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Abstract

Purpose: Because chemoradiotherapy selectively targets proliferating cancer cells, quiescent cancer stem–like cells are resistant. Mobilization of the cell cycle in quiescent leukemia stem cells sensitizes them to cell death signals. However, it is unclear that mobilization of the cell cycle can eliminate quiescent cancer stem–like cells in solid cancers. Thus, we explored the use of a genetically-engineered telomerase-specific oncolytic adenovirus, OBP-301, to mobilize the cell cycle and kill quiescent cancer stem–like cells.

Experimental Design: We established CD133+ cancer stem–like cells from human gastric cancer MKN45 and MKN7 cells. We investigated the efficacy of OBP-301 against quiescent cancer stem–like cells. We visualized the treatment dynamics of OBP-301 killing of quiescent cancer stem–like cells in dormant tumor spheres and xenografts using a fluorescent ubiquitination cell-cycle indicator (FUCCI).

Results: CD133+ gastric cancer cells had stemness properties. OBP-301 efficiently killed CD133+ cancer stem–like cells resistant to chemoradiotherapy. OBP-301 induced cell-cycle mobilization from G0–G1 to S/G2/M phases and subsequent cell death in quiescent CD133+ cancer stem–like cells by mobilizing cell-cycle–related proteins. FUCCI enabled visualization of quiescent CD133+ cancer stem–like cells and proliferating CD133– cancer–like cells. Three-dimensional visualization of the cell-cycle behavior in tumor spheres showed that CD133+ cancer stem–like cells maintained stemness by remaining in G0–G1 phase. We showed that OBP-301 mobilized quiescent cancer stem–like cells in tumor spheres and xenografts into S/G2/M phases where they lost viability and cancer stem–like cell properties and became chemosensitive.

Conclusion: Oncolytic adenoviral infection is an effective mechanism of cancer cell killing in solid cancer and can be a new therapeutic paradigm to eliminate quiescent cancer stem–like cells. Clin Cancer Res; 19(23); 6495–505. ©2013 AACR.

Introduction

Current cytotoxic chemoradiotherapy selectively targets proliferating cancer cells. Quiescent or dormant cancer cells in contrast are often drug-resistant and are a major impediment to cancer therapy (1, 2). Cancer stem–like cells or tumor-initiating cells (3–5) maintain a quiescent or dormant state, which appears to contribute to their resistance to conventional therapies (6–8). Recently, several therapeutic strategies have targeted inhibition of the cancer stem–like cell quiescent state. For example, treatment with arsenic trioxide enhanced the sensitivity of leukemia stem cells (LSC) to cytosine arabinoside through inhibition of LSC quiescence (9). Acute myeloid leukemia stem cells can be induced to enter the cell cycle and apoptosis by treatment with granulocyte colony-stimulating factor (10). However, it is still unclear whether cancer stem–like cells in solid tumors can also be eliminated by inducing them to cycle.

Viruses can infect target cells, multiply, cause cell death, and release viral particles. These features enable the use of viruses as anticancer agents that induce specific tumor lysis (11, 12). Adenoviral E1A, in particular, has been shown to exert tumor-suppressive functions, including enhancement of chemoradiotherapy-induced apoptosis via inhibition of the cellular DNA repair machinery (13).
Translational Relevance

Current chemotherapy and radiotherapy target proliferating cancer cells, while having little effect on dormant cancer cells. Cancer stem–like cells can maintain a quiescent or dormant state, which contributes largely to their resistance to conventional therapies. Recently, several therapeutic strategies have targeted inhibition of the quiescent state in leukemia stem cells. However, it is still unclear whether cancer stem–like cells in solid tumors can also be eliminated by inhibition of their dormant state. Here, we show that a telomerase-specific adenovirus, OBP-301, mobilizes quiescent cancer stem–like cells to cycle and lethally traps them into S-phase. Moreover, we showed by spatiotemporal treatment dynamics that OBP-301 decoyed quiescent cancer stem–like cells in tumor spheres and xenografts into an S-phase trap where they lost viability and cancer stem–like cell properties and become chemosensitive. Thus, our data demonstrated that cell-cycle mobilization and S/G2/M phase trapping induced by adenoviral infection is an effective mechanism of killing cancer stem–like cells in solid cancers.

and inhibition of cell proliferation via suppression of EGFR receptor (EGFR; ref. 14) and HER-2 (15). It has been recently reported that an oncolytic adenovirus efficiently eradicates cancer stem–like cells as well as non–cancer stem–like cells in brain tumors, breast cancer, and esophageal cancer (16–18).

