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Induced pluripotent stem cell-related genes influence biological behavior and 5-fluorouracil sensitivity of colorectal cancer cells*

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Abstract: Objective: We aimed to perform a preliminary study of the association between induced pluripotent stem cell (iPS)-related genes and biological behavior of human colorectal cancer (CRC) cells, and the potential for developing anti-cancer drugs targeting these genes. Methods: We used real-time reverse transcriptase polymerase chain reaction (RT-PCR) to evaluate the transcript levels of iPS-related genes *NANOG*, *OCT4*, *SOX2*, *C-MYC* and *KLF4* in CRC cell lines and cancer stem cells (CSCs)-enriched tumor spheres. *NANOG* was knockdowned in CRC cell line SW620 by lentiviral transduction. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, plate colony formation, and a mouse xenograft model were used to evaluate alterations in biological behavior in *NANOG*-knockdown SW620 cells. Also, mock-knockdown and *NANOG*-knockdown cells were treated with 5-fluorouracil (5-FU) and survival rate was measured by MTT assay to evaluate drug sensitivity. Results: A significant difference in the transcript levels of iPS-related genes between tumor spheres and their parental bulky cells was observed. *NANOG* knockdown suppressed proliferation, colony formation, and in vivo tumorigenicity but increased the sensitivity to 5-FU of SW620 cells. 5-FU treatment greatly inhibited the expression of the major stemness-associated genes *NANOG*, *OCT4*, and *SOX2*. Conclusions: These results collectively suggest an overlap between iPS-related genes and CSCs in CRC. Quenching a certain gene *NANOG* may truncate the aggressiveness of CRC cells.

Key words: Induced pluripotent stem cell, Cancer stem cell, Colorectal cancer, *NANOG*, 5-Fluorouracil
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1 Introduction

The hypothesis that malignant tumors are driven by a minor group of tumor-initiating cells, namely cancer stem cells (CSCs), was proposed long ago and has been evolving for several decades. Prominent parallels have been observed between cancer cells and stem cells, such as self-renewal and similar signaling pathways and surface markers (Lapidot *et al.*, 1994; Reya *et al.*, 2001; Galli *et al.*, 2004; Singh *et al.*, 2004; Bjerkvig *et al.*, 2005; Morrison and Kimble, 2006; Wang *et al.*, 2009; Korkaya and Wicha, 2010). There is growing evidence suggesting that CSCs are indispensable for tumor initiation in a variety of tumor types (Lapidot *et al.*, 1994; Galli *et al.*, 2004; Singh *et al.*, 2004; Wang *et al.*, 2009), though the CSC theory is still controversial (Kelly *et al.*, 2007; Quintana *et al.*, 2008; Shackleton *et al.*, 2009). Besides hunting for valid surface markers or regulators for CSCs in a certain type of tumor, increasing efforts have been made to determine the origin of CSCs and the overall mechanisms controlling oncogenesis driven by CSCs.

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The successful artificial de-differentiation of adult somatic cells (Takahashi *et al.*, 2007; Yu *et al.*, 2007; Park *et al.*, 2008) and cancer cells (Carette *et al.*, 2010; Miyoshi *et al.*, 2010) to an embryonic stem cell-like state by defined reprogramming factors, including *OCT4*, *SOX2*, *KLF4*, *C-MYC*, *NANOG*, and *LIN28*, has drawn growing attention to the putative resemblance between the laboratory generation of induced pluripotent stem/cancer (iPS/iPC) cells in vitro and the spontaneous generation of CSCs in vivo. We speculate that partial reactivation of the reprogramming factor set and inappropriate micro-environments may lead to a disordered self-renewal and differentiation capacity rather than regulated pluripotency. Increasing evidence shows that reprogramming factors are over-expressed in a variety of malignant tumors, including colorectal cancer (CRC), and participate in tumor progression (Almstrup *et al.*, 2004; Ben-Porath *et al.*, 2008; Saiki *et al.*, 2009; Bae *et al.*, 2010; Fang *et al.*, 2010; Meng *et al.*, 2010). However, most studies have been based on analysis of the bulky cells from cell lines or patient samples. In this study, for the first time, we used CSCs-enriched tumor spheres as a subject to investigate the relationship between these reprogramming factors and CSCs in human CRC cell lines.

