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Targeted Elimination of Tumorigenic Human Pluripotent Stem Cells Using Suicide-inducing Virus-like Particles

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Abstract

Sensitization to prodrugs via transgenic expression of suicide genes is a leading strategy for the selective elimination of potentially tumorigenic human pluripotent stem cells (hPSCs) in regenerative medicine, but transgenic modification poses safety risks such as deleterious mutagenesis. We describe here an alternative method of delivering suicide-inducing molecules explicitly to hPSCs using virus-like particles (VLPs), and demonstrate its use in eliminating undifferentiated hPSCs *in vitro*. VLPs were engineered from Q β bacteriophage capsids to contain enhanced green fluorescent protein (EGFP) or cytosine deaminase (CD), and to simultaneously display multiple IgG-binding ZZ domains. After labeling with antibodies against the hPSC-specific surface glycan SSEA-5, EGFP-containing particles were shown to specifically bind undifferentiated cells in culture, and CD-containing particles were able to eliminate undifferentiated hPSCs with virtually no cytotoxicity to differentiated cells upon treatment with the prodrug 5-fluorocytosine.

Keywords

human pluripotent stem cells; protein delivery; virus-like particles

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Supporting Information

The Supporting Information includes methods, 3 tables, 4 movies and 3 figures.

INTRODUCTION

Due to their virtually unlimited self-renewal capacity and differentiation potential *in vitro*, human pluripotent stem cells (hPSCs)—comprising human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs)—remain leading candidates for cell-based regenerative therapies.^{1–5} Despite substantial advances in protocol efficiencies, directed differentiation of hPSCs to a desired cell type usually yields a population containing residual undifferentiated and potentially tumorigenic cells. Before widespread clinical adoption of hPSC-based therapies can be achieved, effective strategies for the removal of hPSCs are needed to eliminate the risk of post-transplantation teratoma formation.^{6–8} To this end, making potentially tumorigenic hPSCs sensitive to prodrugs via expression of suicide-inducing genes is considered a promising approach.^{9–14} Transgenic expression of the bacterial gene encoding cytosine deaminase (CD), which enables the conversion of the negligibly toxic prodrug 5-fluorocytosine (5-FC) to the highly cytotoxic 5-fluorouracil (5-FU), has been used to selectively eradicate pluripotent stem cells derived from mice¹² and primates.¹⁴ However, since transgenic modification of cells poses the risk of deleterious mutagenesis, a safer alternative system for specific intracellular delivery of suicide-inducing macromolecules such as CD to viable hPSCs is desirable for clinical applications.

In this regard, virus-like particles (VLPs) offer a promising way to provide both targeting and cytotoxic functions. VLPs are non-infectious, self-assembling nanostructures derived from the recombinant expression of viral capsid proteins.¹⁵ VLPs derived from the Q β bacteriophage capsid can be engineered to encapsulate specific molecular cargoes¹⁶ and to incorporate multiple ZZ domains on the particle exterior as extensions of the coat protein.¹⁷ Derived from *Staphylococcus aureus* protein A, the Z domain binds the Fc region of IgG antibodies; we used a dimeric version (approximately 200 amino acids) first reported by Nilsson, *et al.*¹⁸ hence the “ZZ” designation. VLPs engineered to display ZZ domains can be labeled with multiple copies of any antibody bearing a protein A-binding Fc domain by simple mixing, and are thereby rendered capable of selectively targeting cells with specific cell-surface markers.^{19, 20} Here we describe the use of this targeting method to bring a prodrug-converting enzyme specifically to the undifferentiated stem cells that must be removed prior to administration.

RESULTS AND DISCUSSION

SSEA-5-labeled Q β (ZZ)₆₀@EGFP₄ VLPs Selectively Deliver EGFP Intracellularly to hPSCs.

VLPs are named here as previously described: for example, Q β (ZZ)_{*n*}@EGFP_{*m*} denotes a Q β VLP in which an average of *n* of the 180 capsid protein subunits have the ZZ domain appended to the N-terminus for display on the external surface, and an average of *m* copies of the EGFP protein are encapsidated inside the particle.²¹ In addition, if the surface ZZ domain on the VLPs is bound to an antibody, a + sign appears followed by the antibody's abbreviation. EGFP packaging was engineered by co-transformation of *E. coli* cells with two plasmids, one [designated pET28-CP@EGFP] coding for both the standard capsid protein (CP) and EGFP fused to the Rev peptide to assist in packaging,²¹ and the other [pCDF-CP(ZZ)] coding for the CP-ZZ fusion (Figure 1a). IPTG-triggered expression from both plasmids routinely yielded particles in a yield of 10–15 mg per L of culture with *n* = 60 and

