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Efficient plasmid-mediated gene transfection of ovine bone marrow mesenchymal stromal cells

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Abstract

Background aims. Given the close similarity between ovine and human cardiomyocytes, sheep models of myocardial infarction and heart failure are increasingly used in studies of stem cell-mediated heart regeneration. In these studies, mesenchymal stromal cells (MSCs) are frequently employed. To enhance the paracrine effects of these MSCs, *ex vivo* transfection with genes encoding growth factors has been proposed. Although viral vectors exhibit higher transfection efficiency than plasmids, they entail the risks of uncontrolled transgene expression and immune reactions that preclude repeated administration. Our aim was to optimize the efficiency of plasmid-mediated transfection of ovine MSCs, while preserving cell viability. *Methods.* Varying amounts of diverse cationic lipids were used to obtain the reagent-to-DNA mass ratio showing highest luciferase activity. Transfection efficiency (flow cytometry) was tested on plasmid-green fluorescent protein-transfected MSCs at increasing DNA mass. *Results.* Lipofectamine LTX 5 μL and Plus reagent 4 μL with 2 μg of DNA yielded 42.3 ± 4.7% transfection efficiency, while preserving cell viability. Using these transfection conditions, we transfected MSCs with a plasmid encoding human vascular endothelial growth factor (VEGF) and found high VEGF protein concentrations in the culture supernatant from day 2 (1968 ± 324 pg/mL per μg DNA) through at least day 12 (888 ± 386 pg/mL per μg DNA) after transfection. *Conclusions.* Plasmid-mediated transfection of ovine MSCs to over-express paracrine heart-regenerative growth factors is feasible and efficient and overcomes the risks and limitations associated with the use of viral vectors.

Key Words: *mesenchymal stromal cell, plasmids, sheep, transfection efficiency, VEGF*

Introduction

In protocols of stem cell-induced cardiac regeneration, one of the most widely used cell types is the mesenchymal stromal cell (MSC), whose regenerative potential has been mostly attributed to paracrine effects of excreted growth factors (1,2). These cells have been engineered to over-express angiogenic and cardiomyogenic growth factors and cytokines (3). To do so, viral and non-viral vectors can be used. Although viral vectors display higher transfection efficiency, non-viral vectors, particularly plasmids, exhibit a better safety profile and prevent the risk of uncontrolled gene expression derived from the insertion of the transgene in the host cell's genome.

To allow a more reliable extrapolation of experimental results to the clinical setting, sheep models of coronary artery disease are being increasingly used (4,5). Ovine biologic features such as a limited potential to develop collateral circulation (6), cardiomyocytes capable of re-entering the cell cycle (7) and similar values for left ventricular function parameters (8) allow reliable extrapolation of experimental results to humans. In addition, their docility, easy housing and slow growth rate facilitate carrying out long-term follow-up protocols.

We aimed to optimize plasmid-mediated transfection of ovine MSCs by assessing the combination of cationic lipids and DNA mass that would yield the highest efficiency while preserving cell viability. We then transfected these cells with a plasmid encoding the 165-amino acid isoform of human vascular endothelial growth factor ($pVEGF_{165}$), a key angiogenic growth factor for stem cell-mediated cardioprotection (9), and confirmed that the culture supernatants contained high VEGF protein concentrations until 12 days after transfection and displayed an intense tube formation effect.

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Methods

Bone marrow extraction

Bone marrow (10–15 mL) was collected aseptically into a heparin-coated syringe from the iliac crest under deep sedation (acepromazine maleate, 0.2 mg/kg intramuscularly, followed by sodium thiopental, 25 mg/kg intravenously). Each sample was diluted in phosphate-buffered saline (PBS) and centrifuged in a Ficoll-Hypaque density gradient (1077 g/mL) at 1500 rpm for 30 min. The mononuclear cell layer was isolated. All procedures on sheep were done in accordance with the Guide for Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Laboratory Animal Care and Use Committee of the Favaloro University.

Ovine primary MSC culture

Single-cell suspensions of bone marrow mononuclear cells were plated in 75-cm^2 culture flasks and cultured at 37°C in the presence of 5% carbon dioxide with low-glucose Dulbecco's modified Eagle medium (Gibco, Grand Island, NY, USA) supplemented with antibiotic-antimycotic (Anti-Anti; Gibco), 20% (v/v) fetal bovine serum (Internegocios S.A., Buenos Aires, Argentina), and 2 mM glutamine (Gibco). After 2–3 days, medium with non-adherent cells was replaced, and half the medium was changed every 3 days. Cells were cultured until 80–90% confluence. Passages 3–5 were used.

