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ORIGINAL RESEARCH

GLUT5 increases fructose utilization in ovarian cancer

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Background: Fructose is one of the most common dietary carbohydrates in the whole world, and recent studies have found that fructose consumption is closely related to the oncogenesis and development of tumors, however, very few studies have focused on the fructose in ovarian cancer. GLUT5 (Glucose transporter type 5), as a specific fructose transporter in mammalian cells, has also been found highly expressed in many cancers.
Methods: In this study, we investigated the abilities of proliferation, colony formation, and migration of ovarian cancer cells in fructose medium, and then silenced GLUT5 in ovarian cancer cells to explore the role GLUT5 in fructose metabolism in ovarian cancer.
Results: The results showed that the ovarian cancer cells had similar abilities of proliferation and migration in fructose medium and glucose medium, but silencing GLUT5 could significantly inhibit these abilities in fructose medium. Meanwhile, we found that GLUT5 was higher expressed in ovarian cancer tissues, and its expression correlated significantly with tumor malignancy and poor survival of ovarian cancer patients. Furthermore, the results of animal experiments also demonstrated that intake too much fructose could prominently increase tumor volume, and silencing GLUT5 could significantly inhibit tumor proliferation.
Conclusion: In conclusion, we demonstrate that ovarian cancer cells could utilize fructose for their growth, and restricting the fructose intake or targeting GLUT5 may be efficacious strategies for ovarian cancer therapy.
Keywords: fructose, GLUT5, ovarian cancer

Introduction

Among female cancers, cancer of the ovary is less common than cancer of the breast and cancer of the uterus. However, since cancer of the ovary is located deep within the pelvis and difficult to assess, and has no early typical symptoms and effective diagnostic methods, 65–75% women with cancer of the ovary are frequently diagnosed with advanced stage disease.[1–3] So cancer of the ovary has the highest case fatality rate, accounting for almost half of the deaths from gynecologic malignancies.[4] After years of study, researchers have discovered several risk factors that might increase a woman's chance of developing ovarian cancer, among them, obesity is the most important risk factor.[5] Obese women have a higher risk of developing ovarian cancer, and obesity also affects the overall survival of a woman with ovarian cancer.[6,7]

With the introduction of high-fructose corn syrup into the food processing industry in the 1970s, the per capita consumption of fructose increased year by year.[8] Epidemiological studies have found that high-fructose intake is associated with obesity, dyslipidemia, and metabolic syndrome.[9] Animal experimental

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studies have also shown that high-fructose diet can cause obesity, so the harm of high-fructose diet is gradually recognized.[10] Although the relationship between high-fructose intake and tumorigenesis and tumor progression is not completely consistent, various metabolic abnormalities caused by fructose, such as obesity, are closely related to tumorigenesis.[11,12]

Recently, more and more studies have found that fructose consumption is closely related to the oncogenesis and development of tumors, however, very few studies have focused on fructose in ovarian cancer. In this study, we investigated the role of fructose on ovarian cancer cell lines, and detected the expression level of GLUT5 (Glucose transporter type 5) in ovarian cancer tissues, and analyzed the correlation of GLUT5 expression with clinicopathological variables and survival of ovarian cancer patients.

Materials and methods

Cell culture

All the cell lines, including OVCAR83 and SKOV3, were obtained from ATCC. OVCAR83, SKOV3, A549, T47D, and AGS were cultured in 1,640 medium, and Hela and LN229 were cultured in DMEM medium, supplemented with 10% FBS (Gibco, Invitrogen Life Technologies, Carlsbad, CA, USA) in 5% CO_2 at 37°C. Glucose-free DMEM and dialyzed fetal bovine serum were obtained from Gibco, and fructose was purchased from Sigma. In this study, Glucose medium was added 25 mM glucose in glucose-free DMEM, and Fructose medium was added 25 mM fructose in glucose-free DMEM.[13]

Antibodies

The primary antibodies GLUT5 (ab36057) and GAPDH (ab8245) used in this study for IHC and Western blot were purchased from Abcam. The secondary antibodies for Western blot were purchased from Santa Cruz Biotechnology, and PV6001 secondary antibody for IHC was purchased from Zhongshan Goldbridge Biotechnology.

Western Blot

All agents were purchased from Santa Cruz Biotechnology. Protein was obtained using a lysis buffer (1% SDS, 10 mM Tris-HCl, pH 7.6, 20 μg/mL aprotinin, 20 μg/mL leupeptin, and 1 mM PMSF). Twenty micrograms of protein were separated on a 10% SDS-PAGE gel and blotted onto a PVDF membrane. Primary antibodies were incubated for 1 hr at room temperature, and then secondary antibody for 1 hr at room temperature. The bands for samples were analyzed with a gel imaging system, and image J was used to quantify the Western blots.

MTT assay for cell proliferation

Cells were seeded in 96 well plates at a density of 3,000 cells/well. At the end of incubation, 20 μL of 5 mg/mL 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added to each well. Then, the plates were incubated in 37°C 5% CO_2 for 4 hrs, following which 150 μL dimethyl sulfoxide was added. The absorbance was measured at 490 nm wavelength.

Clonogenic survival assay

Viable cells were seeded on six-well plates (500/well) and incubated in 37°C 5% CO_2 for 14 days. After these cells were then fixed with methanol and stained with gentian violet. Colonies containing more than 50 cells were scored as surviving cells.

