

Methodology Report

Improved Coinfection with Amphotropic Pseudotyped Retroviral Vectors

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Amphotropic pseudotyped retroviral vectors have typically been used to infect target cells without prior concentration. Although this can yield high rates of infection, higher rates may be needed where highly efficient coinfection of two or more vectors is needed. In this investigation we used amphotropic retroviral vectors produced by the Plat-A cell line and studied coinfection rates using green and red fluorescent proteins (EGFP and dsRed2). Target cells were primary human fibroblasts (PHF) and 3T3 cells. Unconcentrated vector preparations produced a coinfection rate of ~4% (defined as cells that are both red and green as a percentage of all cells infected). Optimized spinoculation, comprising centrifugation at 1200 g for 2 hours at 15°C, increased the coinfection rate to ~10%. Concentration by centrifugation at 10,000 g or by flocculation using Polybrene increased the coinfection rate to ~25%. Combining the two processes, concentration by Polybrene flocculation and optimized spinoculation, increased the coinfection rate to 35% (3T3) or >50% (PHF). Improved coinfection should be valuable in protocols that require high transduction by combinations of two or more retroviral vectors.

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1. Introduction

High rates of infection of target cells with two or more retroviral vectors are often required to achieve a desired biological outcome. In particular, the production of induced pluripotent stem (iPS) cells requires a high rate of coinfection in order to obtain a sufficient fraction of cells that express all four reprogramming factors, *OCT4*, *SOX2*, *KLF4*, and *c-MYC* [1, 2]. Methods are needed to ensure that high coinfection rates are reliably achieved. When introducing reprogramming genes into human fibroblasts, Yamanaka's group used a complex strategy to increase coinfection to a rate sufficient for reprogramming. They first introduced the ecotropic retrovirus receptor into the target human cells using a lentiviral vector, and then infected the cells with retroviruses produced in the ecotropic Plat-E packaging cell line [2]. Plat-E and Plat-A cells are retrovirus packaging cell lines derived from the 293T cell line [3]. While other packaging cell lines used viral LTRs for expression of viral structural genes, Plat-E and Plat-A cells utilize the potent

EF1 α promoter in combination with a Kozak consensus sequence upstream of the initiation codon, resulting in high expression of viral structural proteins [3]. However, the Plat-A cell line, used for amphotropic pseudotyping, produces a titer of only about one-tenth that of the ecotropic packaging cell line Plat-E [4]. Titers from other amphotropic retrovirus packaging cell lines derived from 293T, such as Phoenix-Ampho, are similar [5]. Consistent with a lower rate of vector production by Plat-A than by Plat-E cells, Yamanaka's group showed that human fibroblasts infected with vector from Plat-E cells following introduction of the ecotropic retrovirus receptor were transduced at ~3 times the rate of fibroblasts infected with vector from Plat-A cells [2].

In experiments involving coinfection to achieve cellular reprogramming unconcentrated retroviruses have been used. Generally, unconcentrated preparations produced by amphotropic packaging cells are sufficient for the introduction of single genes into cells. However, higher levels of vector are needed for efficient coinfection with two or more retroviruses. There has been little work on optimizing

conditions for coinfection with amphotropic or ecotropic retroviral vectors. Although it may be assumed that any improvement in the rate of infection will increase the rate of coinfection, this has rarely been tested [6]. We hypothesized that the use of optimized protocols could result in much higher coinfection rates by amphotropic retroviral vectors produced by packaging cell lines like Plat-A.