CSCs are suspected to be responsible for relapse, metastasis, and drug-resistance in malignant tumors (Li *et al.*, 2007; Lacerda *et al.*, 2010; Singh and Settleman, 2010). Anti-cancer agents targeting CSCs and which can be used as potential adjuvants to conventional chemotherapy have yet to be developed (Tang *et al.*, 2007). In a preliminary attempt to explore the potential of these reprogramming factors as anti-CSC targets, we chose the most promising one—*NANOG*, to evaluate its role in the biological behavior of CRC cells. Loss-of-function studies demonstrated that *NANOG* was important in tumor proliferation, tumorigenicity and sensitivity to standard chemotherapy agents. The relationship between reprogramming factor silencing and standard chemotherapy needs further research.

2 Materials and methods

2.1 Cell culture

SW620, SW480 and HT29 CRC cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured according to ATCC's protocols. Tumor spheres were obtained from these cell lines as follows: cells were trypsinized, washed twice with phosphate buffered saline (PBS), and added to low-attachment tissue culture plates; cells were maintained in serum-free (Leibovitz's) L-15 (for SW620 and SW480) or McCoy's 5a (for HT29) growth medium containing 4 U/L insulin, 20 ng/L basic fibroblast growth factor (b-FGF), 20 ng/L epidermal growth factor (EGF), 0.1% bovine serum albumin (BSA). Medium was changed every 2 d and cells were split at a 1:2 ratio.

2.2 Isolation of RNA and real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA from cell lines and tumor spheres was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The transcript levels of *NANOG*, *SOX2*, *OCT4*, *KLF4*, and *C-MYC* were determined by real-time PCR using the Applied Biosystems StepOne™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The PCR reactions were carried out in a total volume of 20 μl per well containing SYBR master mix reagent kit (Applied Biosystems) using published primers (Yu *et al.*, 2007; Park *et al.*, 2008). Human glyceraldehyde phosphate dehydrogenase (*GAPDH*) was amplified as an endogenous control.

2.3 Establishment of stable *NANOG*-knockdown SW620 cell clones

Small hairpin RNA (shRNA) lentiviral particles used for *NANOG* knockdown (sc-43958-v) and mock knockdown (sc-108080) were purchased from Santa Cruz (Santa Cruz, CA, USA). The viral particles were used to infect SW620 cells following the manufacturer's instructions. The infected cells were selected with 3 μg/ml puromycin dihydrochloride 72 h after transduction. The medium was changed every 3–4 d until puromycin-resistant colonies were evident. Surviving colonies were pooled and dispensed into 96-well plates at a density of 0.5 cell/well. About two weeks later, single colonies evident in some wells were picked into 24-well plates, cultured with puromycin selection medium and evaluated for *NANOG* mRNA expression using real-time RT-PCR.

2.4 Cell proliferation assay

Cells were prepared at a concentration of 8×10^3 cells/200 μl and then distributed in 96-well plates at 200 μl/well and cultured overnight. MTT assays were performed every day for up to 5 d. Briefly, 20 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) was added to each well; plates were incubated at 37 °C for 4 h and the supernatants were removed carefully; 150 μl of dimethyl sulfoxide (DMSO) was added to each well and the plates were agitated on a shaker for 5 min. The optical density (OD) was measured with a microplate reader (BioRad, Hercules, CA, USA) at 570 nm. Experiments were performed in triplicate.

2.5 Plate colony formation assay

Cell colony formation rate was measured using a plate colony formation assay. About 2000 cells were added to each well of a 6-well plate. Plates were incubated at 37 °C in an incubator for two weeks and colonies containing at least fifty cells were counted under a microscope.

2.6 Mouse xenograft model

Our animal protocol was approved and performed strictly in accordance with the relevant ethics regulations of Zhejiang Chinese Medical University. SW620 mock-knockdown cells and SW620 *NANOG*-knockdown cells were cultured until 80%–90% confluence before harvesting. Cells were trypsinized, washed twice with PBS, and resuspended in serum-free (Leibovitz's) L-15 medium to a concentration of 2×10^5, 1×10^6, or 5×10^6 per 200 μl. Then, some 200 μl of cells were injected subcutaneously into the dorsal flanks of 5-week-old female nude mice. Tumor sizes were measured in two dimensions with calipers twice a week and tumor volumes (mm^3) were calculated as $L\times W^2\times0.5$ (L is tumor length and W is tumor width).

