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**ARTICLE** *in* ANALYTICAL CHEMISTRY · APRIL 2004

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*Anal. Chem.* **2004,** *76,* 1580−1585

# Mouse Embryonic Stem Cell Sorting for the Generation of Transgenic Mice by Sedimentation Field-Flow Fractionation

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**Mouse embryonic stem (ES) cells are an important tool for generation of transgenic mice and genetically modified mice. A rapid and efficient separation of ES cells that respects cell integrity, viability, and their developmental potential while also allowing purified ES fraction collection under sterile conditions might be of great interest to facilitate the generation of chimeric animals. In this study, we demonstrated for the first time the effectiveness of a sedimentation field-flow fractionation (SdFFF) cell sorter to provide, with a characteristic DNA content, a purified ES cell fraction and with a high in vivo developmental potential to prepare transgenic mice by generation of chimeras having a high percentage of chimerism.**

Introduced in the late 1960s by J. C. Giddings, field-flow fractionation (FFF) methodology is described as one of the most versatile separation techniques.[1,2] This chromatographic-like separation family, in particular, sedimentation-FFF (SdFFF), appears to be particularly well suited for isolation and characterization of micrometer-sized species, such as cells.[2−5] As with all other FFF methods, separation using SdFFF is achieved by the combined action of a parabolic flow profile, generated by flowing a mobile phase through a ribbonlike capillary channel, and of an external field applied perpendicularly to the flow direction.[2] While gravitational FFF (GFFF) uses Earth's gravity, SdFFF, also called centrifugal or multigravitational FFF, uses a multigravitational external field generated by the rotation of the separation channel in a more complex device.[2−5]

The SdFFF elution mode for cells is described as "hyperlayer".[2,4,6−13] In such a mechanism, cell size, density, shape, and rigidity are involved, as are channel geometry and flow rate characteristics. The elution depends on the flow rate/external field balance, which generates hydrodynamic lift forces, which in turn drive particles away from the accumulation wall. Species are then focused into a thin layer at the equilibrium position in the channel thickness dimension where the risk of cell-wall interaction is negligible. At constant flow rate and external field strength, larger or less dense particles are eluted first.[2,4,6−13] Thus, flow rate and external field strength should be selected to promote the biocompatible hyperlayer mode against the "steric" one, which can be defined as a limited case of the hyperlayer elution mode. The steric mode occurs when the external field is sufficiently strong or when the flow rate is sufficiently low to make lift forces negligible compared to the settling forces imposed by the external field. Under steric elution, cells are confined to a very thin layer close to the accumulation wall, which leads to harmful cell−channel interactions;[2,4,6−13] therefore, SdFFF cell separation and sorting requires some specific considerations.[3] As well as for the other cells, embryonic stem(ES) cell separation must respect cell functional integrity. Second, high levels of short- and long-term viability are needed. The maturation and differentiation stages of eluted cells should not be altered. Thus, because SdFFF cell sorting effectiveness is simply based on the intrinsic biophysical characteristics of cells (size, density, shape),[2,3,5] a specific prelabeling (fluorescent or magnetic) is not necessary. Thus, SdFFF is particularly interesting for applications in which labels could interfere with further cell use (culture, transplantation) when labels do not exist or when labels could induce cell differentiation (stem cells). In such cases, SdFFF could provide an advantage

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10.1021/ac030218e CCC: $27.50 
Published on Web 02/14/2004

over flow cytometry (FACS) or magnetic-activated cell sorting (MACS) for stem cell sorting.[5,14,15] Nevertheless, the association of SdFFF separation power with specific biological characterization by flow cytometry could be a very effective tool.[16] Finally, SdFFF cell separation must also provide high repeatability and reproducibility, in particular if routine stem cell preparation is needed. Maximal recovery and sterile collected fractions are also essential if transplantation is needed. To achieve these goals, specific SdFFF methodologies have been developed.[3] SdFFF device setup and elution conditions were optimized concerning injection mode, mobile-phase flow rate and composition, external field strength, composition of channel walls, and cleaning and decontamination procedures. The optimized parameters enabled a better subpopulation separation in association with a drastic limitation of cell accumulation through wall interactions by promoting the safety hyperlayer elution mode. Without such care, interactions lead to channel poisoning with severe consequences for cell integrity.[3,5]

