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# Transformation of human ovarian surface epithelial cells by Krüppel-like factor 8

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## Abstract

Previously we demonstrated that Krüppel-like factor 8 (KLF8) participates in oncogenic transformation of mouse fibroblasts and is highly overexpressed in human ovarian cancer. In this work, we first correlated KLF8 overexpression with the aggressiveness of ovarian patient tumors and then tested if KLF8 could transform human ovarian epithelial cells. Using the immortalized non-tumorigenic human ovarian surface epithelial cell line T80 and retroviral infection, we generated cell lines that constitutively overexpress KLF8 alone or its combination with the known ovarian oncogenes c-Myc, Stat3c and/or Akt and examined the cell lines for anchorage-independent growth and tumorigenesis. The soft agar clonogenic assay showed that T80/KLF8 cells formed significantly more colonies than the mock cells. Interestingly, the cells expressing both KLF8 and c-Myc formed the largest amounts of colonies greater than the sum of colonies formed by the cells expressing KLF8 and c-Myc alone. These results suggested that KLF8 might be a weak oncogene that works cooperatively with c-Myc to transform ovarian cells. Surprisingly, overexpression of KLF8 alone was sufficient to induce tumorigenesis in nude mice resulting in short life span whether the T80/KLF8 cells were injected subcutaneously, intraperitoneally or orthotopically into the ovarian bursa. Histopathological studies confirmed that the T80/KLF8 tumors were characteristic of human serous ovarian carcinomas. Comparative expression profiling and functional studies identified the cell cycle regulators cyclin D1 and USP44 as primary KLF8 targets and effectors for the T80 transformation. Overall, we identified KLF8 overexpression as an important factor in human ovarian carcinoma pathogenesis.

### Keywords

KLF8; transformation; human ovarian epithelial cells; human ovarian cancer



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## Introduction

Ovarian cancer remains the leading cause of death among women cancers due to the lack of early detection methods and effective therapies for late-stage cancers (1). Although various oncogenes including H-Ras (2-4), K-Ras (3, 5), c-Myc (5), Akt (5-12), HER2 (13) and STAT3c (14) and tumor suppressor genes such as p53 (15), pRb (15), pTEN (16, 17), BRAC1 and BRCA2 (18-23) have been reported to contribute to ovarian cancer progression, the causes of ovarian cancer remain largely unknown. Further understanding the mechanisms behind ovarian cancer progression is urgent for developing new diagnosis and treatment strategies to improve patient survival. The vast majority of ovarian cancer derives from the ovarian surface epithelium (OSE). Several experimental models have recently been developed to study the transformation of OSE including genetically modified mouse models (15, 24), *ex vivo* oncogene introduction mouse models (5) and manipulation of cultured human OSE cells (2). The immortalized human OSE cell lines are particularly useful for assessing molecular and signaling mechanisms directly relevant to human patients (2, 4, 13).

Krüppel-like factor 8 (KLF8) is a widely expressed transcription factors and functions as both a transcription repressor (25) and activator (26-28) of a growing list of target genes including γ-globin (25, 29), KLF4 (27), and E-cadherin (30, 31) cyclin D1 (27, 28, 30, 32, 33) and MMP9 (26). The expression and nuclear function of KLF8 are also tightly regulated by important signaling cascades including focal adhesion kinase (FAK) through Src and PI3K signaling pathways (28, 34, 35), the transcription factors Sp1(34), KLF1 (36) and KLF3 (36) and by various types of post-translational modification mechanisms such as SUMOylation (27, 32), acetylation (32, 33), PARylation (37), ubiquitylation (37), phosphorylation (38) and nuclear localization (38). Importantly, recent studies have correlated aberrant overexpression of KLF8 with the malignancy of several types of human cancer including breast (26, 30, 34), ovarian (34, 39), hepatocellular carcinoma (HCC) (31), renal (39, 40), gastric (41) and glioma (42-44). KLF8 has also been shown to play a role in the transformation of the mouse fibroblast NIH 3T3 cells (39). All these lines of evidence have pointed out a potentially causal role of KLF8 for human cancer progression which has not been investigated to date.

In this study, we demonstrate that ectopic overexpression of KLF8 in immortalized non-tumorigenic human OSE cells was sufficient to induce anchorage-independent growth in culture as well as tumorigenesis in mice, the hallmarks of malignant transformation. We also show a strong correlation of aberrant high levels of KLF8 with the aggressiveness of ovarian patient tumors. Our results support a potentially important role for KLF8 in human ovarian cancer development and provide a novel model for ovarian cancer studies.

## Results

### KLF8 protein is highly expressed in human malignant and metastatic ovarian tumors

Our previous reports have demonstrated that KLF8 is aberrantly overexpressed in human ovarian cancer cell lines at both message and protein levels and this aberrant overexpression was confirmed in tumor samples of ovarian patient at message levels (39). To determine the protein expression of KLF8 in ovarian patient tumors, we performed human ovarian cancer

progression tissue array analysis by IHC staining (Figure 1A). We found that KLF8 protein is highly overexpressed in malignant and metastatic ovarian tumors. In borderline and benign tumors, the high levels of KLF8 protein were mostly limited to ovarian surface epithelium. There was rare expression of KLF8 protein in normal tissues adjacent to tumors or in normal ovarian specimens. A gradually increased correlation between KLF8 expression and the multi-step progression of the ovarian tumors was obvious (Figure 1B). These results suggest that aberrant elevation of KLF8 expression may play a critical role in transforming OSE cells into cancer cells, promoting the tumor progression and maintaining the tumor aggressiveness.

