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Lentivirus Transduction of Human Osteoclast Precursor Cells and Differentiation into Functional Osteoclasts

Margaret L. Ramnaraine, Wendy E. Mathews, and Denis R. Clohisy

Department of Orthopedic Surgery, University of Minnesota, 420 Delaware Street SE, MMC 806, Minneapolis, MN 55455, USA

Abstract

Gene transfer into stem cells has been an ongoing priority as a treatment for genetic disease and cancer for more than two decades. Methods described herein, form the basis for providing the cell source to determine if osteoclast precursor cells (OcP) can be used as therapeutic gene delivery systems in vivo. Osteoclasts and tumor associated macrophages or OcP, support survival, tumor progression and osteolysis in bone cancers. Two sources of precursor cells are compared: CD14+ cells, the standard OcP, found abundantly in peripheral blood and CD34+ cells, hematopoietic stem cells that are rare, but which can be expanded into OcP. Our findings characterize cell yield at each step of the transduction process and thus provide essential data for planning future in vivo experiments. In addition we demonstrate that essential functions of OcP are preserved following lentiviral transduction. Specifically, neither the transduction method nor the lentiviral transduction influence the OcP's ability to form osteoclasts, express the marker gene, EGFP, or resorb bone. Finally, we conclude that CD34+ cells yield significantly more transduced cells and form functionally superior osteoclasts in vitro. This study represents a step towards considering human gene therapy for bone cancer by demonstrating successful transduction of human OcP for use as cellular delivery vehicles to sites of bone cancer.

Keywords

osteoclast; lentivirus; gene therapy; hematopoietic stem cells

Introduction

Cell based gene delivery systems are being utilized to develop novel cancer therapies [1, 2]. Strategies for developing these therapies involve transduction of normal host cells ex vivo that, following injection can home to or be provided directly at sites of tumor. The cells, which are utilized to deliver therapeutic genes to sites of cancer, are either "stem cells" or accessory cells, which have an affinity for the cancer tumor microenvironment [3, 4]. Through their actions at sites of tumor these accessory cells are often essential for local progression and even metastasis of cancer. The use of suicide gene delivery to tumors by accessory cells has been demonstrated in vivo for gliomas or brain metastasis, using neural

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Address all correspondence to: Denis R. Clohisy, MD, Professor and Department Head, Department of Orthopedic Surgery, 2512 So. 7th Street, Suite R200, Minneapolis, MN 55454, Office: (612) 273-8032, Fax: (612) 273-7959, clohi001@umn.edu.

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progenitors [5–7] and lung cancer using endothelial precursor cells [8]. More recent work has been with transduction of mesenchymal stem cells (MSC) [2, 9] and in vitro killing of tumors [10, 11]. Most in vivo models use accessory cells of immune [12], neural [11] or endothelial [13] origin.

Osteoclasts and their precursors are accessory cells at sites of primary and metastatic cancers. They serve to support survival and tumor progression within bone and are essential for the successful development of skeletal cancers [14–16]. Previous work using transgenic mice whose osteoclasts expressed cytosine deaminase has shown that osteoclasts can function effectively as cell-based gene delivery systems to treat bone cancers [17] in vivo and in vitro [18]. Having proven that osteoclasts can function as cell-based gene delivery systems and can direct killing of bone cancer, we now seek to develop gene delivery systems using ex vivo osteoclast precursor cells (OcP).

An essential step towards developing gene delivery systems using ex vivo OcP is to establish methods of gene transduction that provide efficient and persistent gene expression in OcP. Previous works directed at transducing OcP have focused on CD14⁺ cells, using electrical [19], chemical [20] or viral [21] methods of transduction. These methods have demonstrated limited to no success and generate insufficient numbers of OcP for in vivo studies. Recent works have demonstrated effective expansion of CD34⁺ hematopoietic stem cells (HSC) to OcP and in vitro osteoclasts [22], production of myeloid precursors in numbers sufficient for clinical trials [23] and efficient methods of transducing CD34⁺ cells [24, 25]. In combination, these methods identify CD34⁺ cells a potential starting point for the generation of OcP suitable for ex vivo gene therapy.