$m = 4$ (Figure 1b, 1c). Characterization by dynamic light scattering revealed a homogeneous hydrodynamic radius (R_h) of 17.2 nm, somewhat larger than the wild-type VLP (14 nm), as expected. Antibody SSEA-5, identified by Weissman, Drukker, and colleagues as a binder of an hPSC-specific fucosylated H-1-type carbohydrate antigen,²² was mixed with these particles to give $Q\beta(ZZ)_{60}@EGFP_4+SSEA-5_{30}$, which showed an increase in apparent R_h to 21.2 nm (Figure 1d), consistent with the presence of no more than a single layer of IgG antibodies on the particle surface. Multiple angle light scattering (MALS) analysis indicated an increase in particle molecular weight from 3.2 MDa to 5.5 MDa, corresponding to occupancy of approximately half of the ZZ domains by IgG molecules (Figure 1e).

$Q\beta(ZZ)_{60}@EGFP_4+SSEA-5_{30}$ particles were incubated (5, 10, and 15 nM in particle, 37°C) with hESC colonies cultured on mouse embryonic fibroblasts (MEFs). After 30 min, EGFP was detected in all the undifferentiated stem cell colonies at all particle concentrations (data not shown). To confirm the antibody-directed nature of EGFP delivery, a blinded experiment was performed in which hESC colonies were again cultured on MEFs and incubated for 30 min at 37°C with 8 nM of unlabeled, IgG1-labeled (κ isotype control), or SSEA-5 labeled $Q\beta(ZZ)_{60}@EGFP_4$. Since MEFs do not express the SSEA-5 binding motif, they were used as a negative cell control. After 30 min, the excess particle-containing supernatant was removed and the cells were rinsed before imaging. Fluorescence images showed green fluorescent stem cell colonies in the wells treated with $Q\beta(ZZ)_{60}@EGFP_4+SSEA-5_{30}$ particles, but virtually no green fluorescence in either the MEFs or the hESCs treated with the unlabeled $Q\beta(ZZ)_{60}@EGFP_4$ or $Q\beta(ZZ)_{60}@EGFP_4+IgG1$ isotype control particles (Figure 1f). In addition, live-cell deconvolution fluorescence microscopy with three-dimensional optical sectioning showed substantial diffuse and intracellular EGFP distribution, when live hESC colonies were incubated with DAPI as a nuclear probe (blue), a membrane-specific wheat germ agglutinin (WGA) dye (red), and 8 nM of $Q\beta(ZZ)_{60}@EGFP_4+SSEA-5_{30}$ particles (green) (Figure 1g). These results demonstrate that $Q\beta(ZZ)_{60}@EGFP_4+SSEA-5_{30}$ particles selectively target, and appear to be taken up by, undifferentiated stem cells while exhibiting negligible non-specific binding.

SSEA-5-labeled $Q\beta(ZZ)_{42}@CD_{13}$ VLPs Selectively Kill hPSCs in the Presence of 5-FC.

Targeted VLPs with cytotoxic capability were generated as above, replacing the packaged EGFP protein with a thermostable variant of yeast cytosine deaminase (CD).²³ The resulting $Q\beta(ZZ)_{42}@CD_{13}$ particles were addressed with SSEA-5 in the same manner, resulting in very similar yield and physical parameters as the EGFP-containing particles (Figure 2a-2d).

The cytotoxic activity of the CD-containing particles was verified first on monocultures of hiPSCs and hESCs, which were incubated for 24 h with titrating concentrations of either 8 nM $Q\beta(ZZ)_{42}@CD_{13}+SSEA-5_{21}$ VLPs in the presence of the prodrug 5-fluorocytosine (5-FC), or its product, 5-fluorouracil (5-FU), alone as a positive cytotoxicity control. Survival percentages for each treatment were determined using a tetrazolium salt-based assay, and decreased in a dose-dependent manner in both hiPSCs and hESCs (Figure 2e). The mean EC_{50} values of 5-FC in the presence of CD-containing particles and 5-FU alone for hESCs and hiPSCs were similar (approximately 1.5 μ M), with negligible cell survival observed at 100 μ M of either small molecule. To test targeting specificity, monocultures of primary

human dermal fibroblasts (hDFs) were treated for 24 h with 8 nM $Q\beta(ZZ)_{42}@CD_{13}+SSEA-5_{21}$ VLPs and either 5-FC or 5-FU at 0.1 μ M to 2 mM. 5-FU was found to kill these differentiated cells with efficiency comparable to the killing of stem cells, but no dose-dependent cell death was observed in the presence of 5-FC and the SSEA-5-coated particles containing packaged CD (Figure 2f).

