Hypoxia and pyruvate/uridine have synergic effect on induction of stemness factors in human esophageal cancer cells

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Abstract: The present study was to examine our hypothesis that hypoxia and pyruvate/and uridine may have synergic effect on the induction of stemness factors in cancer. Esophageal cancer cell lines KYSE450 and KYSE70 were cultivated under different oxygen tensions with/without pyruvate and uridine addition in medium. In comparison to the cells cultivated in 20% O_2 tension, the cells cultivated in 7% O_2 and 1% O_2 showed higher levels of Oct3/4 and SOX2, which were in parallel with increasing HIF-1α, HIF-2α. A stronger induction of these gene expression could be seen in either pyruvate or uridine treatment under hypoxic condition. The strongest induction of the expression of these genes was repeatedly shown under hypoxia with both chemicals. Although the expressions of stemness factors Oct3/4 and SOX2 were higher in hypoxia than that in normoxia, the cells colony formation ability was reduced in hypoxia. However, addition of pyruvate and uridine in the medium, the cells in hypoxia not only showed highest levels of the stemness genes expression, but also high colony formation capability with highest number of colonies. We conclude that hypoxia and pyruvate/uridine synergistically induce the expression of stemness genes and increase the colony formation capability of esophageal cancer cells *in vitro*, indicating that esophageal cancer cells stemness can be upregulated *in vitro* for potential cancer stem cell targeting studies.
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1. Introduction

Several solid tumors including esophageal cancer are considered to contain a small subset of stem-like cells called cancer stem cells, which have the capacity to initiate new tumor. The cancer stem cells particularly have been demonstrated to escape from the radiotherapy and chemotherapy and are able to form metastatic tumor in other organs (Crea et al., 2009;Maitland and Collins, 2008). Investigations on how tumour cells change their stemness are currently demanded.

Low oxygen levels (hypoxia) have been shown to upregulate stemness factors in different types of cells. It is well documented that oxygen plays an important role in development in tissues and cells and hypoxia often occurs in physiological and pathophysiological conditions, especially when rapid growth exceeds the blood supply. For embryonic stem cells, hypoxia helps to maintain cells in stably high stemness and higher levels of oxygen tensions promote cells into differentiation (Forristal et al., 2010;Ma et al., 2009). Similar result has been observed in adult cells like adipocytes, fibroblasts and cancer cells (Kim et al., 2009a;Kim et al., 2009b;Malladi et al., 2007).

The hypoxia inducible factors (HIFs) are the key regulators found in mammalian cells cultured under reduced oxygen tension and play an essential role in cellular and systemic homeostatic responses to hypoxia. HIFs are heterodimers, composed of an alpha subunit and a beta subunit including HIF-1α and HIF-2α which are the major isoform of the α-subunit and share a high degree of sequence identity. Increasing evidence indicates that HIFs regulate a number of genes including glucose metabolism, cell survival, erythropoiesis, stem cell maintenance, angiogenesis related markers and resistance to chemotherapy and radiation therapy(Heddleston et al., 2010).

Oct3/4, also called POU5F1, and SOX2 are known embryonic stem cell markers which are important transcription factors in maintaining the self-renewal of embryonic stem cells and primordial germ cells. They are not only detected in germ cell tumor, but also in different somatic tumors such as lung, gastric, colorectal, rectal, bladder, breast,

ovarian, and esophageal cancers(Ben-Porath et al., 2008;Meng et al., 2010;Nirasawa et al., 2009;Peng et al., 2010;Saigusa et al., 2009;Sotomayor et al., 2009). Comparatively, the expression of these genes is downregulated in all differentiated somatic cell types *in vitro* as well as *in vivo*(Sperger et al., 2003).

Mitochondria are powerhouses of cells and consume up to 90% of inhale oxygen. However, mitochondria DNA (mtDNA) can be depleted *in vitro* by different methodologies and the mtDNA depleted cells (ρ^0 cells) share at least one major feature in common with tumor cells: ATP production is mainly through glycolysis, not through oxygen respiratory chain, because tumor cells, most probably tumour stem cells as well, usually locate in areas with low oxygen niche. It is well proved that the ρ^0 cells become pyruvate and uridine dependent for survival (King and Attardi, 1989;Liu et al., 2009;Olgun and Akman, 2007), indicating that these chemicals play a vital role in maintaining cells in live when cells are forced to obtain energy through glycolysis.

The common issue for cells cultivated in hypoxia condition and tumour cells *in vivo* is their niche of low oxygen tension. Under this condition cells are forced to synthesize ATP via glycolysis in cytoplasm which exhausts less oxygen. To survive better under this hypoxic condition, cells in cultivation may need different culture medium as shown for the ρ^0 cells. We, therefore, hypothesize that pyruvate and uridine addition in culture medium for tumor cells under hypoxic condition may favor their physiological growth and maintain these cells in rather "healthy" status with higher expression of stemness factors.

Therefore, in this study we firstly asked whether hypoxia could influence the expressions of the stemness factors Oct3/4 and SOX2 in the esophageal cancer cell lines KYSE70 and KYSE450. The cells were cultivated under 1%, 7% and 20% oxygen tensions, respectively, and the expressions of these factors and the HIF-1α and HIF-2α were examined by RT-PCR and Western blotting. We did observe that the expressions of Oct3/4 and SOX2 were significantly increased at lower O_2 tensions. We further asked whether pyruvate and uridine had similar effect on these cells and whether synergic effect of hypoxia and these chemicals could be identified in these cells. Our results clearly showed that pyruvate and uridine did have synergistic effect on the expressions of Oct3/4, SOX2, HIF-1α and HIF-2α, and the cells' colony formation capability under this condition was also greatly improved, indicating that the combinational application of low oxygen tension and pyruvate and uridine in esophageal cancer cells cultivation may help to propagate and maintain tumor cells with greater stemness, which may be of importance in cancer stem cell targeting studies.

