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RESEARCH PAPER

Nanomagnetic Activation as a Way to Control the Efficacy of Nucleic Acid Delivery

Bartosz F. Grześkowiak • Yolanda Sánchez-Antequera • Edelburga Hammerschmid • Markus Döblinger • Dietmar Eberbeck • Anna Woźniak • Ryszard Słomski • Christian Plank • Olga Mykhaylyk

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ABSTRACT

Purpose To explore the potential of magnetofection in delivering pDNA to primary mouse embryonic fibroblasts (PMEFs) and porcine fetal fibroblasts (PFFs) and investigate an effect of magnetic cell labeling on transfection efficacy.

Methods The formulation and a dose of the magnetic vector were optimized. The efficacy of the procedure was quantified by vector internalization, transgene expression and cell iron loading upon specific labeling with Ab-conjugated magnetic beads or non-specific labeling with MNPs.

Results Up to sixty percent of PMEF and PFF cells were transfected at low pDNA doses of 4–16 pg pDNA/cell. Specific labeling of the PMEFs with MNPs, resulted in a 3- and 2-fold increase in pDNA internalization upon magnetofection and lipofection, respectively, that yielded a 2–4-fold increase in percent of transgene-expressing cells. Non-specific cell labeling had no effect on the efficacy of the reporter expression, despite the acquisition of similar magnetic moments per cell. In PFFs, specific magnetic labeling of the cell surface receptors inhibited internalization and transfection efficacy.

Conclusions Magnetic labeling of cell-surface receptors combined with the application of an inhomogenous magnetic field (nanomagnetic activation) can affect the receptor-mediated internalization of delivery vectors and be used to control nucleic acid delivery to cells.

KEY WORDS magnetic cell labeling · magnetic nanoassemblies · magnetic nanoparticles · nanomagnetic activation · nucleic acid delivery

ABBREVIATIONS

CPM	Counts per minutes
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
iPSCs	Induced pluripotent stem cells
MNPs	Magnetic nanoparticles
PBS	Dulbecco's phosphate buffered saline
PCS	Photon correlation spectroscopy
PE	Phycoerythrin
PEI	Polyethylenimine
PFF	Porcine fetal fibroblasts
PMEF	Primary mouse embryonic fibroblasts

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B. F. Grześkowiak · Y. Sánchez-Antequera · E. Hammerschmid · C. Plank · O. Mykhaylyk
Institute of Experimental Oncology and Therapy Research, KLinikum rechts der Isar der Technischen Universität München Ismaninger Str. 22, 81675 Munich, Germany

B. F. Grześkowiak · A. Woźniak · R. Słomski
The NanoBioMedical Centre, Adam Mickiewicz University ul. Umultowka 85, 61-614 Poznan, Poland

B. F. Grześkowiak · R. Słomski
Department of Biochemistry and Biotechnology, Poznań University of Life Sciences, ul. Dojazd 11, 60-632 Poznan, Poland

M. Döblinger
Department of Chemistry, Ludwig-Maximilians-Universität München Munich 81377, Germany

D. Eberbeck
Physikalisch-Technische Bundesanstalt Abbestraße 2-12, 10587 Berlin, Germany

O. Mykhaylyk (✉)
Institute of Experimental Oncology and Therapy Research, Technische Universität München, Ismaninger Str. 22, 81675 Munich, Germany
e-mail: olga.mykhaylyk@lrz.tu-muenchen.de

TEM	Transmission electron microscopy
TEOS	Tetraethyl orthosilicate
THPMP	3-(trihydroxysilyl) propylmethylphosphonate

INTRODUCTION

Nucleic acid delivery to somatic cells, such as fibroblasts, is of interest for diverse biomedical applications. Fibroblasts can be reprogrammed into induced pluripotent stem cells (iPSCs) via the delivery of vectors coding for pluripotency factors, and the resulting iPSCs can then be differentiated into variety of specific cell types (1), which is a highly promising development in regenerative medicine. Primary mouse embryonic cells are the most commonly used model cells in this research area. Somatic cell nuclear transfer using fibroblast donor cells, especially porcine cells, has played a central role in the cloning of animals to produce recombinant therapeutic proteins and breed animals for the purpose of xenotransplantation and as a model for human diseases (2). Primary cultured cells, due to their low rate of population doubling, are more representative of the main functional component of the tissue of origin. However, they are considered more difficult to transfect than transformed cell lines (3).

Viral methods to genetically modify somatic cells are highly efficient (1, 4). Despite their high gene transduction efficiency, viral vectors possess disadvantages, such as immunogenicity, the risk of insertional mutagenesis and oncogenesis, and an inability to transfer large genes (5). Therefore, non-viral alternative methods of achieving cell modification are desirable.

Electroporation/nucleofection methods do not require enhancers and can yield a higher proportion of transgene-expressing primary fibroblast cells than can be achieved with lipofection methods, but cell survival is often an obstacle (6, 7). Non-viral delivery methods with cationic lipids or cationic polymers as enhancers (lipofection or polyfection) have the advantages of greater safety and ease of use compared with viral vectors (5). For mouse embryonic fibroblasts (MEFs), transfection with Lipofectamine/plasmid DNA (pDNA) resulted in very low efficacy (approximately 2% transfected cells (8)). Lipofection with Metafectene as an enhancer yielded 40% transfected MEFs (Biontex website). A high transfection efficacy of PMEF cells with retransfections (75% eGFP positivity at a dose of 12.5–50 pg pDNA/cell, with 90% cell viability) was achieved with polyplexes formed with a new bio-reducible poly(amido amine) p(CBA-ABOL) polymer (9). The method was successfully used to induce neuronal transdifferentiation of the cells.

We sought to explore the potential of magnetofection technology to transfect primary mouse embryonic fibroblasts (PMEFs) and porcine fetal fibroblasts (PFFs). In this approach, magnetic (nano)particles" (no interval between (nano) and particles) are associated with nucleic acid vectors, and a gradient magnetic field is applied to sediment the magnetic vectors onto the surface of the cells to be transfected (10). Thus, transfection is synchronized and greatly accelerated, and the vector dose required for efficient transfection is considerably reduced. The efficacy of magnetofection depends on the type of magnetic nanoparticles (MNPs), the magnetic vector composition, and the vector dose per targeted cell. These factors should be optimized for each specific application and cell type. One report is available on the magnetofection of porcine fibroblasts using pDNA complexes with PEI-modified MNPs (11), which resulted in approximately 25% of the cells expressing the transgene under optimized conditions. In MEFs, magnetofection with PolyMag/pDNA duplexes at an applied dose of 10 pg DNA/cell resulted in approximately 53% eGFP-positive cells, with less cytotoxicity than Lipofectamine complexes (12). The magnetofection of NIH 3T3 mouse fibroblasts using nTMag particles associated with pDNA under optimized conditions and the application of an oscillating magnetic field (2 Hz) yielded approximately 25% transgene positivity, similar to the 22% eGFP positivity after 6 h of transfection with Lipofectamine 2000.

Non-specific magnetic cell labeling can increase the efficacy of subsequent transfection or transduction with magnetic delivery vectors (13, 14). In this case, the internalized magnetic particles may enhance local field gradients under applied magnetic fields and serve as a "magnetic seed" to concentrate the vector at the cell surface (15). In the novel Magselectofection technology reported recently (14), a magnetic separation column is used as a magnetic device for magnetofection to associate magnetic delivery vectors with specifically labeled suspended cells directly in the column. This method resulted in further improvement of the results compared with the classical magnetofection procedure. Based on this experience, we investigated whether magnetic labeling can further increase the transfection efficacy of PMEF and PFF cells.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, Dulbecco's phosphate-buffered saline (PBS) and 0.25% trypsin/0.02% EDTA solution were obtained from Biochrom AG (Berlin, Germany). EmbryoMax® ES Cell Qualified Fetal bovine serum (FBS) was purchased from Millipore (Billerica, MA, USA). D-Luciferin was from Synchem OHG (Felsberg, Germany). Luciferase reporter plasmid p55pCMV-IVS-luc+ containing the firefly luciferase cDNA under the control of the cytomegalovirus (CMV) promoter and eGFP plasmid containing eGFP under the control of the EF-1α promoter (BD Biosciences, Clontech, Heidelberg) were amplified and purified by PlasmidFactory, Bielefeld, Germany. The transfection reagents DreamFect Gold and Dogtor were

acquired from OZ Biosciences (Marseille, France). FuGENE HD, X-tremeGENE HP and X-tremeGENE 9 were acquired from Roche (Basel, Switzerland). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Feeder Removal MicroBeads (information about the epitope that is recognized by Feeder Removal MicroBeads is confidential information of Miltenyi Biotec), Anti-PE MicroBeads, PE mouse anti-feeder antibody, PE-conjugated mouse CD90.2 antibody and PE-conjugated mouse CD90.2 were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). PE-conjugated mouse anti-human CD90 antibody was purchased from BD Pharmingen™ (Heidelberg, Germany) and recognizes the Thy-1 antigen (membrane glycoprotein). Na-iodide^{125} (1 mCi in 10 µl) was from Hartmann Analytics. Pierce Pre-Coated Iodination Tubes were purchased from Thermo Scientific. PD-10 desalting columns were acquired from GE Healthcare Life Sciences. All other chemicals were of analytical grade and used without further purification (Sigma-Aldrich, Steinheim, Germany). Tissue culture plates and flasks were obtained from Techno Plastic Products (Trasadingen, Switzerland). A 96-Magnet Plate with a permanent magnetic field with a field strength and gradient of 70–250 mT and 50–130 T/m, respectively, at the cell layer location and a Mega Magnetic Plate were supplied by OZ Biosciences (Marseille, France). OctoMACS Separator and MS Columns were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

Cell Culture

Primary mouse embryonic fibroblasts isolated from CF-1 mouse embryos (Millipore, cat. no PMEF-CFL), hereafter referred to as PMEF cells, were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Millipore), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were split 1:3 every 3–4 days at a seeding density of 1×10^4 cells/cm^2. Porcine fetal fibroblasts isolated from conceptuses, hereafter referred to as PFF cells, were cultured in DMEM supplemented with 20% fetal bovine serum (FBS; Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. Cells were split 1:2 every 3–4 days at a seeding density of 1×10^4 cells/cm^2. Both cell types were cultivated on gelatin-coated flasks (30 min incubation with 0.1% gelatin before cell seeding) at 37°C in a humidified atmosphere containing 5% CO_2.

Magnetic Nanoparticles for pDNA Delivery and Cell Labeling

Core/shell–type iron oxide magnetic nanoparticles (MNPs) were synthesized by precipitating Fe(II)/Fe(III) hydroxide from an aqueous salt solution, followed by transformation into magnetite in an oxygen-free atmosphere, with immediate spontaneous adsorption of the shell components, as described elsewhere (16, 17). For formulation of the magnetic lipoplexes, the particles with a surface coating of 25-kDA branched polyethylenimine (PEI-25_{Br}) combined with the fluorinated surfactant ZONYL FSA (lithium 3-[2-(perfluoroalkyl) ethylthio] propionate) were used, hereafter referred to as PEI-Mag2 (18). For non-specific magnetic cell labeling, the MNPs with a coating resulting from the condensation of tetraethyl orthosilicate (TEOS) and 3-(trihydroxysilyl) propylmethylphosphonate (THPMP), yielding a silicon oxide layer with surface phosphonate groups, were used and will hereafter be referred to as SO-Mag5 (17). The aqueous MNP suspensions were sterilized using ^{60}Co gamma-irradiation at a dose of 25 kGy. The MNP concentration was determined in terms of the iron content of the dry nanomaterial and the iron content in aqueous suspension of the stock nanomaterial as described previously (16). For specific labeling of the PMEF cells, the feeder removal microbeads were used. To label the PFF cells magnetically through a specific interaction, the cells were first labeled by the PE-conjugated mouse anti-human CD90 antibody (BD Pharmingen™), followed by incubation with anti-PE microbeads. The mean iron oxide core size was calculated from the broadening of the X-ray diffraction peaks using the Scherrer formula or evaluated from magnetization data. The static magnetic properties of the particles were evaluated by measuring the quasistatic magnetization in the applied DC-fields or M(H) by using the MPMS commercial susceptometer (Quantum Design, USA).