### KLF8 plays an important role in transforming human OSE cells

We have demonstrated that ectopic expression of KLF8 can partially transform mouse fibroblast cells in culture and enhance the oncogenic effect of v-Src in NIH 3T3 cells (39). To test if KLF8 plays a role in transforming human OSE cells, alone or in cooperation with other ovarian oncogenic proteins, we infected the immortalized non-tumorigenic human OSE T80 cells (2) with retroviruses to generate T80 cell lines constitutively expressing the empty vector (Mock), KLF8, c-Myc, Akt and Stat3c alone or in combination (Figure 2A). The cell lines were then tested for their capability of anchorage-independent growth in soft agar, a hallmark of malignant transformation of cells (Figure 2B). We found that the Mock cells, like the parental T80 cells (2), formed few or no colonies; in sharp contrast, the T80/KLF8 cells formed approximately 2.5 times more colonies than the mock cells did (Figure 2C, compare columns 2 to 1) though a little less than T80/c-Myc, T80/Akt, or T80/STAT3c cells did (Figure 2C, compare columns 3-4 to 1). Interestingly, the cells expressing both KLF8 and c-Myc formed the largest amounts of colonies (column 6), comparable to that formed by the cells expressing the four proteins altogether (column 12), that were even greater than the sum of colonies formed by the cells expressing KLF8 alone and by the cells expressing c-Myc alone. Consistent with the colony formation result, the cell lines showed accelerated BrdU incorporation rate (Figure 2D) and proliferation rate (Figure 2E). Taken together, these results suggest that KLF8 alone may be capable of transforming T80 cells, and more potent when to cooperate with c-Myc, but not Akt or STAT3c, and that aberrant increase in cell cycle progression and proliferation may be a mechanism underlying KLF8-induced T80 transformation (28, 34, 39).

### KLF8 alone is sufficient to induce ovarian tumorigenesis *in vivo*

To further study the oncogenic transforming role of KLF8 in T80 cells, we first injected subcutaneously (s.c.) the T80/KLF8 cells into athymic nude mice and examined the tumorigenesis by the cells. Surprisingly, overexpression of KLF8 alone, like c-Myc or Akt alone, was sufficient to induce tumorigenesis in more than 50% of the mice (Figure 3A, Table 1) although the tumors developed relatively slowly (approximately three to five months after injection) compared to the SKOV3-ip1 positive control tumors (one to two months after injection) (Figure 3B). STAT3c alone induced significantly smaller tumors and the Mock cells formed no tumors as expected (Figure 3A & 3B). Similarly, the T80/KLF8 cells also induced tumorigenesis after intraperitoneal (i.p.) injection (Table 1). Consistent with the tumor formation rate, the average survival time for the mice was about six months for T80/KLF8 compared to approximately two months for SKOV3ip1 (Figure 3C). IHC

staining verified the ectopic over-expression of KLF8 in the tumors (Figure 3D, c & d) and high similarity of the tumors to those in patients as determined by expression of the human ovarian cancer tumor marker proteins cytokeratin, CA125, HE4 and mesothelin (Figure 3D, e-h).

To directly test whether the T80/KLF8 cells can form tumors right in the ovary, we injected the cells into the ovarian bursa of the mice and examined the tumor formation. Interestingly, the cells could form orthotopic tumors in two of six mice after 90 days post injections. SKOV3-ip3 formed tumors in five of six mice in 60 days post-injection while the Mock cells could not form any tumors within 90 days post-injection (Figure 4 A). Despite the smaller tumor sizes, lower incidence and longer latency than the SKOV3ip1 positive control tumors (Figure 4B, Table 1), histological analysis could not distinguish the T80 tumors from tumors of clinical patients (Figure 4C).

Taken together, these results further suggest that KLF8 can likely act alone as an ovarian oncogene.

### KLF8 promotes ovarian cell proliferation by regulating the expression of cell cycle associated genes including cyclin D1 and USP44

To understand the molecular mechanisms by which KLF8 transforms T80 cells, we first compared the gene expression profile in the T80/KLF8 cells to that in the mock cells using cDNA microarray (see Supplemental Table 1). We found that cyclin D1 was among the highly up-regulated genes by KLF8, which is consistent with the results obtained in NIH 3T3 cells (28, 39). Interestingly, ubiquitin-specific protease 44 (USP44), a recently identified de-ubiquitinating enzyme targeting Cdc20 to counteract the ubiquitin E3 ligase APC at the spindle checkpoint of mitosis (45), was among the highly down-regulated targets by KLF8 (see Supplemental Table 2). The regulation of cyclin D1 and USP44 by KLF8 was verified by RT-PCR and qRT-PCR at the mRNA levels and by western blotting at the protein levels in both T80 and SKOV3ip1 cells (Figure 5A-5D). These results suggested that regulation of cyclin D1 and USP44 may be responsible for KLF8 promoted cell cycle progression and subsequent proliferation. Indeed, knockdown of cyclin D1 or re-expression of USP44 reversed the proliferation of T80/KLF8 cells back to the levels of the Mock cells (Figure 5G). Knockdown of cyclin D1, but not re-expression of USP44, reversed the DNA synthesis back to rate of the Mock cells (Figure 5H), consistent with the positive role of cyclin D1 in G1 phase and the negative role of USP44 in M phase of the cell cycle. These results indicate that KLF8 regulation of cyclin D1 and USP44 at both G1 and M phases of the cell cycle plays a critical role for the malignant transformation of immortalized human ovarian surface epithelial cells.

## Discussion

This novel study has demonstrated a close correlation of KLF8 expression with ovarian tumor progression, revealed a potentially significant role of KLF8 for malignant transformation of human OSE, and sheds a new light on the mechanisms of ovarian cancer development.

KLF8 has been shown to transform both mouse fibroblast cells *in vitro* (39) and human cells (this study) both *in vitro* and *in vivo* regardless of implantation sites. We have also observed that extopic expression of KLF8 promotes tumorigenesis of human mammary epithelial cells as well (submitted elsewhere) and that aberrant overexpression of KLF8 is well correlated with the aggressiveness of breast patient tumors (26, 30). These observations highly support an oncogenic role of KLF8 in human ovarian and breast cancer. Given its barely detectable expression in normal tissues, aberrant increase in KLF8 expression may play a causal role for the cancer progression. It will be interesting to test if KLF8 plays a similar oncogenic role in tissue origins of other cancer types that also show abnormally high levels of KLF8 (31, 40, 41, 43).