In the present study, we isolated CD133+ subpopulations from radioresistant cells in human gastric cancer cell lines and characterized them as cancer stem–like cells. By using multicolor cell-cycle imaging that color codes the quiescent cancer stem–like cells and proliferating non–cancer stem–like cells, we showed by treatment dynamics that a genetically engineered telomerase-specific oncolytic adenovirus, OBP-301 (19, 20), eradicates dormant CD133+ cancer stem–like cells via cell-cycle mobilization both in tumor spheres and in subcutaneous tumors.

Materials and Methods

Cell lines and radiation treatment

The human gastric cancer cell lines MKN45 and MKN7 were maintained according to the vendor’s specifications (21). Radioresistant MKN45 and MKN7 cells were established by administration of radiation treatments using an X-ray generator (MBR-1505R; Hitachi Medical Co.).

Recombinant adenoviruses

The recombinant tumor-specific, replication-selective adenovirus vector OBP-301 (Telomelysin), in which the promoter element of the human telomerase reverse transcriptase (hTERT) gene drives the expression of E1A and E1B genes linked to an internal ribosome entry site, was previously constructed and characterized (19, 22).

Isolation of CD133+ and CD133− cells by flow cytometry

After incubation with an anti-CD133/2(293C)-allophycocyanin antibody (Miltenyi Biotec), CD133+ cells were sorted by flow cytometry using a FACSArray flow cytometer (Becton Dickinson). CD133+ and CD133− cells were separated by flow cytometry just before each experiment to ensure that the purity of the CD133+ population was greater than 70%, and the purity of CD133− cells was above 99%.

Cell viability assay

CD133+ and CD133− cells (5 × 10^2 cells/well) in 96-well plates were treated with OBP-301, cisplatin, or radiation at the indicated doses. Cell viability was determined on day 5 after treatment using the Cell Proliferation Kit II (Roche Diagnostics) or athymic nude mice (Charles River Laboratories). Plasmids expressing mKO2-hCdt1 or mAG-hGem were transfected into radioresistant MKN45 cells using Lipofectamine LTX (Invitrogen).

Western blot analysis

The primary antibodies used were: mouse anti-CD133/1(W6B3C) monoclonal antibody (mAb; Miltenyi Biotec); rabbit anti-E2F1 polyclonal antibody (pAb) (Santa Cruz Biotechnology); mouse anti-Ad5 E1A mAb (BD Pharmlin-gen); mouse anti-c-Myc pAb, rabbit anti-phospho-Akt mAb, rabbit anti-Akt mAb, mouse anti-p27 mAb (all from Cell Signaling Technology); mouse anti-p53 mAb, mouse anti-p21 mAb (both from CALBIOCHEM Merck4 Biotechnology); and mouse anti-β-actin mAb (Sigma-Aldrich). Immunoreactive bands on the blots were visualized using enhanced chemiluminescence substrates (ECL Plus; GE Healthcare).

Subcutaneous MKN45 tumor xenograft model

To evaluate the tumorigenicity of CD133+ and CD133− cells, purified CD133+ and CD133− cells from radioresistant MKN45 were inoculated at a density of 1 × 10^5 cells/site on the right and left sides, respectively, of the flank of 5-week-old female NOD/SCID mice (Charles River Laboratories) or athymic nude mice (Charles River Laboratories). To evaluate the in vivo antitumor efficacy of OBP-301, cisplatin, or radiation, the radioresistant MKN45 cells were inoculated at a density of 5 × 10^5 cells/site into the flank of 5-week-old female athymic nude mice. OBP-301 [1 × 10^8 plaque-forming units (PFU)] was injected into the tumors. Cisplatin (4 mg/kg body weight) was intraperitoneally injected and ionizing radiation (2 Gy) was administered to tumors after protection of normal tissues. Mice were treated every 3 days for a total of three treatments.
Imaging of MKN45 cells expressing cell-cycle–dependent fluorescent proteins

Time lapse images of FUCCI-expressing CD133+ and CD133− radioresistant MKN45 cells were acquired using a confocal laser scanning microscope (FV10i; Olympus). Cross-sections of FUCCI-expressing tumors were imaged using a confocal laser scanning microscope (FV-1000; Olympus).

