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Research article

Back to basics: Optimization of DNA and RNA transfer in muscle cells using recent transfection reagents

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ABSTRACT

C2C12 cells are widely used in the muscle field, as they differentiate easily into myotubes and show limited constraints to culture as compared to primary myoblasts. Both C2C12 and primary myoblasts are hard to transfect, which affects downstream experiments. More than 95% of the reports published since 2015 with C2C12 cells have used one gold standard transfectant (*i.e.,* Lipofectamine®), although several studies have suggested less than 30% efficiency of this reagent. In parallel, the capacity of other commercial reagents to transfect muscle cells remains largely unknown. Here, we compared transfection efficiency of five commercial reagents (Lipofectamine® 3000, Viafect™, Fugene® HD, C2C12 Cell Avalanche®, and JetOPTIMUS®) in C2C12 cells. By optimizing DNA:transfectant ratios and cell density, all reagents reached more than 60% transfection efficiency with limited effects on cell growth and viability. GFP-positive myotubes were efficiently generated in cultures transfected with Lipofectamine® 3000, Fugene® HD, C2C12 Cell Avalanche®, and JetOPTIMUS®. Notably, in conditions optimized for DNA transfer in C2C12 cells, these reagents showed low efficiency to transfer siRNA and higher toxicity for primary muscle cells. In conclusion, we reported yet uncharacterized transfection reagents that can serve as a suitable low-cost alternative to the current gold standard in C2C12 cells.

1. Introduction

Muscle fibers are complex cellular entities corresponding to long multinucleated syncytium formed upon the fusion of mononucleated myoblasts. Myogenesis corresponds to the different steps leading to the formation of mature muscle fibers and includes myoblast proliferation, fusion, and differentiation [1]. Recapitulation of these different steps *in vitro* has been widely used to identify the molecules regulating the process. Primary myoblasts, obtained from rodent or human muscle biopsies, are relevant to study myogenesis *in vitro*. However, their constraining isolation and growth limit their use. In contrast, immortalized myoblasts, such as the C2C12 cell line, are easy to grow and differentiate into contractile myotubes upon switching to a low serum medium [2]. Despite being widely used in laboratories working in the muscle field, the C2C12 line is known to be challenging to transfect [3–8], which constitutes a major drawback for most studies.

Cell transfection is a key procedure routinely used *in vitro*. It allows the introduction of genetic materials, *i.e.,* DNA or RNA, into cells and thereby to modulate the expression and/or the activity of proteins of interest. While stable transfection provides a long-term cellular system, transient transfection overcomes unwanted effects conferred by random genomic integration and is often sufficient to assess the function of a gene/protein in cellular processes. Although physical methods, such as electroporation, are efficient in transfecting different cell types, they require expensive instruments and generally remain laborious procedures. In contrast, biochemical methods are easy-to-use and effective procedures to transiently transfect cells [9]. Most, if not all, chemical transfection reagents are made of cationic components (*e.g.,* cationic polymers and lipids), forming complexes with negatively charged nucleic acids. Unsurprisingly, the composition of the large majority of commercial transfection reagents has been made proprietary. Moreover, they generally do not show any cell type specificity, with little information on potential optimization dependent on the different cell lines (*e.g.,* cell density, DNA: reagent ratio). Hence, despite the large choice of commercial reagents developed in the last decades, transfection efficiency remains low or associated with high cytotoxicity for some cell

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lines.

Only electroporation has been shown to allow DNA transfer in differentiated muscle cells [10,11], while transient transfection of C2C12 or primary myoblasts using chemical reagents is feasible. Surprisingly, since 2015, 98% of the articles reporting transient transfection of C2C12 cells (Pubmed: (C2C12[Title/Abstract] and transfection[anyfield]) or (C2C12[Title/Abstract] and overexpression[anyfield])) have used the reagents Lipofectamine® 2000/3000 (ThermoFisher). This monopoly contrasts with the relatively low efficiency of the reagent (20–40%) reported by some groups [5–7,12] and its cost. The large majority of the publications also failed to mention the conditions in which the reagents were used, and the efficiency of the transfection itself.

Here, we optimized and compared the transfection of C2C12 cells with five commercial transfection reagents. Lipofectamine® 3000 (ThermoFisher), as well as the four other reagents tested, *i.e.,* Viafect™ (Promega), Fugene® HD (Promega), C2C12 Cell Avalanche® (EZ Biosystems), and JetOPTIMUS® (Polyplus) led to transfection rates of 60–90% in optimized conditions. Especially, JetOPTIMUS® and Avalanche® transfectants were efficient at low DNA: reagent ratio, and showed a limited effect on cell viability, proliferation, and differentiation. They may thus constitute an alternative to transfect C2C12 myoblasts at a limited cost. Importantly, these reagents were not efficient in transferring siRNA into C2C12 cells, as compared to reagents designed for siRNA transfer. Moreover, transfection of primary mouse myoblasts in conditions set for C2C12 cells was inefficient or led to high cytotoxicity. This points out that different transfection systems or distinct conditions should be adopted to limit the cost and improve the efficiency of routine RNA/DNA transfer in muscle cells.

2. Materials and methods

2.1. Cell culture

C2C12 cells were obtained from the American Type Culture Collection ATCC (CRL-1772). All transfection assays were done with the same batch of cells, with identical number of passages. Myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco™) and 1% penicillin-streptomycin (Gibco™). They were differentiated into myotubes by switching to differentiation medium containing 2% horse serum (HS, Gibco™). Primary mouse myoblasts were obtained as previously described [13]. They were grown in GlutaMAX DMEM (Gibco™) supplemented with 10% HS, 20% FBS, 1% chicken embryo extract (MP Biomedicals™), 1% penicillin-streptomycin (Gibco™) and 0.5 ng/ml β-fibroblast growth factor on Matrigel (Corning®)-coated cell culture dishes. Cells were kept at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. DNA expression plasmids and siRNA

Two GFP encoding plasmids were used: pCMV-EGFP (Nepa Gene, Japan) and pCLX-UBI-GFP (gift from Dr. P. Salmon) of 4.15 and 8.55 kb respectively. Each plasmid vector was amplified in competent DH5α *E. coli* strain and extracted with the endotoxin-free NucleoBond® Xtra Maxi kit (Macherey Nagel). Isolated plasmids were verified on agarose gel after digestion with specific restriction enzymes. TYE563-fluorescent siRNAs were obtained from OriGene Technologies (Rockville, MD).

2.3. Transfection reagents

Five commercially available biochemical reagents were used for myoblast transfection. Fugene® HD (Promega, Madison, WI) is a proprietary non-liposomal blend of lipids and other components. Lipofectamine® 3000 (Invitrogen, Carlsbad, CA) is based on lipid nanoparticle technology. Viafect™ Transfection Reagent (Promega, Madison, WI) is a cationic delivery reagent with proprietary formulation. C2C12 Cell Avalanche® Transfection Reagent (EZ Biosystems™, Baltimore, MD) is a reagent formulated for C2C12 cell transfection, containing both lipids and polymers. JetOPTIMUS® (Polyplus-transfection® SA, New York, NY) is a cationic nanotechnology with proprietary formulation. Lipofectamine® RNAiMAX (Invitrogen, Carlsbad, CA) and INTERFERin® (Polyplus-transfection® SA, New York, NY) are specifically designed for RNA transfer with proprietary formulation.