Flow cytometry analysis of cell surface antigens

Flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) was used to characterize cell surface antigen expression of ovine MSCs using human anti-CD166 (BD Biosciences), ovine anti-CD44 (AbD Serotec, Oxford, UK) and ovine anti-CD45 (AbD Serotec) antibodies. Further immunotypification was not feasible given the scarcity of commercially available antibodies for sheep antigens. Cells (5×10^5) were diluted in PBS with 4% fetal bovine serum and incubated with each antibody or the corresponding isotype control on ice for 30 min. Cells were centrifuged for 5 min at 800 rpm, and the pellet was diluted in PBS. For each sample, 10,000 events were analyzed on the flow cytometer and stored for further processing using Cyflogic 1.2.1 software (Cyflo Ltd., Turku, Finland).

In vitro differentiation assays

For osteogenic differentiation, the STEMPRO Osteogenesis Differentiation Kit (Gibco) was used. Cells were seeded at 5×10^3 cells/cm^2 in a 12-well plate according to the manufacturer's protocol. Medium was replaced every 3–4 days until day 28, when the cells were fixed with 4% formaldehyde and stained with alizarin red S (Sigma-Aldrich, St Louis, MO, USA).

For adipogenic induction, we used the STEMPRO Adipogenesis Differentiation Kit (Gibco). Cells were seeded at 1×10^4 cells/cm^2 in a 12-well plate, according to the manufacturer's protocol. Medium was replaced every 3–4 days. After 14 days under differentiation conditions, cells were fixed with 4% formaldehyde and stained with oil red O (Sigma-Aldrich).

For chondrogenic induction, we employed STEMPRO Chondrogenesis Differentiation Kit (Gibco). We performed micro-mass cultures for 2 h under high-humidity conditions according to the manufacturer's protocol and then added the chondrogenic differentiation medium, which was replaced every 2–3 days. After 21 days, cells were fixed with 4% formaldehyde and stained with alcian blue 8GX (Sigma-Aldrich).

Transfection optimization

Ovine MSCs were cultured in 12 multi-well plates at a density of 80% viable cells/well in low-glucose Dulbecco's modified Eagle medium with 20% fetal bovine serum. After 24 h, cells were pre-incubated in Opti-MEM I (Gibco) for 30 min and then transiently transfected with 1 μg of a plasmid encoding luciferase (pGL3 Control Vector; Promega, Madison, WI, USA).

Varying amounts of different cationic lipids (GeneJuice; Merck Millipore, Darmstadt, Germany; Lipofectamine 2000, Lipofectamine LTX and Lipofectamine Plus reagents; Gibco) were tested. Cells were washed twice with PBS and lysed in 200 μL of 1X Passive Lysis Buffer (Promega) 48 h after transfection, and the cleared extract was assayed for luciferase activity according to the manufacturer's instructions in a GloMax 20/20 Luminometer (Promega). Delay and measurements times were set at 2 sec and 10 sec. Luciferase activity values were normalized by total protein concentration in each well and expressed as relative light units (RLU).

We assessed transfection efficiency (flow cytometry) with a plasmid encoding green fluorescent protein (GFP) (pCruz GFP, Santa Cruz Biotechnology, Santa Cruz, CA, USA) using two transfection conditions: one with the reagent volume-to-DNA mass ratio that had yielded the highest luciferase activity and one with half the amounts used previously. Both transfection conditions were performed in duplicate in two different cell isolates.

Transfection with pVEGF$_{165}$

After optimizing transfection efficiency, we transfected MSCs with pVEGF$_{165}$. The eukaryotic expression vector (pVEGF$_{165}$, deposited as pBSVEK3 at Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; accession number DSM 14346) is a 3930-bp plasmid that includes the human VEGF$_{165}$ cDNA, transcriptionally regulated by the cytomegalovirus promoter/enhancer. A SV40 terminator is located 3′ to the VEGF$_{165}$ coding gene. Preparation, purification and quality control analyses of the plasmid from transformed *Escherichia coli* cultures were performed under Good Manufacturing Practices conditions (Bio Sidus S.A., Buenos Aires, Argentina).