Since the pioneering report of Caldwell et al.,[4] which defined most of the basic rules and methodologies for cell separation, FFF, SdFFF, and related technologies[5,14,17] have shown a great potential for cell separation and purification with major biomedical applications, including hematology,[4,9,16,18,19] cancer research,[15] microorganism analysis,[20,21] and molecular biology.[22−24] More recently, we opened the field of neurosciences.[5,25]

Embryonic stem cells are derived from the inner cell mass of blastocystes. They can be cultured in vitro onto a feeder layer of embryonic fibroblasts in the presence of leukemia inhibitory factor (LIF), the key cytokine that maintains their developmental potential.[26] ES cells can be used as a vehicle for transgenesis. Genetic modifications such as the addition (knock-in) and the inactivation (knock-out) of genes do not impair the ability of these cells to colonize the germ line after introduction into the blastocoel cavity of the blastocyst. The major advantage in using ES cells is the ability to screen for desirable cell clones before reintroduction into the germ line.[27] The major limitation for blastocyst injection is the time needed to obtain germ-line transmission that sometimes requires several experiments and, thus, the sacrifice of numerous mice in order to obtain blastocysts. Actually, ES cell suspensions prepared from cultured ES cells are a mixture of fibroblasts and ES cells at various stages of proliferation. A rapid and efficient separation of ES cells from fibroblasts which respects cell integrity, viability, and their developmental potential while providing a purified ES fraction collection under sterile conditions might be of great interest to facilitate the generation of chimeric animals. These features are easily obtained when SdFFF elution is performed under specific conditions.[3]

In this study, we investigated for the first time the capacity of a SdFFF cell sorter to provide a purified ES cell fraction from an ES cell suspension. Our results demonstrated, first, that SdFFF is an efficient tool for cell separation, as evidenced by the DNA content of the different fractions; and second, the effectiveness of SdFFF to provide in <6 min viable and enriched ES cells with a high in vivo developmental potential.

## MATERIALS AND METHODS

**ES Cell Culture Condition.** Mouse E14 ES cells were routinely grown onto a monolayer of mitomycin-treated primary embryonic mouse fibroblasts as source of cytokines and growth factors. The medium consisted of DMEM with 15% fetal calf serum (Gibco, Cergy Pontoise, France), 100 U/mL penicillin, 100 $\mu$g/mL streptomycin, 1 $\mu$M $\beta$-mercaptoethanol, and $10^3$ U/mL leukemia inhibitory factor (LIF; Gibco). ES cells were grown at 37 °C in a humidified chamber with 10% $CO_2$. ES cells were recovered with trypsin treatment for SdFFF experiments or subcultures (Figure 1A).

**SdFFF Device and Cell Elution Conditions.** The SdFFF separation device used in this study was previously described and schematized.[3,5,16,25] The separation channel was made up of two 870 × 30 × 2-mm polystyrene plates, separated by a Mylar spacer in which the channel (785 × 10 × 0.125 mm with two V-shaped ends of 70 mm) was carved. The measured total void volumes were 960 ± 5 $\mu$L ($n$ = 15), which were calculated after injection and retention time determination of an unretained compound (0.1 g/L of benzoic acid, UV detection at 254 nm). Inlet and outlet 0.254-mm-i.d. Peek tubing (Upchurch Scientific, Oak Harborg, WA) were directly screwed to the accumulation wall. Then polystyrene plates and Mylar spacers were sealed onto a centrifuge basket. The channel-rotor axis distance was measured at $r$ = 13.8 cm. Sedimentation fields were expressed in units of gravity, $g$ = 980 cm/s$^2$, and calculated as previously described.[5] A Spectroflow 400-ABI Kratos chromatographic pump (ABI-Kratos, Ramsey, NJ) was used to pump the sterile mobile phase. A M71B4 Carpanelli engine associated with a pilot unit Mininvert 370 (Richards Systems, Les Ullis, France), controlled the rotating speed of the centrifuge basket. Sample injections were done by means of a Rheodyne 7125i chromatographic injection device (Rheodyne, Cotati, CA). Cleaning and decontamination procedures have been described in a previous report.[3] The elution signal was recorded at 254 nm by means of a Water 484 tunable absorbance detector (Waters Associates, Milford, MA) and a 14-byte M1101 (100-mV input) acquisition device (Keithley Metrabyte, Tauton, MA) operated at 2 Hz and connected to a Macintosh computer. The ES cell suspension elution conditions ranged from 30 to 60$g$ in external field strength and from 0.4 to 1.0 mL/min in mobile phase flow rate. The optimal elution conditions (hyperlayer mode) have