2.4. In vitro transfection

C2C12 cells were plated 24hr before transfection in 24-well culture plates at densities of 6.25×10^3, 12.5×10^3 or 25.0×10^3 cells per cm^2. Cells were switched to growth medium with 10% FBS without antibiotics before transfection. DNA/RNA: reagent complexes were prepared according to suppliers' instructions. Transfection reagents were brought to room temperature and briefly vortexed before use. In all conditions, 0.25 μg of plasmids or 2.5 pmol of siRNA were used per cm^2. For Fugene® HD, Viafect™, Avalanche®, Lipofectamine® RNAiMAX and INTERFERin®, DNA/RNA were added to Opti-MEM™ Reduced Serum Medium (Gibco™) and vortexed briefly, before adding the transfection reagent. For JetOPTIMUS®, Opti-MEM™ was replaced by the JetOPTIMUS® buffer provided in the kit. For Lipofectamine® 3000, DNA was first mixed with the reagent P3000 (ratio of DNA: P3000 of 1:2) in Opti-MEM™ Reduced Serum Medium (Gibco™), and then added to Opti-MEM™ Reduced Serum Medium (Gibco™) containing Lipofectamine®3000. DNA/RNA: reagent complexes were vortexed, kept at room temperature for 5–15 min according to supplier's instructions, and added dropwise on the cell monolayer. In some conditions, culture plates were centrifuged at 300 g for 5 min just after transfection. Unless specified, the medium with transfectants was unchanged for Fugene® HD, Viafect™ and Lipofectamine®, and it was replaced by fresh growth medium without antibiotics after 4 and 5hr for JetOPTIMUS® and Avalanche®, respectively. Transfected myoblasts were cultured for 24–50hr.

2.5. Flow cytometry analysis

Cells were trypsinized and analyzed in BD Accuri™ C6 Flow Cytometer (BD Biosciences) or Attune™ NxT Flow Cytometer for GFP or TYE-563 fluorescence. Cells were first gated based on forward and side scatters (FSC/SSC and FSC-A/FSC-H) to remove debris and doublets. Singlets were then analyzed based on GFP or TYE-563 signals, setting the gate on negative control cells, using FlowJo software (v10.8.1 - BD Biosciences). Transfection efficiency was expressed as the proportion of GFP/TYE-563-positive cells over the total number of cells analyzed. For cell death assay, cells were trypsinized 24hr after transfection and stained with 1 μg/ml propidium iodide (PI). Cells were gated for PI fluorescence based on background signals measured in negative control cells.

2.6. Microscopy analysis

Cells were fixed 24hr or 48hr after transfection for myoblasts and 5 days after differentiation for myotubes, with PBS, 2% paraformaldehyde, 2% sucrose. They were then washed with PBS, 0.1 M glycine, mounted in Vectashield DAPI (Vector) and observed with a Zeiss AxioCam or Zeiss Axio Observer Z1 fluorescent microscopes. Images were captured using Zeiss software. Fusion index and myosin area were quantified with Fiji Software using a macro adapted from ViaFuse [14]. For Incucyte®S3 live cell analysis, cells were seeded in 24-well plate, real-time monitored by Incucyte® system (Sartorius AG). Confluency and area of GFP-positive cells were analyzed with Incucyte® software.

2.7. Statistical analysis

Results are expressed as mean ± SEM of independent samples, with n (number of technical replicates) ≥ 3. Statistical comparison was performed using two-tailed Student's t-test or one/two-way ANOVA test dependent on the conditions, with a 0.05 level of confidence accepted for statistical significance.

3. Results

3.1. Transfection efficiency of C2C12 myoblasts with small GFP-encoding plasmid

To optimize C2C12 transfection, we first compared by flow cytometry the efficiency of five reagents to transfer a 4.15 kb EGFP-encoding plasmid (pCMV-EGFP) in C2C12 myoblasts (Fig. 1A). All transfection assays were conducted with the same batch of C2C12 cells used at the same passage. We started with the maximum volume of reagent recommended by the supplier before adapting the amount of reagent to limit cytotoxicity or increase efficiency. Empirically, we observed that transfection with Lipofectamine® 3000, Viafect™ and Fugene® HD is improved at low cell confluency, while Avalanche® and JetOPTIMUS® require higher cell density to limit cytotoxicity. We therefore compared transfection using cells at 6′250, 12′500 or 25′000 cells/cm^2 (around 40%, 60% or 80% confluency) depending on the reagent.

With Lipofectamine® 3000, 75.2% of cells were detected as GFP-positive by FACS, using a DNA: reagent ratio of 1:3 at low confluency (Fig. 1B). The proportion of positive cells remained at 68.0% with a ratio of 1:1.5 (Fig. 1B), but dropped to 24.3 ± 4.4% with a ratio of 1:1 (*data not shown*). The efficiency was significantly reduced by increasing the number of cells (Fig. 1B). Centrifugation of the plate after the transfection, as suggested by some suppliers, did not improve transfection efficiency (Fig. 1B).

Transfection with Viafect™ at a DNA: reagent ratio of 1:3 gave 52.1% GFP-positive cells, at low confluency, with no major effect of centrifugation (Fig. 1C). Using a higher ratio (1:4) increased transfection efficiency up to 62.8% positive cells when combined with low confluency and centrifugation (Fig. 1C).

For Fugene® HD, the maximum ratio recommended by the supplier (1:4) led to 37.3% GFP-positive cells at low confluency (Fig. 1D). In this condition, centrifugation reduced further transfection efficiency. Interestingly, increasing the ratio to 1:6 strongly improved transfection efficiency, leading to 68.1% GFP-positive cells at low cell density (Fig. 1D).

With C2C12 Cell Avalanche®, 1:1.5 and 1:2 ratios of DNA: transfectant yielded 66.0% and 72.9% GFP-positive cells at around 60% confluency, respectively (Fig. 1E). Efficiency dropped to around 40% positive cells using higher confluency, but tended to increase with centrifugation in this condition (Fig. 1E). Increasing the volume of transfectant led to higher cytotoxicity (*data not shown*).

Lastly, JetOPTIMUS® gave high proportion of GFP-positive cells with low volume of reagent (ratios 1:1 or 1:1.5), reaching up to 90.0% of transfected cells at 60% confluency (Fig. 1F). This proportion dropped when reducing the ratio to 1:0.5 (Fig. 1F). Transfection efficiency remained unchanged with centrifugation, while it tended to decrease at higher cell confluency (Fig. 1F).