As a first step towards developing gene delivery systems using ex vivo osteoclast precursor cells, we examined methods for lentiviral transduction of OcP. Specifically, we compare the efficiency and effect of lentivirus transduction on two distinct methods of OcP transduction using CD14⁺ cells and comparing results to CD34⁺ cells. Findings demonstrate that methods using CD34⁺ cells are superior to methods using CD14⁺ cells as candidates for cell-based gene delivery. Specifically, CD34⁺ cells yield significantly larger pools of transduced OcP, are superior for in vitro expansion of transduced OcP and form functionally superior osteoclasts in vitro.

Materials and Methods

Isolation of CD14⁺ and CD34⁺ OcP

Human peripheral blood was purchased from Memorial Blood Center and the mononuclear cells (MNC) isolated by density gradient centrifugation (Histopaque™, Sigma/Aldrich, St. Louis, MO). CD14⁺ cells from six different donors were purified to >95% using Miltenyi (Auburn, CA) MACS magnetic separation columns. Average CD14⁺ recovery was 15% (range 8–20%). Non-bankable human cord blood (CB) was donated by New York Blood Center or CORD:USE (Orlando, FL) for research purposes. The MNC were isolated and CD34⁺ cells were purified to >98% using RoboSep (StemCell Technologies, Vancouver, BC, Canada) magnetic separation. Eight different CB units were used with an average CD34⁺ recovery of 0.4% of total MNC (range 0.1 – 1.0%).

Preparation and titering of lentiviral vector

Vesicular stomatitis virus envelope glycoprotein (VSV-G)-pseudotyped vectors were generated by three-plasmid cotransfection of HEK 293T cells as previously described (26). Lentiviral vector CSII-EGFP [27] containing the enhanced green fluorescent protein (EGFP) marker, was cotransfected with p ΔNRF to provide lentiviral structural and enzymatic proteins and with pMD.G to provide VSV-G. Viral supernatants were collected 24, 36, and

48 hr posttransfection, pooled and then concentrated 100-fold by high-speed centrifugation (23,000 ×g). Virus was resuspended in serum-free medium and quantitated using NIH 3T3 tk⁻ cells and flow cytometry to determine the percentage of cells expressing EGFP. Briefly, NIH 3T3 tk⁻ cells were transduced with multiple dilutions of vector plus of 8mg/ml Polybrene, and 48 hours later, the cells were harvested for flow cytometry. DNA was also extracted from the transduced cells for real-time quantitative PCR for EGFP. Multiplicity (MOI) was based on these titers, which were on the order of 10⁸ EGFP forming units/ml.

Transduction of CD14⁺ OcP

Lentivirus, was combined with 1–6 × 10⁶ OcP/ml using Minimal Essential media, alpha modification (MEMα) containing 10% FBS (α-10) and 25ng/ml recombinant human macrophage-colony stimulating factor (M-CSF, MØ media) in a total volume of 2ml. An equal volume of media was added after 24h. Transduction of CD14⁺ cells was performed in ultra-low attachment 6-well plates (ULA, Corning Lifesciences, Lowell, MA). Three sets of experiments were performed: first to assess the effect of transduction over time at MOI = 10, with or without a charged bridge; either 2.5 µg/ml DEAE-dextran (d-d) or 10 µg/ml protamine sulfate, or none, and evaluated daily from 18 to 96h, second to evaluate the effect of MOI of 1–20 at 24 and 96h, and third evaluating the effect of the CD34⁺ protocol using CD14⁺ isolated from the same unit as the CD34⁺, run in parallel using MOI of 1–20 and evaluated at 4–11d. Cultures were grown at 37°C in 5% CO₂ and a negative/non-specific control (mock) used MEMα instead of lentivirus.