KLF8 is considered to be widely expressed (25) although its expression in normal cells or tissues is frequently undetectable (30, 34, 39, 46) (also see Figure 1). The mechanisms behind the differential expression f KLF8 between normal and tumor cells or tissues remain largely uninvestigated. Recent studies have demonstrated that the expression of KLF8 can be upregulated by overexpression and/or overactivation of FAK in the human ovarian cancer cell SKOV3ip1 (34) or induced by TGF-β treatment in immortalized non-tumorigenic human breast epithelial cell MCF-10A (30). However, it is premature to consider these mechanisms as primary ones responsible for the aberrant overexpression of KLF8 in human cancer. Other mechanisms could also potentially contribute to the differential expression of KLF8 between normal and cancerous cells such as methylation or acetylation of KLF8 gene promoter, stabilization or destabilization of KLF8 protein and microRNA regulation of KLF8 message and protein translation.

Several other KLF family proteins have been shown to play either a tumor promoting (KLF4, KLF6, KLF9, KLF10, KLF11, and KLF17) or suppressing (KLF5, KLF12) role by intervening in cell signaling associated with proliferation and/or survival (47-49). Regardless of their apposing role in cancer, all the KLFs exert their cancer regulating function via altering the expression of some of their transcriptional target genes. The fact that both cyclin D1 and USP44 are involved in mediating the oncogenic function of KLF8 (see Figure 5) indicates that in the ovarian cells KLF8 promotes cell proliferation by accelerating the cell cycle progression at both G1 and M phase. This novel finding is consistent with a recent report that USP44 is highly expressed in normal ovaries (57). Interestingly, USP44 has been shown to be significantly down-regulated in HCC serum (58), suggesting that USP44 may serve as not only a negative effector of KLF8 in HCC progression but also a biomarker for diagnosis of human cancers associated with elevated expression of KLF8. Since USP44 plays a key role in spindle-assembly checkpoint (45), whether or not KLF8 regulates this checkpoint and chromosomal stability is an interesting future work.

It is widely accepted that transformation process often involves alteration of more than one oncogene as well as tumor suppressor genes. The immortalized (by SV40 T/t antigens and hTERT) feature of the T80 cells suggests that KLF8 could work on top of the loss of p53 and pRb function or gain of the telomerase function to transform the cells. This could explain why KLF8 induces clonogenesis by T80 but not by NIH 3T3 cells (39). Alternatively, this discrepancy could be due to other differences between the T80/KLF8

(human, pooled lines with and constitutive expression) and 3T3/KLF8 cell lines (mouse, clonal lines with inducible expression). Indeed, we found that KLF8 promoted tumorigenesis of an immortalized non-tumorigenic human breast cell line that expresses normal p53 and pRb (submitted elsewhere). The lack of apparent cooperation between KLF8 and Akt or STAT3c (see Figure 2) could be explained by the fact that KLF8 is downstream of both of them (34, 50). It is obvious that KLF8 cooperates with c-Myc in transforming T80 cells *in vitro*. Whether or not KLF8 and c-Myc regulate mutual expression in our experimental conditions is not known. However, a recent report has demonstrated that KLF8 plays a role downstream of Wnt to upregulate c-Myc expression through β-catenin and subsequent expression of cyclin D1 in human liver cancer cells (46, 51). This could be the case in ovarian and/or breast cancer where KLF8 has been demonstrated to regulate cyclin D1 and/or β-catenin (30, 34, 46). It will be interesting to test whether and how co-overexpression of both similarly enhances transformation *in vivo* and increases the tumor aggressiveness such as ascites and metastasis. It is completely uninvestigated whether or not KLF8 regulates tumor suppressor genes or vice versa in ovarian or other types of cancer which could further complicate the role and mechanisms of KLF8 in cancer. Nevertheless, these outstanding questions can be better answered using genetically engineered mouse models for KLF8 and the other oncogenes or tumor suppressor genes in combination with primary human epithelial cells. Experiments in these regards are in progress.

In summary, we have identified KLF8 as a potential ovarian oncogene. Given its barely detectable expression in normal ovarian cells and tissue, KLF8 may represent a novel intervention target against ovarian cancer. The cell and mouse models generated in this work provide new tools for the studies of mechanisms underlying human ovarian cancer progression.

## Materials and Methods

### Cell culture, cell lines and reagents

The immortalized human ovarian epithelial cell line T80 (2) and human ovarian cancer cell line SKOV3ip1 were maintained as previously described (34). pBabe-puro-HA-KLF8 was described previously (30), pLPC-c-Myc was constructed by transferring human c-Myc cDNA from pBS vector (52) to pLPC retroviral vector between Bam HI and Eco RI sites. pBabe-neo-Flag-Stat3c was made by transferring the Flag-STAT3c cDNA from Stat3-c Flag pRc/CMV (Addgene, Plasmid # 8722) by PCR using primers 5′-ATG AAT TCT TCT GCA GAT ATC CAT CAC AC (forward) and 5-ATG TCG ACA GCG AGC TCT AGC ATT TAG G (reverse) and ligation into pBABE-neo (Addgene, plasmid # 1767) between Eco RI and Sal I site), and pLNCX-myr-HA-Akt was from Addgene (plasmid # 9005). After verified by sequencing these retroviral vectors were used to produce viruses and infect T80 cells alone or in combination as previously described (30). Each of the cell lines was established as a pool of positively infected cells selected with either puromycin (KLF8 and Myc) or G418 (Stat3c and Akt). Insertless vector was used to generate mock control cell line (Mock). Constitutive expression of the proteins were confirmed by western blotting using an antibody against HA (F-7), Myc (9E10) or Flag (sc-807) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). USP44 tet-on inducible expression vector was from Addgene (plasmid

#22604 (53)). Anti-cyclin D1 antibody (sc-718) and anti-USP44 antibody (sc-79329) were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Anti-Akt was purchased from Cell Signaling Technology, Inc (Danvers, MA).