MSCs were cultured 1 day before transfection in two 12-well plates at 80% confluence. Each well plate was transfected with 2 μg or 4 μg pVEGF$_{165}$. Culture supernatant was collected before transfection and on days 2, 6, 9 and 12 after transfection (n = 3–5 for each time point) to quantify human VEGF$_{165}$ protein by enzyme-linked immunosorbent assay assay (Quantikine Human VEGF Immunoassay; R&D Systems, Minneapolis, MN, USA).

Tube formation assay

Human mammary epithelial cells were seeded in a 96-well plate pre-coated with Matrigel (BD Biosciences) and incubated for 6 h in basal medium (negative control), medium with recombinant human VEGF$_{165}$ 100 ng/mL (Catalogue No. PF074; Calbiochem, Billerica, MA, USA) as positive control and conditioned media collected from 2-day cultures of

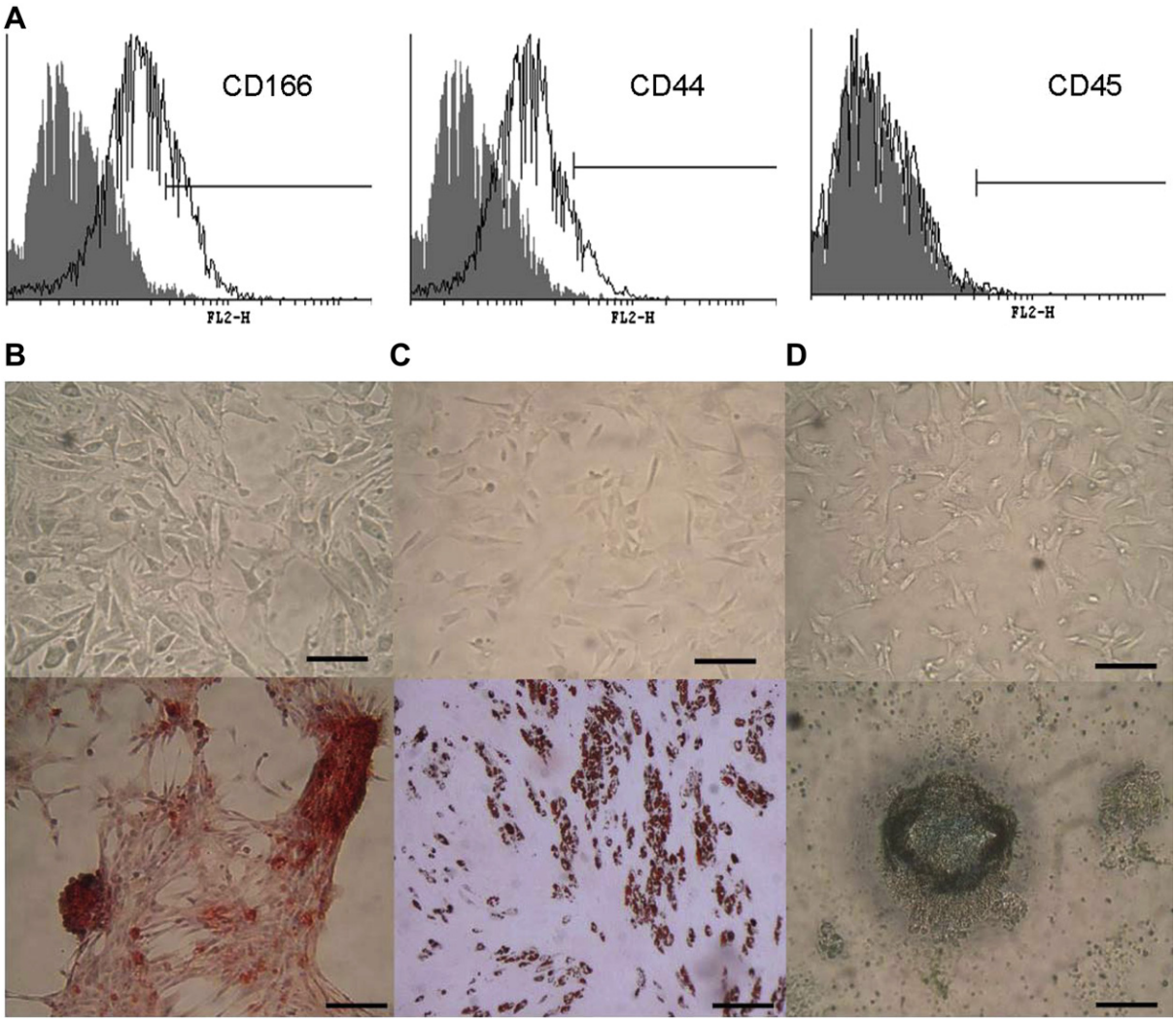

Figure 1. Ovine MSCs characterization. (A) Cells were CD166^{+}, CD44^{+} and CD45^{-}. Isotype control is depicted in gray. (B) Osteogenic differentiation. Alizarin red stain shows the calcium deposits in the extracellular matrix. (C) Adipogenic differentiation. Oil red O stain shows the lipid vacuoles. (D) Chondrogenic differentiation. Alcian blue stain shows proteoglycans. Non-differentiated controls are shown. Bars = 50 μm.

VEGF-transfected and non-transfected MSCs at 37°C in a 5% carbon dioxide humidified atmosphere. The extent of network formation was observed and photographed randomly. Quantification of the fractional area covered with tubes was performed by densitometry using Adobe Photoshop (San Jose, CA, USA) and ImageJ (National Institutes of Health, Bethesda, MD, USA) software and expressed as percent positive area. Each experiment was repeated at least three times.

Statistical analysis

Unpaired *t* tests were used to compare VEGF protein concentrations normalized by DNA mass at each time point, to compare GFP-assessed transfection efficiencies and to evaluate the tube formation capacity of the culture supernatants. Statistical significance was set at $P < 0.05$. Results are expressed as mean ± standard deviation.

Results