Treatment of subcutaneous FUCCI-expressing MKN45 tumors

To evaluate the in vivo antitumor efficacy of OBP-301, cisplatin, paclitaxel, or their combination, the FUCCI-expressing MKN45 and MKN7 human gastric cancer cells. Radioresistant MKN45 and MKN7 human gastric cancer cells. Radioresistant MKN45 and MKN7 cells had a significantly higher percentage of CD133+ cells than parental cells (Fig. 1A and Supplementary Fig. S1A). We hypothesized that CD133+ cells could not produce CD133+ cells (Supplementary Fig. S2). We compared in vitro proliferation of CD133+ and CD133− cells. CD133+ cells produced larger colonies than CD133− cells (Fig. 1B and Supplementary Fig. S3). CD133+ cells made significantly much more tumor spheres than CD133− cells (Fig. 1B). CD133− cells produced tumors in immunodeficient nude mice and NOD/SCID mice, whereas CD133+ cells did not generate tumors in either nude on NOD/SCID mice (Supplementary Fig. S4 and Fig. 1B). Furthermore, CD133+ cells in radioresistant MKN45 and MKN7 cells were significantly more resistant to 5-fluorouracil, cisplatin, paclitaxel, and radiation than CD133− cells (Fig. 1C and Supplementary Fig. S1B). These data indicate that CD133+ cells are cancer stem–like.

Quiescent CD133+ cancer stem–like cells and cycling CD133− non–cancer stem–like cells are independently visualized by fluorescent cell-cycle indicator technology

Sakaue-Sawano and colleagues have reported that the cell-cycle state in viable cells can be visualized using the FUCCI system (23). We established FUCCI-expressing CD133+ or CD133− radioresistant MKN45 cells, in which cell nuclei in G0–G1, S, or G2–M phases exhibit red, yellow, or green fluorescence, respectively. We compared the cell-cycle phase of FUCCI-expressing CD133+ or CD133− cells. Time-lapse imaging showed that most of CD133+ cells were quiescent in G0–G1 phase with red fluorescent nuclei compared with CD133− cells (Fig. 1D). Similar results were also observed in flow cytometric analysis of the cell cycle (Supplementary Fig. S5A and S5B). CD133+ cells had similar proliferation rates as CD133− cells until 3 days after seeding. CD133− cells showed lower proliferation rates than CD133+ cells 5 days after seeding (Fig. 1D). This result was consistent with the cell-cycle status of CD133+ cells which had an increased percentage of cells in G0–G1 phase. Moreover, we examined cell-cycle–related protein (27) expression in CD133+ and CD133− cells. CD133+ cells showed higher expressions of p53, p21, and p27 proteins compared with CD133− cells (Supplementary Fig. S8), suggesting that these proteins are involved in the maintenance of quiescent cancer stem–like cells.

Results

CD133+ cells in human gastric cancer cells are cancer stem–like

Cancer stem–like cells are more resistant to radiotherapy than non–cancer stem–like cells (24–26). To enrich cancer stem–like subpopulations, we established radioresistant MKN45 and MKN7 human gastric cancer cells. Radioresistant MKN45 and MKN7 cells had a significantly higher percentage of CD133+ cells than parental cells (Fig. 1A and Supplementary Fig. S1A). We hypothesized that CD133+ gastric cancer cells would identify cancer cells with stem–like properties, such as asymmetric cell division, in vitro proliferation, dormancy, sphere formation, and in vivo tumorigenicity (5, 6). To investigate the asymmetric division of CD133+ cells, we determined whether CD133+ cells produce both CD133+ and CD133− cells. CD133+ cells generated both CD133+ and CD133− cells, whereas CD133− cells could not produce CD133+ cells (Supplementary Fig. S2). We compared in vitro proliferation of CD133+ and CD133− cells. CD133+ cells produced larger colonies than CD133− cells (Fig. 1B and Supplementary Fig. S3). CD133+ cells made significantly much more tumor spheres than CD133− cells (Fig. 1B). CD133− cells produced tumors in immunodeficient nude mice and NOD/SCID mice, whereas CD133+ cells did not generate tumors in either nude on NOD/SCID mice (Supplementary Fig. S4 and Fig. 1B). Furthermore, CD133+ cells in radioresistant MKN45 and MKN7 cells were significantly more resistant to 5-fluorouracil, cisplatin, paclitaxel, and radiation than CD133− cells (Fig. 1C and Supplementary Fig. S1B). These data indicate that CD133+ cells are cancer stem–like.
CD133+ cancer stem–like cells in human gastric cancer exhibit cancer stem–like cell properties and are more quiescent. A, flow cytometric analysis of CD133 expression in parental (P) and radioresistant (R) MKN45 cells. Representative dot plots (top) and data from 5 experiments (bottom) are shown. B, CD133+ MKN45 cancer cells exhibit cancer stem–like properties. Representative images of colonies from CD133+ or CD133− cells. Histogram shows the size of colonies from CD133+ or CD133− cells (left). Quantitative measurement of the tumor sphere–forming potential of CD133+ and CD133− cells (middle). Representative images of tumor spheres derived from CD133+ and CD133− cells. Histogram shows the numbers of tumor spheres from CD133+ or CD133− cells. Scale bars, 500 μm. C, sensitivity of CD133+ and CD133− cells to 5-fluourouracil, cisplatin, paclitaxel, and irradiation. D, time-lapse imaging of CD133+ and CD133− cells from radioresistant MKN45 cells expressing cell-cycle–dependent fluorescent proteins (FUCCI). The cells in G0–G1, S, or G2–M phases appear red, yellow, or green, respectively. Histogram shows the cell-cycle phase of FUCCI-expressing CD133+ and CD133− cells cultured for 48 hours after sorting (bottom left). The percentage of cells in G0–G1, S, and G2–M phases are shown. Cell proliferation rate of CD133+ and CD133− cells (bottom right). Scale bars, 50 μm. Data are shown as means ± SD (n = 5). *P < 0.01.