Overall, these results show that the five tested transfectants yield high transfection efficiency in optimized conditions, with a maximum

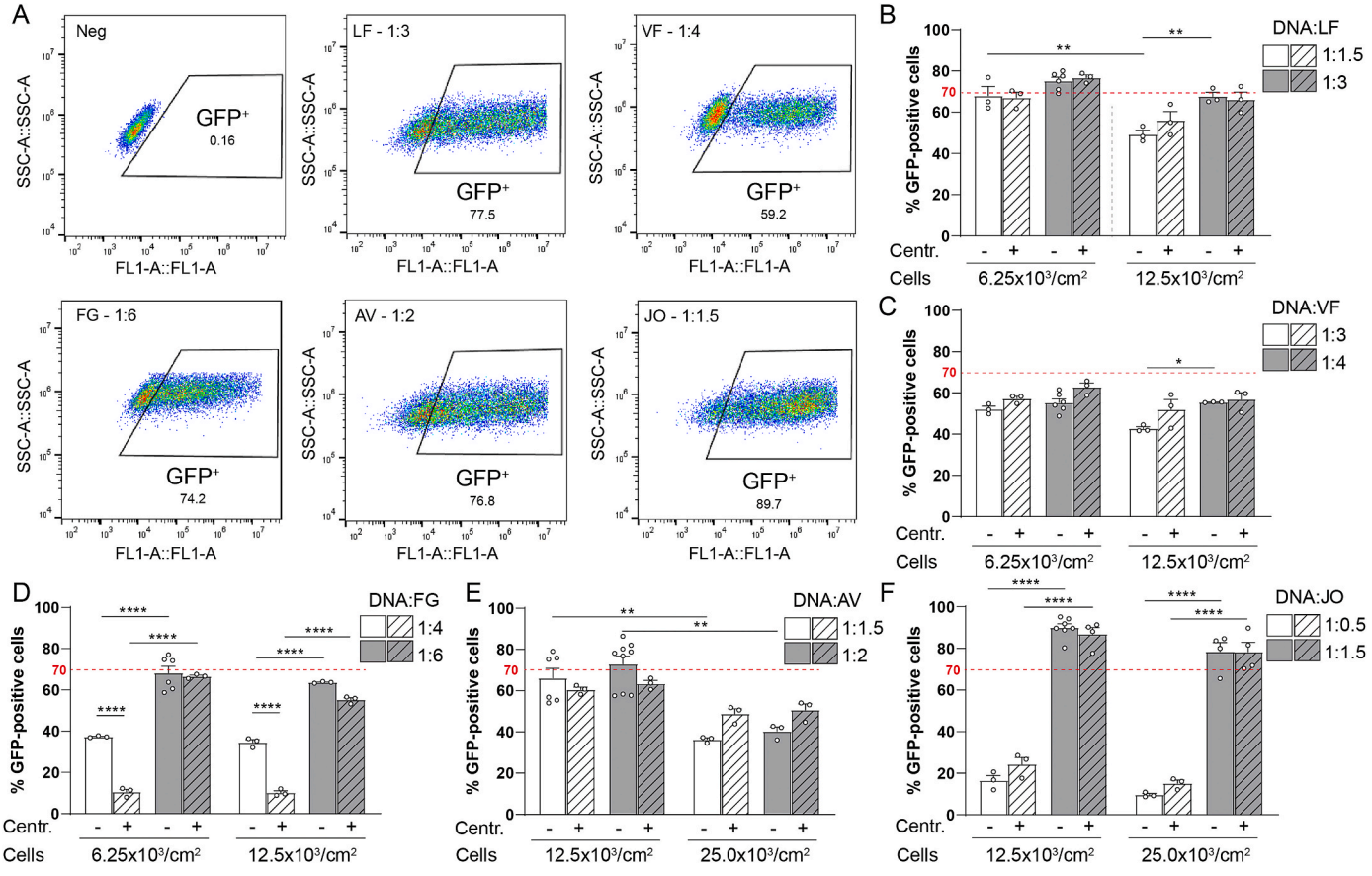

Fig. 1. Optimization of C2C12 cell transfection with pCMV-EGFP plasmid using five commercial reagents. FACS analysis (A) of C2C12 myoblasts transfected with pCMV-EGFP plasmid, using Lipofectamine® 3000 (LF, B), Viafect™ (VF, C), Fugene® HD (FG, D), C2C12 Cell Avalanche® (AV, E) or JetOPTIMUS® (JO, F), with (+) and without (−) centrifugation. Quantification is given for two DNA: reagent ratios and two different cell densities (6′250, 12′500 or 25′000 cells per cm^2 seeded 24hr before transfection). Values are mean ± s.e.m.; n = 3; two-way ANOVA with Tukey's post-hoc test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

reached by JetOPTIMUS®.

3.2. Transfection efficiency of C2C12 myoblasts with a large GFP-encoding plasmid

To further test the transfection capacity of the five reagents, we assessed transfection efficiency using a larger lentiviral plasmid of 8.55 kb encoding GFP (pCLX-UBI-GFP). In this condition, Lipofectamine® 3000 reached 74.0% of GFP-positive cells with a DNA: reagent ratio of 1:3 (Fig. 2A). The efficiency decreased to 62.4% with a ratio of 1:1.5 at low confluency and to 59.0% with a ratio of 1:3 at mid confluency (Fig. 2A).

Viafect™ led to more than 64% GFP-positive cells using a ratio of 1:4 at low confluency, with or without centrifugation (Fig. 2B). Lower ratio or an increased number of cells, as with pCMV-EGFP, tended to reduce transfection efficiency.

Similarly, Fugene® HD gave 64.2% of transfected cells with a high ratio (1:6) at low confluency (Fig. 2C). A reduced ratio (1:4), an increased number of cells, as well as centrifugation, decreased transfection efficiency with Fugene® HD (Fig. 2C).

Avalanche® yielded up to 59.0% of transfected cells using a ratio of 1:1.5 at 60% confluency with centrifugation. As observed with the smaller plasmid, increasing further cell confluency reduced transfection efficiency, which was improved by centrifugation (Fig. 2D).

Lastly, transfection rate of more than 80% of cells was obtained with JetOPTIMUS® at a ratio of 1:1.5 (Fig. 2E and F). The proportion of GFP-positive cells decreased at higher confluency, although it remained higher than 50% (Fig. 2E).

These results indicate that the five tested reagents are efficient in transfecting a large plasmid, with around 60–80% of transfected cells in optimized conditions.

3.3. Impact of transfection reagents on C2C12 myoblast proliferation and viability

Next, we selected the best condition for each reagent (*i.e.* at 6.25 K cells/cm^2: Lipofectamine® 3000, ratio 1:3; Fugene® HD, ratio 1:6; Viafect™, ratio 1:4; and at 12.5 K cells/cm^2: JetOPTIMUS®, ratio 1:1.5; Avalanche®, ratio 1:1.5) and examined the effect on cell dynamics. Using fluorescence microscopy, we confirmed that the transfection rate was high with the five reagents in selected conditions (Fig. 3A). We then evaluated cell death by adding propidium iodide (PI) 24hr after transfection. The proportion of PI-positive cells in transfected cultures was compared to untransfected cultures seeded at the same confluency. There was only a mild increase in cell lethality in transfected cultures as compared to untransfected cells (Fig. 3B). The highest proportion of PI-positive cells was detected with Fugene® HD, which may be related to the large volume of transfectant (ratio 1:6) used in this condition (Fig. 3B). To integrate changes in cell viability and cell proliferation upon transfection, we then followed cell growth over 30hr post-transfection by live microscopy using IncuCyte® S3. Interestingly, both Viafect™ and Fugene® HD increased the number of cells over time, as compared to untransfected culture (Fig. 3C). This increased cell growth may be predominantly linked to a permissive effect of the two reagents on cell proliferation. Inversely, Lipofectamine®3000, and to a lesser extent JetOPTIMUS® and Avalanche®, reduced cell growth (Fig. 3C and D). As cell death was limited with these transfectants, reduction in cell growth may be caused mostly by a repressive effect of the transfectants on cell proliferation.