Dose/response experiments were performed to determine the maximum tolerated dose of lentivirus by OcP and evaluate the effect on osteoclastogenesis. Aliquots were used for cell analysis, marker analysis by flow cytometry (FACS) and osteoclast formation and functional studies. The first experiment determined that use of a charged bridge (d-d) resulted in significantly better transduction (30 ± 4% vs. 41 ± 8%, p < 0.03) while all other parameters were unchanged. All subsequent experiments included 2.5 µg/ml d-d in the transduction.

Transduction and expansion of CD34⁺ OcP

Transduction of CD34⁺ cells was performed in 6-well ULA plates using a previously published defined media [22]; BioWhittaker® X-Vivo-10 (Lonza Walkersville, Inc, Walkersville, MD) supplemented with 0.3% human albumen and 5 recombinant human cytokines; 200ng/ml stem cell factor (SCF), 50ng/ml Flt3L, 10ng/ml Il-6, 35ng/ml MCSF & 20ng/ml granulocyte macrophage colony stimulating factor (GMCSF), (Invitrogen, Carlsbad, CA). CSII-EGFP was combined with 0.5–3 × 10⁵ HSC/ml in a total volume of 2ml. An equal volume of media was added after 24h. Cells were transferred to 10cm Petri dishes on day 7 and demi-depleted every 3–4 days for up to 14 days. Two experiments were performed; 1) dose/response evaluating MOI of 5–100 in a pool of 2 units, and 2) reproducibility using MOI = 100 in 6 separate donor units. Aliquots removed were used for cell analysis, marker analysis, and osteoclast formation and functional studies.

Cell analysis and FACS

Transduction was evaluated for cell yield and viability using a ViCell counter (Beckman Coulter, Inc, Miami, FL). Cells were stained with CD14-PE, CD34-PerCP and CD11b-APC to monitor cell lineage. Analysis was performed using a FACScaliber cytometer (Beckman Coulter, Inc, Miami, FL) for acquisition, and FloJo software (Treestar, Inc, Ashland, OR) used for quantitation of percentage OcP and marker gene expression.

For transduced CD14⁺ cells, backgating of CD11b onto the forward/side scatter plot define the OcP population of viable, single cells and referred to as R1. Cells were evaluated at 1 to 4 days post transduction. For transduced CD34⁺ cells, contour analysis of the forward/side

scatter plot defined a population of viable, single cells and referred to as R1. This population contained both CD34+ and CD11b+ cells by backgating. Cells were evaluated at 4, 7, 11 and 14 days post transduction. Some samples were stained for CD3, CD19 (lymphocytes), CD7, CD56 (NK) or CD11c (dendritic cells) and no significant numbers were found. CD117 (c-kit/SCF receptor) showed a constant value (~5%).

In vitro osteoclastogenesis

Transduced CD14+ cells were cultured at 1.2×10^5 cells/cm² with MØ media for a total of 8 days (including transduction time). Cultures were then switched to osteoclast media, α -10 containing 10ng/ml MCSF plus 50ng/ml recombinant human receptor activator of for 6 NF- κ B ligand (R&D Systems, Minneapolis, MN) and incubated at 37°C in 5% CO₂ additional days. Transduced CD34+ cells were plated 7 days post transduction at 4×10^3 cells/cm². Cells were cultured in MØ media for 8 days, switched to osteoclast media and incubated for 6 additional days. Cultures were fed every 3–4 days. Replicate wells were plated in 96-well plates for both TRAP enzyme activity and histochemical staining or in 16-well chamber slides for fluorescent microscopy. We used Osteologic™ (BD Biosciences, Bedford, MA) 16-well slides to assess osteoclast removal of hydroxyapatite matrix [28, 29]. Osteoclasts were cultured in 6-well plates to obtain RNA.