PCR (qRT-PCR) showed that CD133+ cells had a significant, 3-fold higher expression of hTERT mRNA than CD133− cells (Fig. 2B), suggesting that CD133+ cancer stem–like cells have a higher activity of hTERT than CD133− cells. Next we compared the expression of E1A mRNA and E1A protein in CD133+ cells and in CD133− cells. qRT-PCR showed that the expression of E1A mRNA in CD133+ cells was higher than that in CD133− cells (Fig. 2B). Western blotting showed that the expression of E1A in CD133+ cells was higher than that in CD133− cells (Fig. 2B). Furthermore, we compared the copy number of the E1A gene, which is indicative of viral replication, in CD133+ and CD133− cells after infection with OBP-301. As expected, the copy number of the E1A gene in CD133+ cells was significantly higher than that in CD133− cells (Fig. 2B). These data indicate that OBP-301 is efficiently cytopathic for CD133− cells due to enhanced viral replication.

OBP-301 mobilizes and lethally traps quiescent cancer stem–like cells into S-phase in monolayer culture

To examine whether OBP-301 could change the cell-cycle phase of quiescent CD133+ cells, we treated FUCCI-expressing CD133+ cells with OBP-301. Time-lapse imaging showed that OBP-301 infection significantly decreased the percentage of CD133+ cells in G0–G1 phase, increased the percentage of CD133+ cells in S-phase, and killed them in S-phase (Fig. 2C and Supplementary Movie S1). Similar results were also observed in flow cytometric analysis of the cell cycle (Supplementary Fig. S5C and S5D). These results suggest that OBP-301 induces cell-cycle activation of quiescent CD133+ cells from G0–G1 phase to S-phase and kills them. We next assessed the molecular mechanism by which OBP-301 induces mobilization of the cell cycle in quiescent cancer stem–like cells. OBP-301 increased the expression of E2F1, c-Myc, and phospho-Akt proteins that function as
enhanced viral replication. A, OBP-301 efficiently kills CD133\(^+\) cancer stem–like cells. Left, viability of CD133\(^+\) and CD133\(^-\) MKN45 cells after OBP-301 infection. Right, CD133-positive ratio of MKN45 cells treated with OBP-301, cisplatin, or radiation was analyzed by flow cytometry. B, OBP-301 can replicate more in CD133\(^+\) cells that have more hTERT activity than in CD133\(^-\) cells. Expression of hTERT mRNA in CD133\(^+\) and CD133\(^-\) MKN45 cells assessed by qRT-PCR (top left). The relative levels of hTERT mRNA were calculated after normalization with reference to the expression of PBGD mRNA. Expression of E1A mRNA in CD133\(^+\) and CD133\(^-\) MKN45 cells after OBP-301 infection at an MOI of 10 PFU/cell for 2 hours. Expression of E1A mRNA was analyzed over the following 3 days by qRT-PCR (top right). The relative levels of E1A mRNA were calculated after normalization with reference to the expression of GAPDH mRNA. Western blot analysis of E1A expression in CD133\(^+\) and CD133\(^-\) MKN45 cells treated with OBP-301 for 48 hours (bottom left). Quantitative relative expression level of E1A protein, normalized to \(\beta\)-actin, using NIH ImageJ software (bottom left). Quantitative measurement of viral DNA replication in CD133\(^+\) and CD133\(^-\) MKN45 cells after OBP-301 infection at an MOI of 10 PFU/cell for 2 hours (bottom right). E1A copy number was analyzed over the following 3 days using qPCR. C, time-lapse imaging of FUCCI-expressing CD133\(^+\) and CD133\(^-\) cells treated with OBP-301 at an MOI of 20 PFU/cell. The cells in G\(_0\) were imaged over the following 3 days using FUCCI-expressing CD133\(^+\) cells treated with OBP-301 for 48 hours. The percentage of cells in G\(_0\), G\(_1\), S, and G\(_{2}\)M phases are shown. D, Western blot analysis of E2F1, c-Myc, phospho-Akt, Akt, p53, p21, and p27 expression in CD133\(^+\) cells treated with OBP-301, cisplatin, or radiation for 48 hours. \(\beta\)-Actin was assayed as a loading control for all experiments. Data are shown as means \(\pm\) SD (\(n = 5\)). *, \(P < 0.05\); **, \(P < 0.01\).