2.7 Statistical analysis

For continuous variables, data were expressed as mean±standard error (SE). Results of cell proliferation, plate colony formation assays, and in vivo tumorigenicity assays were analyzed by analysis of variance (ANOVA), with $P<0.05$ in all cases considered statistically significant.

3 Results and discussion

3.1 Differential expression of iPS-related genes in human CRC tumor spheres and bulky cells

To obtain CSCs-enriched samples from CRC cell lines, we used a spheroid-culture system which has been extensively used for various tumor types, including CRC, and has proved to be suitable for enriching cancer-initiating cells (Todaro *et al.*, 2007; Yeung *et al.*, 2010; Cao *et al.*, 2011; Fan *et al.*, 2011; Yin *et al*., 2011). Undifferentiated tumor spheres derived from human CRC cell lines SW620, SW480, and HT-29 were cultured in serum-free medium containing EGF and FGF-2 (Dontu *et al.*, 2003; Ma *et al.*, 2010) (Fig. 1b). We performed real-time RT-PCR to evaluate mRNA expression of the reprogramming factors *NANOG*, *SOX2*, *OCT4*, *KLF4*, and *C-MYC* in both tumor spheres and their parental bulky cells (Fig. 1a). Normal human colon epithelial tissue RNA was used as a normal control (NC). Bulky cells from CRC cell lines showed relatively high expression of *NANOG*, *SOX2*, and *OCT4* compared with NC. However, this alteration was almost negligible compared to the striking elevation in their sphere-like descendants. We did not see significant changes in mRNA levels of the oncogene *C-MYC*, which is involved mostly in cell proliferation and is dispensable in the reprogramming process. These data suggest that the minor CSC subsets may contribute a majority, if not all, of the expression of reprogramming factors in malignant tumors, as previously reported (Almstrup *et al.*, 2004; Ben-Porath *et al.*, 2008; Jeter *et al.*, 2009; Saiki *et al.*, 2009; Bae *et al.*, 2010; Fang *et al.*, 2010; Meng *et al.*, 2010), which was in accordance with our hypothesis that tumor initiation might be achieved by "dysregulated-reprogramming".

KLF4 was found to decrease substantially, which was predictable, since *KLF4* is considered to be a normal regulator in the gastrointestinal tract epithelium (Wei *et al.*, 2006; Yori *et al.*, 2010) and a potential tumor suppressor in CRC (Zhao *et al.*, 2004). Note that *KLF4* expression in tumor spheres was relatively close to normal levels, implying a context-dependent property rather than stemness significance in colon cancer.

Spheroid culture has a heterogeneous property: several studies have shown that the cells in the inner region of a tumor sphere are quiescent (Freyer and