Strategies for increasing the amount of amphotropic or ecotropic vector that can be delivered to target cells include (a) increasing vector production rates by the packaging cells [5], (b) concentrating the vector, and (c) increasing the rate or level of attachment of vector to the target cells. Amphotropic retroviruses can be concentrated by centrifugation, although some loss of titer may be encountered [7, 8]. Retroviruses pseudotyped with vesicular stomatitis virus G (VSV-G) protein can readily be concentrated by centrifugation, but preparation of this type of vector typically requires cotransfection of 293T cells with multiple plasmids [9]. A convenient and efficient method for concentrating amphotropic retroviral vectors was introduced recently; this comprises flocculation of the vector with high concentrations of polymers such as Polybrene followed by a brief centrifugation [10, 11]. This process also separates active viral particles from inactive particles that can compete for receptors on target cells [12]. Polymers, usually Polybrene, are also used in standard infection protocols to increase attachment of viral vectors to target cells [13]. This is particularly effective in established cell lines in combination with low-speed centrifugation of the vector onto the surface of the target cells, a process termed spinoculation or spin-infection [8, 14]. This has not been extensively tested in primary human cell types, however. The two processes should be complementary: separation of the vector from the bulk medium by Polybrene flocculation can be followed by resuspension of the vector in culture medium and optimized infection of the target cells by spinoculation.

In the present experiments we tested whether amphotropic retroviral vectors can be concentrated and efficiently delivered to target cells (primary human fibroblasts and 3T3 cells) by spinoculation. Coinfection rates were determined by the use of retroviral vectors encoding two different fluorescent proteins, EGFP and dsRed2 [15]. We found that the combination of flocculation by Polybrene together with delivery of the precipitated retrovirus-Polybrene complex to target cells by spinoculation raised the coinfection rate by >10-fold over that observed using maximal volumes of unconcentrated viral vector.

2. Materials and Methods

2.1. Cell Culture. Plat-A amphotropic retrovirus packaging cells [3] were obtained from Cell Biolabs (San Diego, Calif, USA). Newborn human skin fibroblasts (CRL-2703, also termed CCD-1137sk) and Swiss mouse 3T3 cells were obtained from the American Type Culture Collection, Manassas, Va, USA. Cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% Cosmic Calf Serum (HyClone Laboratories, Logan, Utah, USA),

0.1 mg/ml penicillin and 0.05 mg/ml gentamicin (referred to here as complete culture medium). Plat-A cells were grown in medium that also contained 10 μ g/ml blasticidin and 1 μ g/ml puromycin [3].

2.2. Retroviral Vectors. A retroviral vector encoding EGFP (pLEGFP-N1) was obtained from Clontech, Palo Alto, Calif, USA. A retroviral vector encoding the red fluorescent protein dsRed2 was derived by cloning the dsRed2 coding region (Clontech) downstream of the LTR in plasmid pMXs [4] to form pMXs-dsRed2.

2.3. Viral Vector Production. Amphotropic retroviruses were generated by transient transfection of Plat-A cells. Plat-A cells were plated at 2×10^6 per 6-cm dish (poly-L-lysine coated; BD Biosciences). The following day, the culture medium was replaced with fresh medium containing 25 μ M chloroquine and 10 mM sodium butyrate [16]. Immediately following this, cells were transfected with 10 μ g retroviral plasmid DNA using the calcium phosphate precipitation procedure [16]. After 16 hours the medium was changed to 3.5 mL fresh medium. After 24 hours the vector-containing medium was removed and filtered through a 0.45 μ m syringe filter. A second 24 hour medium collection was also used for infections.

For each experiment, a single pool of EGFP vector ("green vector") from the Plat-A cells was combined with a single pool of dsRed2 vector ("red vector"). We investigated whether the titers of the green and red vectors used were equivalent when tested on target cells, PHF or 3T3. In each experiment reported here the titers were approximately equal.

2.4. Infection of Target Cells. CRL-2703 cells or 3T3 cells were plated in 6-well plates (BD Biosciences) ($\sim 10^4$ cells per well). Infection was begun when cells were dividing maximally at ~ 60 – 70% confluence. Viral vector-containing medium (3.5 ml EGFP vector plus 3.5 ml dsRed2 vector) was added to cells with the addition of 8 μ g/ml Polybrene [hexadimethrine bromide (Sigma), usually referred to by the trademarked name Polybrene]. After 4 hours, 50% of the medium was replaced with fresh medium. The next day cells were infected a second time using the same procedure. Following this, cells were grown in regular complete medium.