Figure 2. OBP-301 lethally induces S-phase transition of quiescent CD133\(^+\) cancer stem–like cells and decreases cancer stem–like cell frequency via enhanced viral replication. A, OBP-301 efficiently kills CD133\(^+\) cancer stem–like cells. Left, viability of CD133\(^+\) and CD133\(^-\) MKN45 cells after OBP-301 infection. Right, CD133-positive ratio of MKN45 cells treated with OBP-301, cisplatin, or radiation was analyzed by flow cytometry. B, OBP-301 can replicate more in CD133\(^+\) cells that have more hTERT activity than in CD133\(^-\) cells. Expression of hTERT mRNA in CD133\(^+\) and CD133\(^-\) MKN45 cells assessed by qRT-PCR (top left). The relative levels of hTERT mRNA were calculated after normalization with reference to the expression of PBGD mRNA. Expression of E1A mRNA in CD133\(^+\) and CD133\(^-\) MKN45 cells after OBP-301 infection at an MOI of 10 PFU/cell for 2 hours. Expression of E1A mRNA was analyzed over the following 3 days by qRT-PCR (top right). The relative levels of E1A mRNA were calculated after normalization with reference to the expression of GAPDH mRNA. Western blot analysis of E1A expression in CD133\(^+\) and CD133\(^-\) MKN45 cells treated with OBP-301 for 48 hours (bottom left). Quantitative relative expression level of E1A protein, normalized to \(\beta\)-actin, using NIH ImageJ software (bottom left). Quantitative measurement of viral DNA replication in CD133\(^+\) and CD133\(^-\) MKN45 cells after OBP-301 infection at an MOI of 10 PFU/cell for 2 hours (bottom right). E1A copy number was analyzed over the following 3 days using qPCR. C, time-lapse imaging of FUCCI-expressing CD133\(^+\) and CD133\(^-\) cells treated with OBP-301 at an MOI of 20 PFU/cell. The cells in G\(_0\) were imaged over the following 3 days using FUCCI-expressing CD133\(^+\) cells treated with OBP-301 for 48 hours. The percentage of cells in G\(_0\), G\(_1\), S, and G\(_{2}\)M phases are shown. D, Western blot analysis of E2F1, c-Myc, phospho-Akt, Akt, p53, p21, and p27 expression in CD133\(^+\) cells treated with OBP-301, cisplatin, or radiation for 48 hours. \(\beta\)-Actin was assayed as a loading control for all experiments. Data are shown as means \(\pm\) SD (\(n = 5\)). *, \(P < 0.05\); **, \(P < 0.01\).

Three-dimensional tumor spheres maintain a CD133\(^+\) subpopulation by remaining quiescent.