The use of this system requires the elimination of residual undifferentiated hPSCs in a mixed population. Thus, co-cultures of hPSC colonies and MEFs were treated for 15 h with 8 nM of unlabeled, IgG1-labeled (isotype control), and SSEA-5-labeled $Q\beta(ZZ)_{42}@CD_{13}$ VLPs in the presence of 100 μ M 5-FC, and 100 μ M of 5-FU as a positive control. Subsequent staining with ethidium homodimer-1 (a specific red marker of dead cells) and a green fluorescent antibody against the hPSC-specific TRA-1-60 surface marker gave the results shown in Figure 2g. Negligible cell death was induced by unlabeled and isotype control-labeled $Q\beta(ZZ)_{42}@CD_{13}$ VLPs in the presence of 5-FC; in these cases, hPSC colonies remained intact with bright, flat morphology and abundant TRA-1-60 expression. In contrast, hPSC colonies in co-cultures treated with $Q\beta(ZZ)_{42}@CD_{13}+SSEA-5_{21}$ and 5-FC showed extensive ethidium staining; a dark, barely adherent, mounted morphology; and minimal detection of TRA-1-60, likely due to antibody binding to loose fragments of dead hPSC membranes. The surrounding MEFs were unaffected, demonstrating the specificity of VLP-mediated hPSC killing. Co-cultures treated with 5-FU exhibited indiscriminate cell death.

SSEA-5-labeled $Q\beta(ZZ)_{42}@CD_{13}$ VLPs Specifically Eliminate Residual hPSCs without Impeding Cardiomyogenesis.

Currently it is still unclear what stages of hPSC-derived cardiomyocytes are most efficacious for therapeutic application.²⁴ Clinical application of this methodology may require the killing of undifferentiated cells at an early stage to selectively eliminate residual hPSCs without affecting cardiomyocyte differentiation. To test this, such treatment (8 nM VLPs for 1 h + 100 μ M 5-FC for 24 h) was performed at differentiation day 8 or 9 (Figure 3a and Supporting Information Figure 1a). From initial 1×10^6 cells seeded, at least 200–300 colonies with stem cell morphology and positive for the pluripotent stem cell marker TRA-1-60 were detected in cells expanded from progenitors treated with vehicle as a control. In contrast, no hPSC colonies were detected in otherwise identical cultures treated with the antibody-directed VLP+prodrug (Figure 3b and Supporting Information Figure 1b).

To examine if the treatment affected cardiomyocyte differentiation, we maintained the treated cells in RPMI/B27 medium, monitored cell morphology, and examined the expression of cardiomyocyte genes. At differentiation day 14, beating cardiomyocytes were observed in cultures derived from cells that were treated with either the VLPs or vehicle (Supporting Information Movies 1, 2). Similar to cells derived from control cultures, the majority of the cells derived from the VLP-treated cultures were positive for cardiac transcription factor NKX2-5, gap junction protein connexin 43, and cardiac structural proteins including cardiac troponin I, cardiac troponin T and α -actinin as detected by immunocytochemistry (Figure 3c and Supporting Information Figure 1c). In addition, these

cells also expressed similar levels of Ki-67, a marker associated with cells at active phases of cell cycle.(Figure 3c and Supporting Information Figure 1c).

The contractility of cardiomyocytes derived from VLP-treated cultures was found to be indistinguishable from that of vehicle-treated control cultures by video-based analysis²⁵ (Figure 3d), including maximum contraction, maximum relaxation, and beating intervals. Furthermore, intracellular Ca^{2+} transients (observed with the aid of calcium indicator dye Fluo-4) of cardiomyocytes derived from VLP-treated cultures were also very similar in Ca^{2+} transient amplitude, 50% peak time, and 50% decay time to those from control cultures (Figure 3e and Supporting Information Figure 1d). These results suggest that the VLP-based elimination of residual hPSCs from early-stage differentiation cultures did not significantly affect cardiomyocyte differentiation.

SSEA-5-labeled Q β (ZZ)₄₂@CD₁₃ VLPs Specifically Eliminate Residual hPSCs in Co-cultures with Late-stage Cardiomyocytes without Affecting Cardiomyocyte Function.