2.5. Spinoculation. For spinoculation, vector-containing medium (3.5 ml EGFP vector plus 3.5 ml dsRed2 vector) was added to the target cells with addition of Polybrene. Plates were wrapped in Parafilm to minimize pH changes resulting from loss of CO₂ from the medium. Plates were transferred to a plate carrier and centrifuged using a Beckman JS-4.3 swinging bucket rotor for 2 hours at different temperatures and speeds (see Section 3). Following centrifugation, plates were returned to the 37°C incubator. Subsequently, either 50% of the medium was replaced with fresh medium after 2 hours, or 100% was replaced after 4 hours (see Section 3). The following day the spinoculation procedure

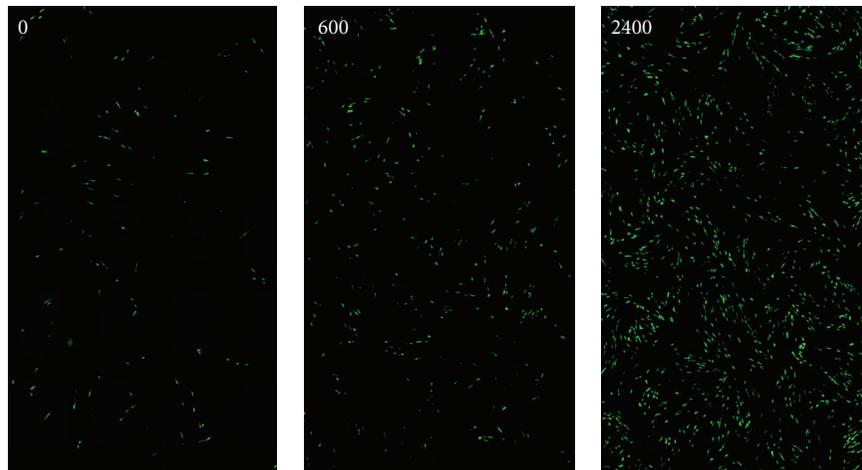


FIGURE 1: Spinoculation increases infection of CRL-2703 human fibroblasts by amphotropic retroviral vectors produced by Plat-A cells. Cells were infected with an EGFP vector without spinoculation or with spinoculation at 600 g and 2400 g (in each case 2 hours at 15°C). Representative microscope fields demonstrating increases in the fraction of infected cells (exhibiting green fluorescence) are shown.

was repeated. Following this, cells were grown in regular complete medium.

2.6. Viral Vector Concentration by Centrifugation. Equal volumes of medium containing EGFP and dsRed2 vectors, of approximately equal titers, were mixed and centrifuged at 10000 g for 3 hours at 4°C [7, 8]. Volumes of vector-containing medium used were equal to those from either 3 or 16 plates of EGFP- and dsRed2-transfected Plat-A cells (see Section 3). Green and red vectors were mixed before centrifugation. The pellet (usually not visible) was resuspended in 7 ml complete culture medium and added to target cells with Polybrene. The following day, medium was changed with addition of the same amount of concentrated vector. Following this, cells were grown in regular complete medium.

2.7. Viral Vector Concentration by Flocculation with Polybrene. Polybrene (320 µg/ml) was added to vector-containing medium of a volume equal to that from 16 plates of EGFP- and dsRed2-transfected Plat-A cells. Green and red viruses were mixed together. The medium was incubated at 37°C for 20 minutes followed by centrifugation at 10,000 g for 10 minutes at 4°C [10]. The pellets were combined into complete culture medium to a final volume of 7 ml which was added to the target cells. The following day, medium was changed with addition of the same amount of concentrated vector. Following this, cells were grown in regular complete medium.