Imaging of osteoclasts and hydroxyapatite removal

Cultured osteoclasts were fixed and either stained with TRAP using a commercial kit (Sigma/Aldrich, St. Louis, MO), or counterstained using DAPI (a nuclear stain) in slo-fade mounting media for fluorescent microscopy (Invitrogen, Carlsbad, CA). Bright-field microscopy was used to visualize TRAP-stained osteoclasts. Images were acquired at 10x, and osteoclast count and area determined using ImagePro Plus (Media Cybernetics, Inc, Bethesda, MD). Multi-nucleated osteoclasts were defined as TRAP+ cells >150 μ m² and counts automated. Manual counts confirmed both the size limit and accuracy of the automated count. Cells were removed from hydroxyapatite coated slides using bleach, according to manufacturer's instructions, and the density or area of hydroxyapatite matrix cleared (pits) determined on replicate 4x images. Pit counts and area measurements were automated using ImagePro Plus. Confocal microscopy (Olympus FluoView 500) was used to visualize green (GFP+) osteoclasts.

TRAP enzyme assay

Cultured osteoclasts were solubilized, incubated with 4-nitrophenyl phosphate substrate and allowed to react for 15min. The reaction was stopped using NaOH and absorbance read at 405nm [30]. Replicate cultures of transduced osteoclasts were compared to mock transduced.

Real-time quantitative RT-PCR (qPCR)

Expression levels of mRNA were determined using a 2-step qPCR. RNA was extracted using RNeasy (QIAGEN, Inc, Valencia, CA) according to the manufacturer's instructions. Single-stranded cDNA was synthesized from total RNA. Expression of NFATc1, calcitonin receptor (CtR), cathepsin K (CtsK), TRAP and hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA was determined using commercial primers (SABiosciences, Frederick, MD). Quantitated mRNA values were normalized by the amounts of HPRT mRNA, and results are given as -fold induction.

Statistics

Data is presented as means $\pm$ SD. Statistical significance was determined by Student's t-test. A P value of less than 0.05 was considered statistically significant.

Results

An essential initial step towards using OcP as an in vivo therapy is to evaluate the efficacy, toxicity and results of transduction procedures. Previous publications describing OcP transduction have provided limited characterization of the cell population initially transduced, the influence of transduction on cell yield, and the phenotype of cells generated [19, 21]. The first step in this investigation was to define these characteristics following CSII-EGFP transduction of CD14+ and CD34+ cells.

Effect of CSII-EGFP transduction on viability, yield, and marker gene expression in CD14+ cells

CD14+ cells subjected to CSII-EGFP transduction methods produced acceptable yields, viability and marker gene expression. Specifically, 4 days after CD14+ cells were exposed to transduction conditions viability was $62 \pm 9\%$. As expected, a high percentage ($94 \pm 3\%$) of cells expressed the OcP marker CD11b and $37 \pm 8\%$ expressed the GFP marker gene. Overall yield of OcP 4 days after CSII-EGFP transduction was $32 \pm 5\%$ of the number of cells originally plated.

Effect of CSII-EGFP transduction on viability, yield, and marker gene expression in CD34+ cells

CD34+ cells were subjected to CSII-EGFP transduction methods and day 4 data were compared to findings from CSII-EGFP transduction of CD14+ cells. Importantly, transduced CD34+ cells, when compared to transduced CD14+ cells, contained a significantly higher proportion of cells expressing the GFP marker gene ($72 \pm 11\%$ vs. 37%) and showed a higher overall yield as a percentage of the number of originally plated cells ($125 \pm 61\%$ vs. 32%) ($p < 0.01$). When compared to data from CD14+ cells, transduced CD34+ cells demonstrated a significantly lower viability, lower proportion of cells in R1, and lower proportion of CD11b+ cells ($p < 0.01$). Specifically viability was $42 \pm 22\%$ (vs. 62% for CD14+), OcP comprised $60 \pm 16\%$ (vs. $86 \pm 3\%$ for CD14+) of the cells in R1 and $21 \pm 10\%$ (vs. 93% for CD14+) of cells expressing the OcP marker CD11b. Eighty-two $\pm 9\%$ of the transduced CD34+ cells in R1 were CD34+. We determined that higher plating number (1 vs. $2.5 \times 10^5/\text{ml}$) resulted in significantly ($p < 0.004$) better viability ($23 \pm 13\%$ vs. $54 \pm 18\%$) and cells in R1 ($47 \pm 12\%$ vs. $68 \pm 12\%$) while transduction efficiency and yield was unchanged. Additionally, these values were no longer significantly lower than transduced CD14+.