Isolation and characterization of ovine MSCs

Ovine MSCs were easily isolated and cultured. Cells were $CD166^+$,$CD44^+$, and $CD45^-$ (Figure 1A) and were able to differentiate into osteocytes (Figure 1B), adipocytes (Figure 1C) and chondrocytes (Figure 1D).

Optimization of transfection efficiency

Table I shows luciferase activity of MSCs using varying amounts of three different cationic lipids. The highest luciferase activity per µg DNA (14,733 ± 996 RLU) was achieved with 2.5 µL of Lipofectamine LTX and 2 µL of Plus reagent. When assessing for increasing DNA masses while keeping constant the relationship between reagent volumes and DNA mass (Table II), luciferase activity was 34,524 ± 5476 RLU for 2 µg of DNA and 54,454 ± 2437 RLU for 4 µg of DNA. However, this mass of DNA required 10 µL of Lipofectamine LTX and 8 µL of Plus reagent, which resulted in manifest cell mortality (see later) and in lower transfection efficiency, as demonstrated by flow cytometry on plasmid GFP-transfected MSCs at 2 µg and 4 µg of DNA mass. This analysis revealed that transfection efficiency was 42.3 ± 4.7% for 2 µg of DNA and 30.1 ± 13.6% for 4 µg of DNA ($P = 0.08$) (Figure 2). Given that cationic lipids have been shown to induce cell toxicity (10–12), we assessed cell viability by propidium iodide stain in one of the samples and found that it was 78.9% for 2 µg of DNA and 30.3% for 4 µg of DNA (Figure 2), confirming that the high volumes of reagents needed for the latter provoked cell mortality.

Table I. Luciferase activities for varying amounts of diverse transfection reagents.

	Transfection condition		
TR	TR (µL)	pLuc (µg)	RLU (mean ± SD)
Lipofectamine 2000	1	1	104 ± 22
	2	1	1547 ± 78
	3	1	1238 ± 63
GeneJuice	1	1	1898 ± 100
	2	1	2434 ± 162
	3	1	2069 ± 138
Lipofectamine LTX/Plus Reagent	1.5/1	1	9419 ± 1361
	2.5/1	1	10,098 ± 696
	3.5/1	1	7683 ± 324
	1.5/2	1	11,802 ± 690
	2.5/2	1	14,733 ± 996
	3.5/2	1	3598 ± 996

Maximal luciferase activity was achieved with 2.5 µL of Lipofectamine LTX and 2 µL of Plus Reagent per µg DNA.
pLuc, luciferase-encoding plasmid; SD, standard deviation; TR, transfection reagent.

