Title: Silencing oncogene expression in cervical cancer stem-like cells inhibits their cell growth and self-renewal ability

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Abstract:

Accumulating evidence supports the concept that cancer stem cells (CSCs) are responsible for tumor initiation and maintenance. They are also considered as an attractive target for advanced cancer therapy. Using a sphere culture method that favors the growth of self-renewal cells, we have isolated sphere-forming cells (SFCs) from cervical cancer cell lines HeLa and SiHa. HeLa-SFCs were resistant to multiple chemotherapeutic drugs and were more tumorigenic, as evidenced by the growth of tumors following injection of immunodeficient mice with 1 x [10.sup.4] cells, compared with 1 x [10.sup.6] parental HeLa cells required to grow tumors of similar size in the same time frame. These cells showed an expression pattern of [CD44.sup.high]/[CD24.sup.low] that resembles the CSC surface biomarker of breast cancer. We further demonstrated that HeLa-SFCs expressed a higher level (6.9-fold) of the human papillomavirus oncogene E6, compared with that of parental HeLa cells. Gene silencing of E6 with a lentiviral-short-hairpin RNA (shRNA) profoundly inhibited HeLa-SFC sphere formation and cell growth. The inhibition of cell growth was even greater than that for sphere formation after E6 silence, suggesting that the loss of self-renewing ability may be more important. We then measured the expression of self-renewal genes, transformation growth factor-beta (TGF-[beta]) and leukemia-inhibitory factor (LIF), in shRNA-transduced HeLa-SFCs and found that expression of all three TGF-[beta] isoforms was significantly downregulated while LIF remained unchanged. Expression of the Ras gene (a downstream component of TGF-[beta]) was also markedly decreased, suggesting that the growth-inhibitory effect could be via the TGF-[beta] pathway. The above data indicate RNA interference-based therapy may offer a new approach for CSC-targeted cancer therapy.

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Keywords: cancer stem cells; sphere-forming cells; RNA interference; HeLa cells; cervical cancer; HPV E6/E7; self-renewal gene; TGF-[beta]; lentiviral vector

Full Text:

Introduction

The concept of cancer stem cells (CSCs) is that tumors contain a small proportion of self-renewal and pluripotent cancer cells that are responsible for tumor initiation and maintenance. (1) The high expression of multiple drug-resistant gene (2) and ATP-binding cassette transporter (3,4) by these cells can also protect them from chemotherapeutic drugs. Therefore, CSCs are considered as an important target for developing future cancer therapies or improving the current therapies. However, as they are multi-drug resistant and also resistant to other current therapies (for example, radiotherapy), (1) conventional methods will not be effective for targeting them and hence alternative approaches are required.
The RNA interference (RNAi) technique has been widely used as a tool for gene-functional studies and also has great potential for developing therapies against viral infection, genetic disorders and cancers. (5-9) Several RNAi therapies have been used clinically, while many more are in clinical trials. (10) For RNAi-based cancer therapy, oncogenes are obviously ideal targets, as they are the driving force of cancer cell growth and are highly expressed in cancer cells. (11,12) Although it is not clear whether oncogene expression in CSCs is different from that in other cancer cells, it is believed that these genes are vital for them. Therefore, oncogenes may offer a new approach for CSC-targeted cancer therapies. There are numerous studies on RNAi-based cancer therapy targeting oncogenes in various cancers; for example, human papillomavirus (HPV) E6 and E7 for cervical cancer, (13-16) and Her-2, (17) c-myc (18) and hdm-2 (19) for breast cancer. However, there is no report on RNAi-based oncogene silencing in CSCs.