Expansion of CSII-EGFP transduced OcP: CD14+ method vs. CD34+ method

With the intention to ultimately utilize OcP for in vivo human therapy it is crucial to determine the influence of transduction variables on expansion of ex vivo OcP. To this end we next determined capabilities of expanding cells using transduction conditions for CD14+ and CD34+ cells and monitored by GFP.

Transduction methods for CD14+ cells expanded GFP+ cells exponentially ($R^2 = 0.98$) to an overall 6-fold increase in GFP+ R1 cells from day 1 to 4 (Figure 1A). Interestingly, the total number of cells at experiments completion was only a fraction of the plated number (32%). Because of the low cell numbers using this method, CD14+ were not cultured beyond 4 days. In contrast, transduction and expansion of CD34+ cells exponentially increased both the number of GFP+ R1 cells from 4 to 14d ($R^2 = 0.95$) and the total number of cells ($R^2 = 0.95$) from the plated number (Figure 1B) to overall 10- and 70-fold increases, respectively. Cell lineage of transduced CD34+ cells showed a linear decrease in HSC (CD34) expression with a concomitant linear increase in OcP (CD11b & CD14) expression, consistent with MCSF and GMCSF in the media (Figure 2). Specifically, from a starting point of 98%,

CD34 expression fell to 17% by day 14, while CD11b and CD14 increased from 0% to 55% and 30%, respectively. It was also noted that the mean fluorescence of transduced CD34+ cells was over a log brighter than transduced CD14+ cells. Over time in culture the higher expressing cells expanded, while lower-expressing cells disappeared (supplemental data 1).

CD14+ and CD34+ were transduced and expanded using the CD34+ method for direct comparison of the two cell types at the same MOI. Analysis of the CD14+ transduced cells revealed that GFP+ OcP could be expanded for up to 7 days in culture. Specifically, the number of GFP+ OcP was 9.4% ($0.47 \pm 5 \times 10^6$) of the number plated after 4 days and increased to 27.8% ($1.39 \pm 5 \times 10^6$) after 7 days. Reduction of GFP+ to 8.5% ($0.42 \pm 5 \times 10^6$) was observed at 11 days in culture (Figure 3). The total number of cells was 50% of the plated number. In contrast, analysis of CD34+ cells resulted in an increase in both the number of GFP+ OcP and the total number of cells at the same MOI. Specifically, the proportion of GFP+ OcP after 4 and 7 days increased from 45% ($0.23 \pm 0.5 \times 10^6$) of the number plated to 158% ($0.79 \pm 0.5 \times 10^6$) (Figure 3) and continued to increase exponentially to 334% ($1.67 \pm 0.5 \times 10^6$) on day 11 ($R^2 = 0.95$). The total number of cells increased 1.5, 6 and 22-fold on days 4, 7, and 11, respectively. To examine the capability and potential for transduction and expansion of CD34+ derived OcP, we performed the same experiment with MOI = 100. Generation of transduced CD34+ cells increased with increasing virus exposure. The proportion of GFP+ OcP increased from 94% to 480% to 775% ($0.47, 2.4 \text{ \& } 3.9 \pm 0.5 \times 10^6$) of initially plated cells on days 4, 7 and 11, respectively.

Effect of MOI on CSII-EGFP transduction: CD14+ method vs. CD34+ method

To further characterize CSII-EGFP transduction of OcP, the effect of MOI on CD14+ and CD34+ OcP populations was compared. CSII-EGFP transduction of both populations was influenced by MOI with increased transduction efficiency seen with increasing MOI. Specifically, transduction of CD14+ cells, measured 24h post transduction, increased linearly by 13-fold ($R^2 = 0.99$) when exposed to MOI of 1 compared to MOI of 20 (Figure 4A). Similarly transduction of CD34+ cells, measured 4 days post transduction, increased linearly by 5-fold ($R^2 = 0.89$) when exposed to MOI of 5 compared to an MOI of 100 (Figure 4B).