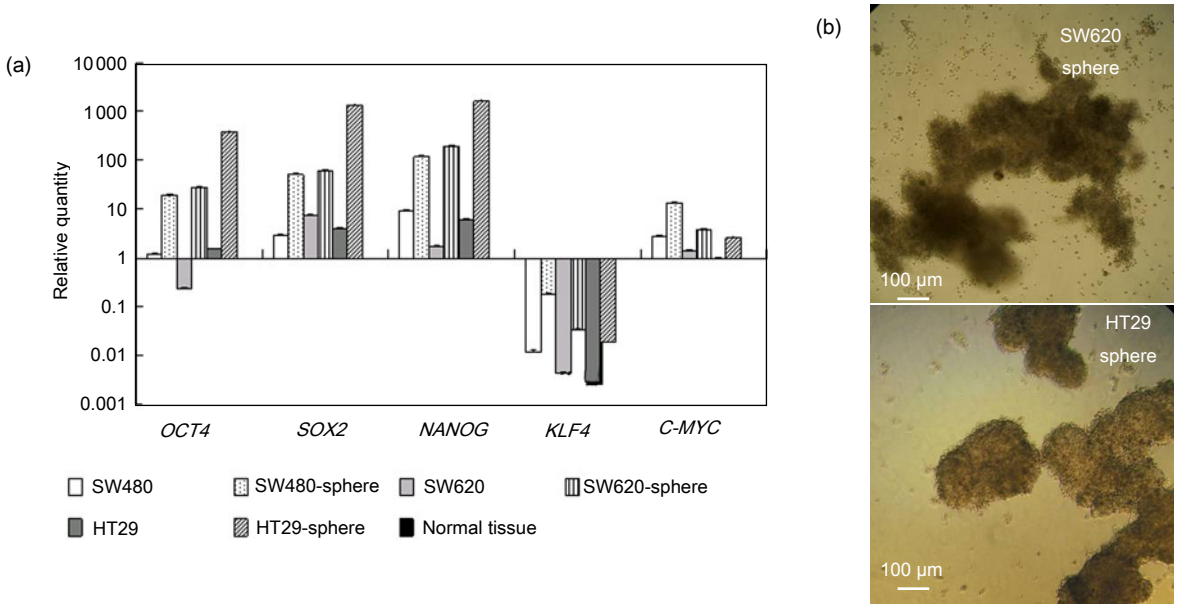


Fig. 1 iPS reprogramming factors expressed differentially in human CRC tumor spheres and bulky cells
(a) A bar chart showing mRNA levels of *NANOG*, *OCT4*, *SOX2*, *C-MYC*, and *KLF4* in CRC tumor spheres and their parental cell lines (*Y*-axis: relative expression levels normalized to normal colon epithelial tissue; $P<0.05$); (b) Morphology of tumor spheres

Sutherland, 1980; Sherar *et al.*, 1987; Tindall and Please, 2007) and more resistant to chemotherapeutics compared with those in the more proliferative outer layer (Wibe, 1980; Waleh *et al.*, 1995). Since the mechanism of sphere formation is still elusive, it will be of great interest to find out which part of the tumor sphere expresses the high level of iPS-related genes. It is more likely that the quiescent inner cells express these "stemness" genes, considering that embryonic stem cells are low-proliferating. Some novel techniques, such as the use of a thermoreversible CyGEL™ reagent that stabilizes live 3D tumor spheroids (Robertson *et al.*, 2010), may help to locate the region highly expressing iPS-related genes.

3.2 Impaired proliferation, colony formation ability and tumorigenicity in *NANOG*-knockdown cells

The roles of reprogramming factors *OCT3/4*, *SOX2*, and *NANOG* in the progression of CRC and other tumor types have been studied extensively (Jeter *et al.*, 2009; Saiki *et al.*, 2009; Bae *et al.*, 2010; Fang *et al.*, 2010; Meng *et al.*, 2010), although there has been limited research on the potential use of anti-CSC agents targeting these genes. We aimed to conduct a preliminary study on the significance of developing a therapeutic agent against a certain reprogramming factor. We chose to focus on *NANOG* because it is scarcely expressed in normal organs or tissues in the human body compared with *OCT4* and *SOX2*, which implies that side effects could be more amenable in prospective therapeutic use. Although we found a prominent increase of *NANOG* in tumor spheres, we used parental bulk cells because the objective of our study was to target the general CRC cells in the human body, rather than only CSCs. CRC cell line SW620 was an ideal cell line to use because of its metastatic property and high tumorigenicity in mouse transplantation.

We established stable *NANOG*-knockdown SW620 cell clones using lentivirus-mediated shRNA gene silencing technology. *NANOG* mRNA expression levels were determined by real-time RT-PCR (Fig. 2a). Clones that showed less than 20% *NANOG* expression compared to the parental SW620 cell line were expanded individually for future use. Clones were mixed together equally before experiments. Interestingly, we noticed that *NANOG*-knockdown cells underwent remarkable alterations in their morphology (Fig. 2b) compared with parental SW620 cells. A typical clone displayed a flatter and rounder