Table II. Luciferase activities for varying DNA mass and constant reagent-to-DNA ratio.

	Transfection condition		
TR	TR (µL)	pLuc (µg)	RLU (mean ± SD)
Lipofectamine LTX/Plus Reagent	2.5/2	1	14,733 ± 996
	5.0/4	2	34,524 ± 5476
	10.0/8	4	54,454 ± 2437
	12.5/10	5	51,341 ± 3867
	15.0/12	6	41,960 ± 764

Peak luciferase activity was achieved with 4 µg of DNA. However, reagent-induced toxicity resulted in high cell mortality (see text).
pLuc, luciferase-encoding plasmid; SD, standard deviation; TR, transfection reagent.

VEGF expression

Figure 3 shows the time course of VEGF protein concentration in the supernatant of cultures of MSCs transfected with 2 µg and 4 µg of $pVEGF_{165}$. The use of 2 µg of DNA yielded higher protein concentrations per µg DNA at all time points; the differences were statistically significant at 9 days (2 µg, 1137 ± 293 pg/mL; 4 µg, 370 ± 93 pg/mL; $P < 0.01$) and 12 days (2 µg, 888 ± 386 pg/mL; 4 µg, 279 ± 53 pg/mL; $P < 0.05$).

Tube formation

Figure 4 shows that the tubulogenic effect of the supernatant of pVEGF-transfected MSC cultures