Soft agar colony formation assay

1×10^4 cells were plated in 0.4% agarose on top of a 1% agarose base supplemented with culture medium in 3.5 cm dishes. A further 1 mL of 1× media was added on top of the growth layer on day 0 and day 14 of growth. Cells were allowed to grow for 4 weeks, and total colonies were counted under the microscope.

Small hairpin RNA (shRNA) and sgRNA plasmids construction and cell infection

shRNA sequence, CCAATCGTTTGAGCTAATAA, was constructed into pLKO.1 plasmids. The day before transfection, 1×10^5 cells were placed in 35 mm dishes in DMEM supplemented with 10% fetal bovine serum and without antibiotics. The transfected cells were cultured for 48 hrs, and puromycin was used for drug screening, then Western blot was utilized to detect protein expression level. The sgGLUT5 sequences 5'-TGTGTCATCTCCT ACGTCAT-3' and 5'-AGATCTCGATGCCACCCGCC-3' were designed according to the Zhang laboratory's protocol[14] and cloned into the lentiCRISPR/CAS9 vector.

Patients and tissue samples

One hundred and thirty-seven cases of ovarian epithelial cancers, including 78 serous carcinomas, 40 endometrioid carcinomas, 11 mucinous carcinomas, and 8 clear cell

adenocarcinomas, and adjacent normal ovarian tissues were obtained from the Department of Pathology, Tianjin Hospital in 2009–2013. Histopathological diagnoses were made using the World Health Organization criteria and examined by specialists. International Federation of Gynecology and Obstetrics (FIGO, 2009) was used to determine cancer stage and grade. This study complied with the Declaration of Helsinki and was approved by the Human Ethics and Research Ethics Committees of the hospital, and all the patients provided written informed consent. Table 1 summarizes all patients' characteristics.

Immunohistochemistry staining and evaluation

Sections were deparaffinized and rehydrated with xylene and graded alcohol solutions. Endogenous peroxidase activity was quenched by 3% hydrogen peroxide, and then boiled in 10 mM citrate buffer (pH 6.0) for 3 mins in an autoclave sterilizer to expose antigen. After that, sections were incubated with GLUT5 antibody (1:100 dilution) overnight at 4°C. Then, sections were incubated with PV6001 for 30 mins at 37°C and stained with DAB for 1–2 mins. Control sections were incubated with PBS instead of a primary antibody. Five high-power fields from each slice were chosen and scored, GLUT5 expression levels of ≤50% in sections were assigned to the low-expression group, whereas those with values >50% were assigned to the high-expression group.

Table 1 Correlation between GLUT5 expression and clinico-pathological variables in patients with ovarian cancer

Variables	n	GLUT5 expression		χ^2	*P*
		Low	High		
Age (years)					
<55	55	24	31	0.20	0.65
≥55	82	39	43		
Clinical stage					
Early (stage I–II)	72	45	27	16.66	**<0.01**
Advanced (stage III–IV)	65	18	47		
Grade					
I	31	19	12	12.14	**<0.01**
II	52	29	23		
III	54	15	39		
Ascites					
No	69	37	32	3.27	0.07
Yes	68	26	42		
Metastasis					
Negative	84	46	38	6.73	**0.01**
Positive	53	17	36		
Histology type					
Serous	78	33	45	4.66	0.20
Endometrioid	40	17	23		
Mucinous	11	8	3		
Clear cell	8	5	3		

Note: Bold values indicate *P*<0.05.

Mice xenograft models

In one animal model, 2×10^6 OVCAR8 cells were injected subcutaneously to the dorsal midline in 10 nu/nu mice to generate murine subcutaneous tumors. When the tumor size reached the size of approximately 60–80 mm^3, these mice were divided into two groups randomly: control group (water without fructose, n=5), fructose-feeding group (water with 15% fructose, n=5), and the standard laboratory chow was given to all the mice. In the second model, 2×10^6 OVCAR8 of sgctrl, sgGLUT5#1, and sgGLUT5#1-GLUT5 cells were injected subcutaneously to the dorsal midline in nu/nu 15 mice, followed by 15% fructose water feeding and standard laboratory chow. All the tumor volumes were calculated every 4 days by the following formula: length*width * width/2, and the tumor in mice for 30 days. All animal studies were followed an approved protocol by the Institutional Animal Care and Use Committee of Tianjin hospital, in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals.

Statistical methods

SPSS 16.0 was used. The standard two-tailed independent samples *t*-test was performed to compare the differences in two groups. The χ^2 test was used to assess the difference in GLUT5 expression and the pathological and clinical factors between normal ovarian tissues and ovarian cancer tissues. Survival was analyzed using the Kaplan–Meier analysis. The significance level was defined as $P<0.05$. All experiments were performed in triplicate.

