

## Research article

# Highly efficient genetic transduction of primary human synoviocytes with concentrated retroviral supernatant

Jianmin Yang, Michael S Friedman, Huimin Bian, Leslie J Crofford, Blake Roessler and Kevin T McDonagh

Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan, USA

**Correspondence:** Kevin T McDonagh, MD, University of Michigan Medical School, 5301 MSRB III, 1150 West Medical Center Drive, Ann Arbor, MI 48109-0640, USA. Tel: +1 734 647 9912; fax: +1 734 764 0101; e-mail: [kmcd@umich.edu](mailto:kmcd@umich.edu)

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## Abstract

We are developing retroviral-mediated gene transfer to human fibroblast-like synovial cells (FLS) as one approach to characterizing genetic pathways involved in synoviocyte pathophysiology. Prior work has suggested that FLS are relatively refractory to infection by Moloney murine leukemia virus based vectors. To determine if viral titer influenced the transduction efficiency of FLS, we optimized a rapid, efficient, and inexpensive centrifugation method to concentrate recombinant retroviral supernatant. The technique was evaluated by measurement of the expression of a viral enhanced green fluorescent protein transgene in transduced cells, and by analysis of viral RNA in retroviral supernatant. Concentration (100-fold) was achieved by centrifugation of viral supernatant for four hours, with 100% recovery of viral particles. The transduction of FLS increased from approximately 15% with unconcentrated supernatant, to nearly 50% using concentrated supernatant. This protocol will be useful for investigators with applications that require efficient, stable, high level transgene expression in primary FLS.

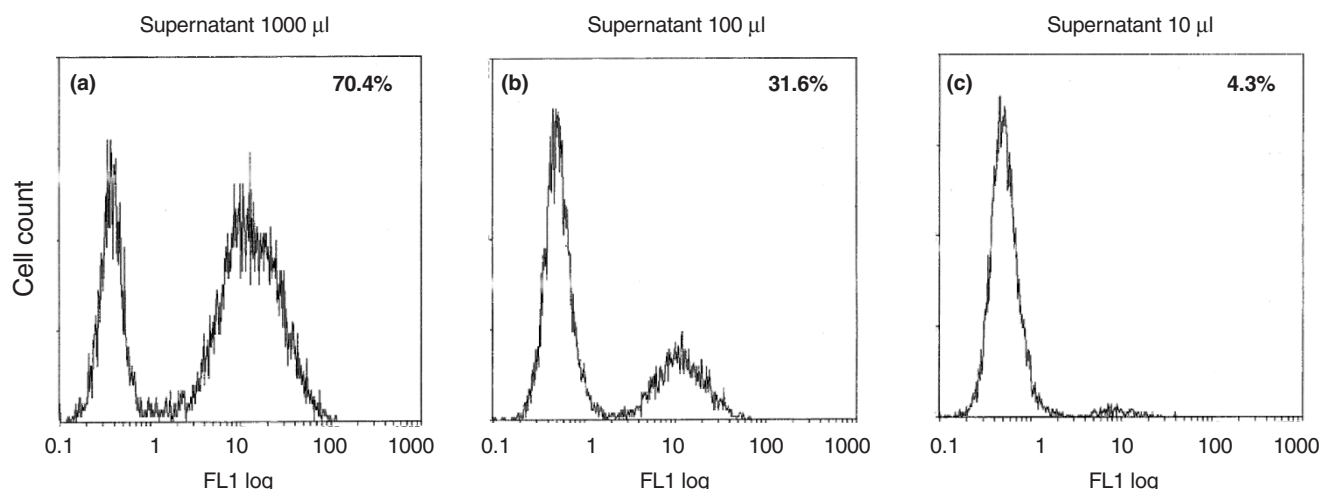
**Keywords:** enhanced green fluorescent protein, fibroblast-like synovial cell, gene therapy, retrovirus, titer

## Introduction

Synovial tissues isolated from patients with rheumatoid arthritis (RA) display biologic properties that differ from 'normal' synovium, and there is a rapidly expanding catalogue of biochemical and molecular changes that underlie this phenotype [1]. We have investigated the feasibility of using Moloney murine leukemia virus (MoMLV) based vectors to constitutively express cloned genes in primary human fibroblast-like synovial cells (FLS), with the long-term objective of defining the contributions of specific signaling pathways and inflammatory mediators to the destructive phenotype of FLS in RA.