Transmission Electron Microscopy

For imaging of PEI-Mag2 and SO-Mag5 particles and feeder removal microbeads, to activate the grid and remove hydrocarbon contamination, a formvar/carbon–coated 400-mesh copper grid was treated by a hydrogen/oxygen plasma of 50 mW for 30 s (Solarus Model 950, Gatan GmbH, USA). A 5 µl drop of the nanoparticle suspension containing approximately 25 µg iron was placed onto the grid and incubated for 10 min. Samples were rinsed carefully with two drops of double-distilled water and air-dried prior to imaging at an accelerating voltage of 80 kV using a TITAN 80–300 S/TEM (FEI Company). For imaging of anti-PE microbeads, a 10 µl drop of the nanoparticle suspension containing 10 µg iron was placed onto a Lacey formvar/carbon-coated 200 mesh copper grid, incubated for 60 min and dried under the vacuum of a 655 Turbo Pumping Station (Gatan) prior to imaging at an accelerating voltage 200 kV using a HRTEM Jeol ARM 200 F (Japan).

Preparation of the Transfection Complexes for Transfection of the Adherent Cells by Self-Assembling

For transfection of the adherent cells in 96-well plates, the magnetic transfection complexes were prepared by mixing

20 µl of a PEI-Mag2 MNP suspension containing 72 µg iron/ml water and 20 µl of the enhancer dilution. The enhancer dilution was prepared by mixing 2.9 µl of the commercial transfection reagent (DreamFect Gold, Dogtor, FuGENE HD, X-tremeGENE HP, X-tremeGENE 9 or Lipofectamine 2000) with 17.1 µl of water just before transfection experiments. Then, 60 µl of the plasmid DNA solution at 12 µg DNA/ml in serum- and supplement-free DMEM was added to the mixture of particles and the enhancer, which resulted in 100 µl of the complex, for a transfection reagent-to-nucleic acid ratio of 4:1 (*v*/*w*), iron-to-plasmid ratio of 2:1 (*w*/*w*) and plasmid concentration of 10 µg/ml. The mixture was further incubated at room temperature (RT) for 20 min to allow the components to assemble. After this time, 260 µl of DMEM was added, resulting in 360 µl magnetic complexes for transfection in triplicate. Lipoplexes used as a reference were prepared using the same method with water instead of MNP suspension. Then, 2:1 dilutions with serum- and supplement-free DMEM were obtained, and 50 µl of the prepared complexes were added to each well, with 3×10^3 seeded cell per well, resulting in applied DNA doses of 32 to 1 pg pDNA/cell.

Preparation of the Transfection Complexes for Transfection of the Magnetically Labeled Cells in Suspension

The magnetic complexes were prepared by mixing 80 µl of a PEI-Mag2 MNP suspension containing 100 µg iron/ml water and 20 µl of the enhancer dilution. The enhancer dilution was prepared by mixing 16 µl of the transfection reagent DF-Gold with 4 µl of water just before the transfection experiment. Then, 200 µl of the plasmid DNA solution at 20 µg DNA/ml in a serum- and supplement-free DMEM was added to the mixture of particles and the enhancer, which resulted in 300 µl of the complex, for a transfection reagent to nucleic acid ratio of 4:1 (*v*/*w*) and iron-to-plasmid ratio of 2:1 (*w*/*w*). The mixture was further incubated at RT for 20 min to allow the components to assemble, and 2:1 dilutions with serum- and supplement-free DMEM were obtained, resulting in DNA doses from 10 to 1.25 pg pDNA/cell after loading of 60 µl of the prepared complexes onto MS columns (transfection in Cell Separation Column) or after adding 26.25 µl complex dilution to each well of a 12-well plate (transfection on Magnetic Plate). Lipoplexes used as a reference were prepared using the same method with water instead of MNP suspension.

Size and Electrokinetic Potential Measurements

The mean hydrodynamic diameter (D_h) and electrokinetic potential (ζ) of the MNP suspension in water and the transfection complexes in DMEM without additives were determined by photon correlation spectroscopy (PCS) using a Malvern Zetasizer Nano Series 3000 HS (UK).

FACS Analysis of Reporter Expression in PMEF and PFF Cells

To enable specific magnetic cell labeling, analysis of PMEF and PFF cells for reporter expression was performed using PE-conjugated mouse anti-feeder and PE-conjugated mouse CD90.2 antibodies and PE-conjugated mouse CD90.2 from Miltenyi Biotec and PE-conjugated mouse anti-human CD90 from BD Pharmingen™. The cells were trypsinized, washed and treated with the antibodies according to the manufacturers' instructions. FACS analysis was performed using a 488 nm argon laser with an excitation maximum of 510 nm, and cell fluorescence was measured using a 585/42 nm bandpass filter (red fluorescence from the cells labeled with PE-Abs).

Analysis of the Non-Heme Iron Content in the Cells

To analyze the associated/internalized iron after cells labeling, the exogenic non-heme iron content was determined using a modified method of Torrance and Bothwell (16, 19). Briefly, approximately 9×10^5 trypsinized cells were washed with PBS. The cell pellet was resuspended in 200 µl of an acid mixture containing 3 M HCl and 0.6 M trichloroacetic acid. After an overnight incubation at 65°C, the samples were centrifuged, and 50 µl of the clear supernatant was analyzed for its iron content by a colorimetric method with 1,10-phenanthroline. To a probe of 50 µl of the supernatant, we added 20 µl 10% hydroxylamine-hydrochloride solution, 100 µl ammonium acetate buffer (25 g ammonium acetate and 70 ml glacial acetic acid, with a total volume adjusted to 100 ml with ddH_2O), and 50 µl of 0.1% 1,10-phenanthroline solution. The mixture was incubated for 20 min, and the optical density was then measured at 510 nm. The iron content was calculated using a calibration curve measured for the freshly prepared dilution series of the iron stock solution (392.8 mg ammonium iron (II) sulfate hexahydrate, 2 ml concentrated H_2SO_4 and 10 ml distilled water were titrated with 0.05 N $KMnO_4$ until a faint pink color persisted, followed by volume adjustment to 100 ml).

Magnetophoretic Mobility of the Magnetic Transfection Complexes and Magnetically Labeled Cells

To evaluate the magnetically induced velocity (magnetic responsiveness) of the magnetic transfection complexes and magnetically labeled cells, we measured the time course of the turbidity of the suspensions when subjected to inhomogeneous magnetic fields, as described previously (20). Briefly, a gradient field was generated by positioning two

mutually attracting packs of four quadrangular neodymium-iron-boron permanent magnets symmetrically on each side of a cuvette holder parallel to the light beam of the spectrophotometer (DU-640, Beckman). The magnetic field between the magnets was measured using a grid of step size 1 mm, and the average magnetic field and resulting field gradient perpendicular to the measuring beam were calculated to be 213 mT and 4 ± 2 T/m, respectively. We diluted aliquots of the suspensions of the complexes to achieve a starting optical density of 0.4–1 at the analytic wavelength. An optical cuvette filled with one of these diluted suspensions was then placed into the holder of the spectrophotometer and exposed to a magnetic field, and the optical density (turbidity) was then recorded at 360 nm in kinetic mode. The resulting clearance curves were drawn by plotting the normalized optical densities at the analytical wavelength (OD_{360}/OD_{360} or $OD_{610\ nm}/OD_{610\ nm}$ for the complexes and labeled cells, respectively) against time. From these curves, the time to magnetic sedimentation of 90% of magnetic complexes/cells $t_{0.1}$ [s] was deduced, and the average magnetophoretic mobility of the free complexes and labeled cells was calculated as $\upsilon_z = 10^3/t_{0.1}$ [μm/s] (accounting for the average path of 1 mm in the experimental setup). In a stationary regime, the hydrodynamic force counterbalances the magnetic force that acts on a particle assembly, such as a magnetic vector or labeled cell comprising multiple magnetic nanoparticles, and the derived magnetophoretic mobility of the complexes and the average hydrodynamic diameter of the objects D_h allowed us to estimate the average magnetic moment M and average number of magnetic nanoparticles associated with the complex or cell $N = M/m_{\mathrm{eff}}$ (accounting for the effective magnetic moment of the insulated MNP in the applied field). This approach was used by Wilhelm et al. when analyzing the magnetophoretic mobility distribution from cell tracking experiments (21). The experimental approach could be useful for experimentally estimating parameters of the magnetic field and exposure to achieve full sedimentation of the complex or enable magnetic targeting.

Transfection of Adherent Cells

For all transfection experiments, the PMEF and PFF cells were cultivated in flat-bottomed plates pre-coated with 0.1% gelatin solution and seeded at a density of 9×10^3 cells/cm^2. For transfection of the adherent cells in 96-well plates, 150 μl of the cell suspension (2×10^4 cells/ml) was transferred into the wells, resulting in 3×10^3 cells/well, providing a confluence of 50% and 30% for PFF and PMEF cells, respectively, on the day of transfection 24 h after seeding the cells. Fifty microliters of the freshly prepared non-magnetic or magnetic complexes was added to each well. The plate was positioned in the 96-element array magnetic plate and incubated for 20 min to sediment the magnetic complexes onto the cell surface. The cell culture plate was then incubated for 48 h until the reporter gene expression analysis. The data are presented as the mean ±standard deviation. Schematic of the transfection procedures are given in Fig. 1.

Transfections were also performed with non-specifically labeled and specifically labeled adherent PMEF cells as shown schematically in Fig. 1. To magnetically label PMEF non-specifically, SO-Mag5 particles suspended in DMEM without additives were added to the cultured cells in a 75 cm^2 culture flask at approximately 75% confluence at an applied iron dose of approximately 10 pg iron/cell, incubated overnight, trypsinized and seeded in 24-well plates at a density of 2×10^4 cells/ml complete medium/well 24 h before transfection. To label PMEFs specifically, the cells were seeded in a 24-well plate at a density of 2×10^4 cells/ml complete medium/well. Twenty-four hours after seeding, the cell culture medium was aspirated and changed to 100 μl/well of magnetic labeling suspension (26 μl of feeder removal microbeads mixed with 1,274 μl of complete cell culture medium), which resulted in approximately 135 pg applied iron/cell. The plate was then incubated for 15 min. Then, the cells were washed with 1 ml of complete cell culture medium to remove unbound beads. Finally, 1 ml of complete medium was added to each well.