Results

Fructose could promote ovarian cancer cells survival in glucose-free medium

According to published reports, many cancer cells, including breast cancer, glioma, and lung cancer, could utilize fructose to

maintain their survival and proliferation.[13,15–17] We first collected many kinds of cancer cell lines (Hela, A549, T47D, LN229, AGS, OVCAR8, and SKOV3), and detected their survival after cultured in medium with added glucose or fructose in 72 hrs. Then we found that, compared to glucose-free medium, fructose could successfully promote all these cell

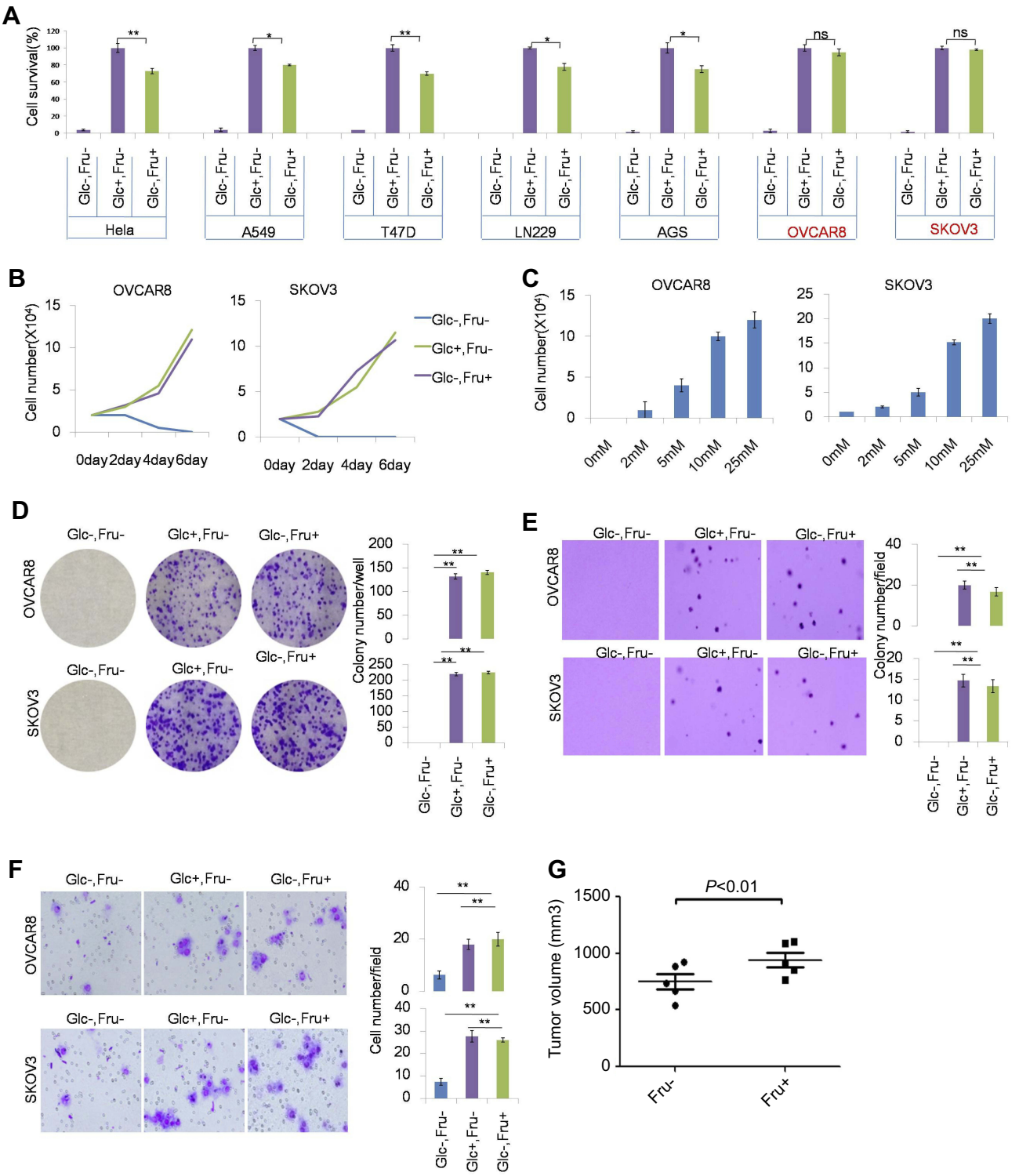

Figure 1 Ovarian cancer cells could utilize fructose for their growth. (**A**) Many kinds of cancer cell lines could utilize fructose. (**B**) Ovarian cancer cells grew at equivalent rates in 25 mM fructose medium and 25 mM glucose medium detected by MTT. (**C**) There was a concentration dependence in fructose utilization for cell growth. (**D, E**) The colony formation of ovarian cancer cells in 25 mM fructose medium and 25 mM glucose medium. (**F**) The migration of ovarian cancer cells in 25 mM fructose medium and 25 mM glucose medium. (**G**) The tumor grew in vivo after feeding fructose 30 days (*P<0.05, **P<0.01).

lines survival (Figure 1A). It is worth noting that there was no significant difference between glucose medium and fructose medium for ovarian cancer cell lines, OVCAR8 and SKOV3, however, this phenomenon was not found in other cell lines (Figure 1A). So this result suggested that fructose was especially important for ovarian cancer. Meanwhile, there was no significant difference between glucose medium and fructose medium in ovarian cancer cell proliferation (Figure 1B). Furthermore, fructose could promote cell proliferation in a dose-dependent manner (0, 2, 5, 10, 25 mM) in the condition of glucose deprivation (Figure 1C). In addition, ovarian cancer cell lines, OVCAR8 and SKOV3, could form clones in the fructose medium, and the number of clone in fructose medium was also similar with the clone number in glucose medium (Figure 1D and E). Furthermore, fructose could also promote ovarian cancer cell migration ability (Figure 1F). To affirm the role of fructose in ovarian cancer, animal experiments were performed. The results showed that, when 15% fructose was added in the water fed to mice for 30 days, fructose could promote tumor growth in vivo (Figure 1G). Recent studies showed that no-tumor cell lines could not utilize fructose to maintain their survival,[13,17] and a normal ovarian cell line, IOSE80, was used to further verify this result in our study. We found that fructose just partly rescues IOSE80 cells in the glucose-free medium, even using higher concentration of fructose (Figure S1A and B). These results suggested that ovarian cancer cells could utilize fructose to maintain their survival, but normal ovarian cells could not.