2. Material and Methods

Cell lines and conventional cell culture

Human esophageal cancer cell lines KYSE70 and KYSE450 were purchased from ATCC (American Type Culture Collection, USA) and maintained in our lab for this study. For conventional cell culture, 2×10^5 cells were seeded in 75 cm^2 culture flasks and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin and 100 µg/ml streptomycin in a humidified 5% CO_2 incubator at 37°C.

Hypoxic cell culture

After 24 hours cultivation in conventional cell culture (allowing cells to attach onto the flasks), the cells were transferred into different incubators with different oxygen tensions. The Xvivo Closed Incubation System (Xvivo system 300C, BioSpherix, New York, USA) was used in this study to obtain accurate different oxygen tensions in different incubators. The cells were simultaneously cultivated in three incubators with 1%, 7% and 20% oxygen tensions, respectively, for variable periods of time before being harvested for additional RT-PCR and Western blotting analyses.

Pyruvate and uridine treatment

Pyruvate (sodium pyruvate) and uridine were purchased from Sigma (Sigma-Aldrich, USA). For cell culture treatment, the concentrations of pyruvate and uridine were determined solely based on our previous experience on mtDNA depleted cell experiment (Liu, Geng, and Suo, 2009). The cells were treated with 100 µg/ml pyruvate and 50 µg/ml uridine, respectively or in combination for 24 hours at 1% O_2 condition. Then the cells were collected for further RT-PCR and Western blotting.

MTT assay (Growth curve)

The KYSE450 and KYSE70 cells were firstly seeded at a density of 2×10^3/ml (180µl/well) into 96-well microplates with complete RPMI-1640 medium and placed into incubators with either 1% O_2, 7% O_2 or 20% O_2 conditions for variable time periods for MTT analyses using the Countess Cell Counter (Electronics Countess automated Cell counter, Invitrogen, USA). After the cells in culture reached their time schedule, the 5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) were added and incubated at 37 °C for 4 hrs before 150µL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was added to each well and mixed thoroughly. The plates were shaken for 15 min and absorbance was determined using a spectrophotometer at a

wavelength of 490 nm (μQuant; Bio-Tek Instruments, Winooski, VT).

RT-PCR procedure

Total RNA was extracted from the cultivated cells using RNeasy Kit (Qiagen, CA, USA) according to the manufacturer's instruction. Dnase I was used in the RNA isolation procedure, in order to eliminate any DNA. The concentrations of RNA samples were quantified using a spectrophotometer (Nanodrop ND-1000, USA) at OD260/280. The RNAs were then reverse-transcribed using the Multiscribe reverse transcriptase (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction. The condition for the reverse transcription was 25°C for 10min, 37°C for 120min and 85°C for 5min and ended with 4°C keeping, and the cDNAs were kept in freezer (-70°C) for later PCR running.

The cDNAs were amplified with a PCR machine (DOPPIO VWR, UK). All PCR programs started with a denaturation step at 95°C for 4 min and terminated with an elongation step at 72°C for 10 min. The primers and PCR conditions for Oct3/4, SOX2, HIF-1α and HIF-2α are shown in Table 1. The amplified PCR products were separated by a 7.5% polyacrylamide gel electrophoresis, stained with gelred (Molecular Probes, Invitrogen) and visualized in a syngene image system (G: BOX Syngene, USA). GAPDH and actin were used as internal controls for normalizing the expression levels in the subsequent quantitative analyses, respectively and the densitometries were analyzed by Syngene software.

Table 1: Primers and amplification conditions used for RT-PCR

Gene	Primers sequences		Ampli-fication length (bp)	Ampli-fication conditions
	Forward primer (5′-3′)	Reverse primer(5′-3′)		
Oct3/4	ACATGTGTAAGCTGCGGCC	GTTGTGCATAGTCGCTGCTTG	297	60°C,32
SOX2	TTGCTGCCTCTTTAAGACTAGGA	CTGGGGCTCAAACTTCTCTC	75	62°C, 35
HIF-1α	AGTGTACCCTAACTAGCCGAGGAA	CTGAGGTTGGTTACTGTTGGTATCA	113	60°C,35
HIF-2α	GACCAGCAGATGGACAACTTGTAC	CAGAAAGATCATGTCGCCATCTT	84	60°C,35
GAPDH	CCTCAAGATCATCAGCAATGC	TGGTCATGAGTCCTTCCACG	101	62°C,28
β-actin	CTTTGATTGCACATTGTTGT	GAAAGCAATGCTATCACCTC	160	62°C,28