Transfection of the Magnetically Labeled Suspended Cells

Transfection of magnetically labeled suspended cells was performed in a plate or in a cell separation column, in both cases under magnetic field application as shown schematically in Fig. 1. For transfection of specifically labeled PMEFs in suspension, 8.75×10^5 PMEF cells in 25 μl of complete cell culture medium were mixed with 5 μl feeder removal microbead suspension, which resulted in approximately 7 pg applied iron per cell. After incubation for 15 min in the dark at 4°C, the cells were resuspended at a density of 2×10^4 cells/ml complete medium.

To magnetically label PFFs specifically, 2.5×10^5 PFF cells in 100 μl of the complete cell culture medium were mixed with 5 μl PE-conjugated mouse anti-human CD90 (BD Pharmingen™), incubated for 15 min in the dark at 4°C, washed with 10 ml of the complete cell culture medium to remove unbound antibodies, centrifuged and resuspended in 100 μl of complete cell culture medium. Subsequently, 10 μl of anti-PE microbeads was added, which resulted in approximately 12 pg applied iron/cell. The suspension was incubated for 15 min in the dark at 4°C, washed with 5 ml of complete cell culture medium, centrifuged and resuspended at a density of 2×10^4 cells/ml complete medium.

To magnetically label PFFs non-specifically, SO-Mag5 particles suspended in DMEM without additives were added to the cultured cells in a 75 cm^2 culture flask at approximately 75% confluence at an applied iron dose of approximately 10 pg iron/cell, incubated overnight, trypsinized and

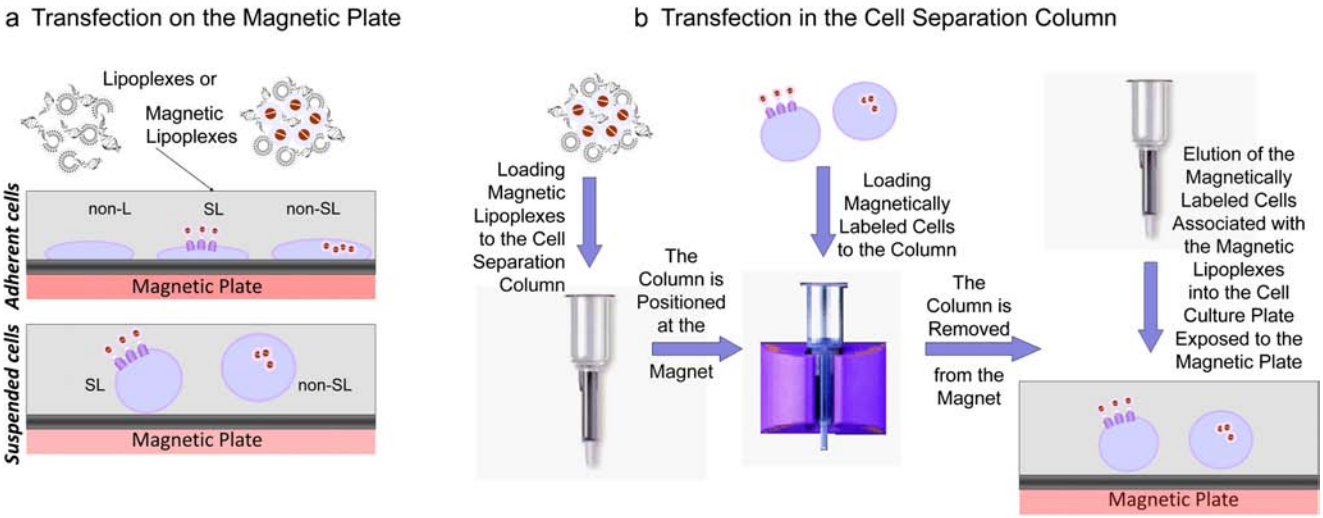

Fig. 1 Schematic of the procedures used to transfect the primary mouse embryonic fibroblasts and porcine fetal fibroblasts. (**a**) For transfection on the magnetic plate, the magnetic lipoplexes (with lipoplexes as a reference control) were associated with non-labeled (*non-L*), specifically magnetically labeled (*SL*) or non-specifically magnetically labeled (*non-SL*) cells adhered to the culture plate or suspended in a well of the cell culture plate, which was exposed to the magnetic plate for 20 min. (**b**) For transfection in the cell separation column, the magnetic lipoplexes were loaded onto the column, the column was positioned in the magnet, and the magnetically labeled cells were added to the column. After 15 min of incubation in the magnet, the column was removed, and the cells associated with magnetic lipoplexes were eluted into the wells of the cell culture plate, which was positioned on the magnetic plate for 15 min. The cells were further cultivated until reporter analysis 48 h later.

resuspended at a density of 2×10^4 cells/ml. The magnetically labeled cells were immediately used for transfection experiments and analyzed for the associated iron content and magnetophoretic mobility. Portions of 1.75 ml of the cell suspension containing 3.5×10^4 cells were transferred to each well of a 12-well cell culture plate. The lipoplexes or magnetic lipoplexes were added at 26.3 μl/well, resulting in pDNA doses of 10, 5, 2.5 and 1.25 pg/cell. The cell culture plate was positioned on the Mega Magnetic Plate for 20 min, and the cells were cultured until the reporter analysis 48 h later.

When the cell separation column was used as a magnetic device, 60 μl (void volume of MS column) of the magnetic lipoplex dilutions were added to the MS Column (Miltenyi) and allowed to seep into the column. The column was placed in the OctoMACS magnet to immobilize the complexes, and 4 ml of cell suspension (8×10^4 magnetically labeled cells) was loaded into the column. After 15 min incubation in the OctoMACS magnet, the column was removed, and the cells associated with magnetic lipoplexes were eluted into wells of a cell culture plate. The plate was positioned on the Mega Magnetic Plate for 15 min, and the cells were further cultivated until the reporter analysis 48 h later. As a reference, transfections of the adherent cells were performed in parallel.

Luciferase Expression Analysis

The expression of luciferase was evaluated by measuring the intensity of the chemiluminescence in luciferin-luciferase light-producing reactions in the cell lysate as described in detail elsewhere (16). Briefly, to prepare the cell lysate, the lysis buffer containing 0.1% Triton X-100 in 250 mM Tris pH 7.8 was added to the wells and incubated for 10 min at RT. Then, 50 μl/well of cell lysate was transferred to the wells of a black 96-well plate and mixed with 100 μl of luciferin buffer (35 mM D-luciferin, 60 mM DTT, 10 mM magnesium sulfate, 1 mM ATP and 25 mM glycyl-glycine-NaOH buffer, pH 7.8). Chemiluminescence was recorded using a TopCount instrument (Canberra Packard, Groningen, Netherlands). The protein content was determined using a Bio-Rad assay as described previously (16). The results are presented in terms of luciferase weight per unit protein weight of the cells. The experiments were performed in triplicate.

eGFP Expression Analysis

The expression of eGFP was characterized by fluorescence microscopy and fluorescence-activated cell sorting (FACS) in a Canto™ II device (Becton Dickinson). The cells were trypsinized, washed and resuspended in PBS supplemented with 1% FBS. FACS analysis was performed using a 488 nm argon laser with an excitation maximum of 510 nm, and cell fluorescence was measured using a 530/30 nm bandpass filter (green fluorescence from the cells expressing eGFP).

Analysis of pDNA Internalization into Cells

To quantify pDNA internalization with transfection complexes into cells, the complexes were formulated using ^{125}I-labeled DNA. For labeling, 100 μg of pDNA in 100 μl of water and 2 μl of Na-iodide^{125} (0.2 mCi) were mixed and

transferred into the Pierce Pre-Coated Iodination Tube. The tube was incubated with a gentle agitation for 10 min. The labeled pDNA fractions were separated from the unbound label by gel filtration using a PD-10 Desalting column pre-equilibrated with water. The specific radioactivity and pDNA concentration of the product were measured using a Wallac 1480 Wizard 3 automatic gamma counter (Finland) and by measuring its optical density at 260 nm. The fraction with a pDNA concentration of 64 μg/ml and specific radioactivity of 2.1×10^6 CPM/μg pDNA was used for experiments. To prepare the complexes for internalization experiments, a mixture of the 80% (*w*/*w*) non-labeled and 20% (*w*/*w*) radioactively labeled plasmid was used. The cells were transfected in a 24-well plate. After 5 h of incubation, heparin solution was added to the wells at a final concentration of 100 U/ml, and the cells were incubated for 10 min to dissociate loosely bound complexes. The medium was then discarded, and the cells were gently washed with PBS. Next, 200 μl of 0.25% trypsin/EDTA solution was added to each well, followed by incubation with 300 μl of lysis buffer. The cells were observed under a microscope, and after complete trypsinization and lysis, the whole material from each well was collected into scintillation vials. Their radioactivity (CPM) was measured using a gamma counter. The applied dose of the radioactively labeled pDNA complexes was used as a reference. The results were recalculated in terms of the internalized pDNA versus applied pDNA dose.

MTT Assay for Cytotoxicity

The MTT assay for cell respiration activity was carried out to assess the cytotoxicity of the complexes. After 48 h of incubation with the complexes, the transfected cells were washed with PBS; then, 100 μl of 1 mg/ml MTT prepared in DMEM-F12 with 5 mg/ml glucose was added to each well of a 96-well plate. The cells were incubated for 4 or 6 h for PMEF or PFF cells, respectively. Afterwards, 100 μl of solubilization solution (10% Triton X-100 in anhydrous isopropanol in 0.1 N HCl) was added to each well, and the plate was incubated at 37°C with shaking to dissolve the formazan crystals. The optical density was measured at 590 nm, and the cell viability was expressed as the respiration activity normalized to untreated cells.

RESULTS

Magnetic Nanoparticles Used for Gene Delivery and Cell Labeling

Some characteristics of the PEI-Mag2 and SO-Mag5 nanoparticles used to formulate gene delivery vectors and label the cells non-specifically, as well as of the commercial magnetic beads (Feeder Removal MicroBeads and Anti-PE MicroBeads) used to label the cells specifically, are listed in Table I. TEM images of these particles are shown in Fig. 2. All of the nanoparticles were of the core-shell type with a core of magnetic iron oxide where a multicore structure of the particles is frequently visible (Fig. 2). The PEI-Mag2 and SO-Mag5 nanoparticles had an average effective magnetic core size of 9 and 6.8 nm, respectively, derived from M(H)-data, assuming a homogeneously magnetised MNP with a certain saturation magnetisation M_s (Table I). On the other hand, both microbeads had two fractions, with a characteristic effective magnetic core size of 6.5 and 16.5 nm, respectively, with the cores of the 16.5 nm nanoparticles accounting for 60% of the total volume. Note, that the difference to the hydrodynamic size (Table I) also hints to a multicore structure of the particles. The iron oxide cores of the particles exhibited high saturation magnetization values of 62–107 emu/g iron. It is predominantly the coating that makes these particles suitable for particular applications. PEI-Mag2 nanoparticles had a surface coating formulated from the fluorinated surfactant ZONYL FSA (lithium 3-[2-(perfluoroalkyl)ethylthio] propionate) and 25-kDa branched polyethylenimine (PEI-25_{Br}). The mean hydrodynamic diameter in aqueous suspension was 28 nm, and the electrokinetic potential was highly positive at $+55.4\pm1.6$ mV. The iron content of the MNPs was 0.56 g iron per g dry weight. Silica iron oxide SO-Mag5 MNPs, described in detail by Mykhaylyk et al. (17), were used for non-specific cell labeling. These nanoparticles were coated with a silicon oxide layer with surface phosphonate groups, yielding a negative ζ potential of -38 ± 2 mV when measured in aqueous suspension, and had a mean hydrodynamic diameter of 40 nm. The dry nanomaterial contained 0.52 g of iron per gram total weight. Both feeder removal and anti-phycoerythrin (PE) microbeads had a mean hydrodynamic diameter of approximately 97 nm and a negative ζ potential in water of approximately −20 mV (see Table I).