### Soft agar colony formation assay

Assays were performed in 12-well plates with 1,000 or 5,000 cells/well, re-suspended as a single cell suspension in 0.4% agar, and layered on top of 0.8% agar. Plates were incubated for 14 days. Colonies were counted manually under 10X objective lenses. The counting was performed for 10 fields in each well, and at least 3 wells per condition were counted in each experiment.

### Cell proliferation assay

Premixed WST-1 cell proliferation Reagent was used and assays were performed based on manufacturer's instruction (Clontech Laboratories, Inc., Mountain View, CA). Briefly, cells were seeded at $2 \times 10^4$ cells/well in 24-well-plate. After 48-72 hours, optical density (OD) values at 450 nm were measured 2 hours after incubation with WST-1, using a multiple bio-reader (Perkin Elmer).

### BrdU incorporation assay, RNA interference, quantitative real-time PCR (qRT-PCR) and western blotting

These assays were performed as previously described (28, 30). OnTarget Plus siRNAs and scramble control siRNAs specific to human cyclin D1 and KLF8 were purchased from Dharmacon. The siRNA was transfected into T80/KLF8 cells or SKOV3ip1 cells using Oligofectamine according to Invitrogen's instructions. Primers for qRT-PCR: cyclin D1, 5′-ccg tcc atg cgg aag atc (forward) and 5′-gaa gac ctc ctc ctc gca ct (reverse); USP44, 5′-ctc aca gaa gcc cag aaa ca (forward) and 5′-aaa gcc aac atg aac acc aa (reverse); GAPDH, 5′-tcg tac gtg gaa gga ctc a (forward) and 5′-cca gta gag gca ggg atg at (reverse).

### Ovarian epithelial tumorigenesis in mice

Four to 5-week-old nude athymic female mice (Taconic, Germantown, New York) were injected subcutaneously (s.c.) or intraperitoneally (i.p.) with $5 \times 10^6$ T80 cell lines stably expressing ectopic KLF8, c-Myc, Akt or Stat3c, alone or in combination. Intrabursal (i.b.) orthotopic injection of the cells ($5 \times 10^5$ in 10 μl) was performed as previously described (15). T80 and SKOV3ip1 cells were used as negative and positive controls, respectively. Subcutaneous tumor development was monitored by palpation twice a week, and tumor sizes were recorded. The tumor volume was calculated by the formula of $V = 0.5 \times L \times W^2$. Resected tumors were measured in three dimensions with a caliper, and their volume calculated using the formula of $V = \pi/6\ (L \times W \times H)$. The mice were housed and maintained in specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the National Institute of Health. Animal care and use was approved by the Institutional Animal Care and Use Committee. Human care of the mice was thoroughly considered.

### Pathological analysis

Resected ovaries and tumors were characterized by microscopic evaluation of paraffin sections with H & E and immunohistochemical (IHC) staining. IHC staining was performed following the manual instructions of Dako North America, Inc (Carpinteria, CA). Briefly, paraffin sections were baked for 1 hour at 62 deg;C for rehydration and microwaved in 0.01M sodium citrate for 5 min for antigen retrieval. After incubated in 3% $H_2O_2$ for 6 min, the sections were serum blocked for 30 min, incubated overnight at 4 deg;C with primary antibodies in phosphate-buffered saline (PBS) and subsequently with biotin-labeled secondary antibodies for 30 min, followed by a peroxidase-labeled avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA) for 30 min. The sections were developed in 3,3-diaminobenzidine tetrahydrochloride for 2 min and counterstained with hematoxylin for 4 min. The stained sections were dehydrated, treated with xylene and mounted for microscopy. Positive staining was displayed in brown or black color. The antibodies used in IHC include anti-KLF8 (30), anti-human CA125 (Clone M11, Dako), anti-human cytokeratin (Clones AE1/AE3, Dako), anti-cytokeratin 8 &18 (Clone Zym5.2, Invitrogen), anti-HE4 (Covance) and anti-mesothelin (Novocastra).

### Tissue Array Analysis

Human ovarian cancer progression tissue array (OV1005) was purchased from US Biomx, Inc (Rockville, MD). IHC staining was performed as previously described (26).

### Statistical Analysis

All the data is summarized and presented as mean ± standard deviation (SD) for continuous variables and frequency counts for categorical variables with a minimum of three observations per group. The ANOVA test or Fisher's exact test was used to examine the statistical differences among treatment groups, as appropriate. The Bonferroni correction was used for multiple comparisons. Animal survival data was analyzed by Kaplan-Meier curve. Significance was determined at the alpha level of 0.05. All analyses were conducted using SAS (V9.2).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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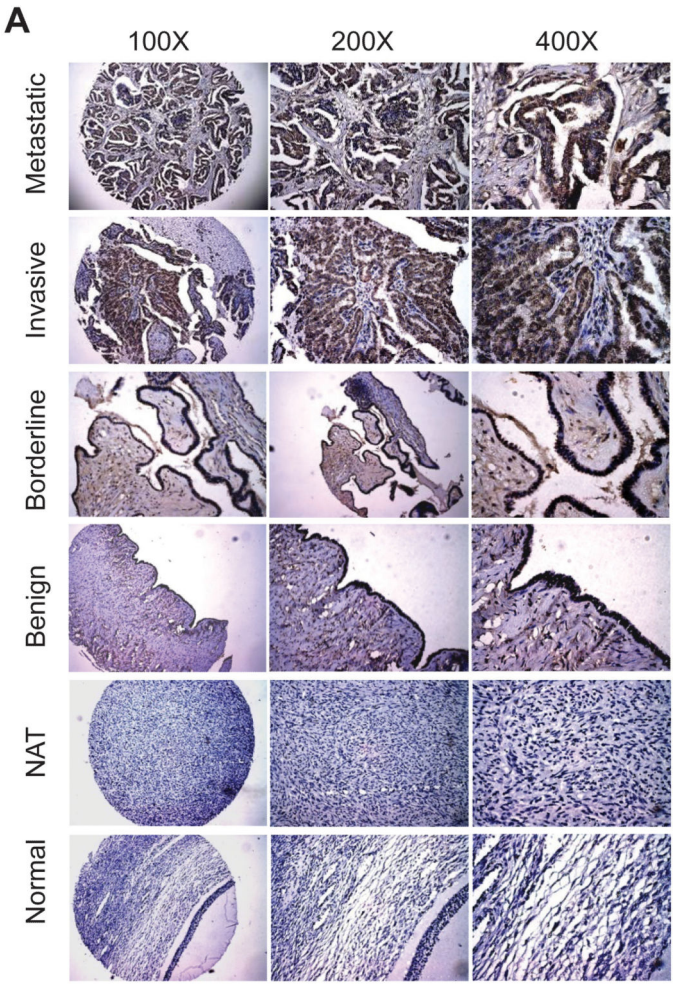