Cervical carcinoma is the second most common cancer in women worldwide and is highly associated (99%) with infections of high-risk types of HPVs. Viral early genes E6 and E7 from high-risk HPV types are responsible for the transformation of epithelial cells, and their continuous expression is essential for ongoing cervical cancer cell survival as they function as oncogenes. (20,21) Therefore, E6 and E7 are ideal targets for RNAi therapy. In recent years, there have been a number of publications showing the potential use of RNAi as a treatment for cervical cancer. (14-16,22-25) We demonstrated that RNAi triggered by short-hairpin RNA (shRNA) targeting a specific site within E7 mRNA could even induce immunity to E7 in immune-competent mice. (26) All the data indicate that RNAi-based therapy can be developed as a promising treatment for cervical cancer. However, no treatment for cervical cancer has yet been developed for clinical use or even for clinical trial. Besides the challenge in the development of a safe in vivo delivery system, the effectiveness of RNAi itself as a mono-therapy to treat cervical cancer patients, especially at late clinical and metastasis stages, is unknown. In addition, the existence of cervical CSCs and their sensitivity to RNAi-based treatments have not been investigated. These are challenging questions and will potentially provide strategic solutions for cancer therapy.

In this study, we firstly isolated sphere-forming cells (SFCs) from cervical cancer cell lines using a method that has been used to successfully isolate or enrich stem cells or CSCs from mammary tissues, (27) primary tumors or cancer cell lines (28-31) Characterization of these SFCs showed CSC-like features, including higher tumorigenicity than parental HeLa cells, expression of biomarkers [CD44.sup.high]/ [CD24.sup.low] and multi-drug resistance. We further demonstrated that these cells were sensitive to E6 oncogene silencing, as evidenced by the profound inhibition of cell growth and sphere formation. We also showed that this effect was likely through a reduction in the self-renewal ability modulated by transformation growth factor-beta (TGF-[beta]).

Materials and methods

Cervical cancer cell lines and sphere culture

Cervical cancer cell lines HeLa (ATCC, CCL-2), CaSki (ATCC, CRL-1550), SiHa (ATCC, HTB-35) and C33A (ATCC, HTB-31) were purchased from American Type Culture Collection and maintained in complete Dulbecco's modified Eagle's medium (Invitrogen, Gladesville, NSW, Australia) + 10% fetal calf serum, as described. (15) The sphere culture medium was prepared as previously described. (27) For the first passage of sphere culture, 2 x [10.sup.4] cancer cells were seeded into a T75 flask with 20 ml of sphere culture medium.
Cells were cultured in suspension for 4 days at 37°C. An additional 10 ml of sphere culture medium was added to the culture on day 4 and the culture continued for another 4-5 days. Spheres were harvested by centrifugation at 300 g for 5 min, and sphere numbers counted after re-suspension in 5 ml medium. For the second or following passage sphere culture, the spheres were treated with 1:1 diluted 2.5% Trypsin-EDTA (Invitrogen) for 5 min at 37°C and washed with sphere culture medium. Spherical cells were separated by repeated pipetting and counted. The separated SFCs were then passed through a cell strainer (40 [micro]M, BD, Brisbane, QLD, Australia) and used for continuous sphere culture or other assays in low-adherence six-well plates (Sigma-Aldrich, Sydney, NSW, Australia).

Drug treatment, cell staining and fluorescence microscopy

Chemotherapeutic drugs cisplatin, doxorubicin and epitoside were purchased from Sigma-Aldrich. Their working concentrations in HeLa cells were cisplatin (1-2 [micro]g [ml.sup.-1]), doxorubicin (0.0625 [micro]g [ml.sup.-1]) and epitoside (0.5 [micro]g [ml.sup.-1]), at which they kill >90% HeLa cells in 24-72 h. For fluorescence-activated cell sorting analysis, SFCs were harvested and dispersed from spheres. The cells were stained with antibodies to CD44 conjugated with fluorescein isothiocyanate and to CD24 conjugated with R-phycocerythrin (Invitrogen) at concentrations of 1:100 (V/V, 1 x [10.sup.5] cells). The cells were washed two times with 1% fetal calf serum/phosphate-buffered saline, then fixed with 2% paraformaldehyde/phosphate-buffered saline for fluorescence-activated cell sorting analysis using Calibur or FACS Canto (BD). For fluorescence microscopy, a small portion of fixed cells was cytopinned onto microscope slides using a Cytospin 4 (Thermo Shandon, Cheshire, UK) and mounted in Fluoroshield mount medium containing 4',6-diamidino-2-phenylindole (Sigma-Aldrich) to stain cell nuclei.