Effect of MOI on osteoclastogenesis: CD14+ method vs. CD34+ method

The effect of MOI on the osteoclastogenic potential of transduced CD14+ and CD34+ was evaluated using TRAP enzyme activity and it was determined that osteoclast formation was superior using the CD34+ transduction method. Specifically, significant reduction in osteoclastogenesis was observed as MOI increased using CD14+ cells but was unchanged using CD34+ transduction methods. Increasing MOI from 1 to 20 during CD14+ transduction reduced osteoclast activity by 42% compared to mock transduced cells (Figure 4A). In CD34+ transduced cells, osteoclast activity was unaffected as MOI increased from 5 to 100 (Figure 4B).

Findings from both the CD14+ and CD34+ transduced OcP were further characterized by determining osteoclast cell counts, osteoclast size and total area covered by osteoclasts (Table 1). There was a significant decrease in both osteoclast number and size when transduced CD14+ OcP (MOI=20) were compared to mock. Transduced CD14+ osteoclast cultures yielded 20% fewer osteoclasts at MOI=10 and 50% fewer at MOI=20 ($p < 0.01$) compared to mock. Additionally, transduced CD14+ osteoclasts were 20% smaller at MOI=20 ($p < 0.01$). There was no adverse change in osteoclast number or size when transduced CD34+ OcP were compared to mock. When compared to transduced CD14+ osteoclast cultures, transduced CD34+ osteoclast cultures yielded significantly larger osteoclasts and increased total osteoclast area ($p < 0.01$). There were significantly larger

osteoclasts and total osteoclast area between mock and MOI=100 ($p < 0.01$) due to the fusion effect of the lentivirus envelope [31]. Overall, CD34+ OcP produced significantly fewer (30%), but significantly larger (10-fold) osteoclasts than CD14+ OcP that covered twice the area.

Characterization of osteoclasts generated from CSII-EGFP transduced culture systems: CD14+ vs. CD34+

To further characterize transduced osteoclast cultures produced by CD14+ and CD34+ OcP, we evaluated mRNA expression, TRAP enzyme activity, microscopic appearance, osteoclast size and hydroxyapatite matrix resorption pits. In sum, findings clearly demonstrate increased activity, size and hydroxyapatite matrix destruction in CD34+, CSII-EGFP-transduced culture systems compared to CD14+, CSII-EGFP transduced culture systems.

Evaluation of mRNA denoting osteoclast differentiation indicated that mRNA sequences expressed during osteoclast differentiation, were detected in both CD14+ and CD34+ osteoclast cultures at levels significantly higher than controls (Hela cells). The mRNA levels for CTR, TRAP, NFATc1 and CtsK were all similarly abundant in each osteoclastogenic culture system and each mRNA was expressed similarly in CD14+ generated, CSII-EGFP cultures compared to CD34+ generated, CSII-EGFP cultures (supplemental data 2). TRAP enzyme activity was detected in all osteoclastogenic culture systems, but was higher in CD34+ CSII-EGFP transduced culture systems.

Microscopic inspection of CD14+ and CD34+, CSII-EGFP-transduced, osteoclastogenic cultures showed dramatic differences between the two systems. While osteoclasts from both culture systems showed high levels of marker gene (GFP) expression (Figure 5A, B), osteoclasts from CD34+, CSII-EGFP-transduced culture systems were significantly larger and showed increased multi-nucleation compared to osteoclasts from CD14+, CSII-EGFP transduced culture systems (Figure 5C, D).

Analysis of hydroxyapatite matrix resorption revealed pit formation in both CD14+ and CD34+, CSII-EGFP-transduced osteoclastogenic culture systems but showed dramatic differences between the two systems. Specifically, inspection of hydroxyapatite matrix following exposure to CD34+ CSII-EGFP-transduced osteoclastogenic cells revealed resorption pits that were significantly larger and deeper than pits from CD14+ CSII-EGFP-transduced osteoclastogenic cells (Figure 5E, F). Due to the small size, CD14+ pits could not be quantified. Pits from CD34+ CSII-EGFP transduced osteoclastogenic cells covered about 50% of the total 9.5mm² field, averaged 300–400 events/field and measured 2000 to 3000 µm² in area. Pit area was significantly increased by an increase in MOI (Table 2) due to larger osteoclast size.