Characteristics of the Magnetic Transfection Complexes

Six commercially available non-viral transfection reagents (DreamFect Gold and Dogtor (OZ Biosciences); Lipofectamine 2000 (Invitrogen); FuGENE HD, X-tremeGENE HP, and X-tremeGENE 9 (Roche)) were used to formulate the lipoplexes and magnetic lipoplexes with PEI-Mag2 nanoparticles at an enhancer-to-DNA ratio of 4:1 (*v*/*w*). Lipofectamine 2000 and X-tremeGENE 9 showed low efficacy in PMEF and PFF cell transfection using both lipo- and magnetic lipoplexes. Based on the results on the luciferase reporter expression and respiration activity after transfection (Fig. S1-S4) and the percentage of GFP-expressing cells (data not shown), DF-Gold and X-tremeGENE HP were used in further

Table I Characteristics of the Iron Oxide Magnetic Nanoparticles Used for the Assembly of The Magnetic pDNA Lipoplexes and Specific and Non-Specific Labeling of the PMEF and PFF Cells

Magnetic nanoparticles	Mean iron oxide core size < d > (nm)	Saturation magnetization of the core Ms (emu/g iron)	Mean hydrated diameter in water D_h/Width (nm)	ξ-Potential in water (mV)	Iron content (g Fe/g dry weight)	Reference
PEI-Mag2	9	62	28/14	+ 55.4 ± 1.6	0.56	(16)
Feeder Removal MicroBeads (Miltenyi Biotec)	6.5 nm (40%) 16.5 nm (60%)	107	96.9/32.6	− 21.8 ± 1.7	–	–
Anti PE MicroBeads (Miltenyi Biotec)	6.5 nm (40%) 16.5 nm (60%)	105	97.5/23.4	− 19.5 ± 1.7	–	–
SO-Mag5	6.8	94	40/14	− 38.0 ± 2.0	0.52	(17)

experiments investigating the magnetofection of PFF and PMEF cells. Two MNP-to-plasmid ratios (2 and 0.5) were tested to formulate the magnetic lipoplexes. The characteristics of the lipoplexes and magnetic complexes at iron-to-DNA ratios of 0.5:1 and 2:1 (*w*/*w*) prepared in DMEM without additives are provided in Table II. The average size of non-magnetic and magnetic complexes with DF-Gold according to the hydrodynamic diameter varied from approximately 600 nm to nearly 1,200 nm, whereas those with X-tremeGENE HP were smaller, with an average size of approximately 500 to 900 nm. All of the complexes had a positive net charge. The ζ-potential of the complexes with DF-Gold was higher than the vectors formulated with X-tremeGENE HP.

To assess the magnetophoretic mobility of the complexes, we applied a method based on the measurement of the velocity of the magnetic lipoplexes under the influence of a magnetic field gradient (20). The time courses of the normalized turbidity of the complexes are plotted in Fig. 3a. The derived magnetophoretic mobility and average hydrodynamic diameter allowed us to estimate the average magnetic moment of the complexes and the number of MNPs associated with each complex (Table II). The complexes formulated at higher MNP-to-pDNA ratios were larger and had significantly higher magnetophoretic mobility and magnetic moment for both enhancers. Furthermore, transfection with 2-to-1 complexes always yielded a higher transfection efficacy in terms of both luciferase expression and the percentage of transfected cells compared with 0.5-to-1 complexes (see examples Fig. S5 and S6). Therefore, we further explored the efficacy of the magnetic 2-to-1 complexes in the transfection of target cells. These complexes had an average of approximately 40,000–60,000 associated MNPs and average magnetic moments of 5.6 femtoA·m^2 and 3.2 femtoA·m^2 for DF-

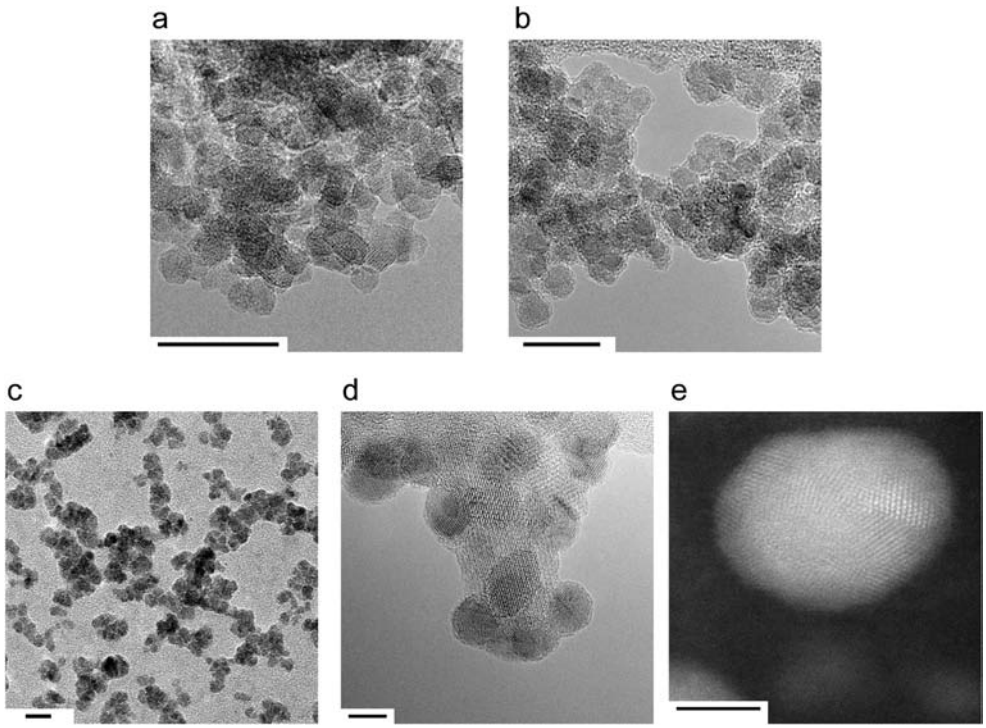

Fig. 2 Transmission electron microscopy images of the nanoparticles. (**a**) PEI-Mag2 nanoparticles used for assembling the magnetic pDNA lipoplexes. (**b**) SO-Mag5 nanoparticles for non-specific labeling of the PMEF and PFF cells. (**c**) Feeder removal microbeads for specific labeling of the PMEF cells. (**d** and **e**) Anti-PE microbeads for specific labeling of the PFF cells in combination with PE-conjugated mouse anti-human CD90 antibody. Bars = 20 nm (**a**, **b** and **c**) or 5 nm (**d** and **e**).

Table II Characteristics of the Transfection Complexes

Complex	Mean hydrodynamic diameter, D_h/Width (nm)	Electrokinetic potential in cell culture medium without serum, ζ(mV) [a]	Time for magnetic sedimentation of 90% the complexes [b,c] $t_{0.1}$ (s)	Magnetophoretic mobility [b,c] u_z (μm/s)	Average magnetic moment, [b] M (10^{-15} Am^2)	Average number of MNPs associated with the complex
DF-Gold/pDNA	781/107	15.9 ± 0.28	–	–	–	–
PEI-Mag2/DF-Gold/pDNA (0.5:4:1)[#]	674/108	20.9 ± 0.28	2,695	0.4	0.5	6.1×10^3
PEI-Mag2/DF-Gold/pDNA (2:4:1)[#]	1,169/116	16.2 ± 0.7	440	2.3	5.6	6.5×10^4
X-tremeGENE HP/pDNA	571/54	1.8 ± 0.4	–	–	–	–
PEI-Mag2/X-tremeGENE HP/pDNA (0.5:4:1) [#]	639/132	5.5 ± 0.2	1,448	0.7	0.9	1.1×10^4
PEI-Mag2/X-tremeGENE HP/ pDNA (2:4:1) [#]	845/131	7.2 ± 1.6	557	1.8	3.2	3.7×10^4

[#] For magnetic lipoplexes, the values of the iron:enhancer:pDNA *w/w/w* ratio are given in brackets

[a] Each value represents the mean ± SD

[b] Measured at an average magnetic field $\overline{B}$ of 0.213 ± 0.017 T

[c] Measured at an average magnetic field gradient $\overline{\nabla B}$ of 4 ± 2 T/m

Gold and X-tremeGENE HP magnetic lipoplexes, respectively (measured under a magnetic field of 213 mT).

Magnetofection versus Lipofection in Adherent Primary Mouse Embryonic Fibroblasts and Porcine Fetal Fibroblasts

Figure 4 shows the dose–response data of magnetofection versus lipofection efficacy in PMEF and PFF cells in terms of the luciferase reporter expression, the percentage of transfected (eGFP-positive) cells, and the respiration activity of the cells after transfection. All of the magnetic complexes were formulated at an iron-to-DNA ratio of 2:1 (*w/w*), which resulted in high complex efficacy and high cell viability. A DF-Gold-to-DNA and X-tremeGENE HP-to-DNA ratio of 4:1 (*v/w*) was used because it was the optimal ratio in numerous experiments. The tested DNA dose ranged from 1 to 32 pg pDNA/cell. As shown in Fig. 4, an advantage in transfection efficiency (as determined by transgene expression) arising from the use of magnetic nanoparticles and the application of an inhomogeneous magnetic field in mouse and porcine fibroblasts was revealed. The optimal dose for magnetofection of the PMEF cells with the magnetic lipoplexes was 16 pg pDNA/cell (Fig. 4a). At this dose, 60% of cells were magnetofected with DF-Gold and approximately 50% with X-tremeGENE HP, and we observed high cell viability after transfection. The PFF cells were significantly more sensitive and exhibited high magnetofection efficacy at a dose of 4–8 pg pDNA/cell. Half of the PFF cells were transfected when using a dose of 8 pg pDNA/cell with DF-Gold as an enhancer (Fig. 4b). Magnetic triplexes formulated with X-tremeGENE HP were also efficient in the magnetofection of PFFs (60% of cells were transfected at a dose of 4 pg pDNA/cell), but they were toxic at higher doses. Higher doses resulted in a decreased efficacy associated with cell toxicity. The MTT assay showed no additional toxicity associated with the particles compared with the non-magnetic lipoplexes within the tested concentration range in the PMEF cells and minimal to no toxic effects up to the plasmid dose of 10 pg pDNA/cell in the PFF cells. Representative microscopy images of magnetofected cells after using 10 pg pDNA/cell and 4 pg pDNA/cell for PMEF and PFF cells, respectively, are shown in Fig. 4a and b.