Animal experiment

HeLa and HeLa-SFCs were trypsinized, washed and re-suspended in phosphate-buffered saline at different cell concentrations (2 x [10.sup.5] to 2 x [10.sup.7] cells [ml.sup.-1]). Female non-obese diabetic, severe combined immunodeficient (NOD/SCID) 6-8-week-old mice were used and subcutaneously injected (three mice per group) with 50 [micro]l of cell suspension to the neck scruff. The tumors were monitored weekly (and size measured with a calipers) and dissected at 22 days post injection for final examination. The animal experiment was approved by the Animal Ethics Committee of Queensland University.

Colony-forming assay

HeLa cells and HeLa-SFCs were harvested as above and counted. A total of 100 cells were seeded in each well of 12-well plates with 1 ml complete Dulbecco’s modified Eagle’s medium; each group had three replicates. The cells were cultured for 7 days and then fixed with 1 ml 95% ethanol for 30min at room temperature and stained with 0.5 ml 0.1% crystal violet for 5 min. The number of colonies in each well was counted and the data were expressed as mean [+ or -] s.d.

Transduction of HeLa cells with lentiviral-shRNA

The production of lentiviral vectors carrying shRNA (LV-shRNA) and the transduction of HeLa cells with LV-shRNA were done as previously described. (15) LVsh-RNA 18E6-1 was
shown to effectively silence HPV E6 and E7 genes, (15) whereas the shRNA control LV-shRNA 16E7-2 was ineffective. (26)

Real-time RT-PCR

Total RNA extraction from transduced HeLa and HeLa-SFCs was done as instructed by the manufacturer using TRIzol reagent (Invitrogen). Reverse transcription reactions were performed with oligo-dT primer using the High Capacity cDNA RT Kit (Applied Biosystems, Melbourne, VIC, Australia). Real-time PCR was carried out with SYBR green master mixture (Promega, Sydney, NSW, Australia) using a Rotor-Gene RG-3000 (Corbett Research, Doncaster, VIC, Australia) with the program pre-heating at 95[degrees]C for 10 min, followed by 40 cycles of 94[degrees]C 15 s, 58[degrees]C 30 s and 72[degrees]C 45 s.
The primers were HPV 18E7: F 5' AAAATGAAAT TCCGGTTGA-3', R5' GGCTGGTAAATGTTGATG AT-3'; HPV 18E6: F 5'-CTGTGACCGGA ACTGAAC AC-3', R5'- TGCA CGCATGGGGGTATCTGT-3'; TGF-[beta]-1: F 5'- CAACAAATTGCCTGGGCAACCC-3', R5'- GA ACCCGTTGATGTCCACTT-3'; TGF-[beta]-2: F 5'-GAGT GCCGAA CAACGGATT-3', R 5'- TGCAGCAGGGGA CAGTGAAG-3'; TGF- [beta]-3: F 5'-GCAACCTTGAG GAGAACTGC-3', R5'- CTGTGGGTTGTGTCTGCA CT-3'; leukemia-inhibitory factor (LIF): F 5'-CCCTGTC GCTCTCTAAGCAC-3', R5'- ATCCTGGACAAGGGT GAGTG-3'; H-Ras: F 5'-GTGGTC AATGTACGGGGA GAC-3', R5'- ACGTCATCCGAGTCTTCAC-3'; and K-Ras: F 5'-AGTCATCCGAGTCTTCAC-3', R5'- GGTAGGGAGGCAAGATGACA-3'. The internal control was 18S rDNA (F: 5'- CCATCGAACGTCTGCC CA-3', R: 5'-TCACCGTGTCACCATTG-3') and it was also used to normalize gene expression.