Western blot analysis

Special attention was paid to quickly rinse the cells by ice-cold phosphate-buffered saline (PBS), and the cells were scraped into RIPA buffer (25 mM Tris HCl pH 7.6, 100 mM NaCl, 1% NP40, 1% Sodium deoxycholate, 0.1% SDS, Thermo Scientific Pierce, Germany) added with protease inhibitors (0.1 uM Aprotinin, 1.0 mM PMSF, 1 uM Leupeptin, 1 uM Pepstatin) immediately before use. The samples were centrifuged at 15,000 *rpm* for 15min at 4ºC and the supernatants were transferred to new tubes. The protein concentrations were measured with Bio-Rad protein assay according to the manufacturer's instruction. After heated with a benchtop heater (Model 111002, Boekel Scientific, USA) at 100ºC for 5min in SDS-loading buffer (500 mM Tris HCl pH 6.8; 10% Glycerol, 2% SDS, 0.6 M DTT, 0.05% Bromphenol blue), equal mount of protein (50 μg) per sample was subjected to 4-10% SDS-PAGE and transferred to polyvinylidene difluoride transfer membrane (BIO-RAD, USA). Membranes were blocked with 5% non-fat dry milk in TBS-Tween for 60 minutes at room temperature and incubated with the primary antibodies at optimal dilution in TBST/ 5% milk overnight at 4ºC, such as: GAPDH (0.2 μg/ml, R&D), Oct3/4 (1 μg/ml, R&D), SOX2 (1 μg/ml, R&D), HIF-1α (1 μg/ml, R&D) and HIF-2α (1 μg/ml, R&D). The membranes were then incubated by secondary HRP-conjugated antibodies including anti-goat, IgG-HRP antibody (Resourced from rabbit, dilution 1:2000) or anti-mouse IgG-HRP antibody (Resource from goat, dilution 1:1000). Immuno-complexes were visualized by enhanced chemiluminescence (GE Healthcare, UK). The western blotting experiments were repeated at least three times.

Colony formation assay

The cells in 80% confluent were dispatched from cell culture flask, harvested and counted with the Countess Cell Counter (Invitrogen). 500 cells per well were plated in plates at 20% or 1% oxygen tension for 14 days, and colonies were fixed with 4% buffered formalin for 15min and then were stained with 1% crystal violet for 30min. The plates were gently washed with PBS and dried before colony evaluation under microscope. Colony number which contained more than 30 cells was counted.

Statistical analyses

Experiments data are shown as the mean ± SD of at least 3 experiments (in duplicates) each; SPSS software (version 16.0) was used for data analysis. Quantification of band densities was performed using Genetools software (version 3.07). Statistical analysis was performed using a one-way ANOVA test and Student *t* test ($P < 0.05$ was considered statistically significant).

3. Results

Hypoxia effect

For both KYSE70 and KYSE450 cell lines, hypoxia inhibition of cell proliferation was repeatedly shown with oxygen tension-dependent manner. As shown in Fig 1, 7% oxygen tension demonstrated proliferation inhibition, 1% oxygen tension showed higher proliferation inhibition, in comparison to the cells cultured in 20% oxygen. There was a statistical significant difference ($P<0.05$).

At mRNA level, the HIF-1α, HIF-2α, Oct3/4 and SOX2 were detected both at hypoxic and normoxic conditions for KYSE70 and KYSE450 cells. At 48 hours cultivation, the expressions of these four factors were significantly increased in 7% and 1% O_2 compared to the expressions in 20% O_2 cultivation, respectively (Figs. 2).

The Western blotting analyses showed weak positive Oct3/4, SOX2, HIF-1α and HIF-2α expressions in both KYSE70 and KYSE450 cells cultivated in 20% O_2. Comparatively, the cells in 7% oxygen tension expressed higher levels of these proteins, and the highest levels of expressions of these proteins could be repeatedly demonstrated in cells cultivated in 1% oxygen tension (Fig 3).

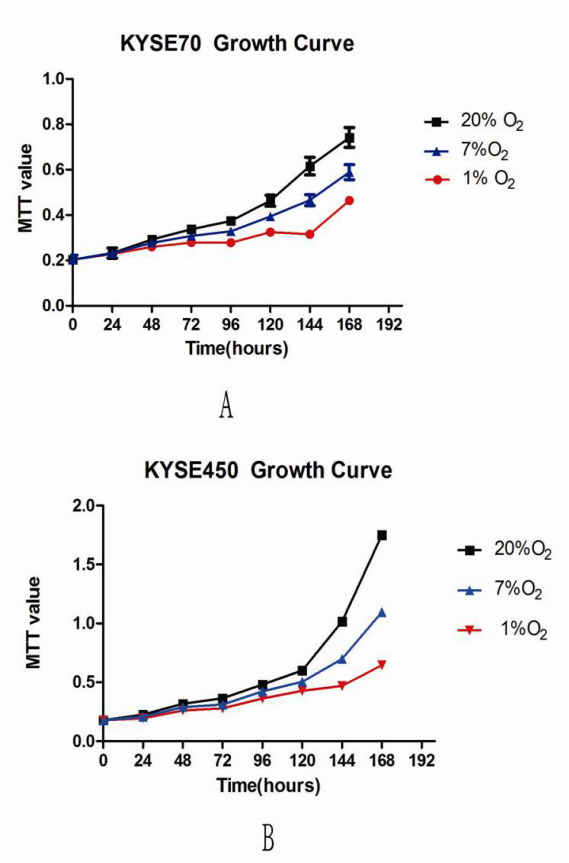


Figure 1. Growth curves of KYSE70 (A) and KYSE450 (B) cells in different oxygen tensions at variable time periods show growth inhibition under hypoxia. MTT values are shown with mean ± SD from 3 separate experiments for both KYSE70 and KYSE450. Statistical differences were observed between different oxygen tensions for both cell lines (*P<0.05*), cells in 7% O_2 tension show lower proliferation rate, while cells in 1% O_2 tension show lowest proliferation rate, in comparison to the cells in 20% O_2 tension.

