

Corning® Ultra-Low Attachment Surface Plates Allow for Lentivirus Transduction of Human Osteoclast Precursor Cells

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Customer Application Note

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Introduction

New methods for treating bone cancer are desperately needed. Survival following treatment of primary bone cancers is approximately 60%, without any significant treatment advances in the last two decades. The prognosis is even worse in patients with cancer metastases to bone (1). The most recent advance in treating bone metastases is bisphosphonate therapy. Unfortunately, this treatment has exhibited no survival advantage and greater than 50% of treated patients develop progression of bone cancers (2). Recent advances in understanding the pathophysiology of bone cancer have confirmed the pathologic role of osteoclasts (1,3). Bone tumors stimulate osteoclast formation and promote osteoclastogenesis by recruitment and differentiation of monocytic lineage cells and their hematopoietic precursors (3).

The use of suicide gene delivery to tumors by accessory cells has been demonstrated in treatment for gliomas and lung cancer using neural (4) or endothelial precursor cells (5). Osteoclasts form from recruited precursor cells in high numbers at sites of bone cancer and stimulate tumor progression. One step toward developing a novel gene therapy for treating bone metastases, is the engineering of osteoclast precursor cells (OcP) to serve as a cellular gene delivery system capable of killing cancer cells. Previous studies in our laboratory have demonstrated that murine OcP can provide a basis for bone cancer-targeted suicide gene therapy (6,7).

Transduction of monocyte lineage cells has proved to be problematic. Previous methods for transducing human macrophages (MØ) or OcP have utilized magnetic separation of CD14⁺ cells and subsequent culturing of adherent monolayers of monocyte/MØ cells (8-10). These methods are time consuming, requiring weeks to grow up and expand, and provide a low yield, about 1% of the input cells are recovered for transduction. Assessing transduction efficiency is difficult on adherent cells *in situ*, compared to flow cytometry of non-adherent cells. The transduction reported was of moderate (20%) efficiency (8,9), or required preliminary sorting to obtain high (60%) efficiency (10). In addition, although no cell yields are published, in house reproduction of these methods show they do not yield sufficient numbers of transduced cells for *in vivo* experiments.

This study evaluates transduction of OcP using a simple, pre-isolation enrichment and maintenance of the cells as a non-adherent culture utilizing a specific hydrophilic and nonionic cell surface. This allows for easier manipulation and evaluation of transduction, cell yields and viability over time. Additionally, a 5-fold scale-up is used to determine feasibility and reproducibility of bulk cultures.

Material and Methods

Isolation of OcP — Human blood was purchased from Memorial Blood Center and the peripheral blood mononuclear cells (PBMC) isolated by density gradient centrifugation. CD14⁺ cells (OcP) were purified to >90%+ using Miltenyi Biotec MACS® magnetic separation columns. Four different donors were used in small-scale experiments with an average CD14⁺ recovery of 5% (range, 2 to 8%). Four different donors were used in large-scale experiments with an average CD14⁺ recovery of 14% (range, 10 to 17%).

Transduction — Transduction was performed in Corning Ultra-Low Attachment Surface plates to prevent adherence using MEM α containing 10% FBS and 25 ng/mL rhM-CSF (MØ media). Two sets of experiments were performed; (i) small-scale experiments were used to assess transduction over time, from 24 to 96 hours, and used 6 well plates in a total volume of 2 mL, and (ii) large-scale experiments were used to assess reproducibility after scaling up, and used 10 cm dishes in a total volume of 10 mL.

Lentivirus, CSIIeG containing the eGFP marker (M.O.I = 10), was combined with 5×10^6 OcP/mL, or a total of 10×10^6 for small-scale experiments and 50×10^6 for large-scale experiments. A charged bridge, either 2.5 μ g/mL DEAE-dextran or 10 μ g/mL protamine sulfate, or none was added. Cells were grown at 37°C in 5% CO₂ for 96 hours with an equal volume of MØ media added after 24 hours. Cultures containing MEM α instead of lentivirus were used as negative/non-specific controls (mock).

Plates with a standard tissue culture treatment (TCT) were used in one experiment, but by comparison gave poor results. Instead of proliferating clusters, OcP grew as attached or single floating cells (Figure 1). There was decreased cell recovery, viability, OcP markers and transduction. Overall significantly fewer CD14⁺ PBMC plated were transduced GFP⁺ OcP, a log decrease compared to using Corning Ultra-Low Attachment Surface plates (Table 1).