Data analysis

Data collected from each (experimental and control) group were expressed as mean [+ or -] s.d. One-way analysis of variance and unpaired Student's t-test (GraphPad Prism 5 program) were used to analyze the differences between groups and distinguish the significant differences (two-tailed, P < 0.05) between experimental and control groups.

Results

Sphere culture of cervical cancer cell lines
We tested four cervical cancer cell lines, HeLa, SiHa, CasKi and C33A, for their sphere-formation abilities; only HeLa and SiHa were able to form cancer cell spheres. We therefore selected HeLa cells for the following studies. When cultured in sphere culture conditions, some HeLa cells formed spheres at day 4 while most HeLa cells adhered to the wall of the culture flask (Figure 1a). At day 9, the spheres became very compact cell clusters (Figures 1b and c). Previous studies showed that breast CSCs from mammo-sphere cells exhibited surface markers of high CD44 and low CD24. As cervical cancer is also a female epithelial cancer, we examined CD44 and CD24 expression in HeLa-SFCs. The results showed that HeLa-SFCs were CD44 positive and CD24 low, whereas parental HeLa cells were CD44 negative and CD24 high (Figure 1d), the same pattern as in breast CSCs.

Tumorigenicity and drug resistance of HeLa SFCs

To examine whether HeLa-SFCs are more tumorigenic than parental HeLa cells, we subcutaneously injected the cells to NOD/SCID mice at different dosages (Table 1). HeLa cells were also injected as controls. In the HeLa-SFC group, 1 x [10.sup.6] cells could form tumors in 3 weeks, whereas 1 x [10.sup.6] parental HeLa cells were needed to grow similarly-sized tumors in the same time frame (Table 1), suggesting HeLa-SFCs are more tumorigenic (100 times) than their parental HeLa cells and that sphere culture is a means of enriching tumorigenic cancer cells. To test whether HeLa-SFCs are also multi-drug resistant, we
cultured the cells in the presence of three clinically used chemotherapy drugs at concentrations that are cytotoxic for HeLa cells. The presence of the three drugs did not affect SFC sphere formation or growth either at passage 1 or at passage 5 (Figure 2a), suggesting HeLa-SFCs are resistant to these drugs. When HeLa-SFCs were cultured in complete Dulbecco's modified Eagle's medium containing fetal calf serum, these cells quickly adopted the morphology of HeLa cells (Figure 2b) and became sensitive to the same drug treatments (Figure 2c). This result suggests that after differentiation, HeLa-SFCs regain their HeLa cell properties. In addition, when they were cultured in complete Dulbecco's modified Eagle's medium, HeLa-SFCs formed more and bigger colonies than HeLa cells (Figure 2d and e), indicating SFCs have a greater ability to grow, and that they grow faster than HeLa cells. Taken together, the above data suggest that HeLa-SFCs have many of the characteristics of CSCs.

Silencing E6/E7 affects cell growth and sphere formation of HeLa-SFCs

As HeLa-SFCs showed the characteristics of CSCs, we then investigated how E6/E7 gene silencing would affect their growth and sphere formation. We first examined the expression of E6 and E7 by HeLa-SFCs. As expected, HeLa-SFCs expressed both E6 and E7. However, to our surprise, HeLa-SFCs expressed 6.9-fold more E6 than the parent HeLa cells, whereas there was no significant difference in E7 expression (Figure 3a). This result indicates that tumorigenic cancer cells may have an oncogene expression profile different from that of differentiated cancer cells and that the investigation of the effect of E6 silencing in HeLa-SFCs is more interesting.