Knockdown of GLUT5 could significantly inhibit cell abilities of proliferation and colony formation in fructose medium

GLUT5, as a specific fructose transporter in mammalian cells, has been found highly expressed in many cancers.[18] To explore if GLUT5 was also highly expressed in ovarian cancer, we detected the expression level of GLUT5 in the normal ovarian cell line, IOSE80, and ovarian cancer cell lines, OVCAR8 and SKOV3, and we found that the GLUT5 was highly expressed in ovarian cancer cell lines (Figure 2A). Then, we silenced GLUT5 using shRNA in ovarian cancer cell lines, OVCAR8 and SKOV3 (Figure 2B), and overexpressed GLUT5 in the normal ovarian cell line, IOSE80 (Figure S1C), to verify if GLUT5 was correlated with fructose utilization in these cells. We found that knockdown of GLUT5 could significantly inhibit the cell proliferation in both OVCAR8 (Figure 2C) and SKOV3 (Figure 2D) in the fructose medium, but there was no effect on the cell proliferation in glucose medium (Figure 2C and D). Using the same method, we detected the abilities of the proliferation of IOSE80 overexpressed GLUT5, and the results showed that overexpressing GLUT5 significantly promoted IOSE80 proliferation in the fructose medium, but not in glucose medium (Figure S1D). Furthermore, knockdown of GLUT5 also significantly inhibited the ability of colony formation in both OVCAR8 (Figure 2E and G) and SKOV3 (Figure 2F and H) in the fructose medium. Meanwhile, knockdown GLUT5 could also significantly inhibit the ability of migration in the fructose medium (Figure 2I and J). These results suggested that GLUT5 was important for fructose utilization in ovarian cancer cells.

Knockout of GLUT5 could also significantly inhibit cell abilities of proliferation and colony formation in fructose medium

To further explore the role of GLUT5 in ovarian cancer, knockout of GLUT5 cells was construction by lentiCRISPR/CAS9 system in OVCAR8 (Figure 3A). The abilities of proliferation, colony formation, and migration of these cells were also detected, and we found that knockout GLUT5 also could significantly inhibit the cell proliferation (Figure 3B), colony formation (Figure 3C) and migration (Figure 3G) in the fructose medium. When GLUT5 was resumed in sgGLUT5 cells (Figure 3D), the cell abilities of proliferation (Figure 3E), colony formation (Figure 3F) and migration (Figure 3G) were also resumed. To affirm the role of GLUT5 in ovarian cancer in vivo, animal experiments were performed, and cells of sgctrl, sgGLUT5, and sgGLUT5-GLUT5 grew in the groin of mice for 30 days. The results showed that the tumor growth rate was much slower in sgGLUT5 tumors than sgctrl, and over-expression GLUT5 in sgGLUT5 cells could rescue the tumor growth (Figure 3H).

Correlations of GLUT5 expression and patients' clinicopathologic variables

We have known that GLUT5 was highly expressed in ovarian cancer cell lines, to further understand the role of GLUT5 in ovarian cancer, we analyzed the expression of GLUT5 mRNA level in ovarian cancer tissues using the data of TCGA in https://xenabrowser.net/datapages/. The results showed that cancer tissues had higher-expression level of GLUT5 mRNA than normal ovarian tissues (Figure 4A). Meanwhile, Kaplan–Meier analysis was also