To mimic a high level of residual hPSCs in a differentiation culture at the late stage, we co-cultured 1–5% undifferentiated hPSCs with late-stage cardiomyocyte differentiation culture from hPSCs, conditions previously used to evaluate specific elimination of stem cells in a mixed population.²⁶ The co-culture was treated with the prodrug-converting VLPs (8 nM) for 1 h and then with 5-FC (100 μM) for 24 h (Figure 4a). Immediately after these treatments or following further culture, the presence of hPSCs and cardiomyocytes was examined by staining with antibodies against markers for stem cells (TRA-1–60) and cardiomyocytes (α -actinin). As expected, TRA-1–60-positive hPSC colonies/cells were detectable in co-cultures treated with vehicle at all time points examined. The amount of TRA-1–60 positive cells decreased over time in the VLP-treated cultures, and the signal for TRA-1–60 was undetectable 3 days after the VLP treatment by fluorescence microscopy (Figure 4b) or by flow cytometry (Supporting Information Figure 2). In contrast, the signal for α -actinin was detectable in the majority of TRA-1–60 negative cells in all cultures and was comparable in the vehicle- and VLP-treated cultures (Figure 4b). Similarly, no significant loss of hPSC-derived cardiomyocytes was observed by flow cytometry following VLP treatments (Supporting Information Figure 2).

A kill curve for 5-FC dose-response was performed in a co-culture of hPSCs and hPSC-derived cardiomyocytes to determine the cytotoxic range of 5-FC. The co-culture was treated for 1 h with the VLPs followed by 24 h incubation with titrated concentrations of 5-FC (from 0.1 μM to 1 mM). Immunochemical analysis showed 100 μM 5-FC to be the minimal lethal concentration for elimination of hPSCs, while hPSC-CMs were not affected even when treated with 1 mM 5-FC (Supporting Information Figure 3). These results show that the VLP+prodrug combination can specifically eliminate undifferentiated hPSCs in the mixture.

In our hands, metabolic selection – the use of glucose-depleted and lactate-supplemented media to purify late-stage hPSC-derived cardiomyocytes from differentiation culture^{27, 28} – was also able to eliminate all detectable TRA-1–60-positive stem cells from co-cultures with hPSC-derived cardiomyocytes (Figure 5). Both VLP+prodrug treated and metabolically selected co-cultures had similar amounts of hPSC-derived cardiomyocytes (Figure 5). Thus,

while both are effective in the presence of late-stage cardiomyocytes, only the VLP-based method is also applicable to early-stage cardiomyocyte differentiation cultures.

To determine if the VLP+prodrug treatment affected molecular and functional properties of cardiomyocytes, the expression of cardiomyocyte genes, cardiomyocyte contractility and intracellular Ca^{2+} transients were examined after the co-cultures were treated with vehicle or VLP + 5-FC. In both vehicle- and VLP-treated co-cultures, beating cells were observed (Supporting Information Movies 3, 4), which expressed cardiomyocyte transcription factor NKX2-5, connexin 43, cardiac troponin I, cardiac troponin T, α -actinin, and proliferation marker Ki-67 to similar extents (Figure 4c). In addition, the expression levels of genes associated with cardiomyocytes (*cTnT*, *NKX2-5*, *MYH6*, *MYH7*, *MYL2* and *MYL7*) were comparable between the vehicle- and VLP-treated samples (Figure 4d). Measurements of cardiac contractility were also similar between vehicle- and VLP-treated cells with no significant differences observed in maximum contraction, maximum relaxation, and beating interval as analyzed by a video-based method²⁵ (Figure 4e). Key parameters of intracellular Ca^{2+} transient recordings were similar between vehicle- and VLP-treated cells (Figure 4f). These results suggest that the VLP treatment does not alter molecular and functional characteristics of hPSC-derived cardiomyocytes.

Overall, prodrug-converting VLPs were able to selectively kill tumorigenic stem cells in mixed differentiation cultures without harming surrounding non-stem cells. The resulting stem cell-derived cardiomyocytes were apparently unaffected by this treatment, rendering it an interesting possibility for improving the safety of stem cell-based therapies. This combination of cytotoxic efficiency and selectivity derives from the production of only small amounts of toxic molecule overall but high enough concentrations to the target cells by virtue of localized delivery and enzymatic turnover. Operationally, this method for the targeted elimination of stem cells is simple to perform, requiring no gene transfection or editing and only a simple procedure involving medium change. In addition, the modular nature of the system should allow one to address different cell targets by simply loading different antibodies on the particle surface. The VLP protects the cargo enzymes from degradation by proteases and other factors, and they can be produced easily and at large scale.

METHODS

Cell Culture.