B

| | KLF8+ | KLF8- | Total | p-Value* |
|---|---|---|---|---|
| Metastatic | 8 | 2 | 10 | 0.001 |
| Invasive | 34 | 11 | 45 | 0.00008 |
| Borderline | 6 | 1 | 7 | 0.0013 |
| Benign | 6 | 12 | 18 | 0.0806 |
| NAT | 1 | 11 | 12 | 0.6 |
| Normal | 0 | 8 | 8 | |

**Figure 1.**

The aberrant overexpression of KLF8 protein is highly correlated with the aggressiveness of human ovarian tumors. *A*, Representative IHC staining images of patient specimens. Human ovarian tissue array (OV1005, US Biomax) was stained with anti-KLF8 antibody (brown). The nuclei were counterstained with hematoxylin (blue). *B*. Summary of the staining result from A. Tumor scoring was described in Supplemental Table 1. *p-value was analyzed by Fisher's exact test, comparing KLF8 expression to Normal. Normal, normal ovarian tissue; NAT, cancer adjacent normal ovarian tissue; Benign, mucinous or serous cystadenoma;

Borderline, borderline mucinous of serous papillary cystadenoma; Invasive, Invasive ovarian carcinoma, adenocarcinoma or cystadenocarcinoma; Metastatic, metastasized ovarian cystadenocarcinoma or adenocarcinoma.

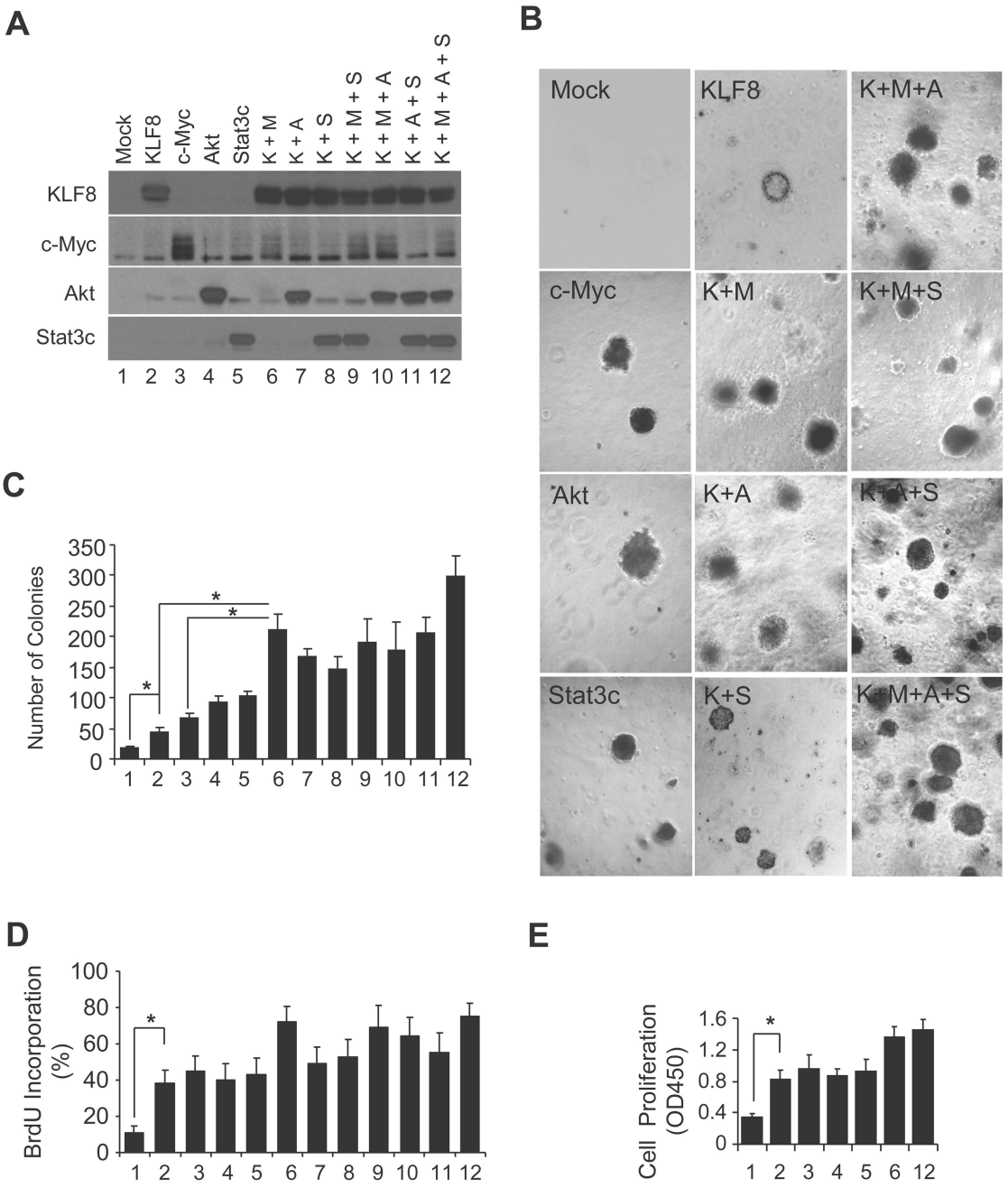


**Figure 2.**

Overexpression of KLF8 transforms human ovarian epithelial cells. *A*, Confirmation of T80 cell lines (shown on the top) constitutively overexpressing KLF8 (K), c-Myc (M), Akt (A), Stat3c (S) or their combinations. The cell lines were generated as described in Materials and Methods. Whole cell lysates were used for western blotting for the indicated proteins shown to the left. *B & C*, KLF8 promotes anchorage-independent growth. The cell lines described in A were grown in soft agar culture and colonies were photographed and counted. *D*, KLF8 accelerates G1 to S phase progression of the cell cycle. After 48 h serum starvation, the cells