Prior studies have suggested that MoMLV-based vectors transduced FLS with relatively low efficiency [2–5]. We designed experiments to determine if viral titer influenced FLS transduction by concentration of retrovirus. In these experiments, we used a modified MoMLV vector (pRET2), designed to improve transcriptional stability in primary cells. We also employed the enhanced green fluorescent protein (EGFP) as a virally encoded transgene to optimize a rapid and efficient superspeed centrifugation technique for concentration of viral supernatant. Viral particles were concentrated to  $>10^8$  colony forming units (cfu)/ml by superspeed centrifugation at 20,000 *g* for four hours. Up

cfu = colony forming units; COX-2 = cyclooxygenase-2; DMEM = Dulbecco's modified Eagle's medium; EGFP = enhanced green fluorescent protein; FACS = fluorescence-activated cell sorting; FLS = fibroblast-like synovial cells; MoMLV = Moloney murine leukemia virus; PCR = polymerase chain reaction; RA = rheumatoid arthritis; RCF = relative centrifugal force.

**Figure 1**

Quantitation of viral titer. Murine fibroblast NIH 3T3 cells ( $2 \times 10^5$ ) were transduced with (a) 1000 µl, (b) 100 µl, or (c) 10 µl of unconcentrated pRET2.EGFP supernatant. The percentage of enhanced green fluorescent protein (EGFP)-positive cells was measured by flow cytometry (% EGFP+ cells indicated in each panel). Titer was calculated using the volume of supernatant yielding <10% EGFP+ cells. In this example: Titer =  $0.043 \times (2 \times 10^5 \text{ target cells}) / 0.01 \text{ ml} = 0.86 \times 10^6 \text{ cfu/ml}$ . For concentrated supernatant, smaller volumes were required to achieve transduction efficiencies <10%.

to 50% of primary human FLS were transduced *in vitro* following a single exposure to concentrated viral supernatant.

## Materials and methods

### Cell Culture

Murine fibroblast NIH 3T3 cells, amphotropic PA317 packaging cells, and Phoenix E ecotropic packaging cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-high glucose (GIBCO-BRL, Grand island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO-BRL, Grand island, NY, USA), 100 U/ml penicillin, 100 µg/ml streptomycin, and 200 mM L-glutamine. The FLS cultures were established from synovial tissues obtained during joint replacement surgery in RA patients [6]. The FLS were cultured in DMEM plus 10% heat-inactivated human AB serum (BioWhittaker, Walkersville, MD, USA), 10% fetal bovine serum, penicillin, streptomycin, and L-glutamine. The FLS were used between the third and tenth passage.

### Construction of retroviral vector and producer cells

The EGFP cDNA was PCR amplified from pEGFP-1 (Clontech, Palo Alto, CA, USA) and subcloned into pRET2, a modified version of the MoMLV-based MFG retroviral vector, designed to optimize gene expression in primary cell lines. The pRET2 incorporates long-terminal repeats from the myeloproliferative sarcoma virus [7], and a point mutation in the primer binding site [8]. A vector expressing the human cyclooxygenase-2 (COX-2) cDNA was constructed in the same backbone (pRET2.COX2).

Amphotropic viral producers were established in PA317 cells (see Supplementary Material).

### Concentration of viral supernatant by superspeed centrifugation

Fresh medium was added to subconfluent producer cell monolayers, collected 24 hours later, and filtered (0.45 µm) prior to use. Centrifugation was performed at 4°C in a Sorval RC-5B centrifuge, using SS-34 or GSA rotors. Following centrifugation, the supernatant was aspirated and saved for analysis. The viral pellet was resuspended in fresh medium by gentle pipetting.

### Quantitation of viral RNA by slot blot hybridization

Viral RNA was quantitated using a slot blot hybridization technique. See Supplementary Material for full details.

### Quantitation of retroviral titer by flow cytometry based expression analysis for EGFP

We developed a flow cytometry assay to rapidly measure the titer of infectious viral particles (Fig. 1). This assay takes advantage of the fluorescent properties of the EGFP transgene. A total of  $2 \times 10^5$  NIH 3T3 cells were transduced with serial dilutions of supernatant. The transduction efficiency was measured by flow cytometry, and viral titer was calculated at limiting dilution according to the following formula:

$$\text{Titer (cfu/ml)} = (2 \times 10^5 \text{ target cells}) \times (\% \text{ EGFP+ cells}) / \text{volume of supernatant (ml)}.$$

See Supplementary Material for full details.

### Transduction of primary human FLS

The FLS were plated in 6-well dishes at  $2 \times 10^5$  cells/well. FLS were cultured with viral supernatant plus protamine sulfate (5  $\mu$ g/ml) for 24 hours. Cells were analyzed for transgene expression 72 hours after infection.

## Results

### Concentration of viral supernatant

To determine if viral titer influenced the transduction efficiency of FLS, we optimized a superspeed centrifugation protocol for concentration of viral supernatant. Prior studies reported improved transduction of primary cells with retrovirus concentrated by centrifugation at 6000 *g* for 16 hours [9–11]. We systematically evaluated different centrifugation parameters to minimize the time required for maximal concentration while preserving viral infectivity. A virally encoded EGFP transgene [12–14] was used to monitor viral concentration and infectious titer. We concentrated viral supernatant 100-fold in as few as four hours by centrifugation at 20,000 *g*, with complete recovery of infectious viral particles. This data is presented in the Supplementary Material (Supplementary Figs 1, 2, 3, and 4).