Human induced pluripotent stem cells [iPS(IMR90)-1, WiCell]²⁹ and human embryonic stem cells (H7, WiCell)³⁰ were cultured either on a feeder layer of irradiated mouse embryonic fibroblasts (MEFs), where they were maintained in hPSC medium (KnockOut DMEM, 20% KnockOut Serum Replacement, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 0.1 mM β -mercaptoethanol, 100 U/mL Penicillin-Streptomycin) supplemented with 8 ng/mL human basic fibroblast growth factor (bFGF), or in feeder-free hPSC conditions as previously described.³¹ Primary human dermal fibroblasts (hDFs) were obtained with informed consent from a patient skin biopsy in a safe and ethical manner as approved by the Emory University Institutional Review Board (IRB). Isolated hDFs were cultured in fibroblast growth medium (DMEM, 10% FBS, 4 mM L-glutamine, 100 U/mL

Penicillin-Streptomycin). All cell culture reagents were from Fisher Scientific unless specified.

Protein Expression and Purification of Q β (ZZ) VLPs.

The hybrid VLP constructs were produced and characterized as previously described.¹⁷ Briefly, ClearColi® BL21(DE3) electrocompetent *E. coli* cells (Lucigen) were co-transformed with one of the following plasmid combinations: pET28-CP@EGFP/pCDF-CP(ZZ) or pET28-CP(ZZ)/pCDF-CP@CD according to the manufacturer's recommended protocol. The ZZ domain was connected to the C-terminal end of the Q β capsid sequence by an 8-amino acid linker; the full sequence of the extended coat protein [(Q β)(linker)(Z)(Z)] was as follows, noting that the N-terminal methionine required for bacterial expression is processed off the capsid structure by cellular proteases:

(AKLETVTLG NIGKDGKQTLV LNPGRVNP TNGVASLSQAGAVPALEKRVTVSVSQPS
RNRKNYKVQVKIQNP TACTANGSCDPSVTRQAYADVTFSTQYSTDEERAFVRTELA
ALLASPL LIDAIDQLNPAY)(GGASESGA)
(AMVDNKF NKEQQNAFY EILHLPNL NEEQRNAFIQSLKDDPSQSANLLAEAKKLND
AQAPKNL NEEQRNAFIQSLKDDPSQSANLLAEAKKLND AQAPK)
(AMVDNKF NKEQQNAFY EILHLPNL NEEQRNAFIQSLKDDPSQSANLLAEAKKLND
AQAPKNL NEEQRNAFIQSLKDDPSQSANLLAEAKKLND AQAPK).

Cells were plated on selective super optimal broth (SOB) agar; after 24 h, single colonies were isolated and transferred to selective SOB media (1% NaCl; w/v) for overnight growth at 37°C. The following morning, cultures were diluted under the same conditions and growth was monitored via optical density (600 nm) until the cultures reached mid-log phase (i.e., O.D.₆₀₀ ~ 0.9). Protein expression was induced by the addition of IPTG at a final concentration of 1 mM. Cultures were maintained at 30°C for 24 h, after which time cells were harvested by centrifugation (6,000 rpm, 10 min). Cell pellets were suspended in 1X Tris-buffered saline (TBS; pH 7.06), and cells were lysed with a probe sonicator in an ice bath (10 min total, 5 s pulse intervals). Cell debris was removed by centrifugation (14,000 rpm, 10 min) and the supernatant was collected. VLPs were precipitated by the addition of 30% (NH₄)₂SO₄ (w/v) for 2 h at 4°C, and the precipitate was pelleted by centrifugation (14,000 rpm, 10 min). The pellet was dissolved in 1x TBS and extracted with a solution of *n*-BuOH:CHCl₃ (1:1, v/v) to remove lipids and aggregates. The aqueous phase was collected following centrifugation (14,000 rpm, 10 min) and VLPs were further purified on 10–40% sucrose density gradients (28,000 rpm, 4 h). VLP bands were isolated via syringe and pelleted by ultracentrifugation (68,000 rpm, 2 h). Subsequent pellets were dissolved in 0.1M potassium phosphate buffer and characterized.

Characterization of Q β (ZZ) VLPs.

VLP concentration was quantified using Bradford assay with BSA standards according to the manufacturer's protocol (ThermoFisher) with average yields of 10–20 mg/L. Particle purity was assessed by fast protein liquid chromatography (FPLC) with a Superose 6 GL column and monitored by absorbance (280 nm). Size of the VLPs was determined by dynamic light scattering (DLS) using a Dynapro II plate reader (Wyatt Technology), and

particle composition was determined by microfluidic gel electrophoresis using an Agilent 2100 Bioanalyzer with Series II Protein 80 chips. The relative particle composition was determined by comparing peak integrations from the electropherograms using the Bioanalyzer software. Due to overlap of the CP(ZZ) and EGFP bands, EGFP concentration was calculated by measuring the sample absorbance at 488 nm and using the Beer-Lambert law ($Abs = \epsilon c l$; $\epsilon_{EGFP} = 56,000 \text{ M}^{-1}\text{cm}^{-1}$). On average, 4–8 molecules of EGFP were packaged, 13–18 molecules of CD were packaged, and 40–60 ZZ-domain subunits were incorporated per particle, respectively.