were stimulated with serum containing BrdU for 12 h and subject for BrdU incorporation analysis. *E*, KLF8 increases cell proliferation. The cells were grown and subject to WST-1 assay as described in Material and Methods. The numbers for the lanes and columns in all the panels label the same cell lines as indicated on the top of panel A. *P < 0.05 compared to Mock by X2-test.

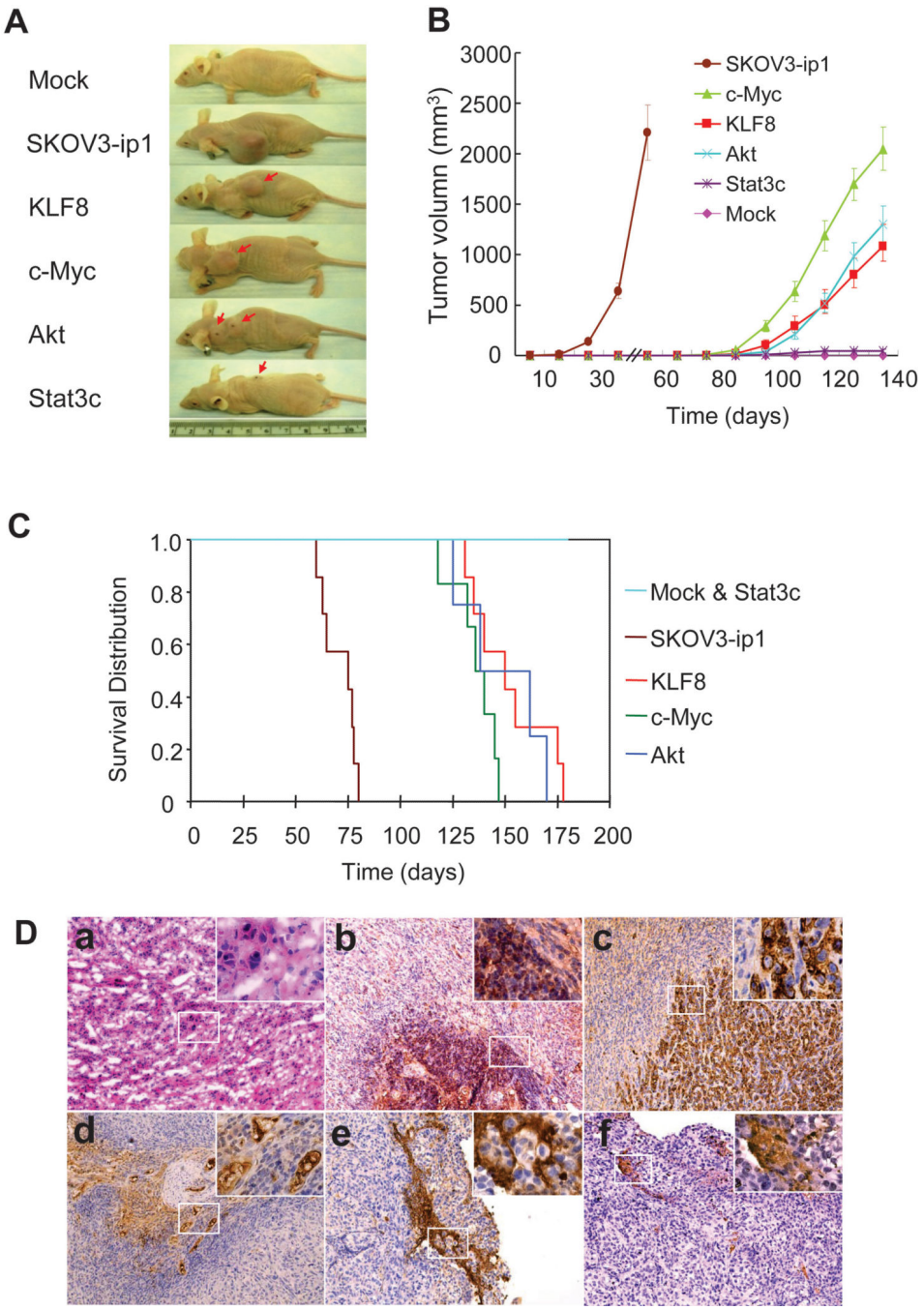


**Figure 3.**

KLF8 alone is sufficient to induce T80 cells to form subcutaneous tumors resulting shortened life. *A*, Representative subcutaneous tumors formed by the indicated cell lines. Mock and SKOV3-ip1 were used as negative and positive controls, respectively. Tumorigenesis experiments were carried out as described in Materials and Methods. Photos were taken on 30 days (for SKOV3-ip1) or 105 days after injection. *B*, Tumor formation rates Tumor volumes were recorded at the indicated time points (see tumor incidence in Supplemental Table 1). *C*, T80/KLF8 tumor formation shortened mouse survival time.