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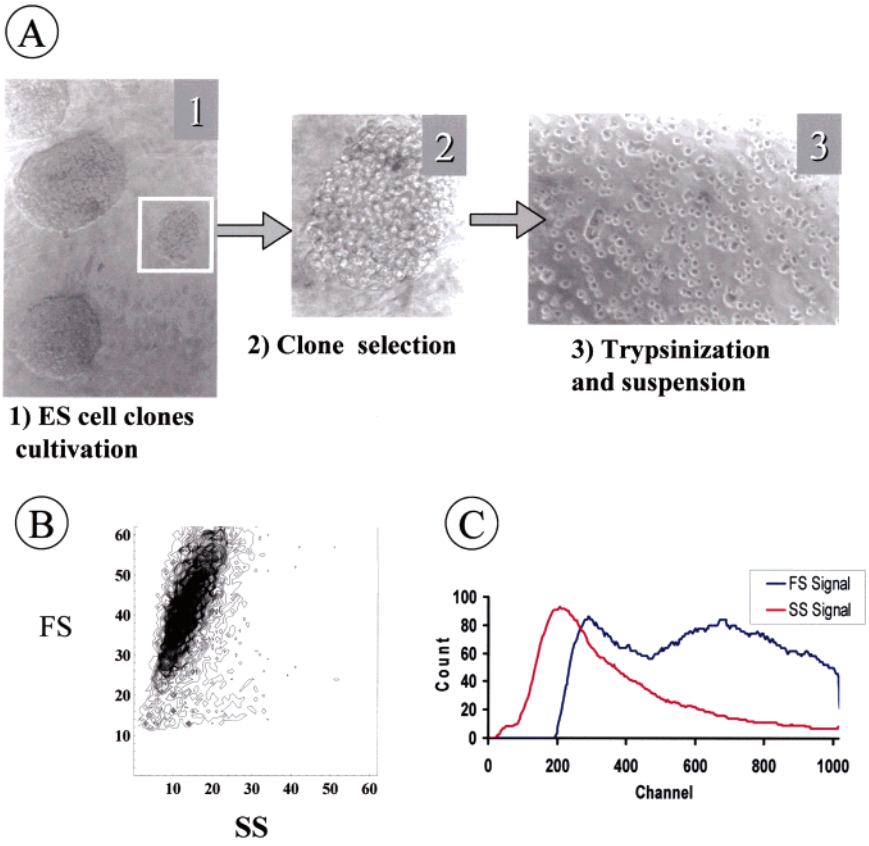


Figure 1. E14 ES cells clones. (A) Microscopic analysis. 1: ES cells ($5 \times 10^2$ cells/cm$^2$) were grown at 37 °C in RPMI 1640 supplemented with 10% (w/v) SVF onto a layer of mitomycin-treated embryonic fibroblasts. Slide shows colony after 7 days growth (Nikon THS inverted microscope, ×10 magnification). 2: characteristic structure of a single ES colony (×20 magnification). 3: ES cell suspension. Colonies were treated with trypsin (5 min, 37 °C) in order to obtain a single cell suspension. (B) Bidimensional FS/SS correlation diagram (resolution, 128/128). Average counted particles, 100 000. Coulter FS gain = 7.06; Coulter SS gain = 47. (C) Flow cytometric profile of part A3 single ES cell suspension in isotonic PBS suspension concentration of $10^6$ cell/mL. FS and SS signals recorded.

been experimentally determined and were flow injection through the accumulation wall of 100 $\mu$L ES cells ($1.5 \times 10^6$ cells/mL); flow rate, 0.6 mL/min; mobile phase, sterile PBS pH 7.4; external multigravitational field strength, $40.0 \pm 0.1g$; spectrophotometric detection at $\lambda = 254$ nm. Three cell fractions were collected, peak fractions 1, 2, and 3 ($PF_n$): $PF_1$, 3 min 40 s/4 min 15 s; $PF_2$, 4 min 20 s/4 min 50 s; $PF_3$, 5 min 00 s/5 min 50 s.