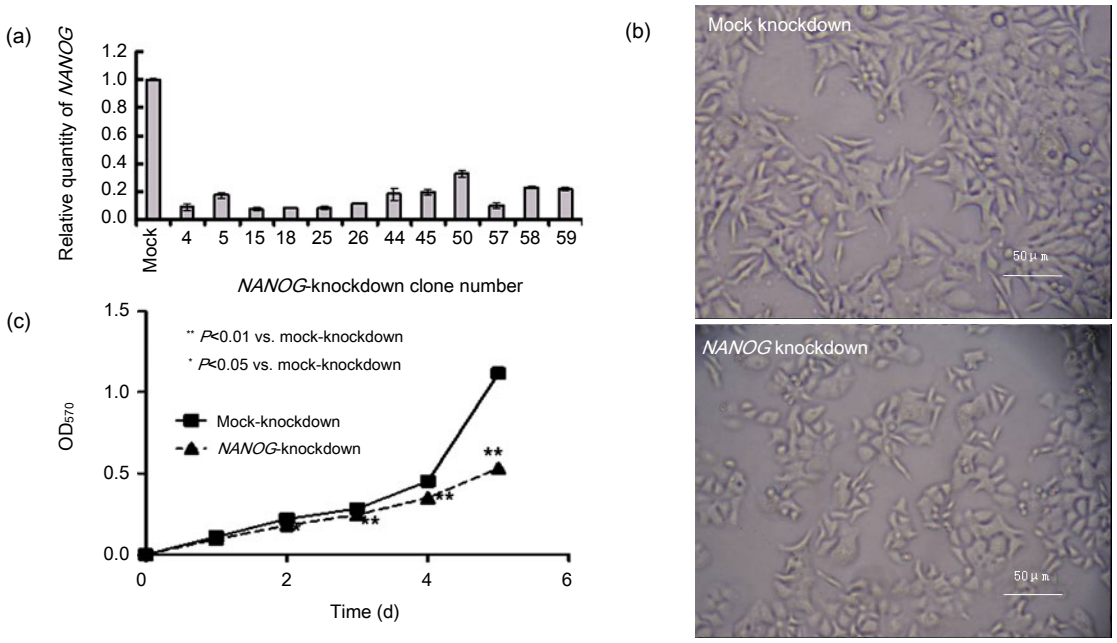


Fig. 2 Changes of cell morphology and growth rate of *NANOG*-knockdown SW620 cells

(a) Expression profile of *NANOG* knockdown clones; (b) Alterations of morphology in *NANOG*-knockdown SW620 cells; (c) Effect of *NANOG* knockdown on cell proliferation determined by MTT assay