To evaluate the expression kinetics of the plasmid with the different transfectants, we next followed GFP-positive area, measured with Incucyte® S3, over 50hr following transfection. We normalized GFP-positive areas to total areas of cells, and to the maximal GFP area detected at 50hr (Fig. 3E). JetOPTIMUS®, Avalanche® and Lipofectamine® 3000 led to maximal relative GFP areas, which was consistent with FACS results. Interestingly, GFP accumulated quicker with JetOPTIMUS® and Avalanche® than with Lipofectamine® 3000 (Fig. 3E). Hence, while a 48–50hr period ensures optimal plasmid expression, Avalanche® and JetOPTIMUS® should allow relevant cell analyses 24hr after transfection.

Lastly, focusing on the three best transfectants, we assessed the consequences of shorter incubation times on transfection efficiency. To this end, we treated cells with JetOPTIMUS®, Avalanche® or Lipofectamine® 3000 for 1–4hr, 1–5hr and 1–48hr, respectively, and analyzed GFP-positive cells after 48hr (Fig. 3F–H). Transfection was improved when treating cells with Lipofectamine® 3000 for more than 24hr, as

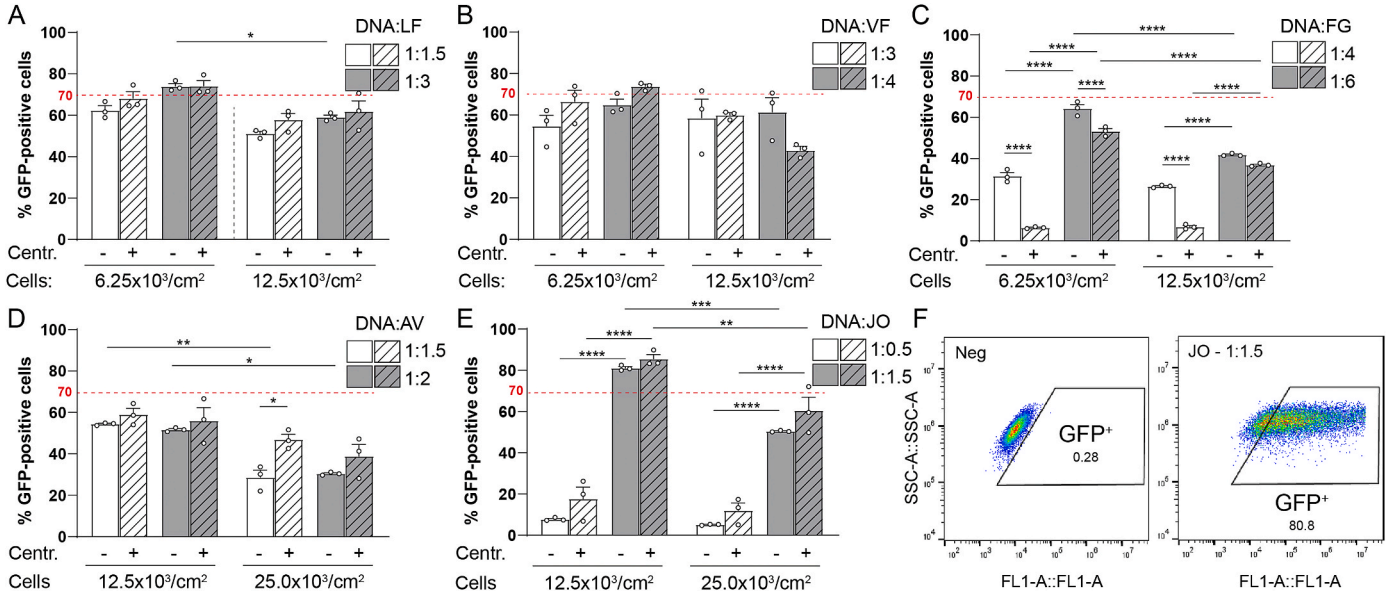

Fig. 2. Optimization of C2C12 cell transfection with pCLX-UBI-GFP plasmid using five commercial reagents. FACS analysis of C2C12 myoblasts transfected with pCLX-UBI-GFP plasmid, using Lipofectamine® 3000 (LF, A), Viafect™ (VF, B), Fugene® HD (FG, C), C2C12 Cell Avalanche® (AV, D) or JetOPTIMUS® (JO, E, F), with (+) and without (−) centrifugation. Quantification is given for two DNA: reagent ratios and two different cell densities (6′250, 12′500, 25′000 cells per cm^2 seeded 24hr before transfection). Values are mean ± s.e.m.; n = 3; two-way ANOVA with Tukey's post-hoc test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