Formation of tumor spheres under serum-free conditions is frequently used to maintain cancer stem–like cell subpopulations (28). The addition of serum makes floating undifferentiated tumor spheres adherent and their cells differentiate into adherent cells (29). Therefore, we hypothesized that tumor spheres maintained their cancer stem–like cell frequency due to quiescence.

cell-cycle accelerators (27) and decreased the expression of p53, p21, and p27 proteins that function as cell-cycle brakes (27) in quiescent CD133\(^+\) cells (Fig. 2D). In contrast, cisplatin and radiation increased the expression of p53 and p21 proteins (Fig. 2D). We further examined whether adenoviral E1A altered the expression of these proteins in CD133\(^+\) cells. E1A-expressing OBP-301 and Ad5, but not E1A-deficient dl312, similarly altered the expression of these proteins in CD133\(^+\) cells (Supplementary Fig. S9). These results indicate that OBP-301 induces cell-cycle progression through upregulation of E2F-related proteins and downregulation of p53-related and p27 proteins by enhanced adenoviral E1A in quiescent cancer stem–like cells.

Three-dimensional tumor spheres maintain a CD133\(^+\) subpopulation by remaining quiescent.

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CD133+ cells aggregated and formed tumor spheres, and arrested in G0–G1 phase (Fig. 3A). Tumor spheres formed from CD133+ cells contained more quiescent cells than those formed from CD133− cells (Fig. 3B). Moreover, established tumor spheres formed from CD133+ cells remained quiescent in 3-dimensional culture without serum (Fig. 3C). In contrast, established tumor spheres, after addition of serum, exited from the quiescent state and began to cycle, divide, and increase (Fig. 3C and Supplementary Movie S2). Flow cytometric analysis showed that CD133+ cells could be maintained in tumor spheres cultured in serum-free medium for 2 weeks, whereas the percentage of CD133+ cells significantly decreased in monolayer cultures or in tumor spheres cultured in serum-containing medium (Fig. 3D). These data indicate that tumor spheres maintain their cancer stem-cell frequency by remaining dormant.

**Real-time imaging spatiotemporally shows OBP-301 eliminates dormant tumor spheres by cell-cycle mobilization and S/G2/M phase trapping**

To further evaluate OBP-301-induced cell-cycle mobilization and S-phase trapping in dormant tumor spheres, we visualized the treatment dynamics of FUCCI-expressing tumor spheres infected with OBP-301. Time-lapse imaging showed that OBP-301 infected quiescent CD133+ cells at the periphery of the spheres and then induced S and G2–M
Elimination of Quiescent Cancer Stem–like Cells by Adenovirus

OBP-301 efficiently eradicates dormant tumor spheres resistant to conventional therapies by mobilizing them into an S/G2/M phase trap.

OBP-301 efficiently kills dormant cancer stem–like cells in established human tumor xenografts by cell-cycle mobilization and S/G2/M phase trapping, thereby reducing cancer stem–like cell frequency

To further confirm whether OBP-301 efficiently reduced CD133+ cancer stem–like cell frequency within tumor tissues (Supplementary Fig. S10A), we investigated the expression of CD133 mRNA and the CD133-positive ratio in subcutaneous tumors derived from radioresistant MKN45 cells after treatment of OBP-301, cisplatin, or irradiation. Suppression of tumor growth by OBP-301 (Fig. 5A) was accompanied by a significant decrease in CD133 mRNA at 2 weeks after the final treatment (Fig. 5B). In contrast, although cisplatin and radiation also suppressed tumor growth to a similar extent as OBP-301 (Fig. 5A), cisplatin did not affect, and radiation significantly increased CD133 mRNA expression at 1 week after the final treatment (Fig. 5B). Immunohistochemistry of CD133-stained tumor sections also showed that OBP-301 reduced the frequency of CD133+ cells, whereas cisplatin and irradiation increased the frequency compared with control (Fig. 5B).

Next, we visualized treatment dynamics in established FLUCI-expressing MKN45 tumor xenografts with or without OBP-301 infection (Supplementary Fig. S10B). FLUCI-expressing MKN45 tumors had a distribution of cancer cells in G0–G1, S, and G2–M phases (Fig. 5A). As tumors grew bigger, cancer cells in G0–G1 phase increased (Fig. 5C), whereas cisplatin and irradiation increased the frequency compared with control (Fig. 5D), indicating the existence of dormant cancer cells.