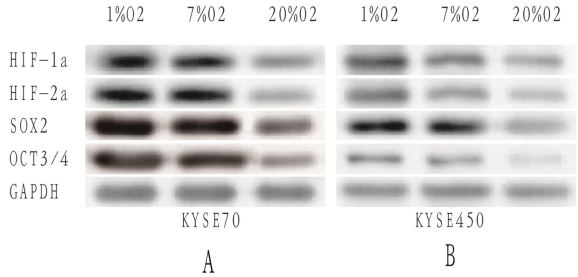


Figure 2. RT-PCR results show increasing mRNA expressions of HIF-1α, HIF-2α, Oct3/4 and SOX2 genes in hypoxic cultivation, in comparison to those in normoxic cultivation in variable time intervals in KYSE70 (A) and KYSE450 (B) cell lines. Representative mRNA expressions of HIF-1α, HIF-2α, Oct3/4 and SOX2 at 1%, 7% and 20% O_2 conditions were showed in the figure. GAPDH was used for internal loading control.

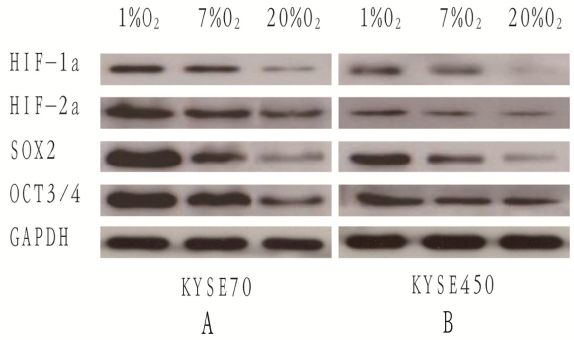


Figure 3. Hypoxia induces the protein expressions of HIF-1α, HIF-2α, Oct3/4 and SOX2 in KYSE70 and KYSE450 cell lines. In comparison to the cells cultivated in 20% oxygen, the cells cultivated in 7% and 1% oxygen tensions show higher levels of HIF-1α, HIF-2α, Oct3/4 and SOX2 as shown in Western blotting results for KYSE70 (A) and KYSE450 (B) cell lines. GAPDH was used as internal loading control. The higher levels of Oct3/4 and SOX2 are in parallel with the higher levels of HIF-1α and HIF-2α in hypoxic conditions.

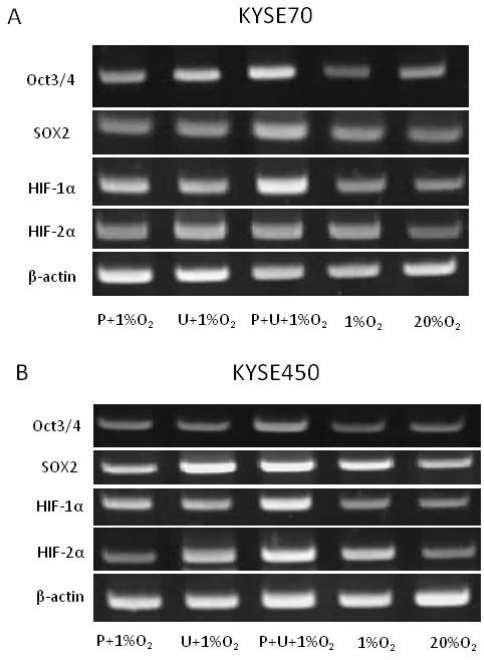
Pyruvate and uridine increased the expressions of Oct3/4, SOX2, HIF-1α and HIF-2α

We next asked whether pyruvate and uridine could have any effect on the expressions of these genes. Since we discovered that the expressions of these genes were significantly upregulated 24 hours in cultivation in 1% O_2 tension, we chose this condition for further pyruvate and uridine test on these cell lines. As shown in Fig 4, on the left panels, in comparison to the expression in 20% O_2, the mRNA level expressions of these genes (Oct3/4, SOX2, HIF-1α and HIF-2α) were upregulated in cells

cultivated in 1% O_2 tension. However, their gene expressions were even higher in the cells treated either with pyruvate or uridine or in combination. Similar Western blotting results were repeatedly obtained as shown in Fig 4 at the right panels.

Pyruvate and uridine enhanced colony formation in hypoxic condition

Inhibition of cell growth and up-regulation of the HIFs and transcription factors under 1% O_2 were repeatedly observed in our lab for these cell lines. For one end, upregulation of these factors is ideal for enriching cells with greater stemness, but for another end the cell proliferation inhibition may be a challenge for *in vitro* studies. Since pyruvate and uridine were proved to have synergic effect on the expressions of these factors, we asked how pyruvate and uridine could influence the colony formation of these cells in different O_2 tensions. As shown in Fig 5, for both KYSE70 and KYSE450 cell lines, fewer colonies were formed in 1% O_2 compared to the cells cultivated in 20% O_2 tension. However, both pyruvate and uridine could significantly stimulate colony formation separately in 1% O_2, and the greatest stimulation could be seen for cells treated with both pyruvate and uridine in 1% O_2 tension, reaching to similar colonies as for cells cultivated in 20% O_2.