**Colony Assays.** Colony assays (in duplicate) were performed with fractionated ES cells. For each fraction, cells were counted in hemocytometer chambers. Viability of cells was assessed by trypan blue exclusion. For each fraction, 500 living ES cells were cultured for 7 days as described above. ES colonies were then counted under an inverted microscope. The differences of colony numbers among dishes from fractions 1, 2, or 3 were analyzed using a *t*-test for paired data.

**Assessment of Ki67 Positive Cells.** For each fraction, cells were counted in hemocytometer chambers. Viability of cells was assessed by trypan blue exclusion. For each fraction, $1 \times 10^5$ living ES cells were washed twice in PBS and fixed (1% paraformaldehyde (PFA) in PBS) for 30 min at 4 °C. Cells were recovered and permeabilized (1% PFA with 0.01% Tween 20 in PBS) overnight at 4 °C. After washing, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-Ki67 antibodies (Becton Dickinson, San Diego, CA) or FITC-conjugated isotype control antibodies (Becton Dickinson) for 2 h at 4 °C. Cells were analyzed on a Coulter XL.2 apparatus (Beckman Coulter, Fullerton, CA). The percentages of Ki67 positive cells in fractions 1, 2, and 3 were compared using *t*-test for paired data.

Unlabeled fractionated and control cell fractions were also analyzed by Coulter XL.2 flow cytometer for FS and SS signals as previously described.[16,28]

**Cell Cycle Analysis.** For each fraction, cells were counted in hemocytometer chambers. Viability of cells was assessed by trypan blue exclusion. For each fraction, $1 \times 10^5$ living ES cells were recovered, fixed, stained with propidium iodide, and analyzed for cell status, as previously described.[29] For these experiments, cells were analyzed using an XL.2 flow cytometer (Beckman-Coulter).

**Generation of Transgenic Mice.** Cells of the ES cell line E14 were submitted to SdFFF. Ten living cells from fractions 2 or 3 were injected into C57 BL/6 blastocysts, which were implanted into foster mothers to derive somatic chimeras. The level of chimerism was determined by analysis of coat color. To test for germ line transmission, chimeric males were mated with females. Analysis of coat color of their offspring indicated whether the germ line had been transmitted.

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## RESULTS AND DISCUSSION

During the past decade, SdFFF and related technologies have demonstrated an important potential for cell sorting in major biomedical applications.[2,5,9,14−25] The generation of transgenic mice and gene-modified mice has rapidly advanced the understanding of gene function in mammals and has permitted the development of useful animal models of human diseases. ES cells transfected with a foreign transgene or modified by homologous recombination were injected into mouse blastocysts, which were implanted into foster mothers to derive somatic chimeras. Chimeras were then bred with wild-type animals to obtain heterozygous F1 mice carrying the genomic manipulation. One of the major technical problem remains the screening among the pool of cultured ES cells of the best ES cells for injection into blastocysts to obtain chimeras with the highest percentage of chimerism and, thus, with the highest capability of germ line transmission. At this time, this operation is based on a difficult, time-consuming, and subjective microscopic cell selection, which may lead to a low-efficiency chimera production.

The goal of this work was the use of SdFFF cell sorting to standardize this process. First of all, different subpopulations of ES cells (fractions) were prepared by using SdFFF under strictly defined conditions (hyperlayer elution mode). Second, we investigated the in vitro and in vivo developmental potential of each fraction, highlighting that SdFFF was efficient to provide a purified ES fraction having the highest potential for the generation of transgenic mice.

**Biophysical Characteristics and SdFFF Elution of ES Population.** Mouse ES cells were grown as described (see the Materials and Methods Section) on a mitomycin-treated fibroblast layer, and colonies were trypsinized before use, as described in Figure 1A. ES cell suspensions appeared relatively homogeneous in shape, but more heterogeneous in size (Figure 1A), with the presence of small, large, or very large cells, which could be residual fibroblasts. As previously described,[16,28] two principal characterization methods can be used in order to physically determine cell parameters by flow cytometry (Figure 1B). The first one, described as "forward scattering" (FS signal, Figure 1B,C), exploits low angle deviation (<10°) of the laser beam by cells. In these conditions, diffraction predominates, and intensity is a complex (log linear) function of cell volume. It is possible to calibrate the FS signal in terms of size (volume) by means of standard spherical latex beads.[28] Thus, the higher the cell volume or diameter, the higher the FS channel displayed in Figure 1B,C. In constant flow cytometry, measurement conditions comparing cell FS channel profiles (Figure 1C) allowed comparison of cell characteristics in terms of size.[16,28] The second main parameter, the "side scattering" (SS signal, Figure 1B,C) corresponded to the cell reflected and refracted light collected at large angle (90°) and provides a complex signal function of particle size, refractometric index, internal composition, and surface characteristics. Convolution of both FS and SS signals could be used to provide cell population-specific bidimensional FS/SS plots (Figure 1B).