Antibody-labeling of Q β (ZZ) VLPs.

Q β (ZZ)@EGFP or Q β (ZZ)@CD VLPs were labeled with anti-SSEA-5 antibodies. Briefly, VLPs were diluted to 0.1 mg/mL and 20 μg VLP was incubated with 50 μg purified anti-human SSEA-5 mouse IgG1, κ antibody (Clone 8e11, BioLegend) for 2 h at 37°C. A antibody:ZZ-domain mole ratio of 0.75 was used for all labeling reactions and was sufficient for uniform labeling of the particles. To serve as a control, the same procedure was repeated with purified mouse IgG1, κ isotype control antibody (Clone MG1–45, BioLegend).

Quantification of Antibody Loading by Multi-angle Light Scattering.

Empty Q β (ZZ) VLPs were split into two samples at 0.1 mg/mL, and one sample was labeled with mouse IgG1, κ isotype control antibody as previously described. Samples were separated over a Superose 6 10/300 GL size exclusion column (GE Healthcare) using an Agilent HPLC to maintain a 0.2 mL/min flow rate of 0.1M KPO₄ buffer (pH 7.4). Samples of 100 μL were injected onto the column. Samples were detected using a UV-vis detector (Agilent), a Viscotek SEC-MALS 20 multi-angle light scattering detector (Malvern), and a Viscotek VE3580 refractometer (Malvern). The number-average molecular weight, M_n , was calculated with OmniSEC 5.0 software and plotted across the elution peak. The increase in molecular weight for the antibody-labeled sample was related to the number of bound antibodies using the average molecular weight for IgG antibodies (~150 kDa).

Cardiomyocyte Differentiation.

hPSCs were induced for cardiac differentiation as described previously.^{32, 33} hPSCs were first dissociated using Versene and plated on Matrigel-coated wells plate at density of 1.6×10^5 cells/cm². Cells were cultured in conditioned medium from MEF (MEF-CM) for 2 days and treated with 100 ng/mL activin A (R&D Systems) from differentiation day 0 till day 1 and 10 ng/mL bone morphogenic protein-4 (BMP4, R&D Systems) from day 1 till day 5 in RPMI/B27 insulin-free medium. Cells were cultured successively with RPMI/B27 medium with insulin and the medium was changed on alternate days. Cardiomyocytes showed beating activity at day 9/10 of differentiation.

Treatment of Cardiac Progenitors with VLPs.

One day before the treatment of cardiac progenitors derived from hPSCs (at day 7 or 8), the medium was replaced with a blocking solution (medium with 20% KnockOut Serum Replacement) to prevent non-specific binding of VLPs. After cultured for 24 h (at day 8 or 9), the cells were then treated for 1 h with medium containing 8 nM SSEA-5-labeled

Q β (ZZ)@CD VLPs or vehicle (PBS as a control) followed by 24 h incubation with 100 μ M 5-FC.

Treatment of Co-culture of hPSCs and Cardiomyocytes with VLPs.

Cardiomyocytes at day 14 to 18 were plated on a Matrigel-coated plate at density of 1.6×10^5 cells/cm² and cultured in RPMI/B27 medium until they regained beating activity.

Undifferentiated hPSCs were then plated at density of 1–5% compared to cardiomyocytes (1.6×10^3 to 8×10^3 cells/cm²) and cultured in MEF-CM medium. Cells were treated for 1 h with medium containing 8 nM SSEA-5-labeled Q β (ZZ)@CD VLPs or PBS (vehicle as a control) followed by 24 h incubation with 100 μ M 5-FC.

Statistics.

For calcium imaging analysis and video-based analysis of contractility, significant differences between vehicle- and VLP-treated cells were assessed by two-sample *t*-test. All data were expressed as mean $\pm$ SD and *p* values <0.05 were considered as significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

VLP	virus-like particle
EGFP	enhanced green fluorescent protein
CD	cytosine deaminase
5-FC	5-fluorocytosine
5-FU	5-fluorouracil
hPSC	human pluripotent stem cell

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Figure 1. SSEA-5-labeled $Q\beta(ZZ)_{60}@EGFP_4$ VLPs selectively deliver EGFP intracellularly to hPSCs.