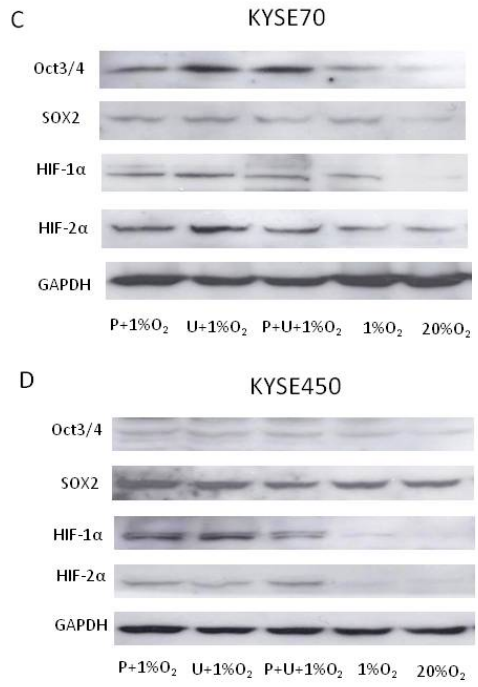
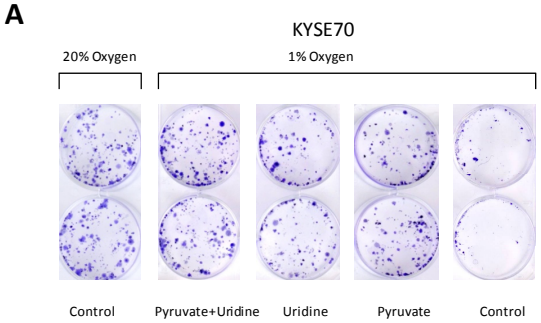


Figure 4. Pyruvate and uridine show synergistic effect on the expression induction of HIF-1α, HIF-2α, Oct3/4 and SOX2 in esophageal cancer cell lines. Representative RT-PCR results show increasing levels of Oct3/4, SOX2, HIF-1α and HIF-2α mRNA expressions in hypoxia, and even higher levels expressions in hypoxia with pyruvate and uridine, in comparison to normoxia in KYSE70 (A) and KYSE450 (B) cells. Representative Western blotting results show increasing protein level expressions of Oct3/4, SOX2, HIF-1α and HIF-2α in hypoxia, and even higher when pyruvate and uridine were added in the media, in compared to the expressions in normoxia in KYSE70 (C) and KYSE450 (D) cells. β-actin was used for internal loading control for all the RT-PCR examinations and GAPDH was used as internal loading control for all the Western blotting analyses. P+1%O_2 is for 1% O_2 with 100 μg/ml pyruvate; U+1%O_2 is for 1% O_2 with 50 μg/ml uridine, P+U+1%O_2 is for 1% O_2 with 100 μg/ml pyruvate and 50 μg/ml uridine; 1%O_2 is for 1% O_2 only; 20%O_2 is for 20% O_2 only.



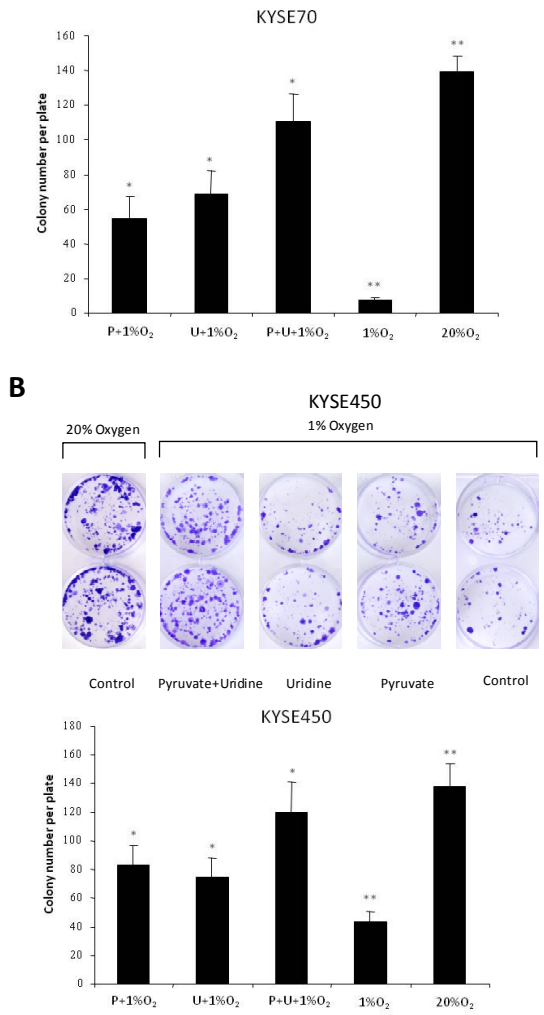


Figure 5. Pyruvate and uridine increase the ability for colony formation of esophageal cancer cells. Representative photomicrographs of colony formation assay of KYSE70 (A, up panel) and KYSE450 (B, up panel) show that cells under 1% O2 form less colonies, in comparison to the cells under 20% O2, while either pyruvate or uridine alone could significantly increase the number of colonies for both cell lines and cells with both pyruvate and uridine added in the medium show greater number of colonies similar to the cells under 20% O2. The down panels in both A and B show the histograms which were created with the means ± SD of three independent experiments. P+1%O2 is for 1% O2 with 100 μg/ml pyruvate; U+1%O2 is for 1% O2 with 50 μg/ml uridine, P+U+1%O2 is for 1% O2 with 100 μg/ml pyruvate and 50 μg/ml uridine; 1%O2 is for 1% O2 only as control; 20%O2 is for 20% O2 only as another control. * means P<0.05, ** and means P<0.01.

4. Discussions

According to the cancer stem cells definition, these cells may generate tumors through the normal stem cell processes of self-renewal and further differentiation upon appropriate stimulation. These cells may persist in tumors as a distinct population and cause relapse and metastasis after general cancer therapies like radiotherapy and chemotherapy. It is believed that the higher stemness the tumour cells show *in vitro*, the more likely that the cells are closer to tumour stem cells. Furthermore, the stemness of tumour cells can be reflected by their expressions of the transcription factors or stemness factors (Cesselli et al., 2009;Takahashi and Yamanaka, 2006).

