from three individual experiments and shown as the mean±S.D. Statistical significance at $^{*}P<.05$.