Non-viral engineered human mesenchymal stem/stromal cells to promote angiogenesis

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Mesenchymal stem/stromal cells (MSC) have generated a great interest because of their inherent potential clinical use. Nevertheless, gene delivery to MSC represents a very promising strategy to improve their therapeutic potential. This study explores non-viral engineered MSC to overexpress vascular endothelial growth factor (VEGF) to promote angiogenesis. To this end, VEGF-encoding and VEGF-GFP-encoding plasmids and minicircles were produced and purified and umbilical cord matrix (UCM) and bone marrow (BM) MSC were transfected using lipofectamine. Transfected UCM cells showed higher cell viability and recovery, as well as superior transfection yields compared to BM cells (4 fold). Despite viability loss and low cell recoveries observed, it was possible to increase VEGF expression up to 66 times with pVAX-VEGF. Furthermore, engineered-MSC retained their immunophenotypic profile and multilineage differentiation capacity along adipogenic and osteogenic lineages. Overall, this work is expected to encourage the development of novel strategies for cardiovascular diseases (CVD) treatment.

Introduction
Cardiovascular diseases (CVD) are the number one cause of death in Europe and in the United States and represent major economic costs. Despite improvements in the treatment and the increasing amount of drugs available, given the population aging and the increasing prevalence of cardiovascular risk factors, CVD will continue to be a significant health concern. Moreover, restoration of cardiac function requires not only replacement of lost cardiomyocytes, but also revascularization of the injured tissue.

Mesenchymal stem/stromal cells (MSC) are multipotent stem cells present in several adult tissues, namely in bone marrow, adipose tissue and umbilical cord. Due to their immunomodulatory, trophic and homing abilities, MSC are very promising for therapeutic use. Furthermore, MSC can be used in allogeneic transplantation without HLA matching need and without being immunologically rejected.

Studies have shown that VEGF, a key regulator in blood vessel formation, is produced by MSC. Since VEGF expression by MSC is insufficient, it would be possible to further enhance MSC therapeutic efficacy through genetic modifications: MSC overexpressing VEGF could enhance angiogenesis and myocardial function.

Viral delivery systems are the most efficient DNA delivery systems, but as they raise safety concerns and are costly, substantial efforts in the
development of non-viral delivery systems have been made \(^\text{14}\). Since nuclease degradation is one of the main causes of inefficient nuclear delivery by conventional plasmids, a plasmid with increased resistance to nucleases might achieve higher transfection efficiencies \(^\text{15}\). Studies have shown that the plasmid’s bacterial DNA was responsible for transgene silencing, and could also trigger immune responses \(^\text{16,17}\). To overcome these problems associated with conventional plasmids, minicircles (MC) devoid of bacterial backbone (BB) were developed. Moreover, smaller plasmids have higher transfection efficiencies \(^\text{18,19}\). In this study, VEGF-encoding and VEGF-GFP-encoding plasmids and minicircles were developed and tested in UCM and BM MSC.

**Materials and Methods**

**Plasmids and minicircles construction**

pVAX-VEGF-GFP (4273 bp) was obtained by pVAX-VEGF-IRES-GFP (4905 bp) digestion with PsiI (NEB) and HpaI (Promega) to remove the IRES, followed by an overnight ligation with T4 DNA ligase (Promega).

pVAX-VEGF (3681 bp) was obtained by pVAX-VEGF-IRES-GFP (4905 bp) digestion with ApaI (Promega) to remove IRES and GFP gene followed by an overnight ligation with T4 DNA ligase.

pMini-VEGF-GFP (5278 bp) was obtained from pMini-GFP (4702 bp) by digestion with EcoRI (Promega) and HindIII (Promega) followed by a VEGF gene insertion and an overnight ligation with T4 DNA ligase. The VEGF gene was obtained from pVAX-VEGF-GFP digestion with EcoRI and HindIII.

pMini-VEGF (4551 bp) was obtained by pMini-GFP (4702 bp) digestion with BamHI (Promega) and ApaI (Promega) to remove the GFP gene, followed by the insertions of the VEGF gene and an overnight ligation with T4 DNA ligase.