### Retroviral transduction of primary human synoviocytes

Concentrated virus was tested for its ability to transduce primary FLS. As shown in Figure 2 and Table 1, concentration of viral supernatant increased FLS transduction. We found that  $14.2 \pm 8.2\%$  of FLS expressed EGFP following transduction with unconcentrated supernatant, compared with  $41.3 \pm 14.7\%$  for 10X concentrated supernatant ( $P < 0.01$ , compared with unconcentrated supernatant), and  $47.3 \pm 14.8\%$  for 100X concentrated supernatant ( $P < 0.01$ , compared with unconcentrated supernatant).

To provide confirmation that improved transduction of FLS was associated with an increase in the intracellular expression of a virally encoded transgene, FLS were transduced with a vector encoding human COX-2 (pRET2.COX2). The expression of COX-2 was measured by western blot on whole cell lysates [6]. A substantial increase in net COX-2 expression was observed following transduction with both 10X and 100X concentrated viral supernatant (Fig. 3).

## Discussion

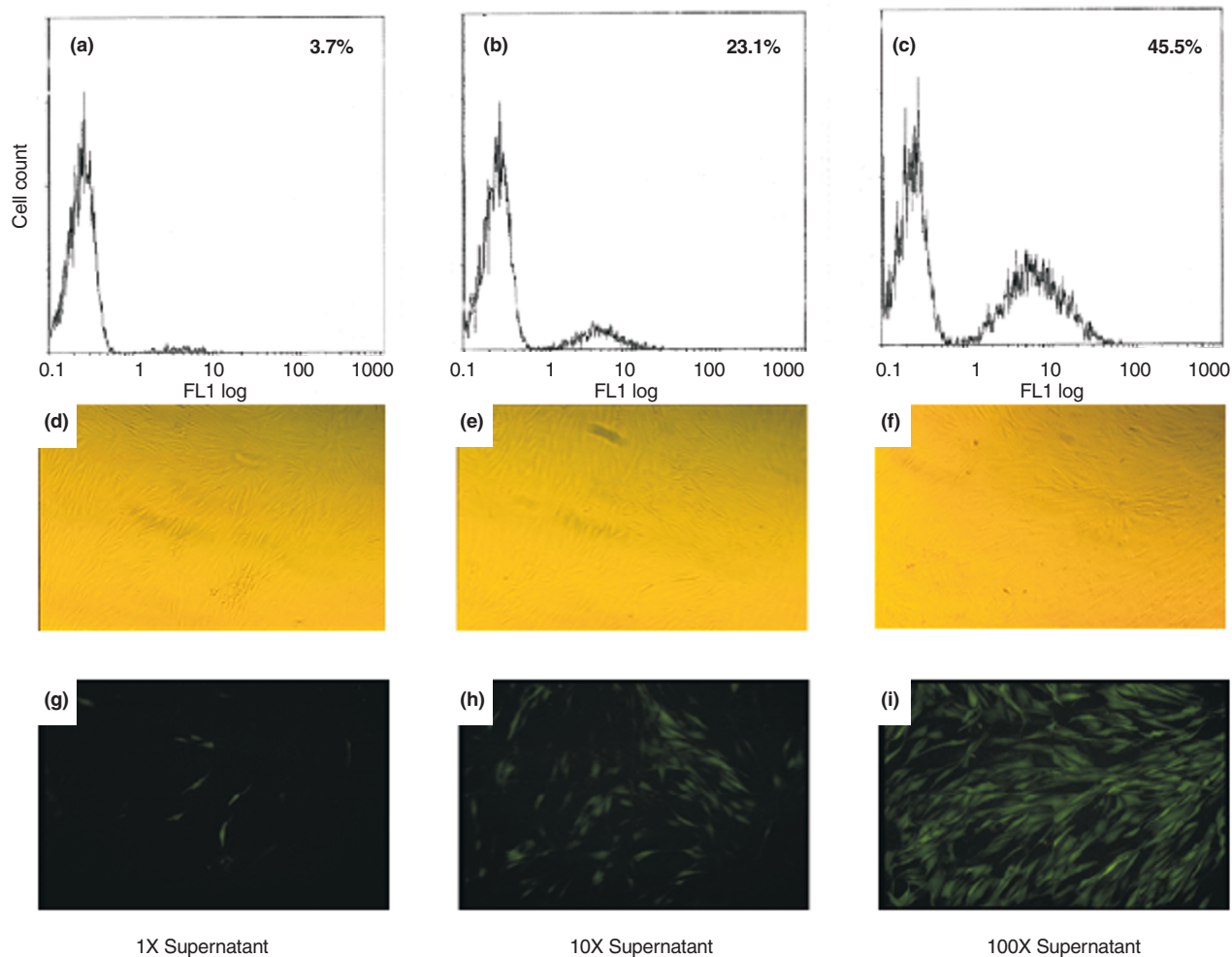
We are characterizing molecular pathways involved in synovial pathophysiology by overexpression of biologically relevant transgenes and dominant negative inhibitors in FLS. The limited expansion potential of FLS, combined with the low efficiency of existing methods, stimulated a systematic examination of various transduction techniques to identify a rapid and efficient method for stable genetic modification of FLS. In this manuscript, we report a retroviral vector system and transduction protocol with the capacity to