(a) Schematic of $Q\beta(ZZ)_{60}@EGFP_4$ viral coat protein expression and particle assembly, followed by VLP labeling with SSEA-5 antibodies. (b) Electrophoretic analysis of $Q\beta(ZZ)_{60}@EGFP_4$ VLPs. (c) Fast protein liquid chromatography analysis of $Q\beta(ZZ)_{60}@EGFP_4$ VLPs. (d) Dynamic light scattering histograms of $Q\beta(ZZ)_{60}@EGFP_4$ VLPs (red) and $Q\beta(ZZ)_{60}@EGFP_4+SSEA-5_{30}$ VLPs (blue); % = % dispersity; R_h = hydrodynamic radius. (e) Multiple angle light scattering curves show the change in refractive index (blue peak) of the VLPs as a function of the elution volume through a size-exclusion column. A comparison between the log MW (red trace) of unlabeled (left) and antibody-labeled (right) VLPs indicates approximately half of the ZZ domains are occupied by antibodies. (f) Representative microscopy images of hPSC colonies grown on mouse embryonic fibroblasts incubated with 8 nM unlabeled, IgG1-labeled, or SSEA-5-labeled $Q\beta(ZZ)_{60}@EGFP_4$ VLPs for 30 min at 37°C. Only hPSCs treated with SSEA-5-labeled $Q\beta(ZZ)_{60}@EGFP_4$ showed detectable EGFP fluorescence. Scale bars = 100 μ m. (g) Live-cell deconvolution fluorescence microscopy of hPSCs incubated for 30 min at 37°C with 8 nM $Q\beta(ZZ)_{60}@EGFP_4+SSEA-5_{30}$, blue DAPI nuclear probe, and membrane-specific red wheat germ agglutinin (WGA). Left = EGFP and DAPI only; right = EGFP, DAPI, and WGA. XY optical sections are shown above orthogonal YZ cross-sections corresponding to the white dashed line in the XY plane. Cross-sections show prominent cytoplasmic co-localization of EGFP and DAPI along with punctate expression of the membrane-specific WGA dye. XY plane scale bars = 15 μ m; YZ scale bars = 5 μ m.

Figure 2. SSEA-5-labeled $Q\beta(ZZ)_{42}@CD_{13}$ VLPs selectively kill hPSCs in the presence of 5-FC.

(a) Schematic of $Q\beta(ZZ)_{42}@CD_{13}$ viral coat protein expression and particle assembly, followed by VLP labeling with SSEA-5 antibodies. (b) Electrophoretic, (c) FPLC, and (d) dynamic light scattering analyses of $Q\beta(ZZ)_{42}@CD_{13}$ VLPs. (% = % dispersity; R_h = hydrodynamic radius). (e) EC₅₀ curves showing dose-dependent decreases in human induced pluripotent stem cell (hiPSC) and human embryonic stem cell (hESC) survival after 24 h treatment with both 8 nM $Q\beta(ZZ)_{42}@CD_{13}$ +SSEA-5₂₁ VLPs in the presence of the prodrug 5-fluorocytosine (5-FC) or the cytotoxin 5-fluorouracil (5-FU). (f) EC₅₀ curves show dose-dependent decreases in human dermal fibroblast (hDF) survival percentages after 24 h treatment with 5-FU, but negligible cell death after treatment with 8 nM $Q\beta(ZZ)_{42}@CD_{13}$ +SSEA-5₂₁ VLPs in the presence of 5-FC. (g) Representative microscopy images show co-cultures of hPSC colonies and MEFs that were treated for 15 h with 8 nM of unlabeled, IgG1-labeled (isotype control), and SSEA-5-labeled $Q\beta(ZZ)_{42}@CD_{13}$ VLPs in the presence of 100 μ M 5-FC as test conditions, and 100 μ M 5-FU as a positive control. Red fluorescence indicates dead cells and green fluorescence indicates expression of the pluripotent stem cell-specific TRA-1-60 marker.

Figure 3. SSEA-5-labeled Qβ(ZZ)₄₂@CD13 VLPs selectively kill residual hPSCs during CM differentiation in the presence of 5-FC.