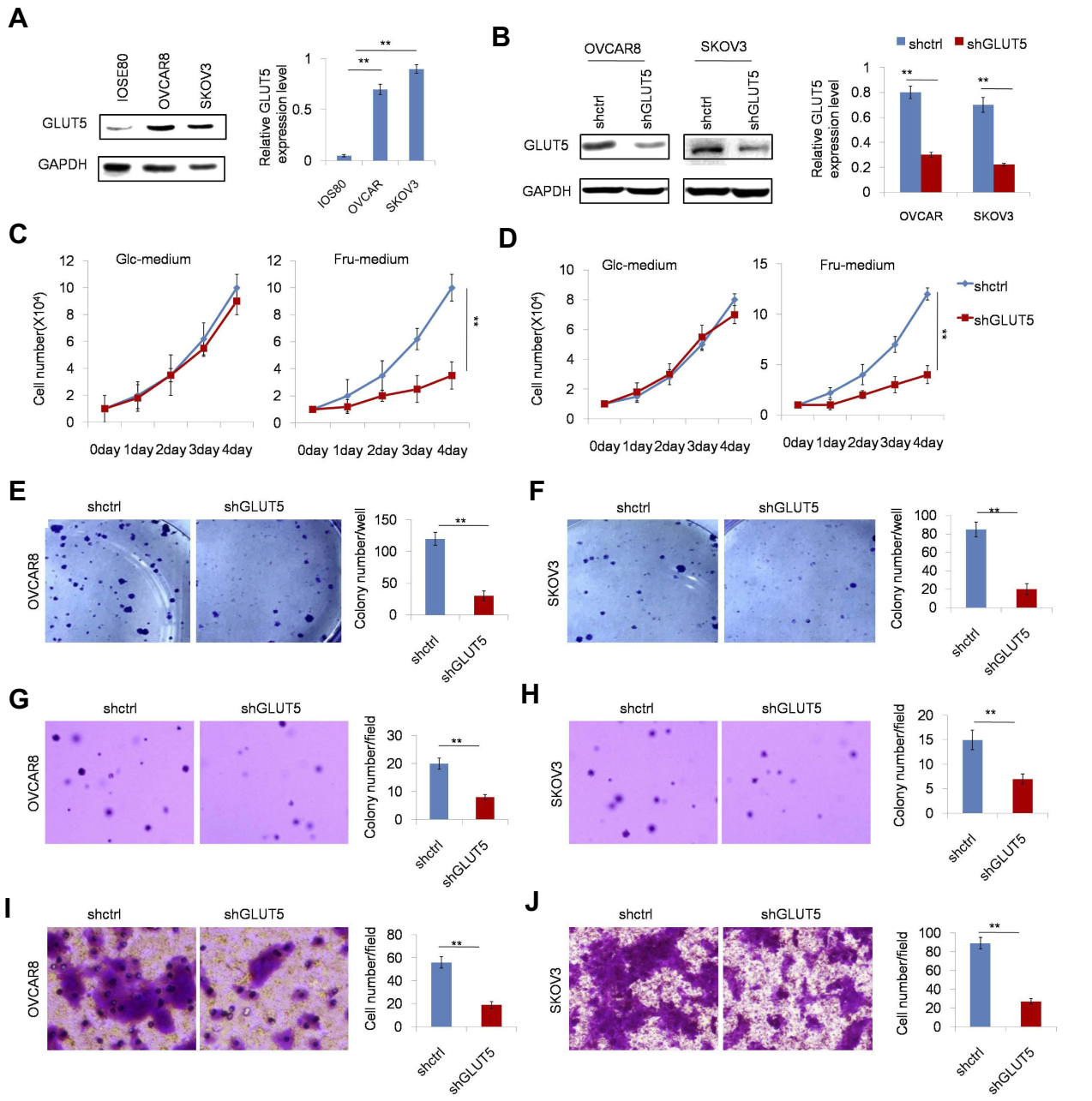

Figure 2 The affects of shGLUT5 in ovarian cancer cells. (**A**) The expression level of GLUT5 in ovarian cancer cells. (**B**) shRNA was used to knockdown GLUT5 in ovarian cancer cells. (**C**) The affect of shGLUT5 in OVCAR8 proliferation in 25 mM fructose medium and 25 mM glucose medium. (**D**) The affect of shGLUT5 in SKOV3 proliferation. (**E, F**) The affect of shGLUT5 in two-dimensional colony formation in 25 mM fructose medium. (**G, H**) The affect of shGLUT5 in 3D colony formation in 25 mM fructose medium detected by soft agar. (**I, J**) The affect of shGLUT5 in cell migration in 25 mM fructose medium. (**P<0.01).

performed using the TCGA database, and the level of GLUT5 mRNA was also associated with poor progression-free survival (Figure 4B).

Then, we detected the expression levels of GLUT5 protein in 137 cases of ovarian cancer and the adjacent normal ovarian tissues utilized the means of IHC, and we found that GLUT5 was always localized to the cell membranes and cytoplasm, and the expression level of GLUT5 protein was also higher in cancer cell than the adjacent normal tissues (Figure 4C). To value the role of GLUT5 in ovarian cancer, we analyzed the correlation of GLUT5 expression level and the patients' clinicopathologic variables, including age, clinical stage, histology grade, ascites production, and metastasis status. For statistical purposes, we divided all cases into two groups according to the GLUT5 expression level, then we found that there was no correlation between GLUT5 expression and ovarian cancer histology type (Figure 4D), but higher