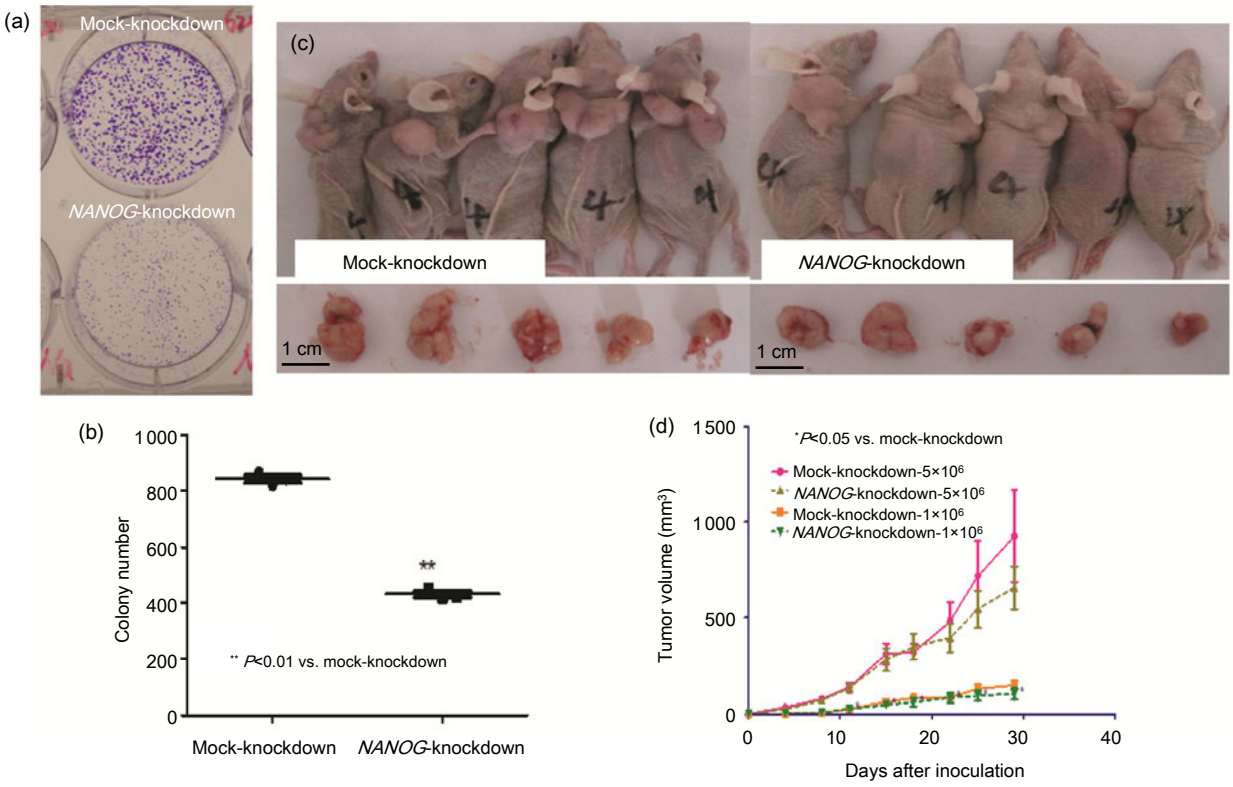


Fig. 3 Suppression of plate colony formation ability and in vivo tumorigenicity of *NANOG*-knockdown SW620 cells

(a) Representative pictures of colonies on plates; (b) Number of colonies formed from 2000 cells (data are representatives of three independent experiments); (c) Representative pictures of tumor samples; (d) Growth curve of mock-knockdown and *NANOG*-knockdown cells in in vivo mouse model

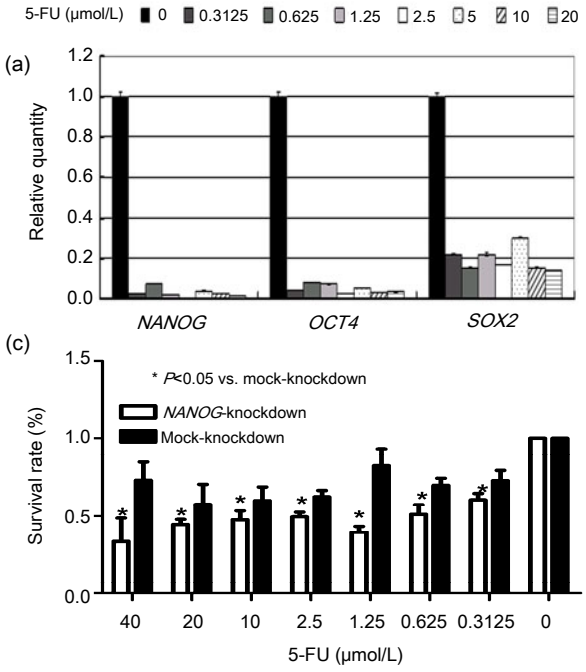
shape, while mock cells were protuberant and spindle-like, resembling typical SW620 cells. This alteration may predict attenuated motility, which is consistent with the previous finding that overexpression of *NANOG* could increase the migratory ability of SW480 cells in a wound healing assay (Meng *et al.*, 2010). MTT assays illustrated a decreased proliferation in *NANOG*-knockdown cells (Fig. 2c).

NANOG silencing significantly impaired the ability of SW620 cells to form colonies in the plate colony formation assay. *NANOG*-knockdown cells generated colonies with an apparent reduction in size and number (Figs. 3a and 3b). Moreover, the xenograft model showed that *NANOG* played a negative role in tumorigenesis of SW620 cells in vivo. Equal numbers of mock-knockdown and *NANOG*-knockdown cells were injected into the dorsal flanks of 5-week-old female nude mice. Tumor growth curves indicated that *NANOG* knockdown inhibits proliferation of SW620 cells in vivo (Fig. 3d). Furthermore, mice injected with mock-knockdown cells exhibited a morbid status both physically and mentally whereas mice in the *NANOG*-knockdown group looked healthier and more vigorous (Fig. 3c). This study provided a prophylactic model of *NANOG* interference in vivo. We will try to establish a therapeutic model using shRNA gene silencing after tumor cell inoculation in a future study.

3.3 Synergism of chemotherapy agent 5-FU treatment and *NANOG* silencing in suppressing SW620 cells in vitro

To investigate the effect of the standard chemotherapy agent 5-FU on stemness-associated genes, we treated SW620 cells with 5-FU at different concentrations (Fig. 4). After 5 d, mRNA levels of *NANOG*, *SOX2*, *OCT4*, and *KLF4* were measured by real-time RT-PCR. We speculated that administration of 5-FU would kill the common bulky cells and enrich stem-like cells. Surprisingly, the levels of *NANOG*, *SOX2*, and *OCT4* decreased dramatically, especially those of *NANOG* and *SOX2* (Fig. 4a), whereas that of *KLF4* went up slightly (Fig. 4b). These results collectively imply that a 5-d 5-FU treatment will suppress these stemness-associated genes.