2.8. Combining Flocculation with Polybrene and Spinoculation. For the combination of concentrating the vector together with spinoculation, viral pellets were resuspended in 7 ml complete culture medium for transfer to target cells, which were subjected to the spinoculation procedure described above (1200 g at 15°C for 2 hours). This was

repeated the following day. Following this, cells were grown in regular complete medium.

2.9. Determination of Coinfection Rate. The two retroviral vectors used encode fluorescent proteins, EGFP and dsRed2, enabling detection of transduced cells by fluorescence microscopy. At 4 days following infection, photographs of random fields of cells were obtained using a 12-bit CCD camera attached to a Zeiss Axiovert microscope. Three images were obtained for each cell field: a phase-contrast image, a “red” image and a “green” image (the latter two images are gray-scale images obtained using appropriate specific filters for EGFP and dsRed2). Each cell that was scored as positive in the red image was located on the phase-contrast image and marked as such. The process was repeated separately for the green image. This results in two copies of the phase-contrast image, one with green cells marked and the other with red cells marked. We then made three totals, those cells that are red but not green, those that are green but not red, and those that are both red and green. The percentage coinfection was determined as $[\text{Number of cells that are both green and red}] / [\text{Number of cells that are either green or red}] \times 100$. In coinfection experiments, each procedure was performed in triplicate. Data are presented as averages and standard deviations. Statistical analysis was carried out by one-way ANOVA and post hoc analysis by the Neuman-Keuls multiple comparison test (Prism software, GraphPad Software, San Diego, Calif, USA).

3. Results

We tested whether spinoculation is a suitable procedure for increasing infection of PHF by amphotropic pseudotyped retrovirus produced by Plat-A cells. In preliminary tests using literature protocols [14] we found that low-speed centrifugation at 25°C or above was detrimental to PHF