express a viral transgene in 50% or more of primary human FLS after a single exposure to virus. We have subsequently used this methodology to successfully express a panel of transgenes in FLS (L Crofford and K McDonagh, unpublished observations). We believe this approach will be of value to investigators addressing similar mechanistic questions in FLS.

Previous studies exploring the use of recombinant MoMLV vectors concluded that FLS were relatively resistant to transduction [2–5], limiting enthusiasm for this approach. The basis for this resistance was unclear, but could be attributable to many factors including vector design, viral titer, or biologic features inherent to FLS. Our experiments differ from prior studies of retroviral gene transfer to FLS in several important respects that may impact on the observed results. First, our viral backbone is a modified MoMLV vector that incorporates genetic elements (myeloproliferative sarcoma virus long-terminal repeats and B2 mutation) associated with resistance to transcriptional silencing following proviral integration in primary cells [7,8]. While we did not perform a detailed comparison of EGFP expression in FLS using the modified and unmodified vector backbones, preliminary experiments suggested that the modified vector was superior (J Yang, unpublished observations). A similar, modified MoMLV vector has been used to stably express EGFP in human marrow stromal cells [15], another fibroblast-like primary cell type. A second distinction is the use of EGFP as a transgene, whereas prior studies relied on lacZ or beta-galactosidase. The expression of EGFP is readily detectable in living cells by fluorescence microscopy or flow cytometry, and expression can be monitored serially over time in a single culture. In contrast to staining for lacZ, which is often complicated by background staining from endogenous galactosidase activity, there is no significant background staining with EGFP. We do not know if analysis of EGFP expression is more or less sensitive than analysis for lacZ expression, although we believe it provides more reproducible and quantitative data due to the absence of background staining.

Using this vector system, we observed a low *ex vivo* transduction efficiency ( $14.2 \pm 8.2\%$ ) of FLS with unconcentrated supernatant (titer of  $10^6$  cfu/ml) that was roughly comparable to prior reports. Centrifugal concentration of viral supernatant by 10- to 100-fold significantly increased the efficiency of viral transduction, with 50% or more of FLS expressing EGFP in several independent experiments using FLS lines from separate donors. Concentration of supernatant to viral titers exceeding  $10^7$  cfu/ml appeared to have the greatest quantitative impact on improving transduction efficiency. Increasing viral titer to  $10^8$  cfu/ml yielded an additional increase in transduction efficiency in some, but not all experiments. This observation suggests that factors in addition to viral titer may limit the maximum

Figure 2



Transduction of fibroblast-like synovial cells (FLS) with pRET2.EGFP. The FLS from patients with rheumatoid arthritis (RA) were transduced with (a) (d) (g) unconcentrated, (b) (e) (h) 10X concentrated, or (c) (f) (i) 100X concentrated pRET2.EGFP supernatant. (a–c) The percentage of EGFP-positive FLS was determined by flow cytometry. (d–f) Light and (g–i) fluorescence microscopy images of cultures following transduction are shown. These results are representative of data using FLS isolated from 5 RA patients.

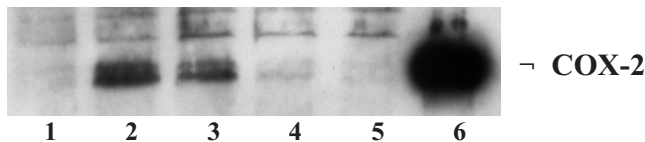
Table 1

Viral transduction of fibroblast-like synovial cells

FLS Line	Negative control	Unconcentrated supernatant	10X Concentrated supernatant	100X Concentrated supernatant
RA16	0.1	8.2	33.3	25.4
RA25	0.6	23.7	62.2	63.5
RA30	0.0	15.8	40.7	58.2
RA31	0.2	3.7	23.1	45.5
RA32	0.2	19.6	47.3	43.9
Mean $\pm$ SD	0.2 $\pm$ 0.2%	14.2 $\pm$ 8.2%	41.3 $\pm$ 14.7%*	47.3 $\pm$ 14.8%**

Fibroblast-like synovial cells (FLS) were transduced with pRET2.EGFP retroviral supernatant. The values represent the percentage of enhanced green fluorescent protein-positive cells by flow cytometry. \* $P < 0.01$  compared with unconcentrated supernatant; \*\* $P > 0.05$  compared with 10X supernatant.