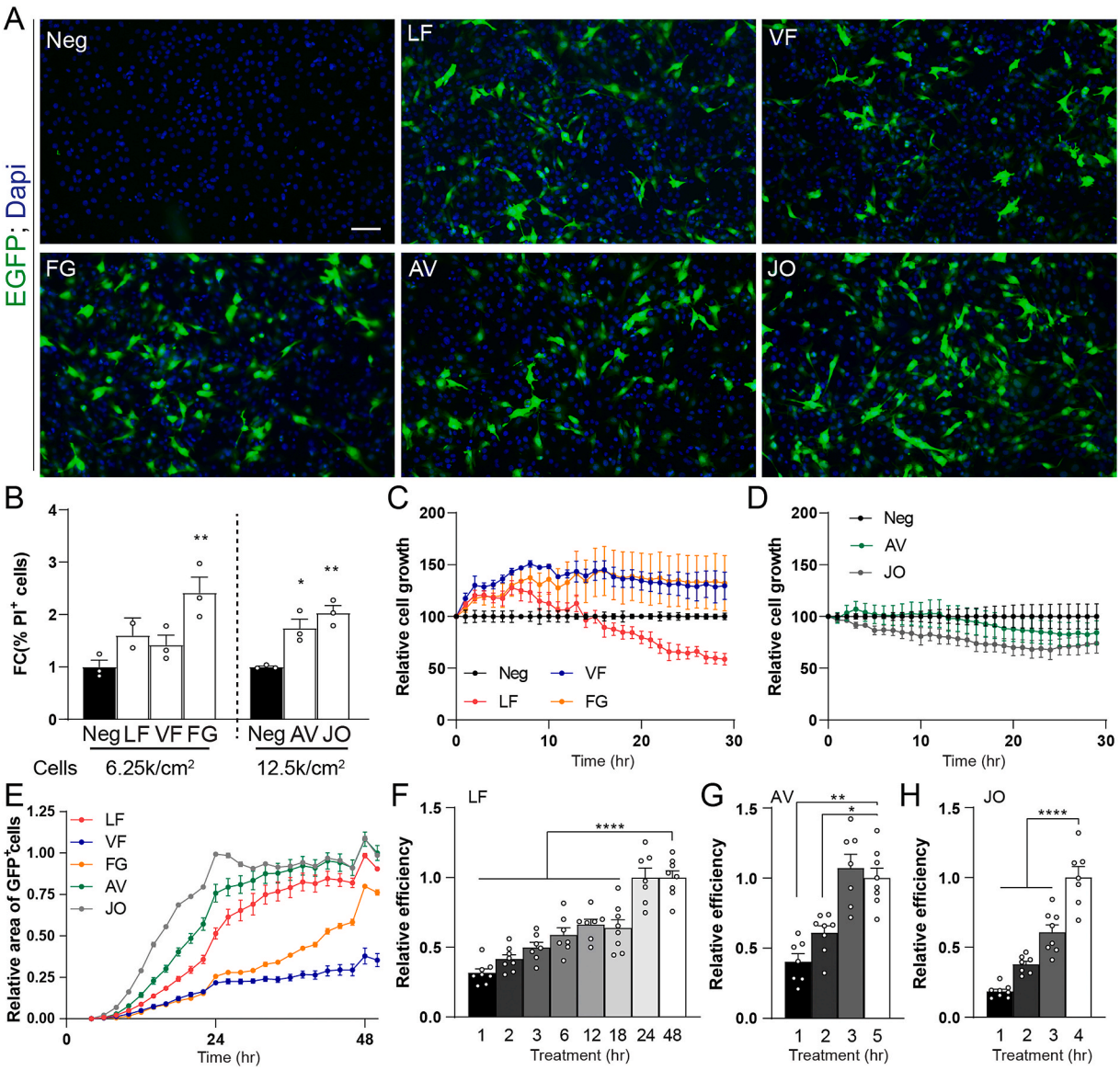

Fig. 3. C2C12 myoblast viability and growth upon transfection. A) Representative staining of GFP-positive myoblasts 48hr after transfection with Lipofectamine® 3000 (LF, ratio 1:3), Viafect™ (VF, ratio 1:4), Fugene® HD (FG, ratio 1:6), Avalanche® (AV, 1:1.5) and JetOPTIMUS® (JO, 1:1.5). Scale bar, 100 μm. B) FACS analysis of C2C12 myoblasts stained with propidium iodide (PI), 24hr after transfection with LF, VF, FG, JO and AV. Values are mean ± s.e.m.; n = 3; one-way ANOVA with Dunnett's post-hoc test, *p < 0.05, ****p < 0.0001 compared to untransfected cells at the same confluency (FC, *Fold Change*). C, D) Relative cell growth upon transfection with LF, VF and FG (C), JO and AV (D): changes in cell number, relative to initial confluency, were normalized to cell growth in untransfected culture seeded at the same cell density 24hr before transfection (*i.e.* 6′250 cells/cm^2 in C and 12′500 cells/cm^2 in D). E) Changes in GFP-positive areas, 4–50hr after transfection, normalized to total cell areas and to the maximal GFP-positive area detected at 50hr (with JetOptimus). Values are mean ± s.e.m.; n = 3. F–H) Transfection efficiency was evaluated for cells incubated for 1–48hr with LF (F), 1–5hr with AV (G) and 1–4hr with JO (H). The proportion of GFP-positive myoblasts was normalized to the proportion obtained with the longer incubation time (*i.e.,* 48hr for LF, 5hr for AV, 4hr for JO). Values are mean ± s.e.m.; n ≥ 3; one-way ANOVA with Dunnett's post-hoc test, *p < 0.05, **p < 0.01, ****p < 0.0001 compared to the longer incubation time.

compared with shorter treatments (Fig. 3F). While transfection efficiency remained unchanged when treating cells with Avalanche® for 3hr or 5hr (Fig. 3G), reducing the incubation time from 4hr to 3hr with JetOPTIMUS® decreased transfection efficiency (Fig. 3H). Hence, the time to complete DNA transfer depends on the transfectant and imposes distinct minimal incubation times with the reagents. Overall, these results indicate that the five reagents efficiently transfect C2C12 myoblasts, although with distinct impacts on cell viability, proliferation and growth and with different kinetics of DNA transfer and plasmid expression.

3.4. Impact of transfection reagents on C2C12 muscle cell differentiation

To assess whether transfection affects the myogenic function of C2C12 cells, we evaluated the capacity of transfected myoblasts to generate transfected myotubes. In the best condition selected for each reagent, we submitted transfected C2C12 cells to differentiation 24hr post-transfection. After 5 days, we examined the overall effect of transfection on myoblast differentiation and fusion, and the formation of GFP-positive myotubes (Fig. 4A). The proportion of myonuclei in cells with more than two myonuclei (fusion index) was strongly reduced in cultures transfected at low confluency (6'250 cells/cm^2) with Lipofectamine®3000, as compared to untransfected cultures (Fig. 4B). Milder reduction in the fusion index was observed with the other transfectants