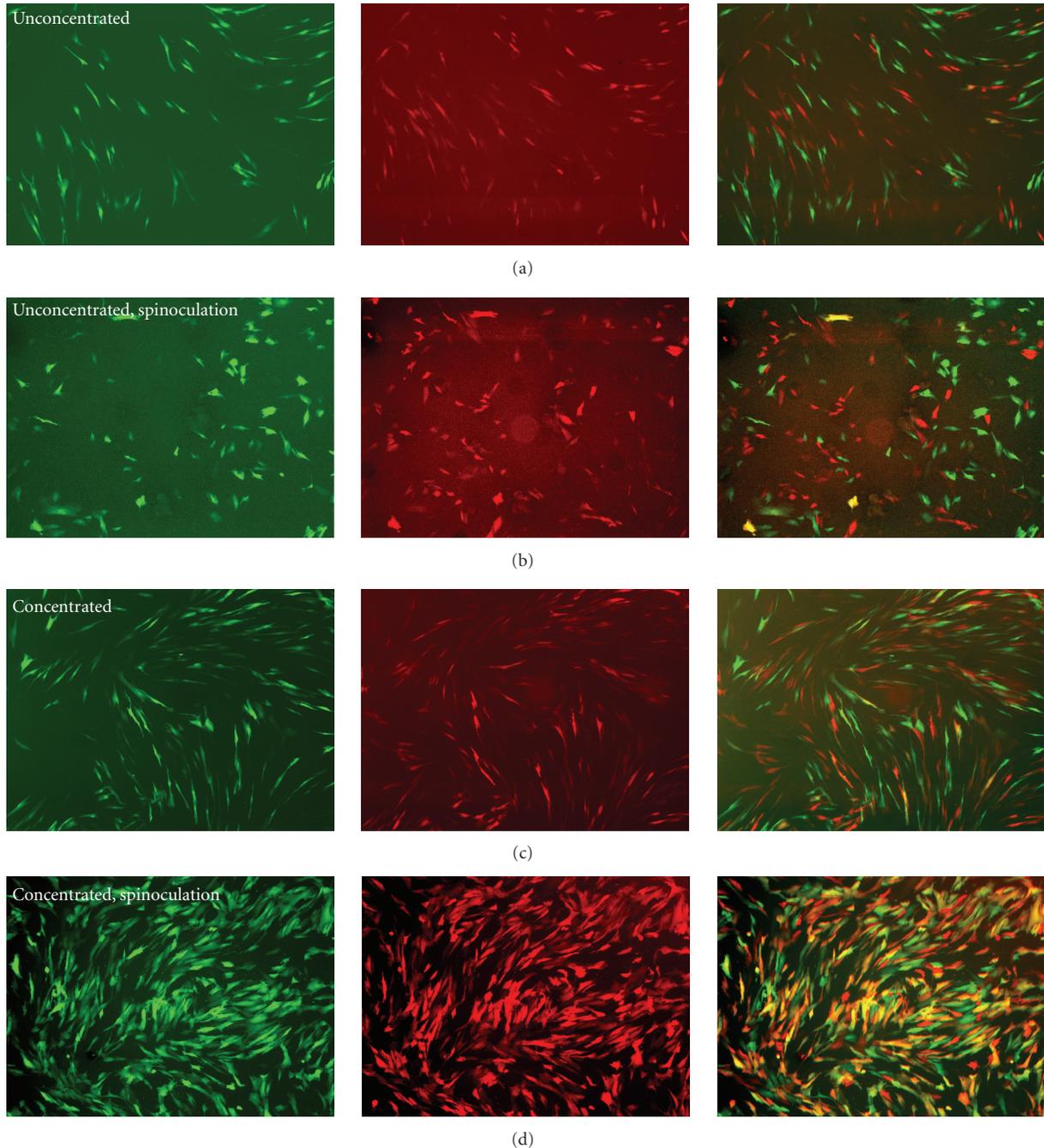


FIGURE 2: Visualization of coinfection using green and red fluorescent proteins. CRL-2703 human fibroblasts were infected with a 1 : 1 mixture of amphotropic retroviral vectors encoding EGFP and dsRed2. Vectors were used *unconcentrated* (maximal volume for a well of a 6-well plate: total 7 ml); the same with *spinoculation* at 1200 g, 15°C, 2 hours; *concentrated* by flocculation with Polybrene from 16 sets of Plat-A cell plates; or *combined* concentration with Polybrene and spinoculation. Cells were photographed 4 days after infection. Green and red images of representative fields of cells (left and middle) were merged (right) to show coinfecting cells (yellow).

cell viability. We tested spinoculation at lower temperatures and found that infection was increased by ~5-fold when cells were centrifuged at 1200 g for 2 hours at 15°C, whereas it was increased ~4-fold at 10°C. Because of the apparent susceptibility of PHF to damage by spinoculation at room temperature, we also investigated whether a lower speed (600 g) might be more effective than conventional speeds (≥ 1200 g).

We found that spinoculation at 600 g for 2 hours at 15°C increased infection (~3-fold over no spinoculation control) but was less effective than higher speeds. Additionally, we found that 1200 g, 2400 g and 4200 g (2 hours at 15°C in each case) produced similar increases over the no spinoculation control. In Figure 1, we show an example of the increase in infection in PHF when spinoculation was used at 600 g and