(a) Schematic of experimental design for treatments of cardiac progenitors with VLPs. (b) Colony assay for the detection of undifferentiated hPSCs. Day 9 differentiated cells were treated with 8 nM Qβ(ZZ)₄₂@CD13+SSEA-5₂₁ or PBS (vehicle as a control) for 1 h and washed to remove excess VLPs. Both groups of cells were then treated with 5-FC (100 μM) for 24 h, washed to remove excess prodrug, and cultured on MEF feeders for the detection of residual hPSCs. After 10 more days, cell were examined by immunocytochemistry (top panels: blue, DAPI nuclear stain; green, TRA-1-60; scale bars = 100 μm). ND = not detected. (c) Expression of cardiomyocyte proteins. Beating cells derived from day 9 vehicle- and VLP-treated cultures were examined by immunocytochemistry for their expression of cardiac transcription factor NKX2-5, gap junction protein connexin 43 (Cx43), cardiac structural proteins including cardiac troponin T (cTnT), α-actinin, cardiac troponin I (cTnI), and proliferation marker Ki-67. Scale bars = 100 μm. (d) Video-based

analysis of contractility. Representative traces show beating velocity recording of vehicle- and VLP-treated cells. Contraction parameters are presented as mean $\pm$ standard deviation of n=3 biological samples. Blue dots denote contraction, and red triangles denote relaxation. (e) Ca^{2+} transient analyses. Representative line-scan images show calcium transients in cardiomyocytes derived from day 9 vehicle- and VLP-treated cells from the area indicated by the white lines. Scale bars = 5 μm . Ca^{2+} transient parameters are presented as mean $\pm$ standard deviation of n=20 cells. NS = no significant difference between groups (p value >0.05). D8 = Day 8; D9 = Day 9.

Figure 4. SSEA-5-labeled Q β (ZZ)₄₂@CD₁₃ VLPs selectively kill hPSCs in co-cultures of hPSC and cardiomyocytes in the presence of 5-FC.

(a) Schematic of experimental design for the VLP treatment for co-cultures of hPSCs and cardiomyocytes. (b) Detection of hPSCs and cardiomyocytes in co-cultures. Co-cultures of hPSCs and cardiomyocytes were treated with 8 nM Q β (ZZ)₄₂@CD₁₃+SSEA-5₂₁ (test) or PBS (control) for 1 h and washed to remove excess VLPs. After the treatment of both groups with 5-FC (100 μM), cells were examined by immunocytochemistry of hPSC-specific marker TRA-1-60 (red) and cardiomyocyte-associated protein α -actinin (green). Scale bars = 100 μm . (c) Detection of cardiomyocyte proteins. Cells from vehicle- and VLP-treated co-cultures were examined for the expression of cardiac transcription factor NKX2-5, gap junction protein connexin 43 (Cx43), cardiac structural proteins including cardiac troponin T (cTnT), α -actinin and cardiac troponin I (cTnI), and proliferation marker Ki-67. Scale bars = 100 μm . (d) Detection of the expression of cardiomyocyte genes by qRT-PCR. Data are presented as mean $\pm$ standard deviation of $n=3$ biological samples. (e) Video-based analysis

of contractility. Contraction parameters are presented as mean $\pm$ standard deviation of n=6 biological samples. (f) Ca^{2+} transient analyses. Representative line-scan images show calcium transients in vehicle- and VLP-treated cells in co-cultures from the area indicated by the white lines. Scale bars = 5 μm . Ca^{2+} transient parameters are presented as mean $\pm$ standard deviation of n=22 cells. NS = no significant difference between groups (p value>0.05).

Figure 5. Comparison of SSEA-5-labeled Q β (ZZ)₄₂@CD₁₃ VLPs with metabolic selection method.

Flow cytometry was performed to compare metabolic selection with VLP treatment. hPSC-CMs at day 18 were dissociated using Trypsin/EDTA and plated onto a Matrigel-coated 24 well plate at a density of 600,000 cells per well, cultured until they recovered beating activity. Undifferentiated hPSCs were then plated at a density of 2% compared to cardiomyocytes (12,000 cells per well) and further cultured in MEF-conditioned medium. For the VLP method, cells were treated for 1 h with medium containing 8 nM SSEA-5-labeled Q β (ZZ)₄₂@CD VLPs followed by 24 h incubation with 100 μ M 5-FC in RPMI/B27 medium. After 5-FC incubation, cells were maintained in RPMI/B27 medium for 2 more days before harvesting on Day 3. Medium was changed on alternate days. For metabolic selection, the co-culture of hPSCs and hPSC-CMs was cultured in RPMI/B27 medium without glucose and with 5 mM sodium DL-lactate 60% syrup (Glu-/Lac+). Medium was changed on alternate days, and cells were harvested after 3 days of metabolic selection. In

controls wells, cells were maintained in RPMI/B27 medium, which was changed on alternated days, and collected at Day 0 (before the start of both VLP-treatment and metabolic selection) and Day 3. Following harvesting, cell numbers were estimated by the hemocytometer, and $\sim 1.5 \times 10^6$ cells aliquot for each treated group was prepared for flow cytometry analysis of cardiac marker α -actinin and stem cell marker TRA-1-60. Summary (**a**) and representative histograms (**b**) of all groups for each marker are shown. ND = Not detectable.