**Figure 3** The effect of E6/E7 silencing on HeLa-SFC growth and sphere formation. (a) HPV E6 and E7 expression levels in HeLa and HeLa-SFCs were measured using real-time PCR and the data were representative for at least two independent assays. (b) Sphere growth of HeLa cells transduced with LV-16E6-1 and -16E7-2 in sphere culture; the sphere growth of LV-16E6-1 (16E6-1-sphere) was obviously inhibited compared with control LV-16E7-2 (16E7-2-sphere), as evidenced by their smaller-sized spheres. (c) The transduced HeLa-SFCs were analyzed by FACS for their enhanced green fluorescent protein (eGFP) expression (onciviral vector carrying an eGFP gene); the majority of them were positive. (d) The transduced HeLa-SFCs were measured for E6 and E7 gene expression using real-time PCR. Compared with control t16E7-2, 16E6-1-transduced SFCs had significantly lower levels of E6 and E7 expression. *P<0.05, **P<0.01.
We treated HeLa-SFCs with lentiviral-shRNA (18E6-1) targeting HPV E6 (as well as E7, as the two genes are expressed bicistronically (15)). The data showed that this significantly inhibited sphere formation (sphere numbers) and also cell growth (cell number within each sphere; Figure 3b and Table 2). As sphere formation and growth represent the self-renewal ability of the cells, these data suggest that E6/E7 may have a profound impact on SFC self-renewal. Thus, further investigations into the molecular mechanism are required. We then measured enhanced green fluorescent protein expression in the SFCs by flow cytometry. Figure 3c shows that the majority of SFCs were green fluorescent protein positive, confirming that the SFCs were transduced by the lentiviral vector. To confirm the gene-silencing effect, we also measured E6/E7 expression levels in transduced HeLa-SFCs. The results proved that transduced HeLa-SFCs had lower E6 and E7 levels, compared with the control (Figure 3d). It was observed that the lentiviral vector itself had some non-specific effects on sphere growth and formation (Table 2, 16E7-2). However, compared with the non-specific LV-shRNA control (16E7-2), 18E6-1 treatment had significant effects on both sphere formation (total spheres in Table 2) and growth (total SFCs in Table 2). The data also showed that the inhibition of cell growth (P< 0.001) was even more profound than sphere formation (P< 0.05, Table 2), suggesting that the gene silencing of E6/E7 in HeLa SFCs has a major inhibitory effect on SFC growth (smaller spheres), with a less-degree effect on causing cell death (fewer spheres).

**[FIGURE 1 OMITTED]**

![Table 2 Silencing of E6/E7 affects sphere formation and cell growth (ratio)](image)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Total spheres</th>
<th>Total SFCs (× 10^6)</th>
<th>Sphere %</th>
<th>SFC:SP ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>3650 ± 95.7</td>
<td>75.5 ± 1.95</td>
<td>17.25 ± 2.47</td>
<td>207 ± 5.74</td>
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<tr>
<td>HeLa-16E7-2</td>
<td>2130 ± 130.4</td>
<td>56.9 ± 1.57</td>
<td>10.05 ± 0.67</td>
<td>195.3 ± 14.58</td>
</tr>
<tr>
<td>HeLa-18E6-1</td>
<td>1467 ± 110.3</td>
<td>16.9 ± 1.27</td>
<td>7.975 ± 0.05</td>
<td>115 ± 7.79**</td>
</tr>
</tbody>
</table>

*Abbreviations: SFC, sphere-forming cell; SP, sphere.
*P< 0.05; **P< 0.001.

Notes: The experiment was repeated 3-4 times and the data were expressed as mean ± s.e. The initial cell numbers plated into the culture was 2 × 10^6.

**[FIGURE 2 OMITTED]**

Silencing E6/E7 downregulates self-renewal gene TGF-[beta] expression in HeLa-SFCs

TGF-[beta] and LIF are two well-documented self-renewal genes in stem cells, with TGF-[beta] also shown to increase the self-renewal ability of CSCs. (33,34) To understand whether silencing of E6/E7 would affect HeLa-SFC growth through self-renewal pathways, we examined the expression of TGF-[beta] and LIF genes in transduced HeLa-SFCs. Real-time PCR results showed that expression of all three TGF-[beta] isoforms was significantly decreased (Figure 4a) but the LIF gene was not affected (Figure 4b), suggesting that the self-renewing ability of HeLa-SFCs is associated with the TGF-[beta] pathway. The TGF-[beta] family has a very close relationship with human Ras genes and they interact with each other (reviewed by Grusch et al (35)). To further confirm the downregulation of TGF-[beta], we measured H-Ras and K-Ras expression in LV-shRNA-transduced HeLa-SFCs. The results showed that both H-Ras and K-Ras were significantly decreased in the 18E6-1 group compared with the control 16E7-2 (Figure 4c), supporting the reduction of TGF-[beta] after E6/E7 silencing with 18E6-1 shRNA.