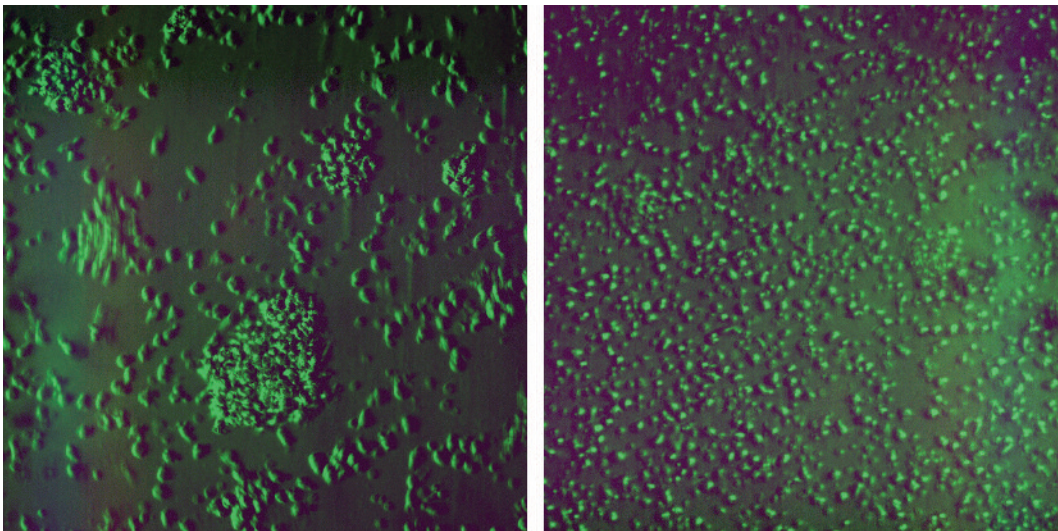


Figure 1. Darkfield images of transduced OcP grown in Corning® Ultra-Low Attachment Surface plate (left) vs. tissue-culture treated (TCT) plate (right).

Verification — Transduction was evaluated for cell yield and viability using a ViCell™ counter. Cells were stained with CD14-PE and CD11b-APC to monitor OcP lineage. Analysis was performed using a BD FACScaliber™ cytometer for acquisition, and FlowJo software used for quantitation of percentage OcP and marker gene expression. Back-gating of CD11b onto the forward/side scatter plot defined the OcP population of viable, single cells, denoted R1.

Osteoclastogenesis — Transduced cells were cultured in quadruplicate in 96 well plates at 5×10^4 cells/well with MØ media for a total of 8 days (including transduction time). Cultures were then switched to osteoclast media, MØ media containing 50 ng/mL rhRANKL and incubated at 37°C in 5% CO₂ for 6 to 13 additional days. Half-volume media changes to replace cytokines occurred every 3 days.

Imaging of osteoclasts — Cultured osteoclasts were fixed and either stained with tartrate-resistant acid phosphatase (TRAP), a marker for osteoclasts or counterstained using DAPI in a SlowFade® mounting media for fluorescent microscopy. Brightfield microscopy was used to visualize TRAP-stained osteoclasts. Confocal microscopy was used to visualize green (GFP⁺) osteoclasts.

Results and Discussion

Yield, viability and OcP markers. In small-scale experiments the number of recovered OcP (%R) decreased from 24 to 48 hours, where it held stable to 96 hours. Viability (%V) also decreased from 24 to 48 hours but was back up by 96 hours. The OcP marker CD11b increased throughout the transduction period; CD14, however, decreased drastically, reaching a low of 11% at 96 hours. R1, the subpopulation of viable OcP steadily increased from 24 to 96 hours. There were no significant differences between charged bridges or none for CD11b, CD14 and R1. Results are summarized in Table 2.

Charged bridge enhances OcP transduction. At every time point, 24 to 96 hours, there were significantly more GFP⁺ (transduced) cells in both the total population and the OcP subpopulation (R1) using a charged bridge. At 24 hours, total GFP was $4.8 \pm 2.3\%$, $8.6 \pm 1.8\%$ and $13.2 \pm 5.6\%$ for no bridge, protamine and DEAE-dextran, respectively. Similar percentages were measured in R1. At 96 hours, the total GFP had increased to $19.7 \pm 2.2\%$, $25.8 \pm 3.9\%$ and $26.9 \pm 5.9\%$, and GFP in R1 to $30.5 \pm 3.6\%$, $39.3 \pm 6.1\%$ and $42.1 \pm 10\%$.

Table 1. Improved Performance Using Corning Ultra-Low Attachment Surface versus TCT Plates

Surface	% of OcP		% of Live Cells				% in R1 GFP ⁺	% CD14 Plated
	Yield	Viability	CD11b ⁺	CD14 ⁺	R1=OcP	GFP ⁺		
Ultra-Low Attachment Surface: mock	26.10%	82.00%	95.20%	69.80%	90.50%	2.10%	1.90%	0.45%
Ultra-Low Attachment Surface: Tfx	32.40%	73.80%	97.60%	70.10%	92.00%	23.70%	23.70%	7.06%
TCT: Tfx	14.40%	40.40%	86.70%	6.90%	46.00%	5.85%	10.00%	0.66%

Transduced OcP proliferate and form osteoclasts.

Proliferation was determined by calculating the absolute number of GFP⁺ OcP using the following: # viable cells x %R1 x %GFP in R1. GFP⁺ OcP increased by 1.8-fold/day for all three conditions (†p < 0.05), a total expansion from 24 to 96 hours of 6-fold. At every time point there were significantly more GFP⁺ OcP using DEAE-dextran (*p < 0.05) (Figure 2). The net result was 3.4 ± 1.6% of CD14⁺ PBMC plated became transduced GFP⁺ OcP.