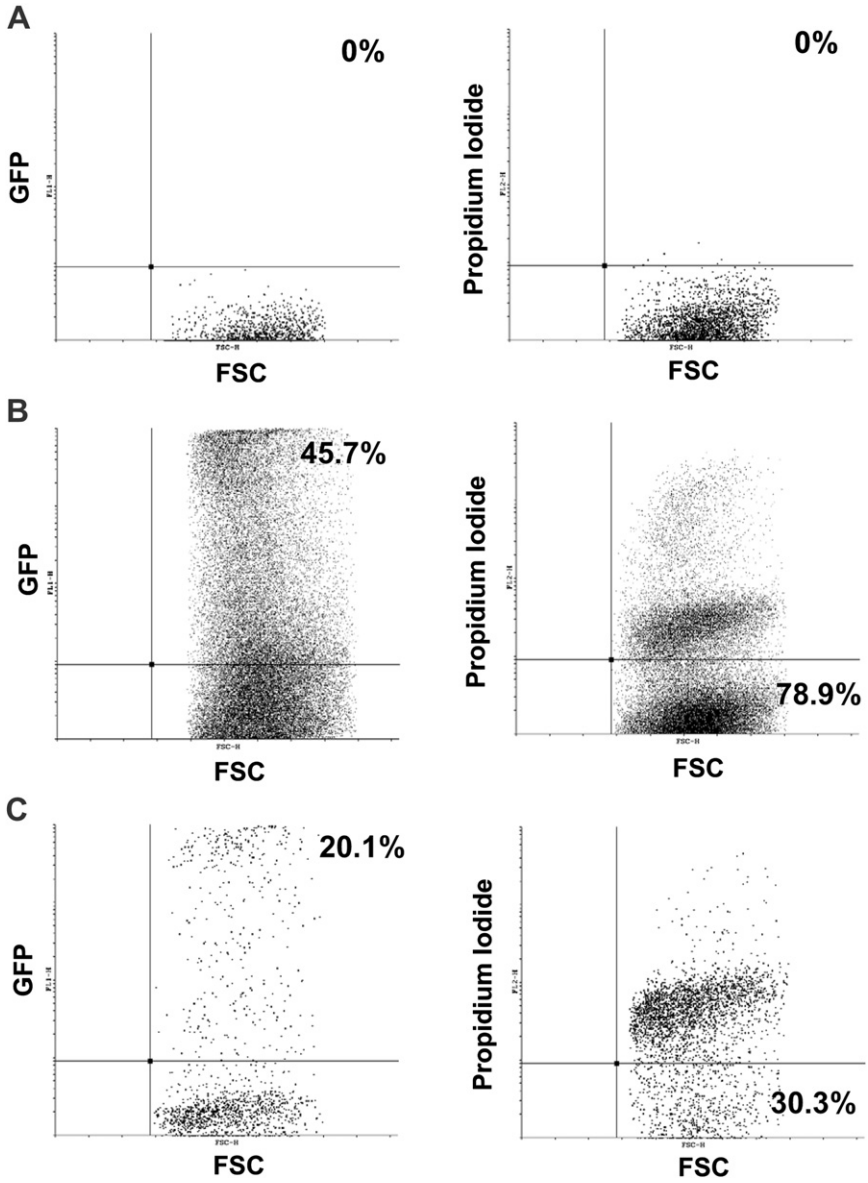

Figure 2. Representative flow cytometry plots of transfection efficiency and cell viability of ovine GFP-transfected MSCs. (A) Negative controls (cells not transfected with plasmid GFP and cells not stained with propidium iodide). (B) With 5 mL of Lipofectamine LTX, 4 mL of Plus reagent and 2 mg of DNA, transfection efficiency was 45.7% (A), and cell viability was 78.9%. (C) With 10 mL of Lipofectamine LTX, 8 mL of Plus reagent and 4 mg of DNA, transfection efficiency was only 20.1% (C), and cell viability 30.3%.

was intense and significantly higher than that achieved with the supernatant of cultures of non-transfected MSCs.

Discussion

Over the past few years, interest in the use of MSCs as carriers of therapeutic transgenes for cardiovascular disease has increased (3). One of the aims of this strategy is the overexpression of growth factors and cytokines that would enhance their regenerative potential. Of the viral vectors employed for transduction, vectors inducing genomic incorporation of the transgene, such as retrovirus and lentivirus, entail the risk of uncontrolled expression and oncogenic insertional mutagenesis. Viral vectors inducing episomal (and transient) expression, such as adenovirus, have been found to be inefficient at transducing MSCs, the rate of transduced cells being approximately 19% (13). To attain transient expression, plasmid-mediated transfection is an alternative. The advantage of plasmid vectors is their excellent safety profile; the disadvantage is their low transfection efficiency. Delivering plasmid DNA to MSCs using traditional transfection techniques, such as the one used here, has shown poor results so far, consistent of low transfection efficiency and significant cell mortality (14). We aimed to optimize

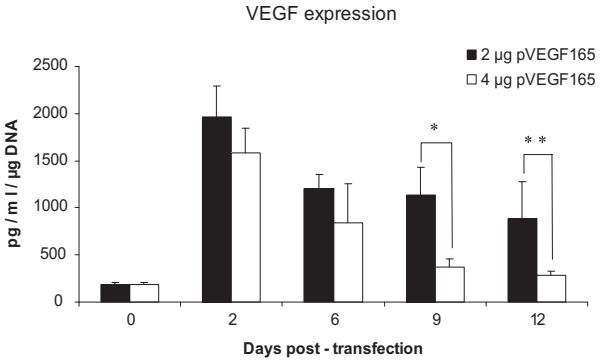

Figure 3. Serial assessment of human VEGF protein in the supernatant of cultured ovine MSCs transfected with 2 µg and 4 µg of $pVEGF_{165}$. The steeper decline over time for 4 µg was associated with manifest cell mortality induced by the transfection reagents (see text). $^{*}P < 0.01$; $^{**}P < 0.05$.

plasmid-mediated transfection of ovine MSCs by combining varying amounts of reagents and DNA. We found that combining 5 µL of Lipofectamine LTX and 4 µL of Plus Reagent with 2 µg of DNA yielded a transfection efficiency of 42.3 ± 4.7%, while preserving cell viability. When using this combination of reagents and DNA mass to transfect ovine MSCs with a plasmid encoding human $VEGF_{165}$, we found that the culture supernatant displays high concentrations of the VEGF protein for at least 12 days after transfection and a tube formation effect significantly exceeding that of the supernatant of non-transfected MSC cultures. The use of twice the mass of DNA (i.e., 4 µg), and hence twice the volume of the transfection reagents, induces manifest cell mortality, which results in lower transfection efficiency and, most importantly, a much steeper and highly significant over time decay of VEGF protein concentration in the culture supernatant.