Discussion

This work identifies CD34+ cells as a feasible candidate for delivery of OcP-directed ex vivo gene therapy. Experiments evaluated and compared the results of lentiviral transduction of two distinct populations of OcP. CD14+ and CD34+ cells were exposed to lentiviral transduction procedures and findings identified CD34+ cells as a superior starting point for generation of large numbers of transduced OcP. Specifically, when transduction efficiency and in vitro experiments were assessed, CD34+ cells demonstrated higher transduction efficiency (73% vs. 37%) and far superior expansion (3-fold increase) compared to a reduction in CD14+ cultures. In addition, increasing transduction efficiency by increasing MOI, proved to be a limiting factor in the transduction of CD14+ cells, as the formation and function of osteoclasts declined at higher MOI.

The finding of increased transduced cells and superior expansion of CD34+ vs. CD14+ transduced cells are not surprising. Expansion of CD34+ into CD14+ monocytes [32], OcP [22] or HSC [23] has been well documented. It has been previously suggested, to transduce CD34+ and differentiate them into monocytes/macrophages, but has yet to be published. In the past it has proven difficult to transfect human monocytes and macrophages, in part due to their phagocytic and poorly proliferative nature [33]. CD14+ cells have been transduced as dendritic cell precursors; however, the transduction efficiency (up to 90%) is generally measured after their differentiation into dendritic cells [34, 35] and no information on yield or viability has been published.

Lentiviral techniques were selected over adenoviral, retroviral, or plasmid DNA techniques for a variety of reasons. Viral approach was preferred over plasmid DNA techniques because lipid [20] or nucleoporation [19] techniques have demonstrated poor transduction efficiency and insufficient yields with large populations of cells. Lentivirus was chosen over other viral techniques based on the knowledge that higher MOI's yield superior viral transduction efficiency. Since lentivirus can be concentrated to achieve higher titers than retroviruses, we reasoned that lentivirus techniques were preferred. Finally, we selected a lentiviral approach over the adenoviral approach as we believe stable integration has advantages and future opportunities for delivering cell-based gene therapies in clinical circumstances requiring prolonged gene expression.

Evaluation of osteoclast size and function identified CD34+ culture as superior to CD14+ culture. While both culture systems formed osteoclasts and displayed the expected panel of osteoclast genes, CD34+ derived culture systems produced osteoclasts that were significantly larger (10-fold) and removed dramatically more hydroxyapatite matrix than cells from CD14+ derived systems. It has been shown [36, 37] that CD34+ cells can form in vitro osteoclasts, however methods have only recently been modernized [38] to utilize commercial purification techniques and defined cytokine media. This is the original report to quantify the area as well as number of human-derived osteoclasts, and to compare CD14+ with CD34+ derived osteoclasts.

With the long term goal of developing ex vivo systems for OcP-directed, cell-based gene delivery to sites of bone tumors, we determined that CD34+ cells seem well suited as the starting point for ex vivo transduction and expansion. Following transduction methods, these early OcP maintain high levels of gene expression, expand perhaps limitlessly in culture and demonstrate a robust osteoclast phenotype in vitro. In addition, the methods described utilize defined media, which may be suitable for the constraints clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

CD cytosine deaminase

TRAP	tartrate resistant acid phosphatase
OcP	osteoclast precursor cells
HSC	hematopoietic stem cells
MNC	mononuclear cells
CB	cord blood
VSV-G	vesicular stomatitis virus envelope glycoprotein
EGFP	enhanced green fluorescent protein
MOI	multiplicity
MEMα	Minimal Essential media, alpha modification
MCSF	macrophage colony stimulating factor
d-d	DEAE-dextran
FACS	flow cytometry
ULA	ultra-low attachment plates
SCF	stem cell factor
qPCR	Real-time quantitative RT-PCR
CtR	calcitonin receptor
CtsK	cathepsin K
HPRT	hypoxanthine guanine phosphoribosyl transferase