Magnetic Cell Labeling and Characteristics of the Labeled Cells

We examined the possibility of labeling both fibroblast cell types magnetically. FACS analysis of the cells after treatment with phycoerythrin-labeled anti-feeder antibody and different CD90 antibodies revealed that PE-conjugated mouse anti-feeder antibody, but not PE-conjugated mouse CD90.2, labeled the PMEFs efficiently (Fig. 5a), which enabled us to use

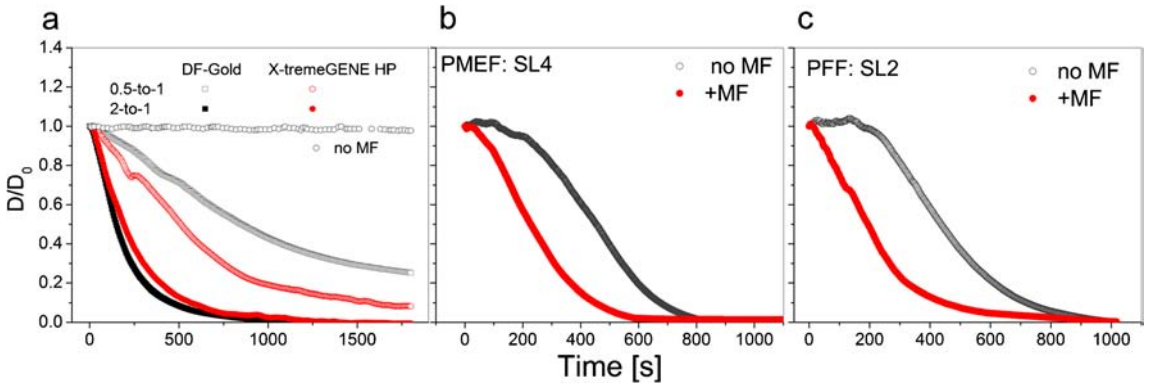

Fig. 3 Magnetophoretic mobility of the magnetic pDNA lipoplexes and magnetically labeled cells. Time course of the normalized turbidity (D/D_0, $\lambda = 360$ nm) recorded in the applied gradient magnetic field (*+MF*) and without magnetic field application (*no MF*) for (**a**) magnetic pDNA lipoplexes formulated with DF-Gold or X-tremeGENE HP as enhancers at iron-to-pDNA ratios of 0.5-to-1 and 2-to-1, respectively, in specifically labeled (*SL*) (**b**) PMEF and (**c**) PFF cells.

the feeder removal microbeads for direct specific labeling of these cells. However, these antibodies did not label the PFF cells. Only PE-conjugated mouse anti-human CD90 antibody labeled the PFFs efficiently (Fig. 5b and c), which enabled two-step labeling of the PFFs by first binding the PE-conjugated mouse anti-human CD90 antibody to the PFFs, followed by

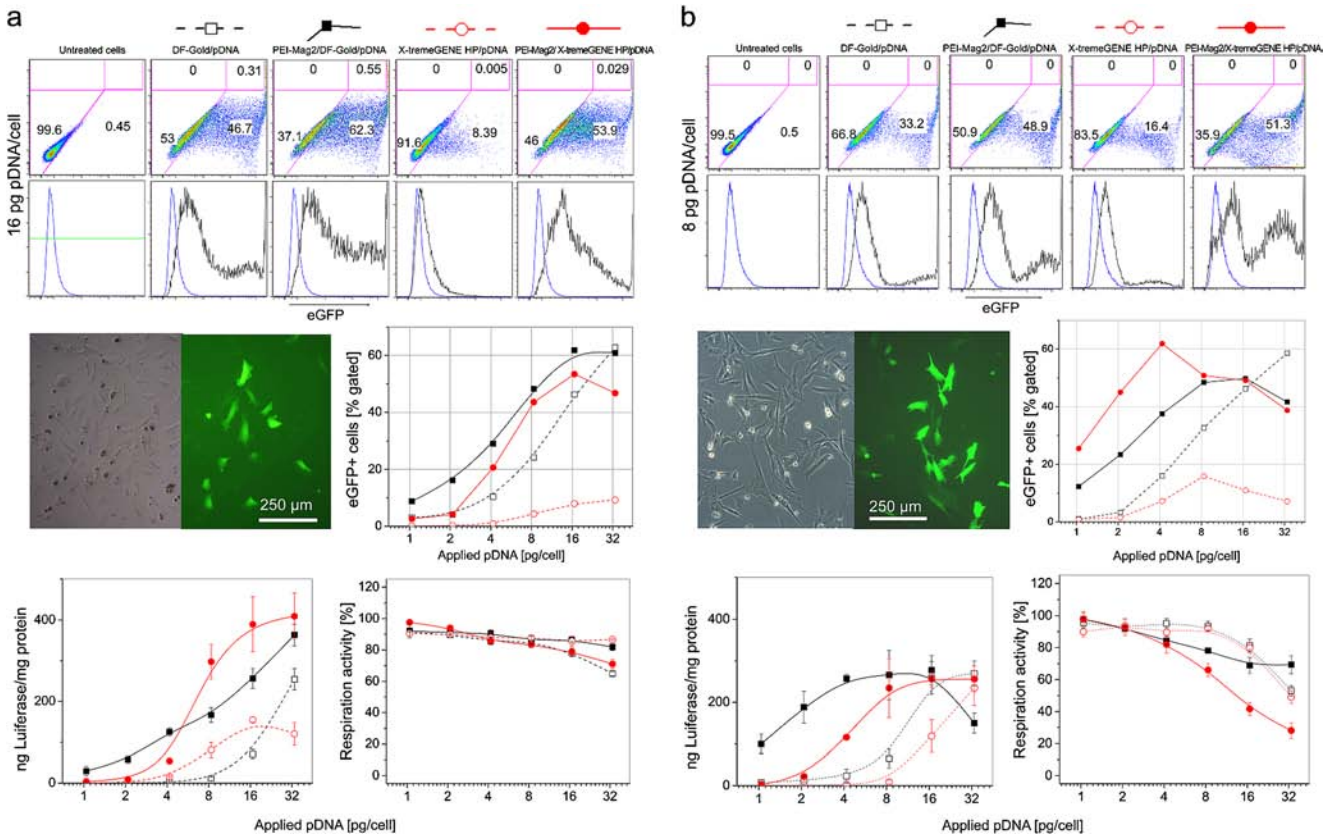

Fig. 4 Magnetofection versus lipofection in adherent (**a**) primary mouse embryonic fibroblasts (PMEFs) and (**b**) porcine fetal fibroblasts (PFFs). The cells were transfected with DF-Gold/pDNA or X-tremeGENE HP/pDNA lipoplexes and magnetic triplexes with PEI-Mag2 magnetic nanoparticles formulated at an iron-to-pDNA ratio of 2:1 (*w/w*) and an enhancer-to-pDNA ratio of 4:1 (*v/w*) under a steady-state magnetic field as described in the Materials and Methods. Forty-eight hours after transfection, the cells were analyzed for transfection efficiency using FACS for eGFP-positive cells or chemiluminescence assay for firefly luciferase, depending on the reporter. The figures at the top show density plots of the cells transfected with lipoplexes or magnetic lipoplexes at a plasmid dose of 16 and 8 pg/cell for PMEF and PFF cells, respectively. FACS data on the percentage of the eGFP-expressing cells and data on luciferase reporter expression in absolute units, normalized per total protein, in cell lysates are provided in the plots for the applied plasmid doses of 1 to 32 pg/cell. Bright field and fluorescence images (490/509 nm) for eGFP green fluorescence were taken after magnetofection of the cells with magnetic PEI-Mag2/DF-Gold/pDNA lipoplexes at an applied plasmid dose of 10 and 4 pg/cell for PMEF and PFF cells, respectively. Cell viability in terms of respiration activity measured 48 h after cell transfection is provided at the bottom.

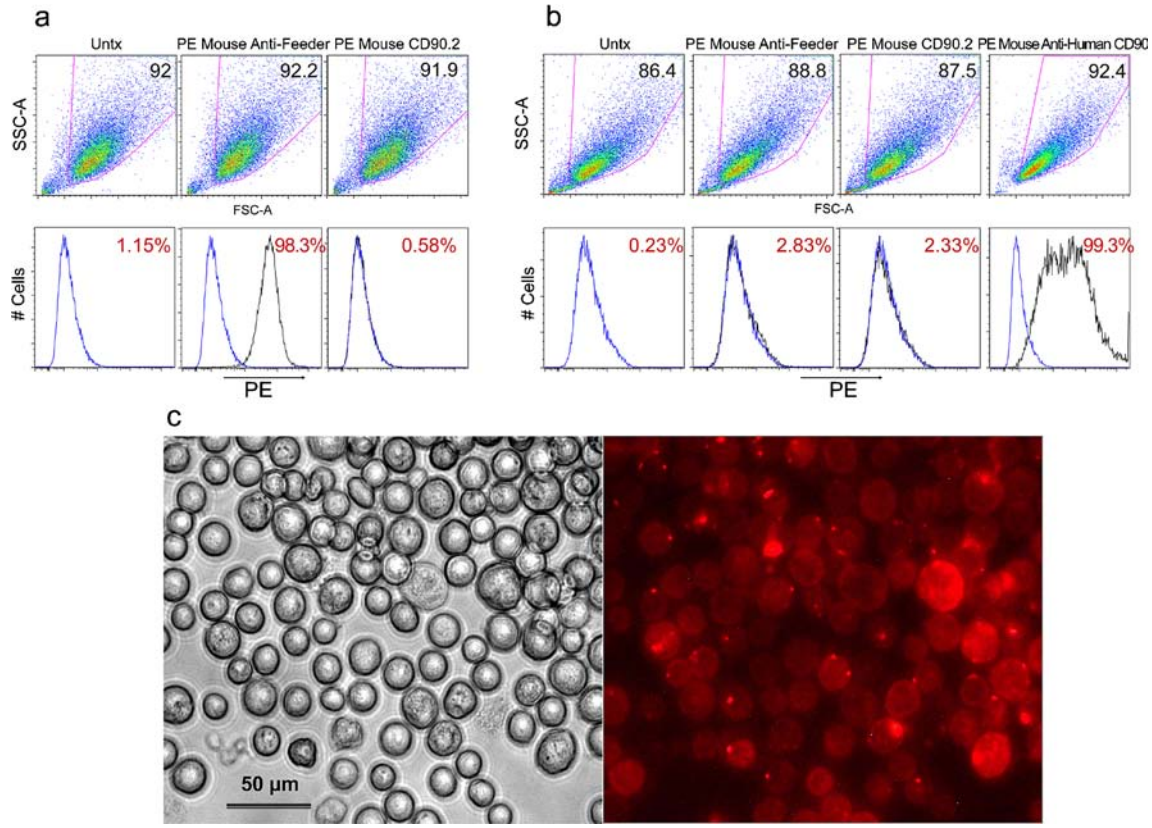

Fig. 5 FACS data on the efficiency of the PMEF and PFF cell labeling with selected antibodies. (**a**) Scatter plots and histograms for PMEF cells treated with the PE-conjugated mouse anti-feeder or PE-conjugated mouse CD90.2 antibody. (**b**) Scatter plots and histograms for PFF cells treated with PE-conjugated mouse anti-feeder antibody and PE-conjugated mouse CD90.2 or mouse anti-human CD90 antibody. (**c**) Phase contrast and fluorescence (510/650 nm) microscopy images of the PFF cells specifically labeled with PE-conjugated CD90 antibody, followed by magnetic labeling with anti-PE-conjugated MB.

magnetic labeling with anti-PE microbeads. The fluorescence image in Fig. 5c shows the distribution of the PE-labeled CD90 at the surface of the PFF cells after treatment with anti-PE microbeads and magnetization in a permanent magnetic field. For non-specific labeling of both cell types, SO-Mag5 nanoparticles were used.