In order to upregulate stemness of tumour cells *in vitro*, hypoxia has been examined in different cell or tissues types during the recent years. Hypoxia often occurs inside solid tumor and exhibits more severe at the undifferentiated parts compared to surrounding tumor or normal tissues. The hypoxia inducible factors HIF-1α and HIF-2α are important factors activated under hypoxia and sometimes happen under normoxia as well in tumour tissues (Khandrika et al., 2009;Peng, Maihle, and Huang, 2010). It has been observed that HIFs greatly influence on the phenotypes of tumors by regulating a number of target genes such as glucose transporters, glycolic enzyme, vascular endothelial cell growth, and growth factors (Heddleston, Li, Lathia, Bao, Hjelmeland, and Rich, 2010;Keith and Simon, 2007;Pescador et al., 2010). HIF-1α and HIF-2α share some target genes such as VEGF, GLUT1, and ADM-1, but they have their unique targets as well: the glycolytic enzymes (PGK1, ALDA) are only targets of HIF-1α and TGF-α, cyclin D1 appear to be HIF-2α targets, at least in certain cell type (Heddleston, Li, Lathia, Bao, Hjelmeland, and Rich, 2010;Keith and Simon, 2007). HIF-1α also regulates the adoptive signaling including Notch signaling, Wnt signaling, c-myc and p53 factors and HIF-2α has an interaction with Oct3/4, c-myc and maybe KIF4 and Sox2.

The HIF-1α and HIF-2α were increased in several cancer cells like lung, gastric, colorectal, breast, ovarian and esophageal cancers (Bryant et al., 2010;Cao et al., 2009;Wan et al., 2009;Jung et al., 2009;Li et al., 2009;Pipinikas et al., 2008;Daponte et al., 2008;Kolev et al., 2008;Shaida et al., 2008;Shi et al., 2007). Li,Y. etc. found that hypoxia induced HIF-1α and HIF-2α expressions in BE1 and A549 cells and also demonstrated that hypoxia-HIF-1α, 2α - CCR7-ERK1/2 pathway could regulate the migration and invasion of lung cancer cells under hypoxic conditions and promote metastasis of lung cancer (Li, Qiu, Zhang, Zhang, and Wang, 2009;Wan, Ma, Mei,

and Shan, 2009). It is reported that HIF-1α increased expression in ovarian cancer cell lines MDAH-2774 and SKOV-3 in response to hypoxia and further investigation showed that inhibition of HIF-1α expression by specific siRNA resulted in a significant decrease in VEGF production and angiogenesis. Other groups chose the ovarian cancer cell lines ES-2 and SKOV3 under hypoxia to induce HIF-1α overexpression (Bryant, Munkarah, Kumar, Batchu, Shah, Berman, Morris, Jiang, and Saed, 2010;Hua et al., 2009). HIF-1α over expression was observed in primary esophageal cancers, compared to normal esophageal epithelium, esophageal cancer bone metastases and esophageal cell lines KYSE70 and KYSE450 as well (Natsuizaka et al., 2012;Zeng et al., 2011). In a clinical observation of high-grade esophageal intraepithelial neoplasia lesions, the precursor of a majority of invasive esophageal adenocarcinoma, increased HIF-1α expression was found in relative to the respective normal epithelium, stromal cells, and benign prostatic hyperplasia (Monsef et al., 2007).

In our study, both HIF-1α and HIF-2α were weakly revealed in 20% O_2 tension cultivation and higher levels expressions were repeatedly demonstrated under hypoxic conditions, typically peaked at the time period 24 hours incubation at 1% and 7% O_2 in both KYSE70 and KYSE450 cells, with the highest level expressions in cells in 1% O_2 cultivation (as shown in Fig 2-4).

Previously, direct molecular links have been established between HIFs and stem cell factors such as Oct3/4, c-Myc and β-catenin. Oct3/4 occupies promoters for many developmental regulators in human embryonic cells, and with SOX2, forms a transcriptional network which has capacity in maintaining the self-renewal of embryonic stem cells and primordial germ cells (Mazumdar et al., 2009). Oct3/4 and SOX2 are not only detected in embryonic cell and germ tumor cell, but also in different somatic tumors such as lung, gastric, colorectal, rectal, bladder, breast, ovarian, and esophageal cancers (Ben-Porath, Thomson, Carey, Ge, Bell, Regev, and Weinberg, 2008;Meng, Zheng, Wang, Liu, Sui, Wu, Zhou, Ding, and Li, 2010;Nirasawa, Kobayashi, Tsuji, Kuribayashi, and Watanabe, 2009;Peng, Maihle, and Huang, 2010;Saigusa, Tanaka, Toiyama, Yokoe, Okugawa, Ioue, Miki, and Kusunoki, 2009;Sotomayor, Godoy, Smith, and Huss, 2009). Comparatively, the expression of these genes is downregulated in all differentiated somatic cell types *in vitro* as well as *in vivo* (Wang et al., 2009).

In consistence with the enhanced HIF-2α expression under hypoxia, the KYSE70 and KYSE450 cells in our study were repeatedly shown with higher level Oct3/4 expression, both at RNA and protein levels, a link supported by the finding that HIF-2α binds to promoter of Oct3/4 and induces its expression and activity directly (Covello et al., 2006). In addition to induction of Oct3/4 expression under hypoxia, we observed also an induction of SOX2 expression.