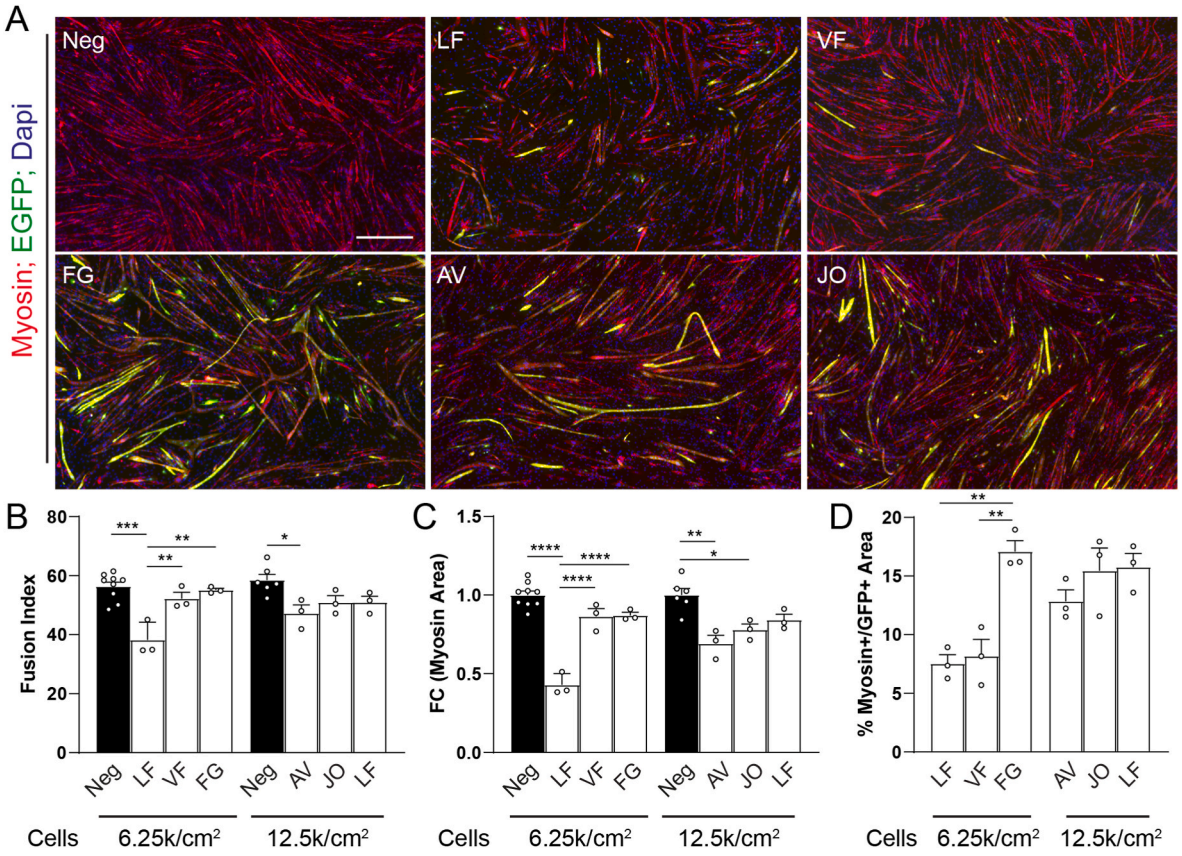

Fig. 4. Differentiation capacity of transfected C2C12 myoblasts. A) Representative staining of myotubes stained with myosin in untransfected culture (Neg) or cultures transfected with pCMV-EGFP with Lipofectamine® 3000 (LF, ratio 1:3), Viafect™ (VF, ratio 1:4), Fugene® HD (FG, ratio 1:6), Avalanche® (AV, 1:1.5) and JetOPTIMUS® (JO, 1:1.5). Scale bar, 500 μm. B) Fusion index quantified 5 days after differentiation induction in untransfected (Neg) and transfected (LF, VF, FG, JO and AV) cultures. Cell density 24hr prior transfection is indicated. Values are mean ± s.e.m.; n ≥ 3; one-way ANOVA with Dunnett's post-hoc test, *p < 0.05, **p < 0.01, ***p < 0.001 compared to untransfected cells at the same confluency. C) Myosin area measured in transfected cultures (LF, VF, FG, JO and AV) and normalized to myosin area quantified in untransfected cultures seeded at the same cell density. Values are mean ± s.e.m.; n ≥ 3; one-way ANOVA with Dunnett's post-hoc test, *p < 0.05, **p < 0.01, ****p < 0.0001 compared to untransfected cells. D) Proportion of the field area occupied by GFP-positive myotubes (myosin-positive) in transfected cultures 5 days after differentiation induction. Values are mean ± s.e.m.; n = 3; one-way ANOVA with Tukey's post-hoc test, **p < 0.01.

(Fig. 4B). Consistently, the area corresponding to myosin-positive cells (*i.e.,* differentiated cells) was reduced by half with Lipofectamine®3000 (confluency at 6'250 cells/cm^2), while it represented more than 69% of the untransfected cultures with other transfectants (Fig. 4C). Of note, at 5 days of differentiation, the density of nuclei in Lipofectamine®3000-treated cultures seeded at low confluency was half the nuclear density in untransfected cultures (0.47 ± 0.02 fold change from control). All other transfected cultures had higher nuclear densities (Viafect™, 1.09 ± 0.31; Fugene® HD, 0.79 ± 0.03; Avalanche®, 0.69 ± 0.04; JetOPTIMUS®, 0.76 ± 0.02). The repressive effect of Lipofectamine®3000 on cell growth may hence contribute to the reduced differentiation capacity of transfected cultures. To overcome the negative effect of Lipofectamine®3000 on cell proliferation, we evaluated differentiation when transfecting cells seeded at higher confluency (*i.e.,* 12'500 cells/cm^2), for which Lipofectamine®3000 still showed good transfection efficiency. In this condition, Lipofectamine®3000 had minor effect on fusion index (Fig. 4B) and myosin-positive area (Fig. 4C).

Importantly, the formation of GFP-positive myotubes generated upon transfection and differentiation was limited in Viafect™-transfected cultures, as well as in cultures transfected with Lipofectamine®3000 at low confluency (Fig. 4D). The area of GFP-positive myotubes was the highest with Fugene® HD, followed by JetOPTIMUS®, Lipofectamine®3000 used at higher confluency, and Avalanche® (Fig. 4D). Of note, transfecting cells at higher confluency (*i.e.,* 12'500 cells/cm^2) with Viafect™ and Fugene® HD did not change differentiation capacity (*data not shown*) but strongly reduced the formation of GFP-positive myotubes (relative area (%): Viafect™, 2.78 ± 0.58; Fugene® HD, 7.04 ± 1.11). Inversely, reducing cell confluency (*i.e.,* 6'250 cells/cm^2) with Avalanche® and JetOPTIMUS® decreased differentiation capacity (*data not shown*) and the area of GFP-positive myotubes (relative area (%): Avalanche®, 3.93 ± 1.58; JetOPTIMUS®, 5.09 ± 1.83). Hence, the use of Fugene® HD, JetOPTIMUS® or Avalanche® with the selected transfection conditions, as well as of Lipofectamine®3000 at higher cell confluency as compared to optimal transfection conditions, is recommended for follow-up studies upon differentiation.

3.5. *Conditions for DNA transfection are not optimal for siRNA transfer in C2C12 cells*

To determine if conditions optimized for DNA transfer can be extended to RNA, we evaluated the capacity of each reagent to transfect siRNA in C2C12 myoblasts. We used the conditions delineated for DNA transfer for Viafect™ (volume 1 μl/cm^2), Fugene® HD (volume 1.5 μl/cm^2), Lipofectamine®3000, Avalanche® and JetOPTIMUS® (volume 0.375 μl/cm^2). Cells were transfected with 2.5 pmol/cm^2 of TYE-563-fluorescent siRNA and analyzed after 24hr by fluorescent microscopy (Fig. 5A). We compared the efficiency of these reagents to the efficiency of Lipofectamine®RNAiMAX (volume 0.375 μl/cm^2) and INTERFERin® (volume 0.5 μl/cm^2), which are specifically designed for siRNA transfer (Fig. 5B). Cytoplasmic fluorescent puncta corresponding to siRNA accumulation were detected in 79.2% and 98.3% of cells transfected