Survival distribution was presented by Kaplan-Meier curve. *D*, The T80/KLF8 tumors are highly similar to tumors of ovarian cancer patients. H & E and IHC staining of the tumors were performed as described in Materials and Methods for expression of the KLF8 and human ovarian cancer marker proteins. a, H & E (100X); b, KLF8 (100X); c, pan-cytokeratins (100X); d, CA 125 (100X); e, mesothelin (100X); f, HE4 (100X). Inlets, 400X

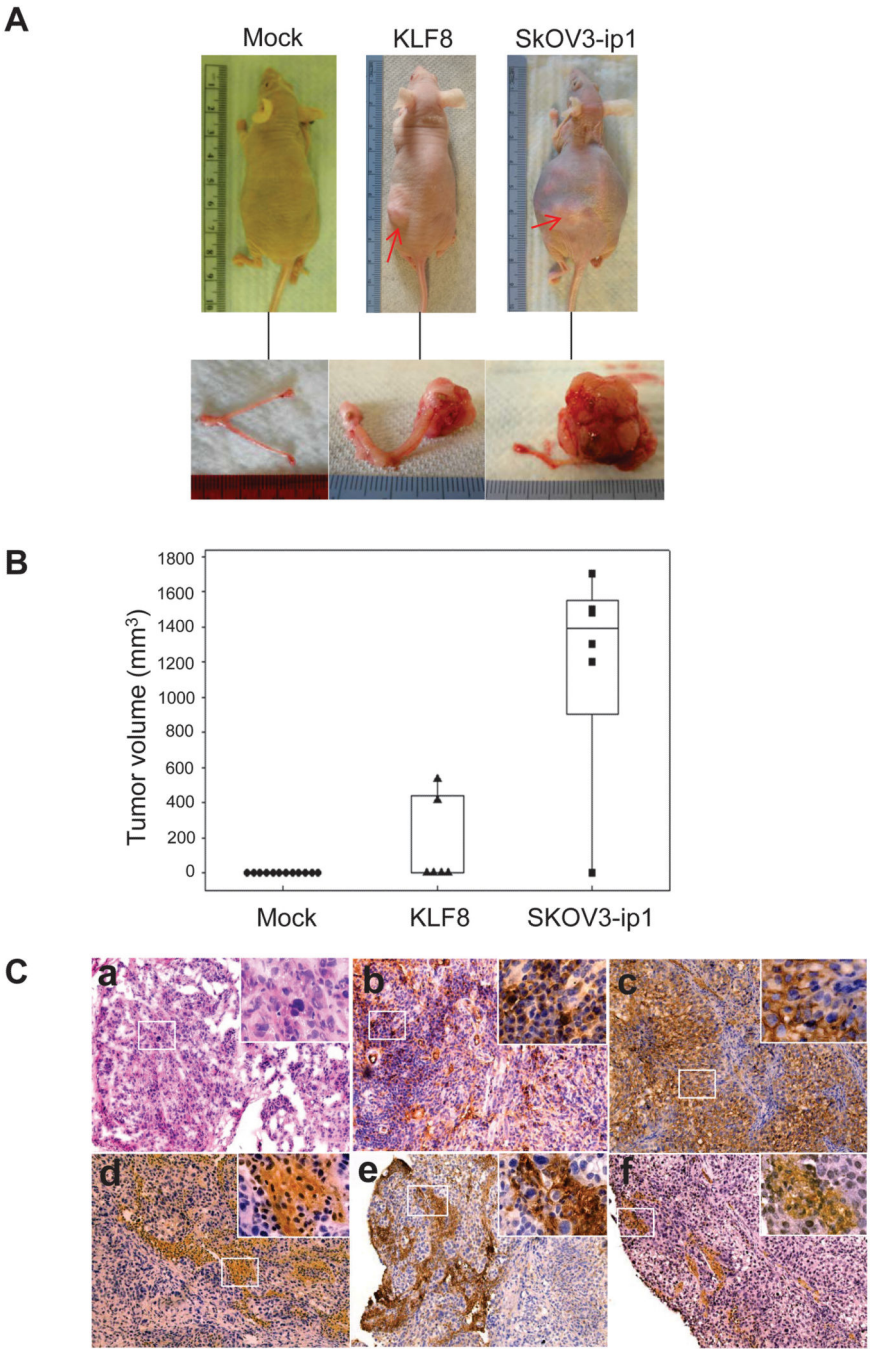


**Figure 4.**

KLF8 alone is sufficient to induce to induce T80 cells to form orthotopic ovarian tumors. *A*, Representative ovarian tumors formed by KLF8 expressing T80 cells. Mock and SKOV3-ip1 were used as negative and positive controls, respectively. 5 x $10^5$ cells per cell lines were injected into the ovarian bursa. Photos of representative mice (top) and tumors (bottom) were taken 60 days or (SKOV3-ip1) 90 days (mock and KLF8) after injection. *B*, Tumor formation rate. The ovarian tumor volume recorded at the time of euthanasia is presented by box-plot (see tumor incidence in Supplemental Table 1). *C*. The T80/KLF8

ovarian tumors are highly similar to tumors of ovarian cancer patients. . H & E and IHC staining of the tumors were performed as described in Materials and Methods for expression of the KLF8 and human ovarian cancer marker proteins. a, H & E (100X); b, KLF8 (100X); c, pan-cytokeratins (100X); d, CA 125 (100X); e, mesothelin (100X); f, HE4 (100X). Inlets, 400X.