Pyruvate and uridine have been used for maintaining the survival of cells with mtDNA defect or depletion. MtDNA is more sensitive to damage than nuclear DNA when DNA intercalating agents like ethidium bromide (EB) *etc*. is applied in cell culture resulting in cells without mtDNA or called ρ^o cells. These cells are therefore forced to synthesize ATP through glycolysis. Many researches show increased tumor cell glycolysis when the cells exposure to low oxygen levels (external pressure) or even under normoxia (internal pressure). The later is due to mtDNA defect or depletion as discussed above. We hypothesize that tumor cells may need pyruvate and uridine for better survival under hypoxic conditions. We found in our study that addition of pyruvate and uridine to the medium for the esophageal cancer cell lines in 1% O_2 cultivation dramatically induced the expressions of HIF-1α, HIF-2α and also the stem cell marker Oct3/4 and SOX2 as shown in Fig 4.

In most cancer cells, glycolysis is responsible for about 60% of ATP production. The raised blood or serum pyruvate was frequently tested in leukemia, lymphoma, and renal cancer carcinoma. In addition, the pyruvate reduces DNA damage during hypoxia which might protect cancer stem cells. Recent studies exhibited that the pyruvate importation was enhanced in hypoxic tumor which could help to maintain pyruvate level and stabilize HIF-1α (Roudier et al., 2007;Roudier and Perrin, 2009). Therefore, it is indicated that the cancer cells cultivated *in vitro* under hypoxia may need extra pyruvate for better growth.

We unexpectedly found that the KYSE70 and KYSE450 cells showed growth inhibition under hypoxia, although there was upregulation of the HIFs and transcription factors under hypoxic conditions. Furthermore, we also discovered that pyruvate and uridine had synergic effect on the expressions of these factors. That the better survival effect of pyruvate and uridine on mtDNA depleted cells encouraged us to investigate whether these chemicals could stimulate colony formation of these cells under hypoxia. In line with our speculation, colony formation capability of both KYSE70 and KYSE450 cells was greatly enhanced with addition of these chemicals, reaching to a similar level of the cells in 20% O_2 cultivation. The synergic effect of hypoxia and pyruvate and uridine results in, therefore, cells with greater stemness *in vitro*.

In conclusion, hypoxia induces the hypoxia inducible factors HIF-1α and HIF- 2α and stem cell factors in esophageal cancer cell lines KYSE70 and KYSE450; Pyruvate and uridine not only have synergic effect on the expressions of these factors in these cell lines, but also enhance the colony formation capability under hypoxia, resulting in cells with greater cell stemness and good propagation possibility, indicating that combinational application of low oxygen tension and pyruvate and uridine in tumor cells *in vitro* may help to propagate and maintain tumor cells with greater stemness, which should be of importance in further cancer stem cell targeting studies.

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References

1. Crea F, Mathews LA, Farrar WL, et al. Targeting prostate cancer stem cells. Anticancer Agents Med Chem, 2009, 9(10):1105-1113.
2. Maitland NJ and Collins AT. Prostate cancer stem cells: a new target for therapy. J Clin Oncol, 2008, 26(17):2862-2870.
3. Forristal CE, Wright KL, Hanley NA, et al. Hypoxia inducible factors regulate pluripotency and proliferation in human embryonic stem cells cultured at reduced oxygen tensions. Reproduction, 2010, 139(1):85-97.
4. Ma T, Grayson WL, Frohlich M, et al. Hypoxia and stem cell-based engineering of mesenchymal tissues. Biotechnol Prog, 2009, 25(1):32-42.
5. Kim Y, Lin Q, Glazer PM, et al. Hypoxic tumor microenvironment and cancer cell differentiation. Curr Mol Med, 2009, 9(4):425-434.
6. Kim Y, Lin Q, Zelterman D, et al. Hypoxia-regulated delta-like 1 homologue enhances cancer cell stemness and tumorigenicity. Cancer Res, 2009, 69(24):9271-9280.
7. Malladi P, Xu Y, Chiou M, et al. Hypoxia inducible factor-1alpha deficiency affects chondrogenesis of adipose-derived adult stromal cells. Tissue Eng, 2007, 13(6):1159-1171.
8. Heddleston JM, Li Z, Lathia JD, et al. Hypoxia inducible factors in cancer stem cells. Br J Cancer, 2010, 102(5):789-795.
9. Ben-Porath I, Thomson MW, Carey VJ, et al. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat Genet, 2008, 40(5):499-507.
10. Meng HM, Zheng P, Wang XY, et al. Overexpression of nanog predicts tumor progression and poor prognosis in colorectal cancer. Cancer Biol Ther, 2010, 9(4):
11. Nirasawa S, Kobayashi D, Tsuji N, et al. Diagnostic relevance of overexpressed Nanog gene in early lung cancers. Oncol Rep, 2009, 22(3):587-591.
12. Peng S, Maihle NJ, and Huang Y. Pluripotency factors Lin28 and Oct4 identify a sub-population of stem cell-like cells in ovarian cancer. Oncogene, 2010, 29(14):2153-2159.
13. Saigusa S, Tanaka K, Toiyama Y, et al. Correlation of CD133, OCT4, and SOX2 in rectal cancer and their association with distant recurrence after chemoradiotherapy. Ann Surg Oncol, 2009, 16(12):3488-3498.
14. Sotomayor P, Godoy A, Smith GJ, et al. Oct4A is expressed by a subpopulation of prostate neuroendocrine cells. Prostate, 2009, 69(4):401-410.
15. Sperger JM, Chen X, Draper JS, et al. Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors. Proc Natl Acad Sci U S A, 2003, 100(23):13350-13355.
16. King MP and Attardi G. Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. Science, 1989, 246(4929):500-503.
17. Liu Y, Geng L, and Suo Z. Differentiation effect of pyruvate and uridine on cultured U937-rho degrees cells. Ultrastruct Pathol, 2009, 33(4):160-164.
18. Olgun A and Akman S. Mitochondrial DNA-deficient models and aging. Ann N Y Acad Sci, 2007, 1100(241-245.
19. Cesselli D, Beltrami AP, Rigo S, et al. Multipotent progenitor cells are present in human peripheral blood. Circ Res, 2009, 104(10):1225-1234.
20. Takahashi K and Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell, 2006, 126(4):663-676.
21. Khandrika L, Lieberman R, Koul S, et al. Hypoxia-associated p38 mitogen-activated protein kinase-mediated androgen receptor activation and increased HIF-1alpha levels