**Figure 3**

Expression of cyclooxygenase-2 (COX-2) in transduced fibroblast-like synovial cells (FLS). The FLS from patients with rheumatoid arthritis were transduced with retrovirus. Lane 1: 100X concentrated RET2.EGFP; lane 2: 100X concentrated RET2.COX2; lane 3: 10X concentrated RET2.COX2; lane 4: unconcentrated RET2.COX2; lane 5: post-centrifugation supernatant RET2.COX2. Whole cell lysates were analyzed for COX-2 by western blot (lane 6: purified COX-2 protein). The experiment was repeated using FLS lines from different patients with similar results.

number of transduced FLS observed using these culture conditions. Lentiviral vectors have the capacity to transduce nonreplicating cells [16], and may represent an alternative to MoMLV-based vectors for some applications.

## Conclusion

We report a retroviral vector system and transduction methodology that achieve stable transgene expression in primary human FLS with efficiencies of approximately 50%. These results establish the feasibility of using widely available retroviral gene transfer techniques to study the biologic impact of overexpression of specific regulatory and inflammatory molecules in primary FLS.

## Acknowledgements

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## Supplementary material

### Supplementary Introduction

Synovial cells play a central role in the pathophysiology of inflammatory arthritis. Much of our understanding of this biology has been derived from the study of primary fibroblast like synovial cells cultured from arthritic joints after arthroscopic biopsy or surgery. Stable genetic modification of primary synovial cells is an approach that may be useful in defining the roles that specific signaling pathways or inflammatory mediators play in the joint destruction associated with rheumatoid arthritis. As our understanding of this biology improves, investigators have also proposed that gene transfer to primary synovial cells could be developed as a therapeutic approach to the treatment of patients with inflammatory arthritis [2,3].

Recombinant retroviral vectors are widely used in the laboratory, and in experimental clinical applications, to introduce new genetic material into the host genome in a stable form. Retroviral packaging cells routinely yield viral supernatants with titers in the range of  $10^5$  to  $10^6$  cfu/ml or higher, and titers of up to  $10^7$  cfu/ml may be achieved in some cases. Physical methods to concentrate viral supernatants have been pursued with mixed results. Ultracentrifugation can be used to physically concentrate MoMLV-based retroviral particles, but viral infectivity is

impaired secondary to damage to the envelope protein. Pseudotyped retroviruses containing the vesicular stomatitis virus G protein are more robust, and can be concentrated more than 100-fold by ultracentrifugation without significant loss of viral infectivity. However, because of the toxicity of the vesicular stomatitis virus G glycoprotein, only transient methods of virus production have been described [S1,S2]. Bowles *et al.* previously reported a superspeed centrifugation technique for concentration of recombinant retrovirus [9]. A MoMLV based recombinant retrovirus was concentrated over 100-fold by centrifugation at 6000 *g* for 16 hours.

## Supplementary Materials and methods

### Cell culture

The murine fibroblast NIH 3T3 cell line (CCL 92) and the amphotropic retroviral packaging cell line PA317 (CRL 9078) were obtained from the American Type Culture Collection (Rockville, MD, USA). The Phoenix-E ecotropic packaging cell line was obtained from Dr Gary Nolan (Stanford University, USA).

### Isolation of amphotropic producer cells

A transfection technique was used to rapidly establish a polyclonal amphotropic producer line of moderate to high titer. The pRET2.EGFP or pRET2.COX2 plasmids were transfected into ecotropic Phoenix E packaging cells by the calcium phosphate precipitation method, using the Profection kit (Promega, Madison, WI, USA). Retroviral supernatant was collected 48 hours after transfection, filtered through a 0.45  $\mu$ m filter (Nalgene, Rochester, NY, USA), supplemented with 5  $\mu$ g/ml protamine sulfate (Elkins-Sinn, Inc. Cherry Hill, NJ, USA), and incubated with amphotropic PA317 packaging cells for 24 hours. The transfection procedure was repeated twice. Following transfection with ecotropic viral supernatant, 100% of the PA317 cells were transduced with the pRET2.EGFP vector, as determined by fluorescence microscopy. The successful transinfection of pRET2.COX2 into PA317 was confirmed by G418 selection. These polyclonal populations of PA317 producer cells were used as the source of viral supernatant for subsequent viral transduction and concentration experiments. The presence of replication competent retrovirus was excluded by PCR for viral envelope coding sequence in genomic DNA isolated from virally transduced NIH 3T3 target cells (primers: 5'-AAG-GTGGTAAACCAGGGGGATC-3' and 5'-TGAGCAGCT-TCATGCCGCTATC-3').