**[FIGURE 3 OMITTED]**
Discussion

Because of the special role of CSCs in tumor initiation, maintenance and drug resistance, they are considered as important targets for future cancer treatments. However, as these cells can easily gain resistance to chemo/radiotherapies, these conventional methods will not be effective for further treatment of these cells. RNAi-based therapies have shown great potential for the treatment of diseases such as viral infections, genetic disorders and cancers. We thus investigated whether silencing oncogenes expressed by CSCs would significantly affect their growth and self-renewal ability. To achieve this, we first isolated and characterized cervical cancer HeLa-SFCs and found that they exhibited CSC features. We then showed that they expressed 6.9 times more HPV E6 than their parental HeLa cells, and that silencing the E6 oncogene had a profound effect on their cell growth and self-renewal ability. We also showed that the inhibitory effect was associated with the downregulation of self-renewal gene TGF-[beta] and other oncogenes such as Ras. To our knowledge, this is the first report on CSC oncogene silencing using RNAi. The data suggest the potential of RNAi-based therapy to target CSCs.

[FIGURE 4 OMITTED]

Previously, we showed that silencing of HPV E6/E7 with LV-18E6-1 could effectively inhibit HeLa cell growth in vitro and in vivo and could significantly decrease tumor nodules in a mouse lung metastasis model. (15) According to the current study, the action of LV-18E6-1 in the previous study may have been due to its ability to target cervical CSCs; this may be especially important in the lung metastasis model, as CSCs are reported to be responsible for initiating metastatic tumors. (30) These data suggest that RNAi-based therapy may be used as a sole therapy, as it targets both CSCs and differentiated cancer cells by silencing their oncogenes. Alternatively, it could be used in combinational therapies with chemotherapy or radiotherapy to further target the resistant CSCs and improve treatment outcomes.

To confirm the gene-silencing effect in SFCs, we measured HPV E6 and E7 expression levels in transduced HeLa-SFCs (Figure 3d). Compared with the inhibitory effect on sphere formation and cell growth, the decrease of E6 and E7 levels was not as profound as expected, though the statistical analysis showed significance (P<0.05 or 0.01; Figure 3d). One possibility is the sample was taken after the period of sphere culture (which normally takes 8-10 days) following transduction, and this time point might have passed the peak gene-silencing time; according to our previous study, the peak protein-inhibitory effect of 18E6-1 shRNA was around 1 week. (15) Another possibility is that the shRNA might silence the gene expression by partially blocking protein translation rather than cleaving the mRNA.

The TGF-[beta] family regulates cell survival, proliferation, differentiation and adhesion. (36,37) TGF-[beta] is also reported to be an important self-renewal factor for embryonic stem cells, (38) to promote CSC self-renewal (33) and to increase the self-renewal ability of glioma-initiating cells. (34) In the current study, we observed that after silencing of E6/E7, expression of all three isoforms of TGF-[beta] was significantly decreased in HeLa-SFCs, whereas that of another self-renewal gene, LIF, remained unchanged. This result suggests that E6/E7 silencing has a specific effect on expression of the self-renewal gene, TGF-[beta]. As TGF-[beta] isoforms are reported to have slightly different roles in different cancers, (39) the result reveals that the silencing of E6/E7 may activate a general mechanism that downregulates the expression of all three isoforms of TGF-[beta]. Although the mechanisms...
are not clear, E6 and E7 are known to promote cancer cell growth through the PI3K/ Akt pathway, (40,41) and reduce apoptosis through the p53 pathway. (20,42) TGF-[beta] also promotes CSC growth and one of its downstream targets is PI3K/Akt. (43) We postulate that downregulation of the PI3K/Akt pathway after E6/ E7 silencing is the link that indirectly affects TGF-[beta] expression, but this needs further investigation.