Examination of osteoclast cultures showed abundant mono-, bi-, and multi-nucleated TRAP⁺ cells. GFP was expressed for up to 21 days after transduction in OcP and multi-nucleated osteoclasts.

Scale-up improves transduction and cell yield. In large-scale experiments mock was compared to transduced using DEAE-dextran and evaluated after 96 hours. Recovery was 47 ± 15% of the plating number, with 83 ± 6% viable. Lineage markers were 95 ± 2%, 42 ± 26% and 95 ± 2% for CD11b, CD14 and R1, respectively, with no significant differences between mock and transduced. The large-scale experiments demonstrated significantly (p < 0.01) improved recovery, viability and CD14⁺, with similar results for CD11b and R1 compared to small-scale experiments. Most importantly, total live-cell GFP and GFP in R1 were 27 ± 24% and 22 ± 9%. Overall, 10 ± 6% of CD14⁺ PBMC plated became transduced GFP⁺ OcP, a trend towards improved performance.

Discussion. Gene transfer into stem cells has been an ongoing priority as a treatment for genetic disease and cancer for more than two decades. Efforts focused on HSC have been hampered by the low frequency of precursors (CD34⁺ are about 0.1% of PBMNC), and slow growth and expansion. Additionally, loss of pluripotency is an issue when the goal is a single bone marrow transplant. In this study, we use a high frequency (10 to 25% of PBMNC), limited lineage precursor that can be readily isolated and expanded, making it readily available for multiple treatments. With suicide gene therapy, the hope is to develop a cell-based delivery system

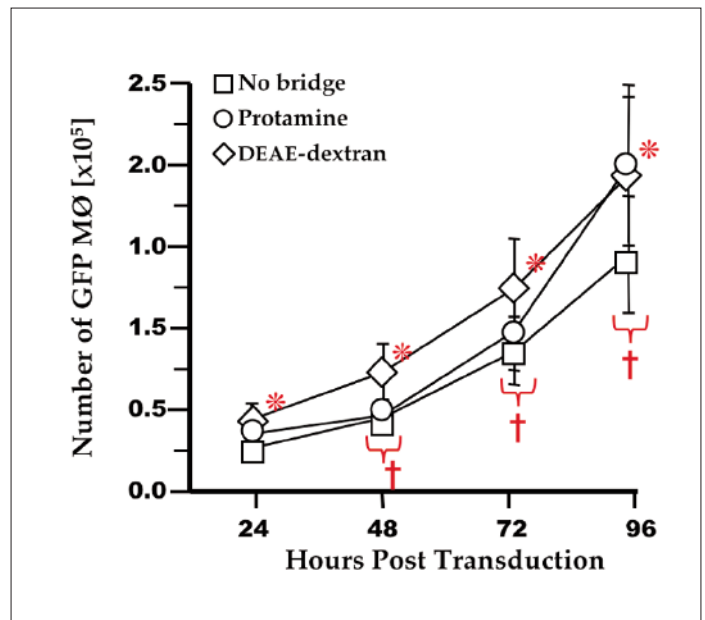


Figure 2. Transduced OcP Proliferate *in vitro*.

that would simultaneously kill tumor cells and eliminate osteoclast-mediated bone destruction.

Conclusion

In this report we introduce the culture and transduction of monocytic lineage OcP as non-adherent cells, instead of the more traditional adherent monolayer, utilizing a specific hydrophilic and nonionic cell surface (Corning® Ultra-Low Attachment Surface). This technique allows for efficient transduction of a marker gene and recovery of large numbers of cells that maintain good viability and OcP lineage markers. Cell number and viability were carefully monitored so that an honest assessment of recovery could be determined. This was also used to demonstrate that bulk cultures could be successfully transduced in the same manner and with the same results. In addition, transduced OcP could be cultured to form osteoclasts that continued to express the marker gene.

Table 2. Effect of Time in Culture on Viability and OcP Parameters

Time (h)	OcP Yield		Viability		CD11b ⁺		CD14 ⁺		R1=OcP	
	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
0	NA		NA		92.2%	5.0%	90.6%	5.5%	87.1%	6.0%
24	52.5%	6.3%	63.9%	5.4%	78.7%	2.5%	21.8%	1.2%	45.7%	2.7%
48	34.5%	2.7%	48.4%	2.9%	88.3%	1.1%	17.2%	5.1%	49.0%	5.8%
72	31.5%	0.8%	55.3%	3.3%	92.2%	1.4%	13.8%	2.3%	54.3%	4.5%
96	32.3%	2.0%	61.6%	3.2%	93.5%	0.8%	11.0%	2.0%	85.9%	2.0%

This study represents a crucial step toward human gene therapy for bone cancer metastasis by demonstrating successful transduction of human OcP as cellular delivery vehicles. We also demonstrate a 5-fold scale-up with no loss of transduction efficiency, and improved OcP markers, cell recovery and viability. Using this method will allow us to obtain sufficient numbers of OcP to use in animal models or a phase I clinical trial.

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