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Figure 4. Visualization of elimination of dormant tumor spheres by virus infection. A, time-lapse images of tumor spheres treated with OBP-301 (5 × 10^6 PFU), cisplatin (10 μmol/L), or radiation (10 Gy). The cells in G0–G1, S, or G2–M phases appear red, yellow, or green, respectively. B, histogram shows the cell-cycle phases of the spheres with OBP-301, cisplatin, or radiation. The percentage of cells in G0, G1, S, and G2–M phases are shown. C, representative images of control, OBP-301-, cisplatin-, or radiation-treated spheres (top). Histogram shows the relative cell viability of treated tumor spheres (bottom). D, the CD133+ tumor spheres treated as above were stained for E2F1, phospho-Akt, p53, and p21. Immunofluorescence staining was visualized by confocal laser microscopy. Scale bars, 100 μm. Data are shown as means ± SD (n = 5). *, P < 0.01.

phase entry, leading to cellular death by viral replication (Fig. 4A). Moreover, OBP-301 penetrated into the deeper layers, tumor spheres gradually shrunk after virus infection (Fig. 4A and C). In contrast, cisplatin and radiation did not affect the cell-cycle phase or the size of tumor spheres (Figs. 4A–C and Supplementary Movie S3). Immunofluorescence staining of tumor spheres also confirmed that OBP-301 infection downregulated CD133, p53, and p21 expression and upregulated E2F1 and phospho-Akt expression in tumor spheres (Fig. 4D). These results suggest that OBP-301 efficiently eradicates dormant tumor spheres resistant to conventional therapies by mobilizing them into an S/G2/M phase trap.
Figure 5. OBP-301 induces cell-cycle progression and efficiently kills dormant cancer cells resistant to conventional therapy in established human tumor xenografts. CD133<sup>+</sup>-rich radioresistant MKN45 cells (5 × 10<sup>6</sup> cells/mouse) were injected subcutaneously into the left flanks of mice. When the tumors reached approximately 6 mm in diameter (tumor volume, 100–120 mm<sup>3</sup>), mice were administered OBP-301 intratumorally (1 × 10<sup>8</sup> PFU/tumor), injected intraperitoneally with cisplatin (4 mg/kg), or exposed to 2 Gy of radiation for 3 cycles every 3 days. A, growth curves of tumors derived from radioresistant MKN45 cells after treatment with OBP-301, cisplatin, or radiation. Black arrows indicate the day of treatment. B, expression of CD133 mRNA in tumors treated with OBP-301, cisplatin, or radiation at 1, 2, and 3 weeks after treatment (top). Representative images of CD133-stained tumor section treated with OBP-301, cisplatin, or radiation (bottom left). Scale bars, 100 μm. Histogram shows the percentages of CD133<sup>+</sup> cells in tumors treated with OBP-301, cisplatin, or radiation (bottom right). The percentage of CD133<sup>+</sup> cells was calculated by dividing the number of CD133<sup>+</sup> cells by the total number of cells. Data are shown as means ± SD (n = 3), *, P < 0.05. C and D, FUCCI-expressing MKN45 cells (5 × 10<sup>6</sup> cells/mouse) were injected subcutaneously into the left flanks of mice. When the tumors reached approximately 7 mm in diameter (tumor volume, 150–180 mm<sup>3</sup>), mice were administered OBP-301 intratumorally (1 × 10<sup>8</sup> PFU/tumor), injected intraperitoneally with cisplatin (4 mg/kg) or paclitaxel (5 mg/kg) for 3 cycles every 3 days. Representative images of cross-sections of FUCCI-expressing MKN45 subcutaneous tumors of control, OBP-301-, cisplatin-, or paclitaxel-treated mice (left). The cells in G<sub>0</sub>–G<sub>1</sub>, S, or G<sub>2</sub>–M phases appear red, yellow, or green, respectively. Histogram shows the cell-cycle phase of FUCCI-expressing MKN45 subcutaneous tumor from control, OBP-301-, cisplatin-, or paclitaxel-treated mice (right). The percentage of cells in G<sub>0</sub>–G<sub>1</sub>, S, and G<sub>2</sub>–M phases are shown. Data are shown as means ± SD (n = 5), *, P < 0.05. Scale bars, 500 μm.
After cisplatin or paclitaxel treatment, the tumor consisted mostly of red fluorescent cells (Fig. 5D), indicating that the cytotoxic agents killed only cycling cancer cells and had little effect on quiescent dormant cancer cells. These tumors regrew, with the quiescent cells re-entering the cell cycle 21 days after last treatment (Fig. 5D). In contrast, intratumor injection of OBP-301 mobilized the cancer cells into the S/G2/M phase trap, leading to elimination of cancer cells in S/G2/M phases (Fig. 5D). These data indicate that OBP-301 could efficiently kill quiescent cancer stem–like cells in tumors by inducing cell-cycle progression.