To prove the downregulation of TGF-[beta] after E6/E7 silencing, we measured expression of both H-Ras and K-Ras genes, as Ras is a downstream component of the TGF-[beta] pathway. (35) The result confirmed that expression of both genes was decreased in transduced HeLa-SFCs. Furthermore, during epithelial-mesenchymal transition, the TGF-[beta] family and Ras have an even closer relationship as TGF-[beta] has been shown to promote tumorigenicity only in Ras-expressing cells. (44) These data further support the conclusion that TGF-[beta] regulates Ras gene expression, especially in the tumorigenic epithelial-mesenchymal transition cancer cells, and the decrease of Ras gene expression is due to the downregulation of TGF-[beta]. The decreased Ras expression could then interfere with the growth of HeLa-SFCs after E6/E7 silencing. Although a previous study showed that TGF-[beta] regulates self-renewal of glioma-initiating cells (or CSCs) through LIF, (34) we did not observe similar changes in LIF, suggesting that different cancer models may utilize different self-renewal pathways.

Since the first report of sphere culture in 2003, (27) this method has been used by many laboratories for isolating CSCs from different cancers, with these cells shown to be multi-drug resistant, more tumorigenic and more metastatic. (28,45,46) An advantage of this method over other methods is that it does not involve the cell-sorting procedure and limits the damages to the cells. Furthermore, as this method is selective for self-renewal, a definitive character of stem cells, (47) there is a better chance to isolate CSCs. As shown in the present study, this method can also be used to isolate CSCs from cancer cell lines, which has some advantages over isolation from primary tumors. For example, cancer cell lines can be easily treated with chemotherapeutic drugs in vitro to increase sphere formation rates. (46) Our data suggest that cisplatin-treated HeLa cells can increase the sphere formation to 27%, whereas doxycyclin-treated HeLa can reach 96% (unpublished data). Another important advantage is that isolating CSCs from cancer cell lines may avoid potential contamination with normal stem cells.

To summarize, this study shows a new approach to target CSCs for future cancer therapy. At least in this cervical cancer model, targeting oncogenes E6/E7 has profound effects on cancer stem-like cell growth and self-renewal ability, which are mediated through the TGF-[beta] pathway. The sphere culture method can be effective in enriching CSCs from cancer cell lines, and this can be further enhanced by treating the cell lines with chemotherapeutic drugs. Future investigations in this direction may lead to a better understanding of CSCs and, ultimately, better cancer treatments by targeting CSCs.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Table 1 Xeno-transplant tumors in NOD/SCID mice at 22 days after injection of HeLa-SFCs

<p>| Injected cell numbers | 1 x [10.sup.3] | 1 x [10.sup.4] | 1 x [10.sup.5] | 1 x [10.sup.6] |</p>
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<td>2/3</td>
<td>3/3</td>
<td>ND</td>
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<tr>
<td>HeLa</td>
<td>0/3</td>
<td>0/3</td>
<td>1/3</td>
<td>3/3</td>
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</tbody>
</table>

Abbreviations: ND, not detected; NOD/SCID, non-obese diabetic, severe combined immunodeficient; SFC, sphere-forming cell.

Table 2 Silencing of E6E7 affects sphere formation and cell growth (ratio)

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<td>115 [+ or -] 7.79 ***</td>
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</table>

Abbreviations: SFC, sphere-forming cell; SP, sphere.
* P < 0.05; *** P < 0.001.

Notes: The experiment was repeated 3-4 times and the data were expressed as mean [+ or -] s.e. The initial cell numbers plated into the culture was 2 x 104.

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