We aimed to achieve similar iron cell loading regardless of whether the cells were labeled non-specifically or specifically. Specific cell labeling resulted in 0.6–0.9 pg iron per cell based on non-heme iron analysis. The magnetophoretic mobility of the specifically labeled PMEF cells was between 1.5 and 2.2 µm/s, which yielded a magnetic moment of 60–95 femtoA·m^2/cell originating from 0.5–1 pg iron per cell (approximately 1.5×10^5 internalized/associated particles) (Table III, Fig. 3b). The data show that the non-heme iron analysis and magnetophoretic mobility assay resulted in similar values for the iron loading of the specifically labeled PMEF cells. Similar results for magnetophoretic mobility, magnetic moment, and iron loading per cell were achieved when the PMEF cells were labeled with SO-Mag5 nanoparticles non-specifically.

Specific, indirect labeling of PFF cells resulted in a magnetophoretic mobility of between 2.5 µm/s and 4.6 µm/s, which corresponded to a magnetic moment of 109–200 femtoA·m^2/cell (approximately $2.2–4.1\times10^5$ internalized/associated particles or 0.9–1.7 pg iron per cell, Fig. 3c and Table III). The PFF cells non-specifically labeled with SO-Mag5 nanoparticles showed a magnetophoretic mobility, magnetic moment, and iron loading per cell close to the values found in labeled PMEFs cells. No toxicity was observed after cell labeling.

Transfection of Specifically Labeled Adherent Primary Mouse Embryonic Fibroblasts Compared with Non-Labeled and Non-Specifically Labeled Cells

To explore whether magnetic cell labeling can further improve transfection efficacy compared with the classical magnetofection procedure, we performed experiments on the transfection of the magnetically labeled cells, as illustrated in Fig. 1. The non-labeled cells or non-specifically labeled cells were seeded in the plates. The specific labeling of the adherent cells was performed directly in the plate immediately before transfection. After either the lipoplexes or magnetic lipoplexes were added to the cells, the cell culture plate was incubated on the magnetic plate. The results for the percentage of

Table III Characteristics of the Magnetically Labeled Cells

Sample	Applied MNPs (pg Fe/cell)	Time for magnetic sedimentation of 90% the cells[a,b] $t_{0.1}$(s)	Magnetophoretic mobility u_z (μm/s)[a,b]	Average magnetic moment M (10^{-15}Am^2)[a]	Average number of MNPs associated with the cell	Iron loading (pg/cell) based on	
						Magnetophoretic mobility measurements	Non-heme iron analysis
PMEF Cells							
non-SL1	20	210	4.8	203	3.5×10^6	2.16	4
non-SL2	10	654	1.5	65	1.1×10^6	0.69	0.75
non-SL3	10	591	1.7	72	1.2×10^6	0.77	–
SL1	7	710	1.4	60	1.2×10^5	0.52	0.8
SL2	7	680	1.5	63	1.3×10^5	0.54	0.9
SL3	7	537	1.9	79	1.6×10^5	0.68	0.56
SL4	10	450	2.2	95	1.9×10^5	0.82	–
PFF Cells							
non-SL1	10	860	1.2	50	8.6×10^5	0.54	0.35
non-SL2	10	710	1.4	61	1.0×10^6	0.65	–
non-SL3	10	640	1.6	68	1.2×10^6	0.72	1
non-SL4	10	690	1.4	63	1.1×10^6	0.67	1
non-SL5	10	720	1.4	60	1.0×10^6	0.64	–
non-SL6	10	520	1.9	83	1.4×10^6	0.89	1.57
non-SL7	10	427	2.3	102	1.7×10^6	1.08	0.34
SL1	9.7	217	4.6	200	4.1×10^5	1.72	0.43
SL2	12	399	2.5	109	2.2×10^5	0.94	–

[a] Measured at an average magnetic field $\langle B\rangle$ of 0.213 ± 0.017 T

[b] Measured at an average magnetic field gradient $\langle\nabla B\rangle$ of 4 ± 2 T/m; hydrodynamic diameters of the suspended PMEF and PFF cells were 20.3 ± 4.2 and 20.7 ± 4.3 μm, respectively

transfected cells are provided in Fig. 6a and b shows microscopy images of the cells taken 48 h after transfection. The data show an improvement of the transfection efficacy after lipofection and magnetofection for specifically labeled cells compared with non-labeled cells. Non-specific labeling had no effect on transfection efficacy.

Transfection of Magnetically Labeled Suspended Primary Mouse Embryonic Fibroblasts

Based on the improvement of the transfection efficacy after lipofection and magnetofection as a result of specific magnetic labeling of the adherent cells, we performed transfections of magnetically labeled suspended cells. The association of the transfection complexes with the cells was carried out in a plate or a cell separation column under a magnetic field as shown schematically in Fig. 1. The percentage of transfected cells (eGFP$^+$ cells) showed a significant improvement of lipofection (approximately 4-fold) and magnetofection (between 2-fold and 4-fold, depending on the dose) of the specifically labeled suspended cells (Fig. 7) compared with the classical transfection of adherent cells (Fig. 4). At a dose of only 2.5 pg pDNA/cell, 70% of the specifically labeled suspended PMEF cells were transfected, and at a doses 5–10 pg pDNA/cell, 75% of cells expressed the transgene. No signs of toxicity were revealed within this dose range by the MTT assay. No additional advantage was observed due to the association of cells with magnetic complexes in the cell separation column compared with the plate ("Magnetofection in the column" curve in Fig. 7). In terms of the transgene expression level, the advantage due to specific cell labeling was revealed only at doses between 1 and 8 pg pDNA/cell. Experiments with radioactively labeled pDNA showed that the specifically labeled suspended cells internalized 2-fold as much of the plasmid with lipoplexes and 3-fold as much as the magnetic complexes compared with non-labeled adherent cells (Fig. 7, bottom).

Transfection of the Magnetically Labeled Suspended Porcine Fetal Fibroblasts

We performed lipo- and magnetofection of the specifically and non-specifically labeled suspended PFF cells in a plate and the cell separation column, as was performed for the PMEF cells. In contrast to the PMEF cells, in PFF cells, the magnetic specific cell labeling inhibited the efficacy of gene delivery (Fig. 8). The association of the non-specifically labeled

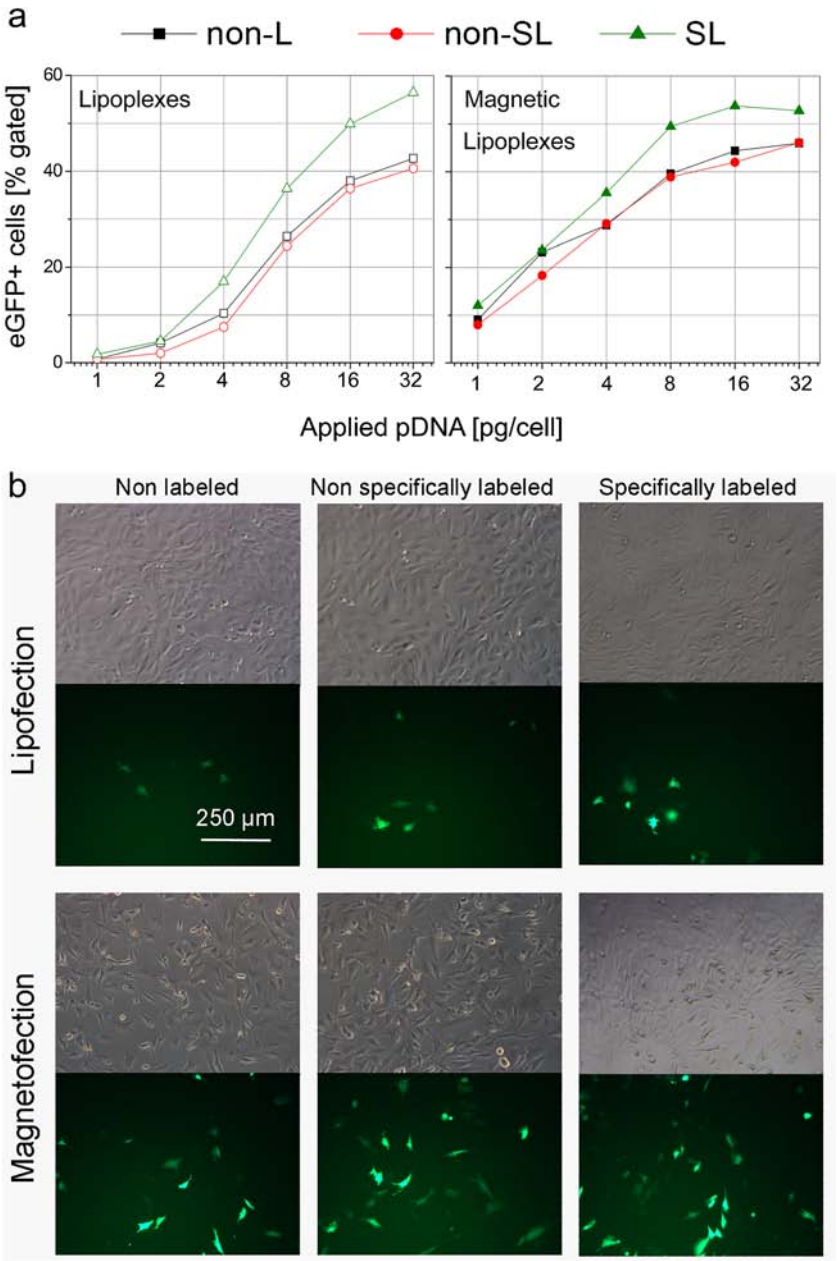

Fig. 6 Specific magnetic labeling of primary mouse embryonic fibroblasts results in increased transfection efficiency with both magnetic and non-magnetic lipoplexes under applied magnetic fields compared with non-labeled and non-specifically labeled cells. PMEF cells that were non-specifically labeled with SO-Mag5 magnetic nanoparticles (*non-SL*) or specifically labeled (*SL*) with feeder removal microbeads were transfected with DF-Gold/pDNA lipoplexes or PEI-Mag2/DF-Gold/pDNA (2:4:1 iron *w/v/w* ratio) magnetic lipoplexes and incubated on the magnetic plate for 20 min. Forty-eight hours after transfection, (**a**) the rate of eGFP positivity was determined by FACS analysis, and (**b**) bright field and fluorescence images (490/509 nm) were taken for eGFP green fluorescence.

cells with magnetic complexes in the cell separation column increased the efficacy to some extent, but there was no advantage over classical magnetofection. This result is in line with the inhibition of internalization that was revealed for both lipoplexes and magnetic complexes after specific or non-specific labeling of PFFs (Fig. 8). In a dose range higher than 8 pg pDNA/cell, toxicity was observed after transfection of the labeled PFF cells (results not shown).

For non-specific cell labeling, we used SO-Mag5 iron oxide nanoparticles. An applied MNP dose of 10 pg iron yielded iron cell loading from 0.5 to 1 pg iron/cell in both PMEF and PFF cells (Table III). The observed magnetic moment was between 50 and 80 picoemu/cell. Specific cell labeling of the cells with commercial magnetic beads yielded a similar iron loading and magnetic moment. Non-specific magnetic labeling with iron loading, which ensured a similar average magnetic moment of the cells compared with specific labeling with feeder removal microbeads (79 versus 65 picoemu/cell), had no effect on the transfection efficacy. These results show that the acquired magnetic moment itself did not induce the transfection enhancement we observed in specifically labeled cells.