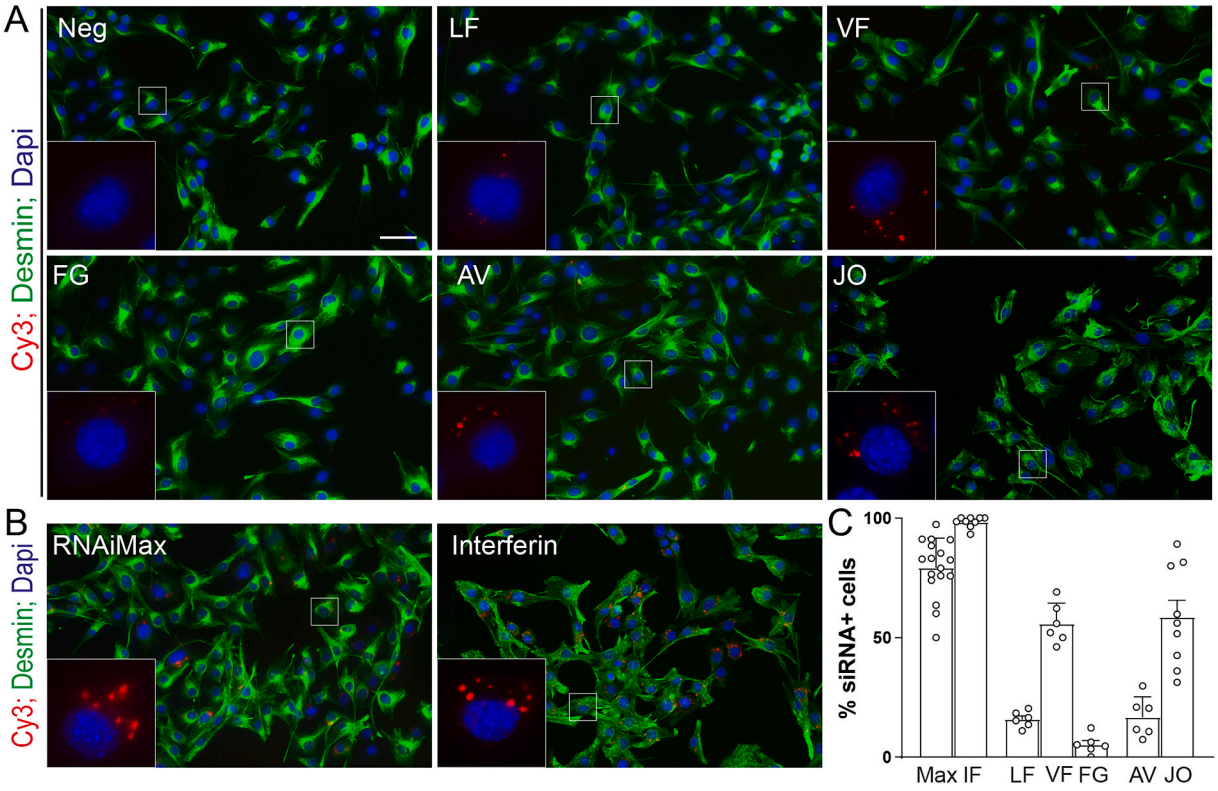

Fig. 5. siRNA transfer in C2C12 cells. A, B) Representative picture of C2C12 myoblasts transfected with TYE-63-siRNA with Lipofectamine® 3000 (LF – 0.375 μl/cm^2), Viafect™ (VF - 1 μl/cm^2), Fugene® HD (FG – 1.5 μl/cm^2), Avalanche® (AV – 0.375 μl/cm^2), JetOPTIMUS® (JO – 0.375 μl/cm^2), and Lipofectamine® RNAiMAX (0.375 μl/cm^2), INTERFERin® (0.5 μl/cm^2). Scale bar, 50 μm. C) Proportion of cells positive for TYE-63-siRNA in transfected cultures 24hr after transfection. Values are mean ± s.e.m.; n ≥ 6 fields.

with Lipofectamine®RNAiMAX and INTERFERin®, respectively (Fig. 5C). In parallel, the proportion of transfected cells with Viafect™ and JetOPTIMUS® yielded more than 50% cells (Fig. 5C). In contrast, very few siRNA-positive cells were observed using Fugene® HD, Lipofectamine®3000, or Avalanche® (Fig. 5C). Hence, transfer of siRNA in conditions optimized for DNA transfer is feasible with Viafect™ and JetOPTIMUS®, although with a limited efficiency as compared to dedicated RNA transfection reagents.

3.6. Transfection conditions set for C2C12 myoblasts are not optimal for primary myoblasts

Primary myoblasts are often considered as a more relevant system, as compared to C2C12 cells, to study myogenesis *in vitro*. Thus, we wondered whether transfection conditions optimized for C2C12 cells are also effective for primary muscle cells. We transfected mouse primary myoblasts with the pCMV-EGFP plasmid and assessed transfection efficiency by FACS after 48hr. While Viafect™ (ratio 1:4) and Fugene® HD (ratio 1:6) led to 10.5% and 0.8% transfected cells, respectively, more than 50% GFP-positive cells were detected with Lipofectamine® 3000 (ratio 1:1.5), Avalanche® (ratio 1:2) and JetOPTIMUS® (ratio 1:1.5) (Fig. 6A and B). However, in these conditions, there was a dramatic decrease in the number of cells per volume unit in transfected cultures, as compared to untransfected cells (Fig. 6C). This suggested high toxicity of Lipofectamine® 3000, Avalanche® and JetOPTIMUS® in the conditions set for C2C12 cells. Reducing the amount of reagents, *e.g.* with Lipofectamine® 3000 at ratio 1:1 (Fig. 6B, C), may be sufficient to reduce cell death while preserving transfection efficiency. Hence, Lipofectamine® 3000, Avalanche® and JetOPTIMUS® may be potent reagents to transfect primary myoblasts, although further optimization of the conditions is required based on the high sensitivity of primary muscle cells.

4. Discussion

C2C12 cells and primary mouse myoblasts are widely used cell systems to study myogenesis *in vitro*. Both cell types are known to be difficult to transfect. Transfection with Lipofectamine® has been reported in the very large majority of studies conducted in C2C12 cells. However, its efficiency was suggested to be low in this cell line [5–7,12] and its cost remains high for routine procedures. Here, we delineated other commercially available transfectants, such as JetOPTIMUS® and Avalanche® that constitute efficient alternatives to transfect C2C12 cells at a limited cost. However, conditions optimized for C2C12 cells cannot be directly applied for RNA transfer or transfection of primary muscle cells.

Among the five reagents tested in the study, only C2C12 Cell Avalanche® reagent was designed specifically for C2C12 cells. In our study, we initially used the highest concentration of reagent suggested by suppliers, and then reduced or further increased it to limit toxicity or increase efficiency. We first observed that Lipofectamine® 3000, Viafect™ and Fugene® HD show higher efficiency when reducing cell confluency, while higher cell density was required to limit cytotoxicity conferred by Avalanche® and JetOPTIMUS®. Previous studies reported transfection efficiency lower than 40% in C2C12 cells with Lipofectamine® reagents, even at high ratio of DNA: reagent [7]. Higher transfection efficiency of Fugene® HD, as compared to Lipofectamine®2000, has also been reported [12]. Here, we showed that Lipofectamine®3000 gives more than 70% transfected cells with small and large plasmids, using a DNA: reagent ratio of 1:3, which corresponds to the highest volume recommended by the supplier. Although decreasing the ratio to 1:1.5 reduced transfection efficiency, this ratio may still be used (efficiency higher than 60%) to reduce the cost of the procedure. For Viafect™ and Fugene® HD, high ratios of 1:4 and 1:6, respectively, had to be used to get more than 60% transfected cells. The main issue