This may be because once CSCs sense adverse changes in the niche, they will trigger a self-protecting system which allows them to stop self-renewal, inactivate most non-essential transcript activities, and step into dormancy. Another possibility is that 5-FU is able to interfere with the transcription stability of these genes. Several studies have indicated that chemotherapeutic agents like 5-FU are likely to enrich cancer cells with a CSC phenotype, based on elevated CSC surface marker levels or clonogenic ability (Dylla *et al.*, 2008; Dallas *et al.*, 2009). According to these findings, we could speculate that it is



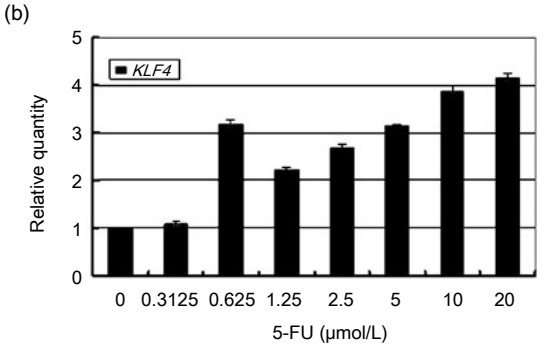


Fig. 4 Synergism of 5-FU treatment and *NANOG* silencing in suppressing SW620 cells in vitro

Transcript levels of (a) *NANOG*, *OCT4*, *SOX2*, and (b) *KLF4* after a 5-d 5-FU treatment (*Y*-axis: relative expression levels normalized to SW620 cells without 5-FU treatment; $P<0.05$); (c) Survival rates of *NANOG*-knockdown and mock-knockdown cells after 72 h 5-FU treatment determined by MTT assay (*Y*-axis: relative absorbance normalized to *NANOG*-knockdown or mock-knockdown cells without 5-FU treatment)

more likely that 5-FU disturbs the stability of these genes rather than shrinks the CSC population. This hypothesis needs support from in vivo experiments since a lack of a stem cell niche, such as in angiogenesis, could also explain the instability of CSCs. Another interesting study showed that the levels of *SOX2* and *OCT4* in residual cancer cells from patients after chemoradiotherapy were positively associated with distant recurrence (Saigusa *et al.*, 2009). This result implies that the impact of chemoradiotherapy on cancer cells in vivo is a more context-dependent and patient-specific process. It will be more meaningful to conduct further studies on multiple primary cancer cell lines than on established cell lines.

The suppression of the stemness-associated gene *NANOG* raised the sensitivity of SW620 cells to 5-FU. We treated both mock-knockdown cells and *NANOG*-knockdown cells with 5-FU for 72 h and determined the survival rate by MTT assay. *NANOG*-knockdown cells displayed a decreased survival rate compared to mock cells when exposed to the same concentration of 5-FU.

These results suggest that regular chemotherapy and silencing of the stemness-associated factor *NANOG* act in synergy, and are not simply complementary. The addition of anti-CSC agents to regular chemotherapy may greatly enhance therapeutic efficiency and reduce the drug dose, which means fewer side effects. Systemic administration of anti-CSC agents after chemotherapy may be used as a strategy to prevent tumor relapse and metastasis.

The antimetabolite 5-FU is one of the standard chemotherapy regimens for colon cancer. Adjuvant chemotherapy has also been conducted with oxaliplatin and irinotecan in the last decade. However, persuasive evidence for including molecular targeted therapy is still lacking. Current molecular targeted therapy agents, like bevacizumab and cetuximab, function mainly by inhibiting angiogenesis. As a putative prime candidate for refractoriness of malignant cancer, CSCs warrant more attention. We have cast a brick to attract jade, hopefully encouraging the emergence of valuable research on therapy targeting CSCs in the coming decade.

4 Conclusions

For the first time, we have found that transcript levels of iPS-related genes *OCT4*, *SOX2*, and *NANOG* are strikingly high in CSCs-enriched human CRC tumor cells. RNA interference of *NANOG* could truncate the aggressiveness of CRC cells by suppressing proliferation, invasion, tumorigenicity and by increasing their sensitivity to 5-FU. Therefore, these reprogramming factors may play an important role in tumor progression. Strategies to interfere with these genes may have great clinical significance. Hopefully, we can draw more attention to anti-cancer therapies targeting these reprogramming factors.

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