### Quantitation of viral RNA by slot blot hybridization

A nylon transfer membrane (Micron Separations Inc. Westborough, MA, USA) was soaked in 10X SSC for 10 min and inserted into a BRL convertible filtration manifold system (BRL Life Technologies Inc. Gaithersburg, MD, USA). Each well was washed twice with 200  $\mu$ l of 10X SSC immediately before sample loading. Retroviral

supernatant samples were directly loaded onto the membrane without further preparation. After application of the sample to the membrane, the wells were washed three times with 200  $\mu$ l of 10X SSC. The membrane was cross-linked with UV light (Stratalinker 1800, Stratagene, La Jolla, CA, USA) and stored for analysis by hybridization. An EGFP probe fragment (~800 base pairs) was prepared by PCR and labeled with  $^{32}$ P-dCTP (Amersham Life Science Inc., Arlington Heights, IL, USA) using a kit (Prime-It RmT, Stratagene, La Jolla, CA, USA). The membrane was prehybridized for 2 hours at 42°C in 10 ml of hybridization buffer (final concentrations: 50% formamide, 5X Denhardt's solution, 0.1% SDS, 5X SSPE, 150  $\mu$ g/ml denatured herring sperm DNA), and hybridized with the denatured probe overnight in 5 ml of hybridization buffer at 42°C. The membrane was washed twice with 2X SSPE at room temperature for 10 min, three times with 0.1X SSPE/0.5% SDS at 55°C for 30 min, and twice with 0.1X SSPE at room temperature for 10 min. The autoradiograph was visualized by exposing the membrane to X-ray film at -80°C with an intensifying screen.

### Quantitation of retroviral titer by FACS based expression analysis for EGFP

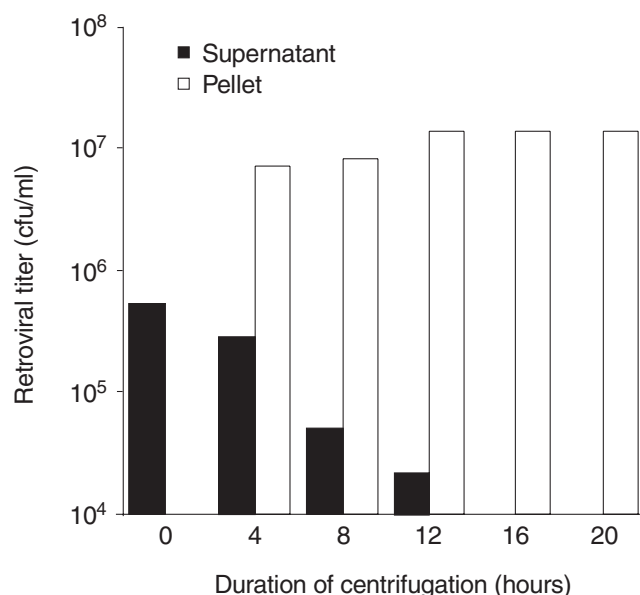
The NIH 3T3 cells were plated in 6-well tissue culture dishes at a density of  $10^5$  cells per well. The following day, the medium was replaced with 2 ml of fresh medium containing a defined volume of viral supernatant, supplemented with protamine sulfate (5  $\mu$ g/ml). After exposure to viral supernatant for 24 hours, the medium was replaced with fresh, virus-free medium and the cells were cultured for an additional 48 hours. At the conclusion of the experiment, the cells were trypsinized and analyzed by flow cytometry on an EPICS XL (excited by 488 nm light, using a  $530 \pm 15$  nm bandpass filter to detect the signal on FL1) to determine the percentage of cells expressing EGFP. In all cases, serial dilutions of viral supernatant were tested.

## Supplementary Results

### Optimization of the centrifugation protocol

#### Duration of centrifugation

Supernatant collected from the RET2.EGFP producer cells was centrifuged at 6000 *g* for time periods varying between 1 and 20 hours. After centrifugation, the supernatant was collected and saved for quantitation of residual viral particles. The viral pellets were resuspended in a thirtieth of the original volume of the supernatant. As measured on NIH 3T3 cells by flow cytometry, viral titer increased 14-fold after four hours of centrifugation, and appeared to plateau after 12 hours of centrifugation at  $1.34 \times 10^7$  cfu/ml (Supplementary Fig. 1). There was a proportional decline in the viral titer of the post-centrifugation supernatant. Even following concentration for as long as 20 hours, the infectivity of the recombinant virus was preserved.