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Figure 1. CSII-EGFP transduced OcP expand Ex Vivo

[A] GFP+ OcP from transduced CD14+, increased exponentially [$R^2 = 0.98$] and significantly by 1.8-fold/day, for up to 4 days in culture, while the total number of cells decreased to a fraction of the number of cells plated [$R^2 = 0.70$]. [B] Transduced CD34+, showed an exponential [$R^2 = 0.95$] increase in both GFP+ OcP and total cell number, with log increases from the number plated, for up to 14 days in culture. •••• number of cells plated. * $p < 0.05$ vs. the previous time point. † $p < 0.01$ vs. day 4.

Figure 2. CSII-EGFP transduced CD34+ cells change from HSC to OcP lineage over time in culture

Transduced CD34+ demonstrate a linear decrease [$R^2 = 0.94$] in CD34+ and linear increases in CD11b+ and CD14+ cells with increased time in culture [$R^2 = 0.93$ and 0.84].

Figure 3. Expansion of CSII-EGFP transduced OcP

Transduced CD34+ (MOI = 25) demonstrate an exponential increase in GFP+ OcP.

Transduced CD14+ (MOI = 20) expand initially. Graph represents cells purified from the same donor and cultured in parallel.

Figure 4. Effect of MOI on CSII-EGFP transduction and osteoclastogenesis
A) Transduced CD14+ OcP %GFP+ cells and TRAP activity (* $p < 0.02$ vs. mock). B)
Transduced CD34+ OcP %GFP+ cells and TRAP activity. •••• TRAP activity of mock
transduced OcP.

Figure 5. Effect of CSII-EGFP transduction of CD14+ or CD34+ OcP on osteoclast gene expression, formation and function

Confocal images of day 14 osteoclasts from A) CD14+ or B) CD34+ CSII-EGFP_transduced osteoclastogenic culture systems. Gfp+ (green), multinucleated (blue) osteoclasts. TRAP stained osteoclasts from C) CD14+ or D) CD34+ OcP. Two osteoclasts are outlined (••••) to demonstrate size. Resorption (white/cleared areas) in osteoclasts from E) CD14+ or F) CD34+ OcP. Scale bar = 100 μ m.

Table 1
Characterization of CD14+ and CD34+ osteoclasts following CSIIeG transduction

Cell	MOI	# Osteoclasts ^a		Oc Area [μm^2]		Oc total [mm^2] ^b	
		mean ^c	$\pm$ SD	mean ^c	$\pm$ SD	mean ^c	$\pm$ SD
CD14+	0	330	11	640	82	0.21	0.21
CD14+	10	281	93	588	131	0.17	0.17
CD14+	20	167 [*]	66	512 [*]	92	0.09 [*]	0.06
CD34+	0	105 [†]	29	4145 [†]	1742	0.40	0.10
CD34+	50	95 [†]	15	6130 [†]	1937	0.56	0.11
CD34+	100	77 [†]	24	9471 ^{†*}	3905	0.66 [*]	0.09

^aNumber of TRAP+ multinucleated cells.

^bTotal area (mm^2) in 10x field covered by osteoclasts.

^cValues are mean $\pm$ SD of 5 replicate wells.

[†] p < 0.01 CD14+ vs. CD34+.

* p < 0.01 mock [0] vs. transduced.

CD34+ pits following CSIIeG transduction

Table 2

Cell	MOI	#Pits ^a		Area [μm ²] ^b	
		mean	±SD	mean	±SD
CD34+	0	312	30	2233	197
CD34+	50	366	32	2637*	257
CD34+	100	387	63	3033*	400

^a Number of cleared resorption pits/9.5mm² [4x] field.
^b Average area of resorption pits in 4x field.
* p < 0.05 mock [0] vs. transduced.