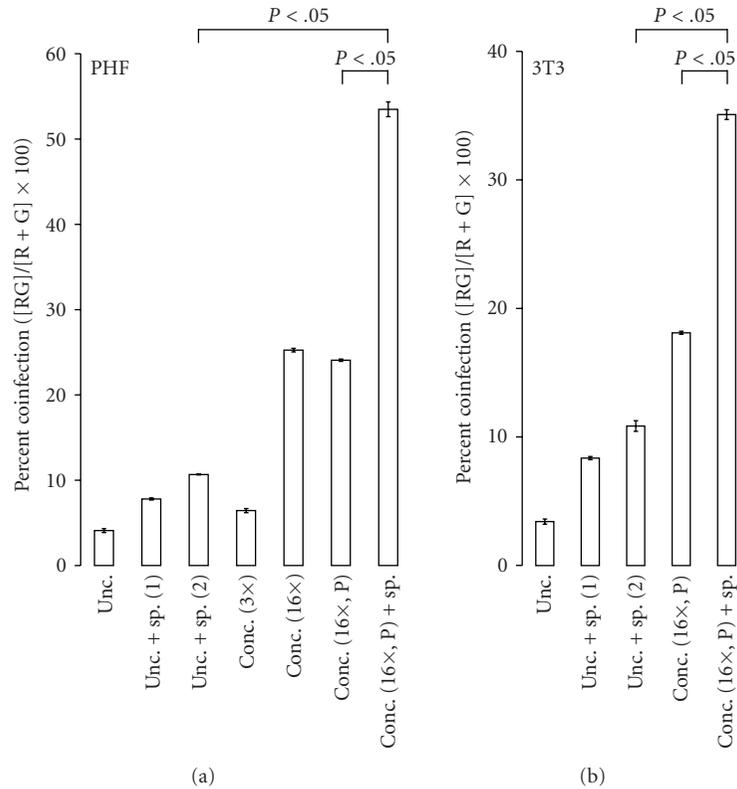


FIGURE 3: Coinfection with two retroviral vectors as a function of treatment. Coinfection rates were calculated as cells that are both green and red as a percentage of cells that are either green or red. (a) Mixed green and red vectors (1 : 1) were added to PHF (CRL-2703) in unconcentrated (*unc.*) form; with spinoculation (*sp.*; 1200 g, 15°C, 2 hours; (1) = with replacement of 50% of viral supernatant after 4 hours; (2) = complete change of medium after 4 hours); with concentration (*conc.*) from 3 sets of Plat-A plates or 16 sets, using 10,000 g for 3 hours; concentrated using Polybrene flocculation (P); or combined concentration with Polybrene and spinoculation. (b) Mixed green and red vectors (1 : 1) were added to 3T3 cells in unconcentrated (*unc.*) form; with spinoculation (*sp.*; 1200 g, 15°C, 2 hours; (1) = with replacement of 50% of virus after 4 hours; (2) = change of medium after 4 hours); concentrated using Polybrene flocculation (P); or combined concentration with Polybrene and spinoculation. For both CRL-2703 and 3T3, coinfection rates were calculated 4 days after infection. Averages from three experiments are shown \pm standard deviation. Significant differences ($P < .05$) are indicated for the combination of spinoculation and Polybrene flocculation versus either procedure alone.

2400 g. We decided to use 1200 g, 15°C as a standard protocol because speeds >1200 g gave results similar to 1200 g, and thus it was unnecessary to use higher speeds. In repeated experiments, spinoculation of PHF at 1200 g for 2 hours at 15°C resulted in increases in infection in the range of 5- to 10-fold.

Having confirmed that spinoculation is a suitable method for infecting PHF with vector produced by Plat-A cells, we then combined optimized spinoculation with vector concentration. Because our aim was to improve coinfection, we measured coinfection rates for unconcentrated vector, spinoculation, vector concentrated by ultracentrifugation or Polybrene flocculation, and the combination of spinoculation and concentration. To measure coinfection rates, cells were infected with a 1 : 1 mixture of retroviruses encoding green (EGFP) and red (dsRed2) fluorescent proteins of approximately equal titers. Representative fields of infected cells are shown in Figure 2. Merged green and red images reveal the coinfecting cells. It was noticeable that relatively few cells were coinfecting with unconcentrated vector, despite the

fact that there were many cells that had been infected with either the green or the red vector. Images of cells infected by spinoculation or with vector concentrated by Polybrene flocculation show greater numbers of coinfecting cells. However, the combination of concentration and spinoculation produced an obvious and dramatic increase in coinfection rates (Figure 2).