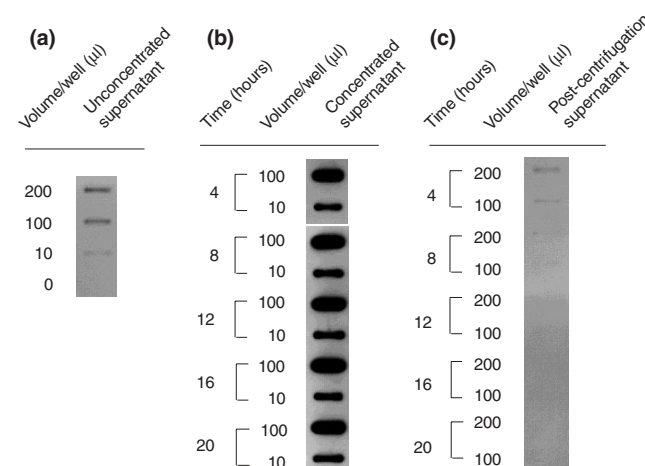
**Supplementary Figure 1**

Quantitation of functional viral titer following time course optimization. Viral supernatant was centrifuged at 6000 *g* for the time periods indicated. The viral pellet was resuspended in a thirtieth of the original volume. The viral titer of the post-centrifugation supernatant (solid bars) and the resuspended viral pellet (open bars) were measured on NIH 3T3 cells by the FACS-based limiting dilution expression assay. Data are representative of three similar experiments.

To confirm the viral titer derived by expression analysis, we performed slot blot hybridization analysis on viral RNA in the postcentrifugation supernatant and the resuspended viral pellet (Supplementary Fig. 2). Following centrifugation at 6000 *g* for four hours, most retroviral RNA was concentrated in the viral pellet. Almost no retroviral RNA remained in the postcentrifugation supernatant after centrifugation for 12 hours.

#### Relative centrifugal force

To further optimize the concentration procedure, we examined a range of relative centrifugal force (RCF). The time of centrifugation was fixed at four hours and the RCF was varied in a range from 6000 to 30,000 *g*. Following centrifugation, the viral pellet was resuspended in a hundredth of the original volume. Viral titer was quantitated by expression studies in NIH 3T3 cells (Supplementary Fig. 3) and slot blot hybridization analysis (Supplementary Fig. 4). We observed a progressive rise in viral titer as RCF was increased from 6000 to 20,000 *g*. At a RCF of 20,000 *g*, the titer of the resuspended pellet reached a plateau value of  $1.3 \times 10^8$  cfu/ml. Further concentration of viral particles was not achieved by increasing RCF above 20,000 *g*. Viral particles were not detectable by expression assay or by slot blot hybridization analysis in the post-centrifugation supernatant at an RCF of 20,000 *g* or

**Supplementary Figure 2**

Quantitation of viral RNA by slot blot hybridization analysis after concentration of virus by centrifugation at 6000 *g*. Viral supernatant was centrifuged at 6000 *g* for the time periods indicated. The viral pellet was resuspended in a thirtieth of the original volume. The indicated volumes of (a) uncentrifuged supernatant, (b) the resuspended viral pellet, and (c) the post-centrifugation supernatant were loaded onto a nylon membrane in a 48-well slot blot format, hybridized with an enhanced green fluorescent protein probe, and exposed to film. Experiments were repeated three times with similar results.

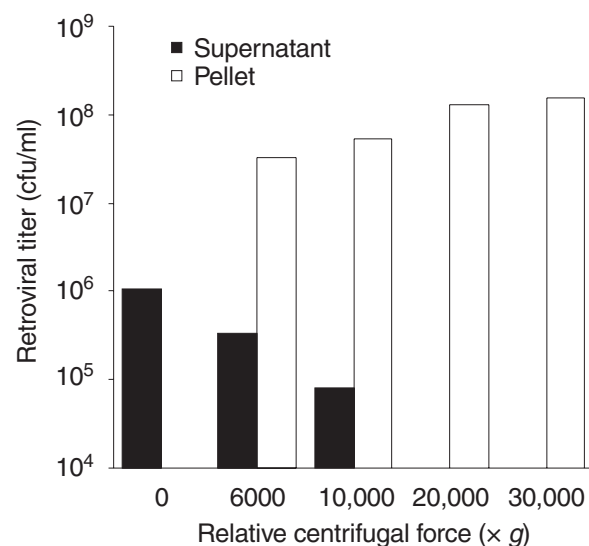
higher. The expression data also suggested that centrifugation at a RCF as high as 30,000 *g* for four hours did not affect viability of the recombinant retrovirus.

### Supplementary Discussion

The FLS are the principal cell type of sublining synovial tissue. Proliferation of FLS is observed in RA, a debilitating condition that affects as many as 1–2% of adult individuals worldwide. Primary FLS cultures can be established following arthroscopic biopsy or surgical resection of synovium from the joint. Protease digested synovial tissues placed in culture rapidly yield fibroblast-like cells. After three passages, these primary cultures are depleted of macrophage-like type A synoviocytes [S3]. Doubling time is stable between the third and the tenth passages, but marked reduction in proliferation rate occurs in later passage cells [S4].