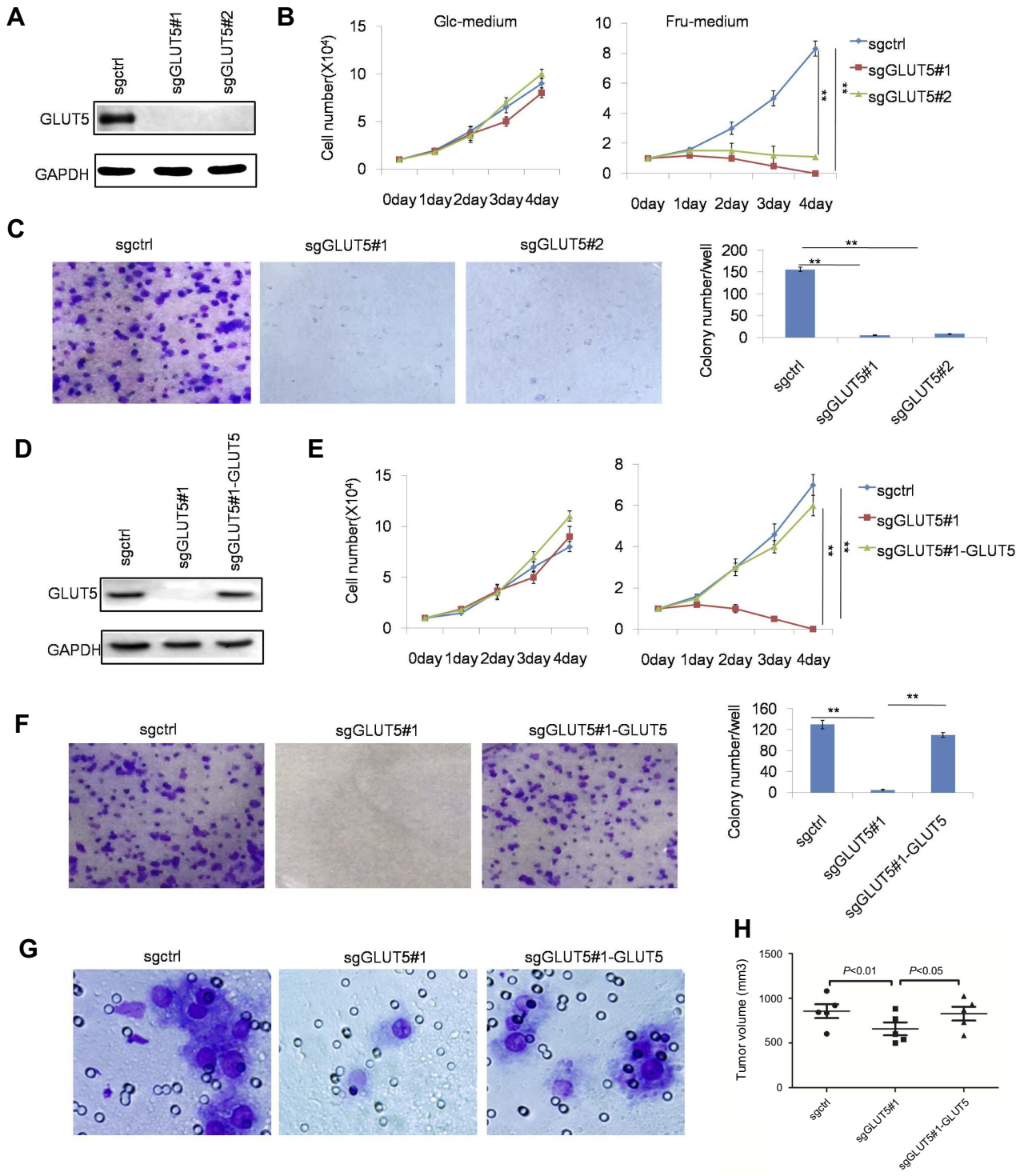

Figure 3 The affects of sgGLUT5 in ovarian cancer cell line OVACR. (**A**) sgRNA was used to knockout GLUT5 in ovarian cancer cells. (**B**) The affect of sgGLUT5 in cell proliferation. (**C**) The affect of sgGLUT5 in two-dimensional colony formation. (**D**) Resuming GLUT5 in sgGLUT5 cells. (**E**) The proliferation rates of cells sgctrl, sgGLUT5, and sgGLUT5-GLUT5. (**F**) The colony formation of cells sgctrl, sgGLUT5, and sgGLUT5-GLUT5. (**G**) The migration ability of cells sgctrl, sgGLUT5, and sgGLUT5-GLUT5. (**H**) The cells of sgctrl, sgGLUT5, and sgGLUT5-GLUT5 growth for 30 days in vivo (**P<0.01).

GLUT5 expression was significantly associated with higher pathology grade (χ^2=12.12, P<0.01) (Figure 4E), a higher rate of metastasis (χ^2=6.73, P=0.01) (Figure 4F), and higher clinical stage (χ^2=12.14, P<0.01) (Figure 4G), which suggested that GLUT5 high expression was associated with aggressive clinical features in ovarian cancer. In addition, Kaplan–Meier survival analysis was performed and the results showed that both the overall