**OBP-301 sensitizes quiescent cancer stem–like cells to chemotherapy by cell-cycle mobilization and S/G2/M phase trapping**

As we previously showed that OBP-301 enhances the sensitivities to chemotherapeutic agents in various types of human cancer cells (30, 31), we further evaluated whether OBP-301 sensitizes quiescent CD133+ cancer stem–like cells to chemotherapy by inducing cell-cycle progression and S/G2/M phase trapping. OBP-301 infection significantly enhanced the inhibitory effect of chemotherapy on cell viability and tumor sphere formation of CD133+ cells (Fig. 6A and Supplementary Data).
Cancer stem–like cells have been shown to be highly resistant to chemotherapeutic agents (32, 33) and ionizing radiation (24–26). As expected, CD133⁺ human gastric cancer cells were more resistant to conventional therapies than CD133⁻ cells; OBP-301, however, efficiently reduced the viability of CD133⁺ cells, similar to their reduction of viability of CD133⁻ cells. Moreover, we showed that OBP-301 significantly reduced the stem cell properties of CD133⁺ cells in vitro and in vivo compared with conventional chemoradiotherapy and further sensitized CD133⁺ cancer stem–like cells to chemotherapy. These findings indicate that OBP-301 is a promising anticancer therapy to eliminate cancer stem–like cells more efficiently than conventional therapy in the clinical setting.

Recent studies have showed that p53 and p21⁰/waf1 maintain the quiescent state in hematopoietic stem cells (34, 35). Moreover, p21⁰/waf1 has been suggested to be involved in suppression of the transition from the G₀ phase to G₁–S phases (36, 37). Cancer stem–like cells maintain a more quiescent state than non–cancer stem–like cells, which is associated with cancer stem–like cell resistance to conventional therapies (9, 10). OBP-301 induced S and G₂–M phase entry and subsequent cell death in quiescent CD133⁺ cells through upregulation of E2F1-related proteins and downregulation of p53-related and p27 proteins in an E1A-dependent manner. A recent report suggested that suppression of the p53-mediated G₁ checkpoint is required for E2F1-induced S-phase entry (38). Furthermore, adenoviral E1A has been shown to suppress p53-mediated cell-cycle arrest after DNA damage (39). Thus, OBP-301 can inhibit cancer stem–like cells from maintaining a quiescent state and force them into cycling by not only upregulating E2F-related proteins but also downregulating p53-related and p27 proteins (Supplementary Fig. S15B), leading to the sensitization to chemotherapy.

FLUCCI (23) is a powerful tool to visualize the quiescent state in cancer stem–like cells and the treatment dynamics of OBP-301. When tumor spheres were formed, CD133⁺ cells maintained a quiescent state, which was defined by red fluorescent nuclei expressed in G₀–G₁ phases. In contrast, S and G₂–M phase entry induced by OBP-301 could be clearly visualized as yellow and green fluorescent nuclei, respectively. Our data indicate that 3-dimensional cultures are extremely important for the maintenance of the quiescence of CD133⁺ cells. FLUCCI-based real-time imaging of the cell cycle provides a platform for the screening of candidate therapeutic agents that modulate the quiescent state of drug-resistant cancer stem–like cells.

In conclusion, we have clearly shown that a genetically engineered oncolytic adenovirus, OBP-301, efficiently eradicates quiescent cancer stem–like cells in solid tumors by cell-cycle mobilization and S/G₂/M phase trapping. A phase I clinical trial of intratumoral injection of OBP-301 in patients with advanced solid tumors was recently completed and OBP-301 monotherapy was well tolerated by these patients (20). However, the difficulty of adenoviral delivery to inaccessible primary and metastatic tumor tissues is a major obstacle for clinical translation of this treatment modality. In this study, the combination therapy of OBP-301 with chemotherapy was highly effective antitumor therapy to eliminate both cancer stem–like and non–cancer stem–like cells in a xenograft model. Future clinical trials of intratumoral injection of OBP-301 in combination with conventional antitumor therapy are suggested by the results of the present study.

Disclosure of Potential Conflicts of Interest

Y. Urata is President & CEO of Oncoly BioPharma, Inc., the manufacturer of OBP-301 (Telomelyn®). H. Tazawa and T. Fujiwara are consultants of Oncoly BioPharma, Inc. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Yano, H. Tazawa, Y. Hashimoto
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