Figure 1C shows, through the FS signal, an important size polydispersity of the ES population. The count-versus-FS channels histogram of the sample had a roughly bimodal distribution, with a first population in the 200−400 channel range (small size) and the second in the range of 500−800 channels (Figure 1C). The presence of two populations with an important size dispersity was in accordance with microscopic observations (Figure 1A). In comparison, judging from the SS signal, ES cells were relatively homogeneous (Figure 1C).

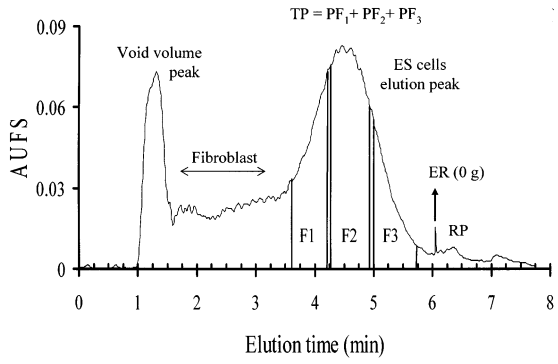


Figure 2. Representative fractogram of ES cell suspensions after SdFFF elution. Elution conditions: flow injection of 100 $\mu$L ES (1.5 $\times$ $10^6$ cells/mL); flow rate, 0.6 mL/min (sterile PBS pH 7.4); external multigravitational field, 40 $\pm$ 0.1*g*; spectrophotometric detection at $\lambda$ = 254 nm. Fractions were collected as follows: $PF_1$, 3 min 40 s/4 min 15 s; $PF_2$, 4 min 20 s/4 min 50 s; $PF_3$, 5 min 00 s/5 min 50 s. ER correspond to the end of channel rotation. In this case, the mean externally applied field strength was equal to zero gravity; thus RP, a residual signal, corresponds to the release peak of reversible cell-accumulation wall sticking.

Figure 2 shows a specific and representative ES cell elution fractogram. Two major peaks were seen. The first corresponded to the elution of unretained species. In this case, $R_{obs}$, which is the ratio of the void time versus the retention time,[30] was ~1 ($n$ = 15). The second corresponded to ES cells with $R_{obs}$ = 0.359 $\pm$ 0.003 ($n$ = 15). The part of the fractogram between void volume and ES peak corresponded to the elution of residual fibroblasts on which ES cells were cultured. At the end of the fractogram when channel rotation was stopped (ER, Figure 2) and mean gravity was equal to 0 (external field applied = 1*g*), we observed a residual signal (RP, Figure 2) which represented cell release from the separating channel.[3,5]

As shown in Figure 2, cell fractions were collected as a function of time and designated as peak fractions 1, 2, and 3 ($PF_n$). Gaps without collection were introduced between PF1, 2, and 3 to increase the selectivity of cell fraction collection.

To determine if cells migrated under the hyperlayer mode, we measured the pattern of the retention ratio, $R_{obs}$, for the specific cell elution peak under these various elution conditions. In the hyperlayer elution mode, micrometer-sized species show an $R_{obs}$ that is flow-rate and external-field-dependent. At a constant field 40.0 $\pm$ 0.1*g*, the increase in flow rate induced an increase in $R_{obs}$: $R_{obs}$ = 0.303 $\pm$ 0.004 at 0.4 mL/min, and $R_{obs}$ = 0.420 $\pm$ 0.003 at 1.0 mL/min (mean $\pm$ SD for $n$ = 15). An increase of field at a constant flow rate (0.6 mL/min) decreased $R_{obs}$, with $R_{obs}$ = 0.391 $\pm$ 0.006 at 30.0*g*, and $R_{obs}$ = 0.330 $\pm$ 0.005 at 60.0*g* (mean $\pm$ SD for $n$ = 15).