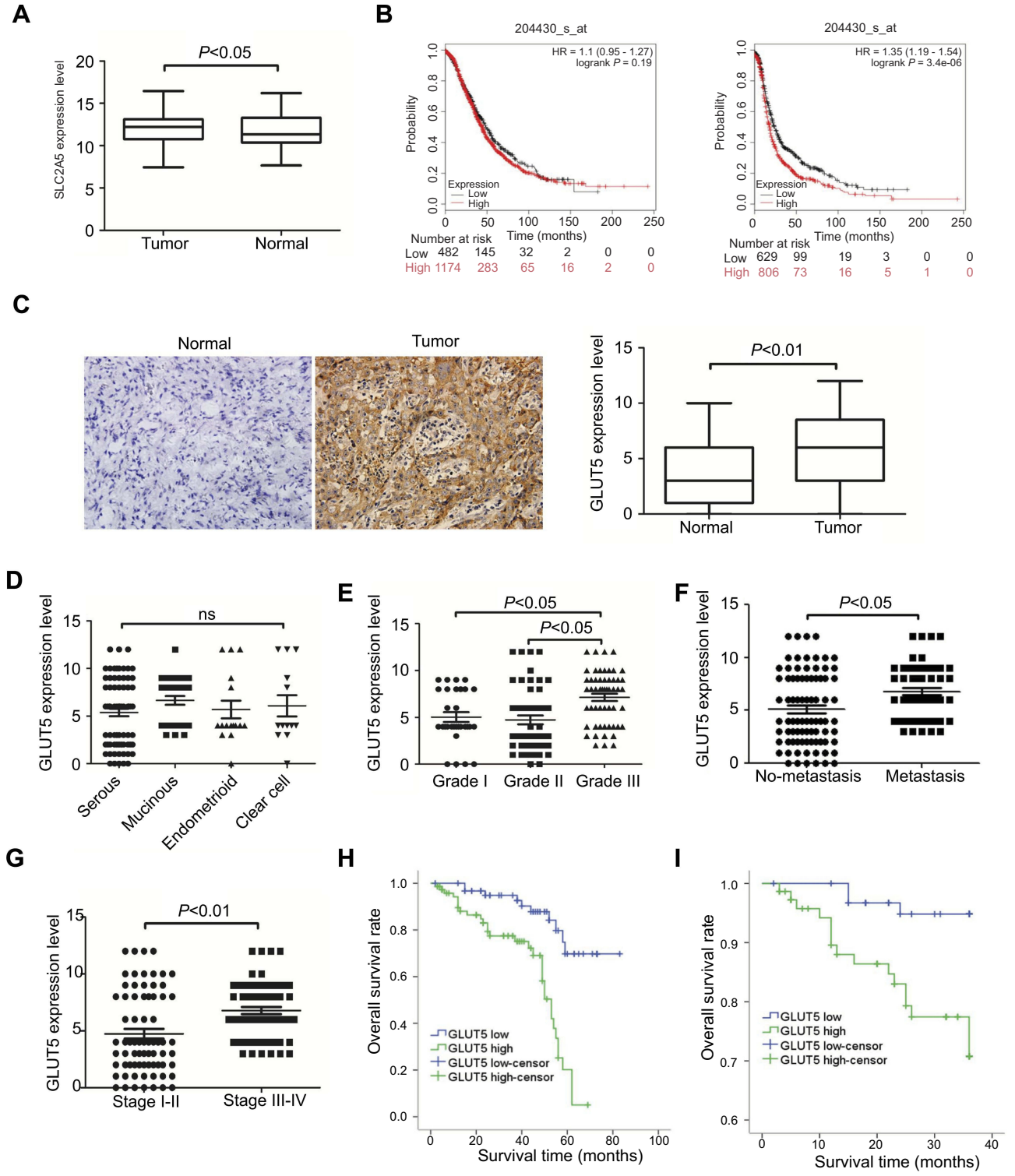

Figure 4 The expression of GLUT5 in ovarian cancer. (**A**) The expression level of GLUT5 mRNA in ovarian cancer tissues and normal ovary tissues in the TCGA database. (**B**) The effect of GLUT5 mRNA on the free progress survival and overall survival of glioma patients in the TCGA database. (**C**) The expression level of GLUT5 in ovarian cancer tissues and normal ovary tissues detected by IHC. (**D**) The expression level of GLUT5 in different types of ovarian cancer. (**E**) The expression level of GLUT5 in different grades of ovarian cancer. (**F**) The expression level of GLUT5 in metastasis ovarian cancer and no-metastasis. (**G**) The expression level of GLUT5 in different clinical stages of ovarian cancer. (**H**) The effect of GLUT5 on the overall survival of ovarian cancer patients. (**I**) The effect of GLUT5 on 3-year survival of ovarian cancer patients.

survival rate (Figure 4H) and 3-year survival (Figure 4I) were significantly lower in the GLUT5 higher-expression group. All these results suggested that GLUT5 could serve as a candidate biomarker for ovarian cancer.

Discussion

Infinite proliferation is one of the most prominent characteristics in cancer, and material, such as amino acids and carbohydrates, must be doubled to adjust their metabolism.[19] The rapid use of soluble sugars and the fast proliferation always caused a low-glucose environment within the tumor. Fructose is hexoses monosaccharides, and is isomers of glucose.[20] Unlike glucose, fructose metabolism is not regulated by rate-limiting enzyme, and fructose kinase is insulin-independent, suggesting that tumor cells are more likely to utilize fructose.[11]

The results of epidemiological investigation have shown that high intake of fructose was associated with bladder cancer and leukemia in women, but it was not closely related to male patients. In addition, fructose intake can promote the development of small intestinal and pleural tumors, and there is no gender difference.[21] Nielsen et al, examined the relationship between fructose consumption and breast cancer risk in 23,850 Danish postmenopausal women (50–65 years old), but they found no relationship between fructose consumption and breast cancer risk.[22] However, the experiment study showed that the fructose could promote breast cancer cells malignancy.[17] Meanwhile, a large number of animal and cell experiments also confirmed that fructose could replace glucose to promote the proliferation of other tumor cells, such as pancreatic cancer and colorectal cancer cell lines.[22,23] In addition, fructose can promote the invasion of breast cancer cell lines such as MDA-MB-468 by changing the glycosylation and cytoskeletal structure of certain proteins on the cell surface.[24]

Epidemiological investigations and experimental studies have also revealed significantly closed correlations between excessive fructose consumption and tumor progression. However, very few studies have focused on the fructose utilization in ovarian cancer. In this study, we detected the abilities of proliferation, colony formation, and migration of ovarian cancer cells in fructose medium. The results showed that the ovarian cancer cells were able to grow at equivalent rates in fructose medium even though no glucose was available; meanwhile, there was no significant difference in cell abilities of colony formation and migration between fructose medium and glucose medium, demonstrating that fructose could serve as an alternate carbohydrate substrate for ovarian cancer. But non-tumor ovary cells could not grow well in fructose medium, and such experiment result is not new. The similar results were reported in breast cancer and acute lymphoblastic leukemia, and normal monocytes and breast cells could not grow in fructose medium.[17,25]