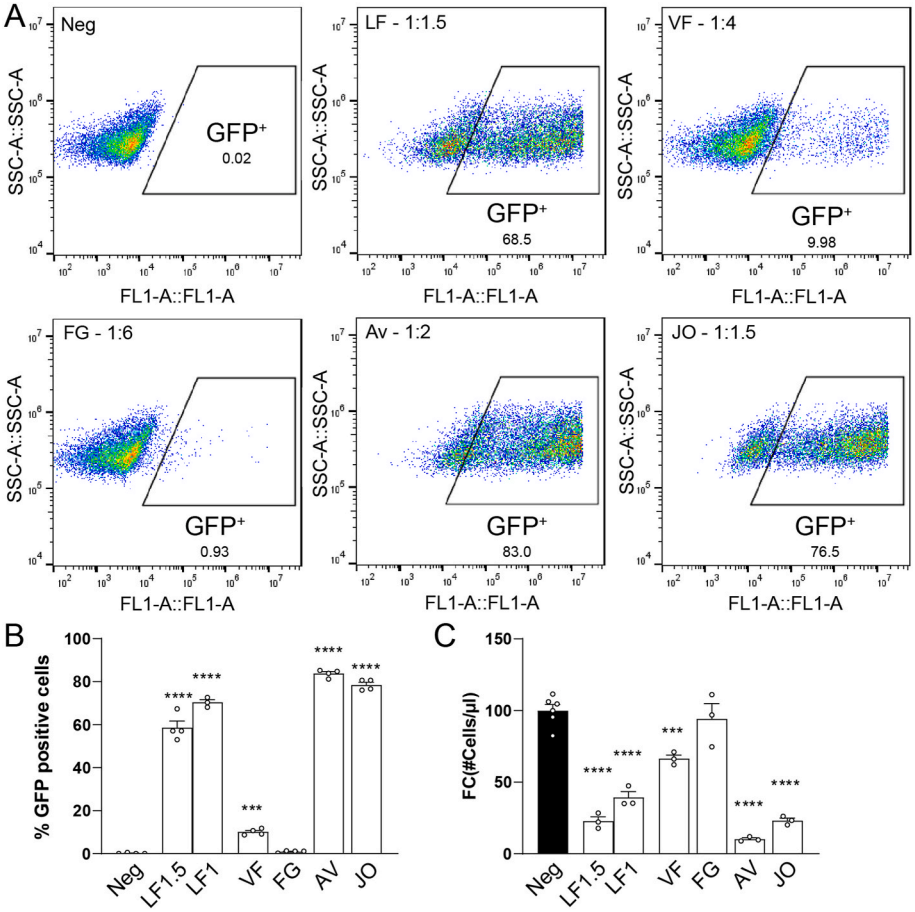

Fig. 6. Efficiency and cytotoxicity of transfection reagents in primary mouse myoblasts. A, B) FACS analysis of primary mouse myoblasts transfected with pCMV-EGFP plasmid, using Lipofectamine® 3000 (LF, 1:1.5), Viafect™ (VF, 1:4), Fugene® HD (FG, 1:6), C2C12 Cell Avalanche® (Av, 1:2) or Jet-OPTIMUS® (JO, 1:1.5) 48hr after transfection without centrifugation. Quantification is given in (B). C) Changes in cell number upon transfection with LF, VF, FG, Av and JO, as compared to untransfected cells (Neg). Values are mean ± s.e.m.; n = 3; one-way ANOVA with Dunnett's post-hoc test, ***p < 0.001, ****p < 0.0001.

conferred by such DNA: reagent ratios is the associated cost of the procedure, which may be incompatible with routine experiments. In contrast, for Avalanche® and JetOPTIMUS®, ratios were reduced to 1:1.5 yielding more than 60% transfected cells. Overall, JetOPTIMUS® led to the highest percentage of transfected myoblasts, regardless of plasmid size, with the smallest amount of reagent used.

Interestingly, based on propidium iodide assay, cell viability remained largely unaffected in all conditions, with higher cell lethality detected for Fugene® HD at a 1:6 ratio. Despite this cytotoxic effect, Fugene® HD tended to increase cell growth suggesting a permissive effect on cell proliferation. Moreover, the effect on cell viability and proliferation did not impinge cell differentiation, as Fugene® HD led to the largest area of GFP-positive myotubes. Viafect™ also tended to increase cell growth and did not interfere with efficient differentiation of myoblast cultures. However, it did not lead to efficient generation of GFP-positive myotubes, likely because of the limited efficiency of myoblast transfection. In parallel, Lipofectamine® 3000 at a ratio of 1:3 strongly limited cell growth over time, and it reduced the capacity of C2C12 myoblasts to differentiate efficiently. Increasing the initial cell confluency, however, restored cell differentiation capacity and increased the formation of GFP-positive myotubes. Finally, Avalanche® and JetOPTIMUS® had a limited effect on cell viability and cell growth. Moreover, they were compatible with cell differentiation and the generation of GFP-positive myotubes. Of note, none of these reagents gave GFP-positive myotubes when performing the transfection after inducing differentiation (*data not shown*). Hence, considering their limited effect on cell dynamics and their transfection efficiency at limited cost (volume used/cost of the reagent), JetOPTIMUS®, and to a lesser extent Avalanche®, constitute the best option to transfect C2C12 myoblasts. Notably, limiting Lipofectamine® amount (ratio of 1:1.5) reduced its deleterious effect on cell growth, while preserving an efficiency similar to Avalanche®. For the same volume of reagent used (*i.e.* ratio 1:1.5), the procedure with Lipofectamine®3000 remains, however, more expensive than with JetOPTIMUS® or Avalanche® (dependent on regional offers from suppliers). JetOPTIMUS®, as well as Viafect™, led to the highest proportion of cells transfected with siRNA, when tested in conditions optimized for DNA transfer. However, their efficiency was lower as compared to Lipofectamine® RNAiMax or INTERFERin®, two reagents specifically designed for siRNA transfer. Further optimization of the conditions for siRNA transfer with the different reagents may allow selecting a single transfection reagent to conduct efficient DNA/siRNA transfection.

The response of C2C12 cells and primary mouse myoblasts to the transfection reagents also strongly differed. Both Viafect™ and Fugene® HD led to minimal transfection of primary myoblasts, even using high concentration of transfectants. Of note, high transfection efficiency has been reported with Fugene® HD at 1:2 ratio in primary myoblasts using single-stranded oligonucleotides [15]. Surprisingly, Avalanche®, and to a lesser extent JetOPTIMUS® and Lipofectamine® 3000 led to major toxicity upon transfection, suggesting higher sensitivity of primary muscle cells to these reagents as compared to C2C12 cells. Adapting the conditions set for C2C12 cells, especially by reducing the amount of transfectant used, is hence required to optimize transfection of primary myoblasts and select a single reagent compatible with both cell types.

In conclusion, we here delineated alternative reagents to efficiently transfect C2C12 cells, with a major interest in procedures limiting permissive or repressive impacts on cell growth, viability, and differentiation, at a limited cost. Conditions set for C2C12 cells with these reagents require specific optimization for distinct contexts, such as primary muscle cells or siRNA transfer, to improve efficiency and reduce cytotoxicity.

Credit author statement

Ilaria Cocchiararo: Conceptualization, Investigation, Formal analysis, Writing - Review & Editing, Mélanie Cornut: Investigation, Writing - Review & Editing, Hadrien Soldati: Conceptualization, Investigation, Writing - Review & Editing, Alessandro Bonavoglia: Investigation, Perrine Castets: Conceptualization, Resources, Writing - Original Draft, Supervision, Project administration, Funding acquisition.

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Author's contributions

IC performed experiments and analyzed the data; MC, HS and AB performed experiments; PC designed experiments, analyzed the data and wrote the manuscript. All authors read, edited and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Raw data are available on: https://doi.org/10.26037/yareta:jyea5x7a4fbcbh4eksm6sxa42y

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