Using a protocol described in detail in Section 2, the increase in coinfection rates with spinoculation and concentration was assessed as the number of cells that are both green and red as a percentage of the cells that are either green or red (i.e., coinfecting cells as a percentage of infected cells). These values varied from a low of ~4% for unconcentrated vector to >50% for the combination of concentration by Polybrene flocculation and spinoculation (Figure 3). We confirmed, as found by others, that concentrating amphotropic retroviruses by centrifugation is feasible. When a single retroviral vector was used, concentrating the vector from medium equivalent to that from 3 or 16 Plat-A plates resulted in approximately the expected increase in infection of target

cells (either HDF or 3T3). When we tested coinfection, vector that had been concentrated from a volume of medium equivalent to that from 3 plates of green vector and 3 plates of red vector resulted in a small increase in coinfection over unconcentrated vector, while concentrating vector from 16 sets of plates increased coinfection to ~25%. We found comparable values for coinfection using vector concentrated by centrifugation alone (10,000 g for 3 hours) and for vector concentrated by Polybrene flocculation. Because Polybrene flocculation is much faster (~30 minutes versus 3 hours) this method is preferable. When Polybrene flocculation was combined with spinoculation, the coinfection rate increased to 53%. This was a significant increase over either concentration alone or spinoculation alone.

To determine whether the results in PHF were reproducible in another cell type, the effects of spinoculation and Polybrene flocculation were tested on coinfection rates in Swiss mouse 3T3 cells. Results were very comparable to those in PHF (Figure 3). Both spinoculation and Polybrene flocculation resulted in increased coinfection, while the combination of Polybrene flocculation and spinoculation resulted in a coinfection rate of 35%. This was significantly more than either spinoculation or Polybrene flocculation alone.

4. Discussion

While high rates of coinfection are needed for efficient delivery of genes to target cells, for example for iPS cell production, few studies have been performed with the aim of improving coinfection rates with retroviral vectors. Here we combined two protocols, spinoculation and concentration, with the result that coinfection of HDF improved from ~4% to >50%. Concentration of amphotropic retroviral vectors can be performed by 10,000 g centrifugation or by Polybrene flocculation, but the latter is preferred because of its rapidity. In these experiments we investigated coinfection by a mixture of two viral vectors. As the number of different types of retroviral vectors in a mixture increases, cells that have been infected with all the viral types in the mixture become increasingly infrequent, thus emphasizing the necessity for optimizing coinfection conditions.

In the context of human iPS cell generation, coinfection rates for viruses encoding the four reprogramming factors were not measured. Yamanaka's group reported a rate of human iPS cell generation of 10 colonies per 50,000 starting cells together with 100 non-iPS-like colonies; when c-MYC was omitted, far fewer non-iPS-like colonies were generated, and 0-5 iPS colonies arose from a population of 500,000 cells [2]. The importance of a high rate of coinfection was also shown recently by another group, who initially reported that four reprogramming factors were insufficient for iPS cell generation from adult human fibroblasts [17]. When a sufficiently high retroviral multiplicity of infection was employed, the other factors were not needed [18]. Another consideration is that it may be necessary to achieve infection rates high enough not only to deliver four reprogramming factors efficiently but also to produce multiple integrations of each viral genome in target cells [1, 2, 19–22]. This is unlikely

to represent a requirement for insertional mutagenesis; probably, only cells with multiple integrations achieve levels of expression of the reprogramming factors that are high enough for iPS cell generation.

Recent results show that iPS cell generation is possible with one or two transcription factors in combination with small molecules that induce reprogramming [23]. For the immediate future, generation of iPS cells may use combinations of viral methods, which require high efficiency coinfection, together with other innovative techniques. The application of the relatively simple methods described here to increase coinfection should be useful for iPS cell generation and for other experiments in which high coinfection rates are required.

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