Flow cytometric analyses of collected fractions during FFF separations determined some characteristics of the three isolated populations, as compared to the original one (Figure 3A,B). Because the FS signal is correlated with size,[16,28] it is possible to compare particle size distributions of the three fractions and the original sample. As already described, the crude sample was

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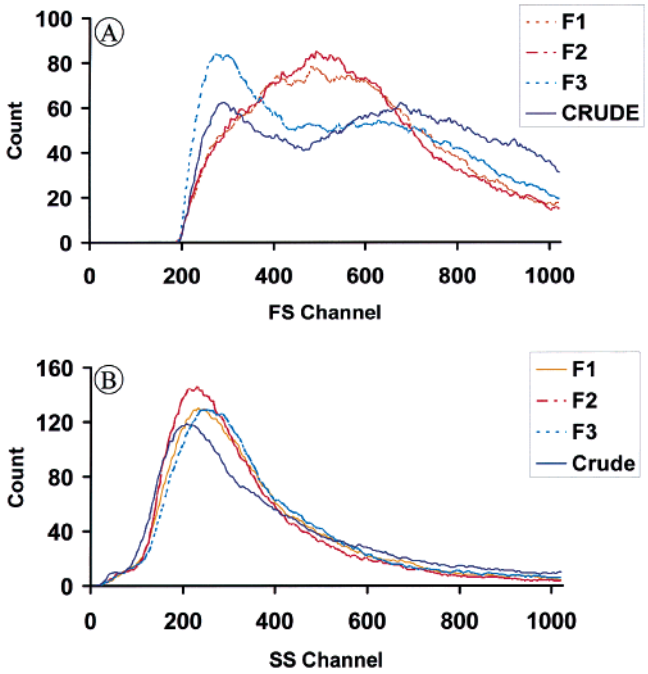


Figure 3. Flow cytometry studies of fractionated ES cells. ES original sample suspension and FFF collected fractions comparating FS (A) and SS (B) flow cytometric signals. Histograms were normalized. Flow cytometric operating conditions described in Figure 1.

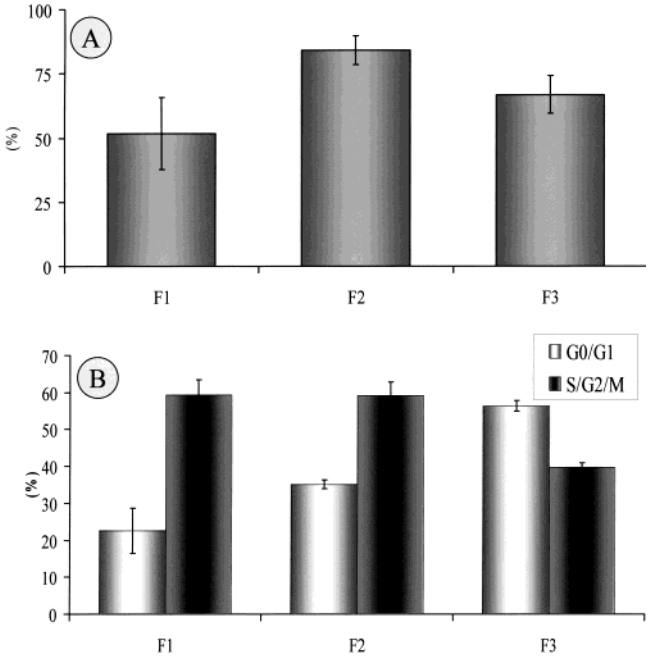


Figure 4. Assessment of the Ki67 antigen (A) and of cell cycle (B) in fractionated ES cells. (A) The Ki67, a nonhistone nuclear protein, is a proliferation-associated antigen found in all phases of the cell cycle, except G0 and early G1. We investigated the presence of this protein in fractionated ES cells. Thus, cells were fixed, permeabilized, and incubated with anti-Ki67 monoclonal antibodies labeled with fluorescein-isothiocyanate (FITC). The stained cells were then analyzed by flow cytometry to determine the percentage of Ki67+ cells in ES cell fractions. Results are expressed as mean ± SEM of five independent experiments. (B) Propidium iodine is an efficient fluorescent DNA probe to ensure cell DNA content. Incubation of cells with a hypotonic solution of propidium iodine results in disruption of the cell membrane and rapid staining of the nuclear chromatin; cells in G2/M phase containing twice the DNA content as cells in the G0/G1 phase. After propidium iodide staining, fractionated ES cells were analyzed on an XL.2 Software to assess the percentage of cells in the G0/G1, S, and G2/M phase. Results are expressed as mean ± SEM of five independent experiments.