Transfection of the Non-magnetically Labeled Primary Mouse Embryonic Fibroblasts

We further investigated whether non-magnetic labeling with antibody (PE-conjugated mouse anti-feeder Ab) to the same specific surface antigene influences transfection efficacy in

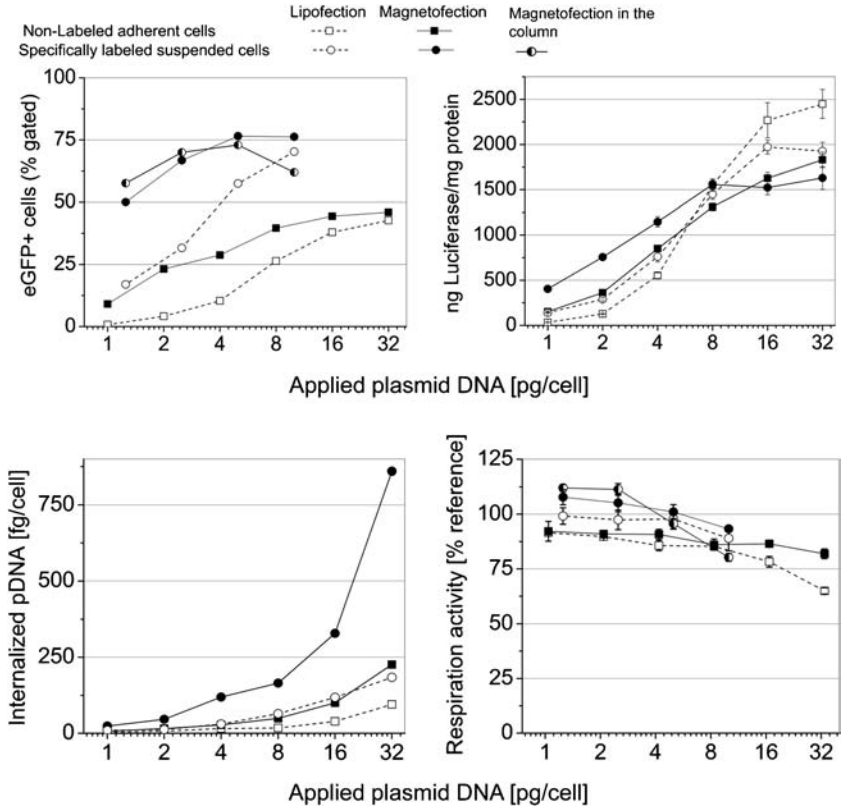

Fig. 7 Specific cell labeling of primary mouse embryonic fibroblasts and association with transfection complexes in suspension results in higher vector internalization, a higher percentage of cells expressing the target protein and higher protein expression (under optimal conditions) compared with the non-labeled adhered cells. The adherent non-labeled cells or suspended cells specifically labeled with feeder removal microbeads (63 femtoA·m^2 per cell) were transfected with DF-Gold/pDNA lipoplexes or magnetic triplexes with PEI-Mag2 magnetic nanoparticles, prepared as described in Fig. 3, under a steady-state magnetic field on the magnetic plate or in the cell separation column. Forty-eight hours after transfection, the cells were analyzed for transfection efficiency using FACS for eGFP-positive cells or chemiluminescence assay for firefly luciferase. To quantify vector internalization, the complexes were formed with ^{125}I-labeled plasmid DNA. Twenty hours after transfection, the cell-associated radioactivity was measured. The respiration activity of the cells according to the MTT assay was recorded 48 h after transfection.

PMEF cells in a manner similar to magnetic labeling. Figure 9 shows the percentage of eGFP-expressing cells 48 h after association of the lipoplexes or magnetic lipoplexes with the cells seeded 24 h before transfection or with the cells trypsinized, labeled, and mixed with the transfection complexes in a suspended state and incubated on the magnetic plate. Figure 9 clearly illustrates that labeling of the receptors with the specific antibody yields some improvement of the transfection efficacy. However, direct specific magnetic labeling of the cell surface receptors results in the highest improvement of the rate of the transfected cells in all setups (lipofection, magnetofection in both adherent or suspended cells) compared to "two-step" magnetic labeling or non-magnetic labeling of the receptors with a specific antibody.

The data from Fig. 9 are plotted in, Fig. S7, in groups according to the type of cell labeling to clearly illustrate the effect of the state of the cells when associated with the transfection complexes (adherent or suspended) for both lipoplexes and magnetic lipoplexes. This figure shows that for the non-labeled cells as well as for the non-magnetically and magnetically specifically labeled cells, transfection of the suspended cells resulted in a higher rate of cells expressing the eGFP transgene, as it is also shown for another setup in Fig. 7.

DISCUSSION

In this study, we aimed to develop magnetic nanoformulations and protocols to transfect primary mouse embryonic fibroblasts and porcine fetal fibroblasts efficiently using magnetofection technology. We used PEI-Mag2 nanoparticles synthesized in our laboratory for assembly into magnetic delivery vectors/complexes with an enhancer and pDNA. These particles associate efficiently with such nucleic acids as pDNA, siRNA, and mRNA in duplexes and triplexes with different enhancers and different viral vectors used for efficient delivery into cells in vitro, ex vivo, and in vivo (16, 22). For non-specific cell labeling, we used SO-Mag5 iron oxide nanoparticles (17) and antibody-modified magnetic microbeads for specific cell labeling. Transfections were performed with non-

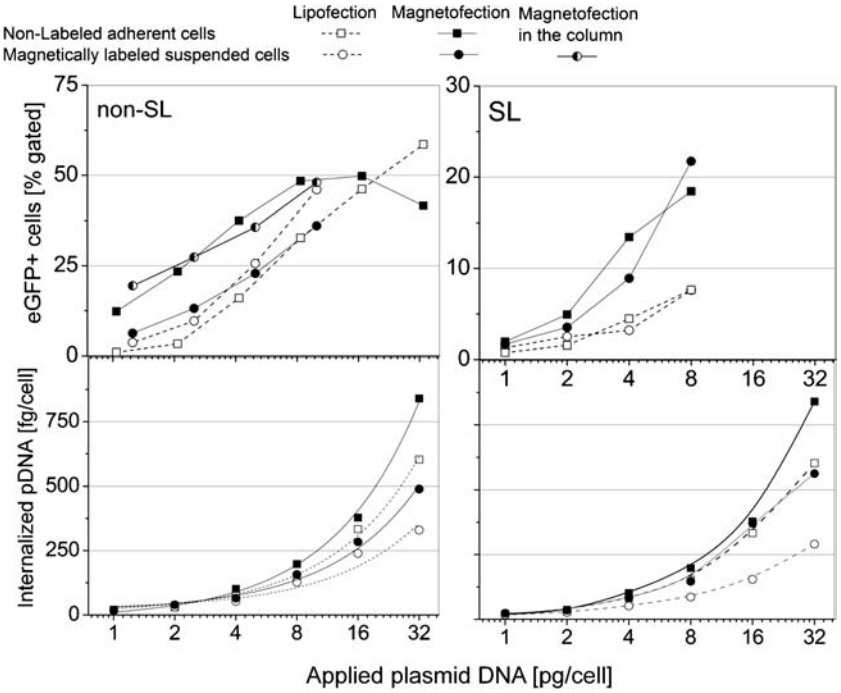

Fig. 8 Magnetic labeling (both specific and non-specific) of primary porcine fetal fibroblasts and association with transfection complexes in suspension results in inhibition of vector internalization and fewer cells expressing the target protein compared with the non-labeled adhered cells. The adherent non-labeled cells, suspended cells non-specifically labeled with SO-Mag5 (*non-SL*) nanoparticles (magnetic moment of 60 femtoA·m^2 per cell) or suspended cells indirectly specifically labeled (*SL*) with mouse anti-human CD90 antibody were further labeled magnetically with anti-PE microbeads (magnetic moment of 200 femtoA·m^2 per cell). Cells were then transfected with DF-Gold/pDNA lipoplexes or magnetic triplexes with PEI-Mag2 magnetic nanoparticles, prepared as described in Fig. 4, under the steady state magnetic field on the magnetic plate or in cell separation column. Forty-eight hours after transfection, the cells were analyzed for transfection efficiency using FACS. To quantify vector internalization, the complexes were formed with ^{125}I-labeled plasmid DNA, and 20 h after transfection, the cell-associated radioactivity was measured.

labeled, non-specifically labeled and specifically labeled adherent and suspended cells, as shown schematically in Fig. 1.

We screened six different enhancers to formulate the magnetic triplexes for magnetofection of the target cells. Our data show that Lipofectamine 2000 and X-tremeGENE 9 had low efficacy in plasmid delivery to both PFF and PMEF cells. This finding is in agreement with the observations of Fouriki et al. (8). To our knowledge, two reports are available on the magnetofection of primary porcine (11) or mouse fibroblasts (12). Both studies used magnetic duplexes with PEI-modified nanoparticles from Chemicell. Cui et al. (11) succeeded in transfecting up to 25% of porcine fibroblasts under optimized conditions. Lee et al. (12) reported a 53% transfection rate of MEFs at an applied dose of 10 pg DNA/cell. Complexes of nTMag particles associated with pDNA under an oscillating magnetic field with a frequency of 2 Hz yielded 25% transgene-expressing cells compared with 22% with Lipofectamine 2000 (8). The best reported results for the transfection of porcine primary fibroblasts have been achieved with optimized nucleofection protocols (6, 23–25). Richter et al. (25) reported a rate of 53% eGFP-positive PFF cells with low cytotoxicity. Nakayama et al. (6) reported optimized nucleofection parameters that yielded survival rates of PEFs derived from male fetuses of Clawn miniature swine of approximately 60%, as assessed by Trypan Blue exclusion 2 h after gene delivery; 79% of surviving cells exhibited transgene expression. Dickens et al. (23) showed that under optimized nucleofection conditions, up to 60% of PFFs were transfected. Transfection by nucleofection had an efficiency of 85% in P16 PFFs in the study of Maurisse et al. (24). The authors reported low (approximately 5%) cytotoxicity.

In the present study, 60% PMEF cells were transfected with the magnetic DF-Gold lipoplexes at a dose of 16 pg pDNA/cell. In PFF cells, we found a 60% transfection rate at a dose of 4 pg pDNA/cell when X-tremeGENE HP was used to formulate magnetic triplexes with PEI-Mag2 MNPs. We conclude that optimized formulations of magnetic pDNA complexes enable the genetic modification of both primary porcine and mouse fibroblasts by magnetofection at low pDNA doses with low toxicity and high efficacy compared with optimized nucleofection protocols.