GLUT5, as a specific fructose transporter in mammalian cells, has also been found highly expressed in breast cancer, glioma, lung cancer and so on, and silencing GLUT5 could significantly inhibit these cancer cells growth in fructose medium.[13,15–18,23] In this study, we used a similar method to silence GLUT5 in ovarian cancer cells to explore the role the GLUT5 in fructose metabolism in ovarian cancer. As expected, silencing GLUT5 could significantly inhibit the abilities of proliferation, colony formation, and migration in fructose medium, which suggested that GLUT5 was necessary for fructose utilization in ovarian cancer cells. Meanwhile, we found that GLUT5 was also higher expressed in ovarian cancer tissues, and GLUT5 expression correlated significantly with tumor malignancy and poor survival of ovarian cancer patients. In addition, it has been found that the glucose transporter GLUT1 is highly expressed in the primary lung cancer, but the fructose transporter GLUT5 is highly expressed in the metastasis, suggesting that fructose plays an important role in the metastasis of lung cancer.[15]

From all the results, we found that fructose could be used by ovarian cancer cells, and GLUT5 was necessary for this utilization. GLUT5 was expressed at a significantly higher level in ovarian cancer tissues, and its higher expression was correlated with malignant progression, and poor survival of patients. Overall, as reported in other tumors,[13,16,25] GLUT5 may also be used as a predictor of prognosis for ovarian cancer patients, and restrict the fructose intake or targeting GLUT5 may be efficacious strategies for ovarian cancer therapy.

Acknowledgment

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

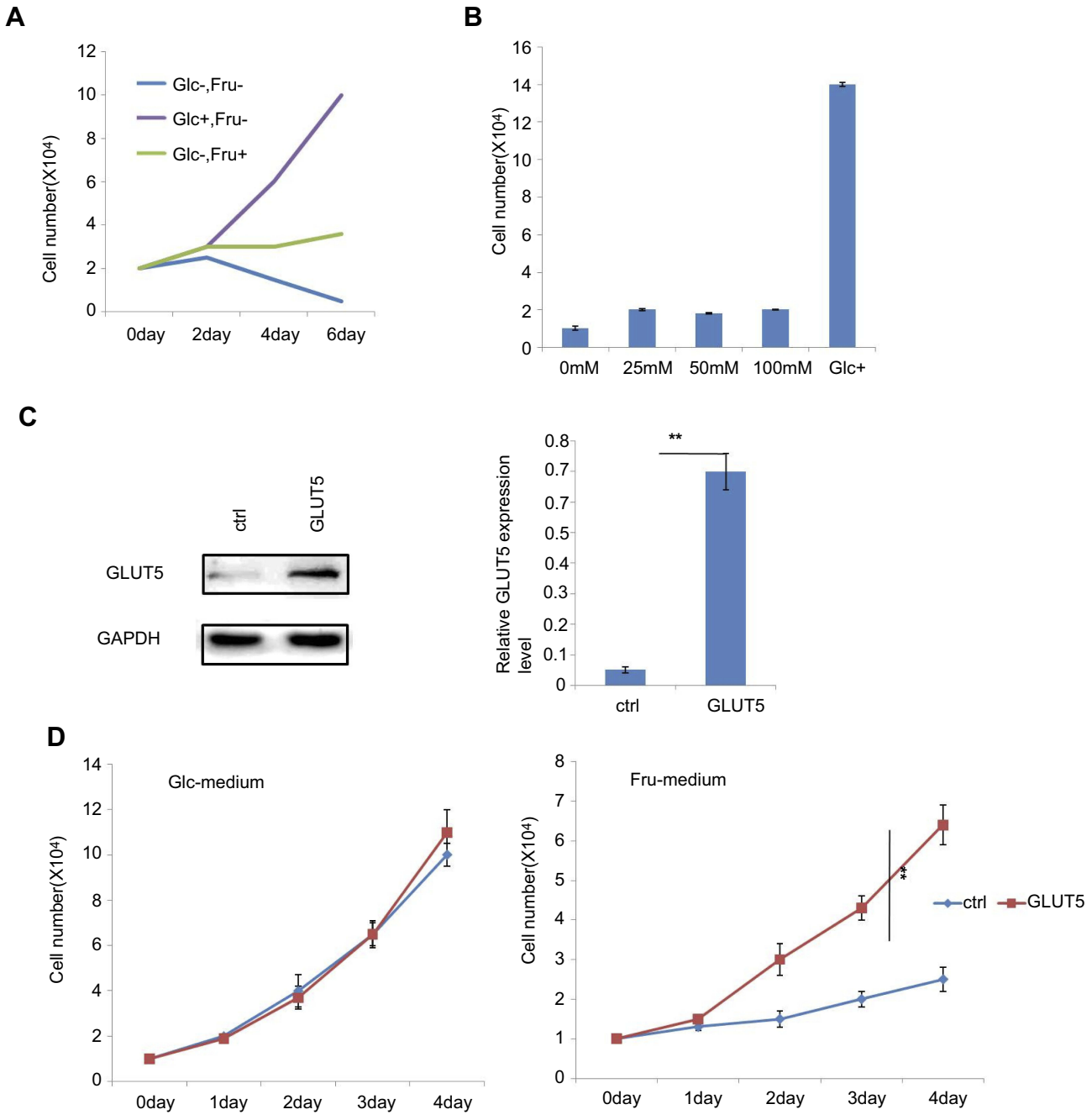

Figure S1 The effect of fructose on normal ovary cell line IOSE80. (**A**) The cell proliferation of IOSE80 in 25 mM fructose medium and 25 mM glucose medium. (**B**) The effect of different dose of fructose on IOSE80 proliferation. (**C**) Over-expression GLUT5 in IOSE80. (**D**) The proliferation of IOSE80 over-expression GLUT5 in fructose medium and glucose medium.
Note: $^{**}P<0.01$.