bimodal. It is observed that Fractions 1 and 2 were associated with monomodal PSD (particle size distribution) of large polydispersity and analogous profile characteristics (Figure 3A). Compared to the original sample, it is observed in Figure 3A that cells from these fractions were of intermediate average size, and they were depleted in cells of smaller as well as larger size. On the contrary, fraction 3 was enriched with smaller cells, while the proportion of large cells was reduced (Figure 3A). Cells of smaller size were eluted last. Thus, according to the SdFFF elution mode description for micrometer-sized species,[2,4,6−13] we can assume that ES cells were eluted under the hyperlayer elution mode. Finally, the effectiveness of this mode to reduce particle-accumulation wall interactions was shown in part by the absence (Figure 2) or the very low level of the corresponding cell release peak at the end of the fractogram.

Concerning SS signal discrimination, Figure 3B shows that all fractionated populations behaved analogously describing a rather homogeneous pattern in terms of "SS" characteristics.

From a granulometric point of view, ES cell selectivity appeared at that stage of characterization to be linked to cell size. However, fractions 1 and 2 showed an analogous and large PSD (FS signal), yet they were eluted at different times, and no SS signal differences were produced, which would explain such elution differences. On the basis of hyperlayer elution mode, density differences may explain this elution order. Unfortunately, average density and density distributions have not been described to account for transgenesis efficiency.

We then studied the respective biological properties of the eluted ES cell fractions (Figure 2). First, fractions were characterized in vitro by using flow cytometry to study cell cycle status and by using cell culture to study viability and clonogenicity, with the goal of selecting the most effective fraction to use for long and difficult in vivo studies.

**Evaluation of in Vitro Developmental Potential of SdFFF-Eluted Cells.** We first assessed whether ES fractions had different in vitro developmental potential by assessing their capacity to generate ES colonies. ES cells from fraction 2 significantly ($p < 0.05$, *t*-test for paired data) generated more ES colonies (153 ± 24, mean ± SEM of three independent experiments) than ES cells from fraction 1 (93 ± 7) and fraction 3 (42 ± 17); this latter cell fraction had the lowest capacity to generate ES clones in vitro. The simplest hypothesis to explain these results was a different proliferation state between ES fractions. G0 is a phase of resting cells outside the cell cycle. The phases of the cell cycle include G1 (gap 1), S (synthesis of DNA), G2 (gap 2), and M (mitosis). During the G1 phase, several growth factors exert their influence. After appropiate signals, the cell enters the S phase and begins to replicate its chromosomes. During the G2 phase, DNA damage repair mechanisms operate. During the M phase, chromosomes segregate if they are intact. After this latter stage, the cell enters the resting state (G0) or remains in the cycling compartment.[31] Thus, S/G2/M cells have an higher DNA content, as compared to G0/G1 cells. To test the hypothesis of a different proliferation state between ES fractions, we therefore investigated the presence