Previous results of plasmid-mediated transfection of MSCs have been reported for rats and humans. In both species, optimal transfection efficiency was achieved using Lipofectamine 2000. In the case of rats, 4 µL of this cationic lipid and 1.6 µg of DNA mass yielded 19.6% efficiency (15). For human MSCs, maximum transfection efficiency (50%) was obtained with 0.9 µL of Lipofectamine 2000 and 0.6 µg of DNA (16). Because the method used to determine transfection efficiency in the study on rats was similar to ours, the disparity with our results is likely attributable to species differences. In the case of the human MSCs, in addition to species differences, transfection efficiency was assessed by cell counting using a Neubauer chamber and fluorescence microscopy. On these grounds, the results from those studies are hardly comparable to ours.

Randomized, placebo-controlled clinical trials of therapeutic angiogenesis in ischemic cardiovascular disease have shown little medium-term and long-term benefit, possibly owing, at least in part, to the fact that the microvascular network induced by pro-angiogenic interventions lacks stability (17) or that it regresses in the absence of VEGF (18). In previous studies on plasmid-mediated VEGF gene transfer in pigs and sheep with myocardial ischemia and infarction, we observed that VEGF gene expression is transient and that the angiogenic and arteriogenic response present at 7 and 10 days after transfection vanishes in specimens studied later on (7,19,20). This observation suggests that, on one hand, strategies to prolong transgene expression, such as the use of MSCs as carriers, are recommendable (21,22) and, on the other hand, these treatments should be repeated to attain positive, stable results (23). If treatments are to be repeated, the use of non-viral vectors, particularly plasmids, is mandatory, given that viral vectors entail the serious risk of adverse immune reactions on readministration.

Finally, we would like to comment on the animal model. Mice and rats are the species most usually employed for pre-clinical research on human angiogenesis and cardiomyogenesis. However, differences

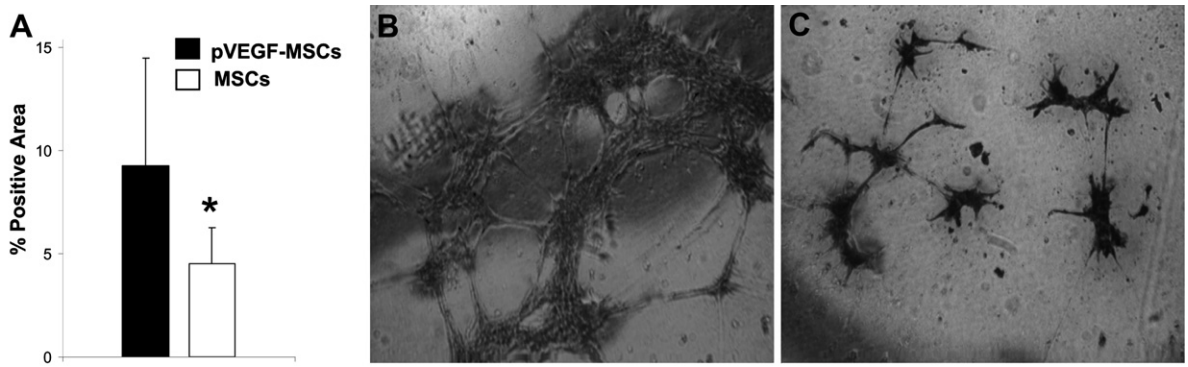

Figure 4. Tube formation assay. (A) The tube formation effect of the supernatant from cultures of ovine MSCs transfected with a $pVEGF_{165}$ was higher than that of non-transfected MSCs. (B and C) Representative tube formation images from pVEGF-MSC and MSC culture supernatants. (Magnification 10×.) $^{*}P < 0.05$.

in time of gestation, life span and heart size preclude reliable extrapolation of results on topics such as vessel growth, myocyte replication and regeneration capacity (24). Of the large mammalian models used, pigs and sheep share with humans their limited capacity to develop collateral circulation. However, as concerns cardiomyogenesis studies, sheep are preferable because, in contrast to pigs, whose cardiomyocytes may have up to 32 nuclei (25), sheep cardiomyocytes have one to four nuclei (26,27), resembling the human cardiac myocyte more closely.

In conclusion, plasmid-mediated transfection of ovine MSCs using 5 μL of Lipofectamine LTX and 4 μL of Plus reagent with 2 μg of DNA yields high transfection efficiency and preserves MSC viability; this results in high transgene expression lasting at least 12 days after transfection. This strategy may prove useful in engineering MSCs to deliver paracrine cardioprotective or regenerative factors efficiently and transiently in the myocardium, while avoiding the risks and limitations associated with the use of viral vectors.

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