contribute to emergence of an aggressive phenotype in prostate cancer. Oncogene, 2009, 28(9):1248-1260.
22. Keith B and Simon MC. Hypoxia-inducible factors, stem cells, and cancer. Cell, 2007, 129(3):465-472.
23. Pescador N, Villar D, Cifuentes D, et al. Hypoxia promotes glycogen accumulation through hypoxia inducible factor (HIF)-mediated induction of glycogen synthase 1. PLoS One, 2010, 5(3):e9644.
24. Bryant CS, Munkarah AR, Kumar S, et al. Reduction of hypoxia-induced angiogenesis in ovarian cancer cells by inhibition of HIF-1 alpha gene expression. Arch Gynecol Obstet, 2010,
25. Cao D, Hou M, Guan YS, et al. Expression of HIF-1alpha and VEGF in colorectal cancer: association with clinical outcomes and prognostic implications. BMC Cancer, 2009, 9(432.
26. Wan J, Ma J, Mei J, et al. The effects of HIF-1alpha on gene expression profiles of NCI-H446 human small cell lung cancer cells. J Exp Clin Cancer Res, 2009, 28(150.
27. Jung HJ, Park JW, Lee JS, et al. Silibinin inhibits expression of HIF-1alpha through suppression of protein translation in prostate cancer cells. Biochem Biophys Res Commun, 2009, 390(1):71-76.
28. Li Y, Qiu X, Zhang S, et al. Hypoxia induced CCR7 expression via HIF-1alpha and HIF-2alpha correlates with migration and invasion in lung cancer cells. Cancer Biol Ther, 2009, 8(4):322-330.
29. Pipinikas CP, Carter ND, Corbishley CM, et al. HIF-1alpha mRNA gene expression levels in improved diagnosis of early stages of prostate cancer. Biomarkers, 2008, 13(7):680-691.
30. Daponte A, Ioannou M, Mylonis I, et al. Prognostic significance of Hypoxia-Inducible Factor 1 alpha(HIF-1 alpha) expression in serous ovarian cancer: an immunohistochemical study. BMC Cancer, 2008, 8(335.
31. Kolev Y, Uetake H, Takagi Y, et al. Lactate dehydrogenase-5 (LDH-5) expression in human gastric cancer: association with hypoxia-inducible factor (HIF-1alpha) pathway, angiogenic factors production and poor prognosis. Ann Surg Oncol, 2008, 15(8):2336-2344.
32. Shaida N, Launchbury R, Boddy JL, et al. Expression of BNIP3 correlates with hypoxia-inducible factor (HIF)-1alpha, HIF-2alpha and the androgen receptor in prostate cancer and is regulated directly by hypoxia but not androgens in cell lines. Prostate, 2008, 68(3):336-343.
33. Shi YH, Bingle L, Gong LH, et al. Basic FGF augments hypoxia induced HIF-1-alpha expression and VEGF release in T47D breast cancer cells. Pathology, 2007, 39(4):396-400.
34. Hua K, Din J, Cao Q, et al. Estrogen and progestin regulate HIF-1alpha expression in ovarian cancer cell lines via the activation of Akt signaling transduction pathway. Oncol Rep, 2009, 21(4):893-898.
35. Natsuizaka M, Naganuma S, Kagawa S, et al. Hypoxia induces IGFBP3 in esophageal squamous cancer cells through HIF-1alpha-mediated mRNA transcription and continuous protein synthesis. FASEB J, 2012, 26(6):2620-2630.
36. Zeng KF, Jin HL, Zhang WF, et al. Effect of RNA interference targeting HIF-1alpha gene on biological behavior of human esophageal squamous cell carcinoma and gastric adenocarcinoma cells in vitro. Zhonghua Zhong Liu Za Zhi, 2011, 33(4):260-264.
37. Monsef N, Helczynski L, Lundwall A, et al. Localization of immunoreactive HIF-1alpha and HIF-2alpha in neuroendocrine cells of both benign and malignant prostate glands. Prostate, 2007, 67(11):1219-1229.
38. Mazumdar J, Dondeti V, and Simon MC. Hypoxia-inducible factors in stem cells and cancer. J Cell Mol Med, 2009, 13(11-12):4319-4328.
39. Wang Q, He W, Lu C, et al. Oct3/4 and Sox2 are significantly associated with an unfavorable clinical outcome in human esophageal squamous cell carcinoma. Anticancer Res, 2009, 29(4):1233-1241.
40. Covello KL, Kehler J, Yu H, et al. HIF-2alpha regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. Genes Dev, 2006, 20(5):557-570.
41. Roudier E, Bachelet C, and Perrin A. Pyruvate reduces DNA damage during hypoxia and after reoxygenation in hepatocellular carcinoma cells. FEBS J, 2007, 274(19):5188-5198.
42. Roudier E and Perrin A. Considering the role of pyruvate in tumor cells during hypoxia. Biochim Biophys Acta, 2009, 1796(2):55-62.

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