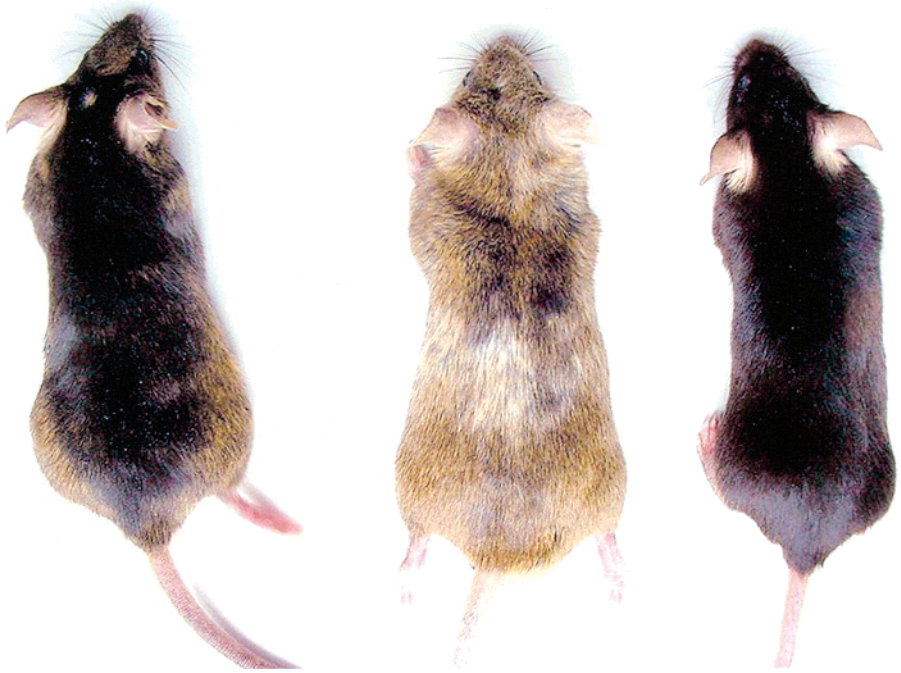

Figure 5. Generation of chimera with fractionated ES cells. Comparison of a wild-type animal (right) and typical chimeras obtained with ES cells from fractions 3 (medium) and 2 (left).

of the proliferation-associated antigen Ki67. It is well-known that its expression is related to the cell proliferation state, the Ki67 protein being expressed in all phases of the cell cycle except G0 and early G1.[32] As reported in Figure 4A, the percentage of Ki67 positive cells was significantly higher ($p < 0.05$, Student *t*-test for paired data) in fraction 2 ($84.0 \pm 5.6$), as compared with fractions 1 and 3 ($66.8 \pm 7.4\%$). To reinforce the hypothesis of a different cell proliferation state between ES fractions, we then analyzed their DNA content. Propidium iodide staining experiments indicated that the percentage of G0/G1 cells was significantly higher ($p < 0.05$, *t*-test for paired data) in fraction 3 ($56.3 \pm 1.4\%$), as compared to fractions 1 and 2 ($35.1 \pm 1.2\%$) (Figure 4B). In agreement with these latter results, the percentage of cells in the S phase (when DNA is synthesized) was significantly ($p < 0.05$) reduced in fraction 3 ($39.5 \pm 1.4\%$), as compared to cells of fractions 1 and 2 ($59.2 \pm 4.2\%$) (Figure 4B). Altogether, these results reinforced our hypothesis that the ES cell proliferation state was different between SdFFF eluted fractions. Finally, to examine if we were able to sort the desired ES fraction for transgenic mouse generation, we used ES fractions 2 and 3 to test their in vivo developmental potential, these fractions having the highest and the lowest in vitro developmental potential, respectively.

**Evaluation of in Vivo Developmental Potential of SdFFF-Eluted Cells.** ES cells from fractions 2 and 3 were, thus, injected into mouse blastocysts to generate somatic chimeras. The frequency of chimeras obtained with ES cells from fraction 3 (9 with 27 injected blastocystes) was not significantly different ($p > 0.05$) from those obtained with ES cells from fraction 2 (9 with 37 injected blastocystes). In contrast, a higher degree ($p < 0.02$, Mann−Whitney *U*-test) of color coat chimerism was obtained with ES cells from fraction 3 ($87 \pm 5\%$), as compared to ES cells from fraction 2 ($63 \pm 9\%$) (Figure 5), showing that SdFFF can be used as an efficient tool in order to separate a purified ES cell fraction with a higher chimeric potential. The ability to colonize the germ line is the ultimate test for any new ES cell chimera technique. Thus, in a last set of experiments, four chimeric males from F2 and F3 were mated with females to test for germ-line transmission. A germ-line transmission was obtained for 4/4 F3 males within 3 months, as compared with 1/4 F2 males after six months. These latter results confirmed that ES cells purified by SdFFF can result in high-level chimerism without compromising normal development. Altogether, these results showed that the primary advantage of SdFFF for chimera production is the ability to obtain chimeras with a high degree of chimerism, including that of the germ line.

## CONCLUSION

Under strictly defined conditions, SdFFF cell sorting in association with two major tools for cell characterization, flow cytometry and cell culture, has enabled us to isolate in <10 min a sterile, purified, and viable fraction of ES cells having high in vitro developmental potential in order to generate chimeras having a high percentage of chimerism. This result is, in particular, based on the capacity of SdFFF to sort cells by their position in the cell cycle.

**Note Added after ASAP Posting.** The article was posted on February 14. In Figure 5, the wild type mouse and the chimera had been misidentified. The corrections were made and the article was reposted on February 25.

## ACKNOWLEDGMENT

This work was supported by a MENRT Grant, FNT ACI TS9-2001-01B0190.

Received for review May 27, 2003. Accepted January 6, 2004.
AC030218E

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