Retroviral mediated gene transfer is a commonly used technique to stably introduce genes into primary cells. The titer of retroviral supernatant is one of several factors that influence transduction efficiency. A variety of strategies have been employed to physically concentrate retroviral particles in an attempt to further increase viral titer and improve the efficiency of target cell transduction. Centrifugation of retroviral supernatant is a potentially attractive approach to viral concentration because of the wide availability of centrifuge equipment, the simplicity of the tech-

Supplementary Figure 3

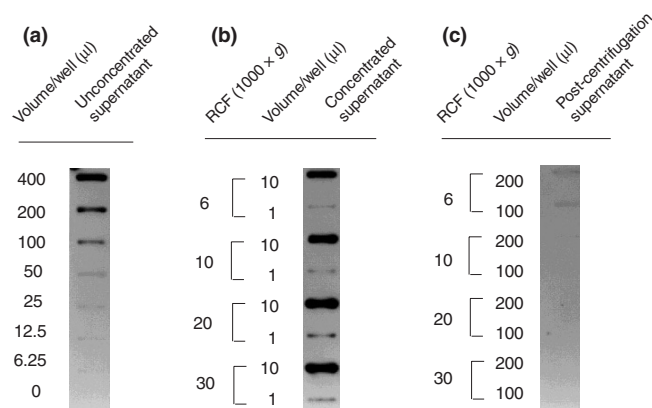


Quantitation of functional viral titer following optimization of relative centrifugal force. Viral supernatant was centrifuged for four hours at the indicated relative centrifugal force. The viral pellet was resuspended in a hundredth of the original volume. The functional viral titer of the post-centrifugation supernatant (solid bars) and the resuspended viral pellet (open bars) were measured on NIH 3T3 cells by the FACS-based limiting dilution expression assay. Data are representative of three similar experiments.

nique, and the theoretical potential for rapid processing of large sample volumes.

Concentrated recombinant retrovirus, generated by super-speed centrifugation of retroviral supernatant, has been used to improve the transduction efficiency of primary cells, including hepatocytes [9] and endothelial cells [11]. In these prior reports, concentration was accomplished by centrifugation for 16 hours at a RCF of 6000  $g$ . We used a recombinant retrovirus encoding the green fluorescent protein to optimize a protocol to rapidly and efficiently concentrate retrovirus by superspeed centrifugation. Our studies indicate that the time necessary to recover essentially all viral particles can be reduced to four hours by increasing the RCF to 20,000  $g$ . The protocol does not appear to adversely affect the infectivity of the viral preparation, as the functional viral titer on NIH 3T3 cells closely matched the titer that was predicted by the degree of concentration. Although it has been reported that centrifugation may result in concurrent concentration of noninfectious viral particles or inhibitors of viral transduction [S5], we have been able to substantially increase the transduction efficiency of primary FLS using concentrated viral supernatant produced by our protocol. This optimized technique may be useful in generating high titer retroviral supernatants from production lots of relatively modest

Supplementary Figure 4



Quantitation of viral RNA by slot blot hybridization analysis after concentration of virus by centrifugation for four hours. Viral supernatant was centrifuged for four hours at the indicated relative centrifugal force (RCF). The viral pellet was resuspended in a hundredth of the original volume. The indicated volumes of (a) uncentrifuged supernatant, (b) the resuspended viral pellet, and (c) the post-centrifugation supernatant were loaded onto a nylon membrane in a 48-well slot blot format, hybridized with an enhanced green fluorescent protein probe, and exposed to film. Experiments were repeated three times with similar results.

titer. We anticipate that this method will be effective in concentrating other pseudotyped MoMLV vectors and lentivirus based vectors, though additional testing will be required to evaluate its suitability for each vector system.

While our studies were not initiated with the objective of developing a therapeutic protocol, these results may also have implications for clinical studies. The *ex vivo* genetic modification of FLS has been proposed as a potential approach to the treatment of arthritis [S6,S7]. In these studies, FLS are cultured from synovial tissue obtained by synovectomy, transduced with retroviral supernatant *ex vivo*, and injected into another joint of the same individual. Approval for these clinical studies was based on *ex vivo* transduction data in preclinical animal models [S8,S9]. Essentially, all data on transduction efficiency of FLS was derived using retroviral vectors that express lacZ or beta-galactosidase. Although most authors have obtained *ex vivo* transduction efficiencies of cultured FLS in the range of 1–5%, some have reported transduction efficiencies up to 20%. Preactivation of FLS with tumor necrosis factor  $\alpha$ , however, may increase transduction efficiency levels to over 30% [S8].

### Supplementary References

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