There are many applications of magnetically labeled and genetically modified cells (26, 27). In our previous studies, we investigated whether preloading cells with magnetic nanoparticles interferes with magnetofection efficiency. We labeled H441, HeLa, and 3T3 cells non-specifically with iron oxide

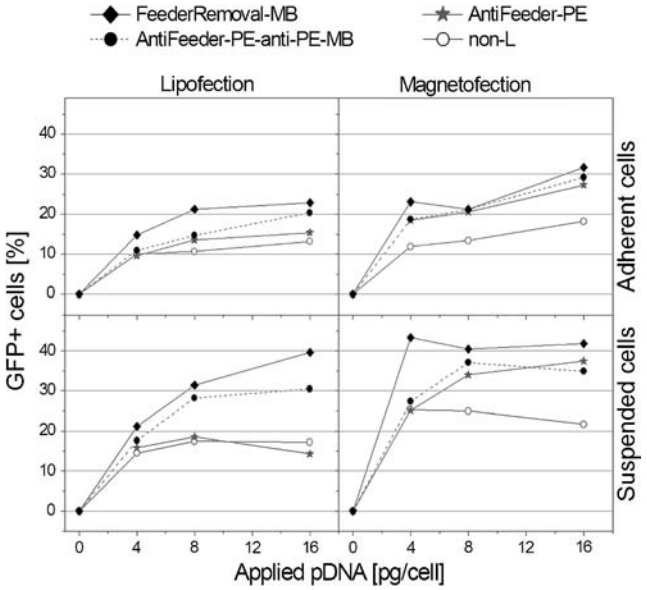

Fig. 9 Direct specific magnetic cell labeling of primary mouse embryonic fibroblasts and association with transfection complexes results in a higher percentage of cells expressing the target protein compared with the non-labeled cells and cells labeled only with the antibody. The adherent or suspended cells specifically labeled with PE mouse anti-feeder antibody (AntiFeeder-PE), specifically labeled with PE mouse anti-feeder antibody followed by magnetic labeling with anti-PE microbeads (AntiFeeder-PE-anti-PE-MB, indirect magnetic labeling), or directly labeled with feeder removal microbeads (FeederRemoval-MB) were transfected in 12-well plates (40,000 cells per well) with DF-Gold/pDNA lipoplexes or magnetic triplexes with PEI-Mag2 magnetic nanoparticles, formulated with DF-Gold as an enhancer. Iron-to-pDNA ratios of 2-to-1 were used under a steady-state magnetic field application on the magnetic plate for 30 min. Forty-eight hours after transfection, the cells were analyzed for transfection efficiency using FACS for eGFP-positive cells.

nanoparticles (7–20 pg iron per cell) and further transfected them with magnetic lipoplexes (13). The acquired magnetic moment of 200–400 picoemu/cell was sufficiently high to manipulate the cells magnetically. The magnetic pre-labeling of cells increased the efficiency of magnetofection without causing cell toxicity. For NIH 3T3 mouse fibroblasts, a 2-fold increase in reporter expression was observed. In another study, non-specific magnetic labeling of human umbilical cord mesenchymal stem cells with magnetic nanoparticles with 50 pg applied iron per cell resulted in a 2-fold increase in transduction efficiency compared with cells labeled with CD105 MicroBeads immediately before magnetofection (14). In this study, magnetic lentiviral complexes were used, and magnetotransduction was performed using a cell separation column as a magnetic device. Apparently, the magnetic moment of the labeled cells in the applied magnetic field increased the local gradient of the field in the vicinity of the cell, and the neighboring magnetic complexes were magnetized and concentrated at the cell surface (the so-called "avalanche effect"). Using this principle, seeds of magnetic nanoparticles have been used to improve magnetic drug targeting by increasing the gradient of the field close to the cell (15, 28). Furthermore, in a recently reported magselectofection procedure (14), specific cell labeling with magnetic nanoparticles followed by association with magnetic complexes directly on the cell separation column under an applied magnetic field resulted in a significant enhancement of the transfection and transduction efficacy of primary mesenchymal and hematopoietic stem cells compared with classical magnetofection on a magnetic plate. Based on these results, we searched for ways to further improve the transfection efficacy of the cells of interest.

Specific labeling of the PMEF cells and their association with magnetic vectors in suspension upon exposure on the magnetic plate further improved the transfection efficacy of both lipoplexes and magnetic lipoplexes. Up to 75% of cells were transfected at 5 pg pDNA/cell. When association with magnetic complexes was performed in the cell separation column to concentrate the "reagents" and enhance the magnetic field gradients, no further improvement in the efficacy was achieved. It is possible that magnetic membrane labeling with nanoparticles through specific interactions combined with a gradient magnetic field (i.e., nanomagnetic actuation) contributed to the observed increase in transfection efficacy through a significantly enhanced internalization of both magnetic and non-magnetic complexes. Indeed, internalization was improved approximately 2-fold for lipoplexes and 3-fold for magnetic complexes in the entire dose range (Fig. 7).

PFFs responded differently. Neither specific nor non-specific magnetic labeling increased the transfection efficacy of the cells, and internalization was inhibited by magnetic labeling (Fig. 8). Additionally, transfection in the cell separation column yielded no improvement and even inhibited gene delivery efficacy and caused some toxicity to these cells. One can speculate that the different response of the porcine fibroblasts to specific magnetic labeling (no improvement in the transfection efficacy) might be due to the labeling of different antigens on the cell membrane of the mouse and porcine fibroblasts and due to the difference in the amount of the antigens on cell surface. The signaling effects as well as a mechanical force applied to the cell membrane via magnetically labeled cell receptors in an applied magnetic fields can be different depending on the type of the cell receptor.

We conclude that specific labeling may be an effective method to control the transfection efficacy, but the observed effects of nanomagnetic activation can be cell type and receptor type dependent.

The activation of endocytosis can occur in response to the activation of certain receptors directly or as a consequence of the signal transduction processes (29, 30). In 2000, Bildirici et al. (31) reported that magnetic beads coated with antibodies and bound to specific cell-surface transmembrane proteins enable the efficient transfection of cells with naked DNA or other macromolecules. The authors called the procedure "immunoporation" (32). In our experiments, we observed a

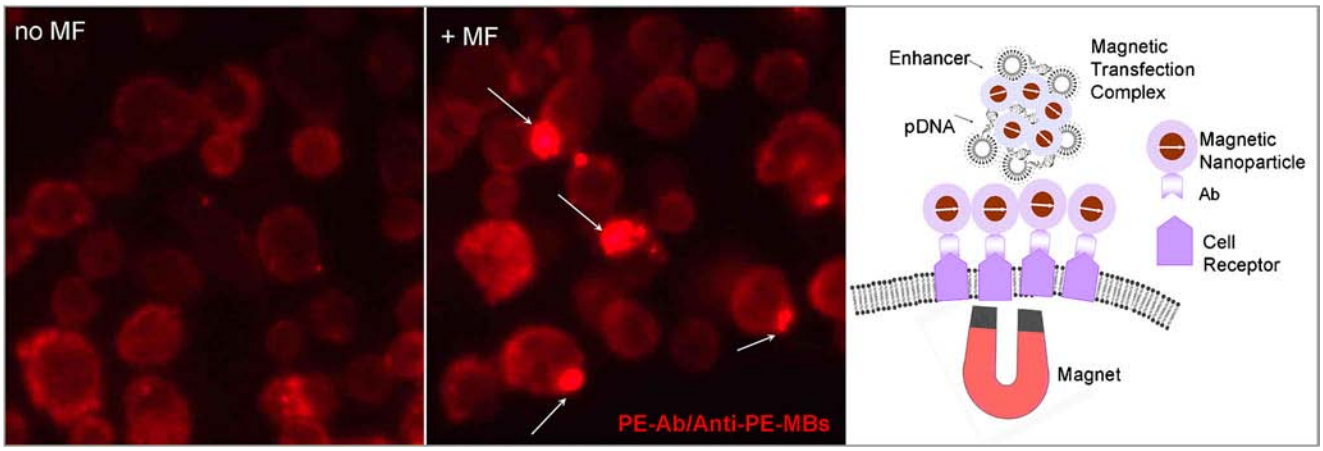

Fig. 10 Aggregation of the magnetically labeled cell receptors in applied magnetic fields. Fluorescence (510/650 nm) microscopy images of the PFF cells specifically labeled with PE-conjugated CD90 antibody, followed by magnetic labeling with anti-phycoerythrin microbeads (PE-Ab/Anti-PE-MB) anti-PE-conjugated MB (*no MF*) and after 20 min incubation of the plate at the Magnetic Plate (*+MF*). Aggregation of the magnetically labeled cell receptors in applied magnetic field is schematically shown in the figure.

3-fold increase in plasmid internalization with magnetic vectors when the suspended PMEF cells were directly labeled with anti-feeder microbeads accompanied by the respective increase in the rate of transgene-expressing cells (Figs. 7 and 9). The internalization of both lipoplexes and magnetic complexes was enhanced under magnetic field application with specifically magnetically labeled cells (Fig. 7). We also observed that specific non-magnetic labeling of the PMEF cells using the same surface receptor (either in the adherent state or in suspension) improves the transfection efficiency of cells by magnetofection (Fig. 9). These effects might be attributed to an activation of endocytosis in response to activation of the specific receptors at PMEF cells, which is apparently more pronounced for magnetically labeled receptors. In this context, it is interesting to note that the efficacy of nanomagnetic activation can be dependent on the availability of suitable receptors and toxicity effects. Thus, the PMEF cells showed no toxicity after nanomagnetic activation.

The manipulation and control of cells and sub-cellular structures through the magnetic nanoparticle-based actuation (nanomagnetic actuation) of cellular processes is a hot research topic (33–35). Recent studies have demonstrated that it is possible to manipulate and control cell function with an external magnetic field and specific magnetic labeling of the cell surface receptors (36, 37). Mannix et al. showed that magnetic beads decorated with a specific receptor ligand can mediate receptor clustering in an applied magnetic field, which further leads to a rapid rise in intracellular calcium (33). Calcium plays an important role in endosomal/lysosomal fusion processes (38). Hoffman et al. recently showed that in the presence of a magnetic field, RanGTP proteins conjugated to superparamagnetic nanoparticles can induce microtubule fibers to assemble into asymmetric arrays of polarized fibers in *Xenopus laevis* egg extracts, and the orientation of the fibers was dictated by the direction of the magnetic force (39). In another recent study, penetrin-modified magnetic nanoparticles were specifically bound to the receptors at the cell membrane, and a magnetic field stimulated clathrin-mediated endocytosis and the subsequent uptake of these particles (40). These results suggest that the magnetic labeling of the cell surface receptors in combination with an applied magnetic field can control different cellular processes and signaling pathways; thus, nanomagnetic activation and actuation can be useful tools for controlling cell behavior. The effects of nanomagnetic actuation may have contributed to the enhanced internalization of the magnetic vectors in magnetically specifically labeled cells observed in our study. Clustering of the magnetically labeled receptors in an applied magnetic field that is evident in the fluorescent image shown in Fig. 10 may be one of the mechanisms involved in the nanomagnetic activation of nucleic acid delivery to cells.

CONCLUSION

Formulated nanomagnetic plasmid DNA assemblies (transfection complexes) in combination with applied inhomogeneous magnetic fields (magnetofection) allowed for improved complex internalization and transfection in both primary mouse embryonic fibroblasts and porcine fetal fibroblasts, yielding up to 60% transfected cells at doses of 4–16 pg pDNA/cell, with high cell viability, compared to the lipofection. No further improvement of the transfection efficacy was achieved upon both "two-step" specific and non-specific magnetic labeling of the PFF cells. In contrast, direct specific labeling of the cell membranes of PMEFs with feeder removal microbeads, which was carried out in seeded adherent cells and

suspended cells, but not non-specific magnetic labeling, further improved the transfection efficacy of both lipoplexes and magnetic lipoplexes. Direct specific magnetic labeling of the cell surface receptors in PMEF cells yielded a greater improvement of the rate of the transfected cells compared to "two-step" magnetic labeling or non-magnetic labeling of the receptors with a specific antibody. The observed effect may be attributed to the nanomagnetic activation of the cell-surface receptors, resulting in an enhanced internalization of the nanoassembled magnetic gene-delivery complexes.

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