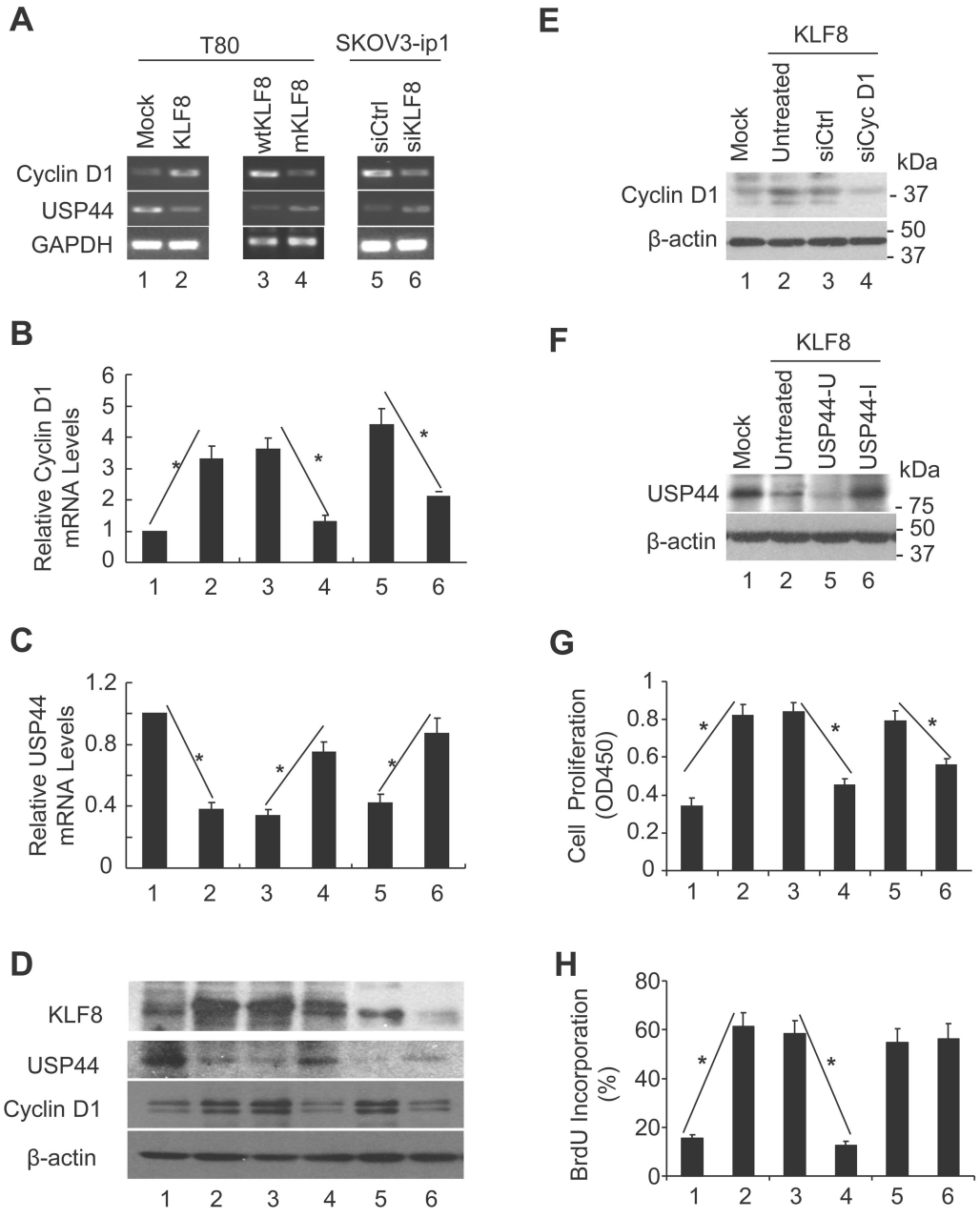


**Figure 5.**

KLF8 promotes proliferation by up-regulating cyclin D1 and down-regulating USP44 expression in the human ovarian cells. *A-D*, KLF8 up-regulates cyclin D1 and down-regulates USP44 expression. mRNA and whole cell lysates were prepared from T80 cells stably expressing empty vector (Mock) or KLF8 (KLF8) (lanes or columns 1 & 2) or T80 cells transiently expressing wild type (wtKLF8) or transactivation-defective mutant (mKLF8) for 48 h (lanes or columns 3 & 4), or the SKOV3ip1 cells transiently transfected with control siRNA (siCtrl) or siRNA against KLF8 (siKLF8) for 48 h (lanes or columns 5

& 6) for RT-PCR (A), qRT-PCR (B & C) or western blotting (D). *E-H*, Knockdown of cyclin D1 or re-expression of USP44 reverses the increased proliferation induced by KLF8 back to the Mock level. T80 cells stably expressing KLF8 (KLF8) were transfected with non-targeting siRNA or siRNA against cyclin D1 for 72 h (lanes or columns 3 & 4, siCtrl & siCycD1) or with tet-on regulated USP44 vector for 24 h followed by induction of USP expression for 48 h (lanes or columns 5 & 6, USP44-U & USP44-I). A fraction of the cells were used for western blotting to confirm the cyclin D1 knockdown (A) and induced expression of USP44 (B). Two other fractions of the cells were used for analysis of cell proliferation (G) and BrdU incorporation (H), respectively. Mock and untreated KLF8 expressing T80 stable cells (lanes or columns 1 & 2, Mock & untreated) were included as controls. U, expression uninduced; I, expression induced. *$P < 0.05$.

**Table 1**

**Tumor incidence of T80/KLF8 cells**

| T80 Cell Line | Genotype* | Tumor Incidence | | | P-value** |
|---|---|---|---|---|---|
| | | (s.c.) | (i.p.) | (i.b.) | |
| Mock | SV40 T/t, hTERT | 0/7 | 0/6 | 0/12 | |
| KLF8 | SV40 T/t, hTERT, KLF8 | 7/13 | 2/6 | NT | 0.0741, 0.6061, 0.0980 |
| c-Myc | SV40 T/t, hTERT, c-Myc | 6/7 | 2/6 | NT | 0.0117, 0.6061, |
| Akt | SV40 T/t, hTERT, Akt | 4/7 | 1/6 | NT | 0.0874, 0.5000, |
| STAT3c | SV40 T/t, hTERT, STAT3c | 3/7 | 0/6 | NT | 0.1923, 1.0000, |
| SKOV3ip1 | - | 7/7 | 5/6 | 5/6 | 0.0029, 0.0152, 0.0014 |

* Ectopic overexpression

** Compared to Mock of the same type of implantation; s.c., Tumor cells were implanted by subcutaneous injection; i.p., Tumor cells were implanted by intraperitoneal injection; i.b., Tumor cells were implanted by intrabursal injection; NT, not tested. All raw exact-p values were adjusted by Bonferroi correction;