**Production and purification of the plasmids**

pDNA was obtained by growing *E. coli* cultures (harbouring the plasmid) during the day in 5 mL of LB (Sigma) medium with 30 µg/mL kanamycin, followed by an overnight growth in 250 mL of LB medium with 30 µg/mL kanamycin at 37°C and 250 rpm \(^\text{10,20}\).

Plasmids’ purification was performed using Endotoxin-free plasmid DNA purification kit (Macherey-Nager), according to manufacturer’s instructions \(^\text{10,20}\).

Purified plasmid solutions’ concentrations were measured by spectrophotometry at 260 nm (NanoVue Plus, GE Healthcare Life Sciences). pDNA integrity was verified by agarose gel electrophoresis stained with ethidium bromide.

**Production and purification of the minicircles**

DNA was obtained by growing *E. coli* cultures (harbouring the plasmid) in 5 mL of LB medium with 30 µg/mL kanamycin and 0.5% glucose overnight at 30°C and 250 rpm overnight, followed by a growth in 250 mL of LB medium with 30 µg/mL kanamycin at 37°C and 250 rpm. At 3.5-4.5 of optical density, 0.01% of L-Arabinose (Merck) was added to the culture for plasmid’s recombination, followed by a 2 hour incubation at 37°C and 250 rpm.

Vectors’ purification was performed using Endotoxin-free plasmid DNA purification kit, according to manufacturer’s instructions. The obtained solution was further purified using techniques that will be patented and therefore cannot be disclosed.

Purified minicircle solutions’ concentrations were measured by spectrophotometry at 260 nm and pDNA integrity was verified by agarose gel electrophoresis stained with ethidium bromide.

**BM and UCM MSC culture**

Cryopreserved cells were thawed and resuspended in 5 mL of Iscove’s Modified Dulbecco’s Medium (IMDM, Gibco) supplemented with 10% of Fetal Bovine Serum (FBS, Gibco) and centrifuged at 1250 rpm for 7 minutes. Cells were resuspended in
Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 10% MSC-qualified FBS and antibiotics. The number of cells and their viability was determined using the trypan blue exclusion method. Afterwards, cells were plated at a 3000 cells/cm² density in cell culture flasks, using DMEM+10% FBS, and kept at 37°C with 5% CO₂ in a humidified atmosphere. The medium was replaced every 3-4 days.

At 80% cell confluence, MSC were washed with Phosphate Buffered Saline (PBS, Gibco) and detached from the flasks using accutase solution (Sigma) for 7 minutes at 37°C. Cell number and viability were determined using the trypan blue exclusion method. After 72 hours of culture, 1 µg of pDNA was transferred to the cells using 1 µL of Lipofectamine 2000™ (Invitrogen), according to supplier’s instructions. Lipofectamine and plasmid stock solutions were prepared with Opti-MEM ® I (Gibco) and mixed together to form the DNA/lipid complexes. Culture medium was exchanged to DMEM (without serum or antibiotics), and the transfection mixture was then added to the cells. After 5 hours of incubation at 37°C, the medium was replaced by DMEM+10% FBS.

Cell recovery and transfection yield were determined using equations previously described. Flow cytometric analysis

Cells were harvested using accutase and washed in PBS. Then, cells were counted and stained for 15 minutes in the dark with 5 µL propidium iodide (PI, Becton Dickinson) to assess cell viability. The percentage of dead and viable GFP-expressing cells was determined by flow cytometry (FACSCalibur, Becton Dickinson Biosciences) using the CellQuest software (Becton Dickinson Biosciences).

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BM and UCM MSC transfection

For transfection, MSC were plated at a density of 3000 cells/cm² in 24-well plates and cultured in DMEM+10% FBS. After 72 hours of culture, 1 µg of pDNA was transferred to the cells using 1 µL of Lipofectamine 2000™ (Invitrogen), according to supplier’s instructions.

Lipofectamine and plasmid stock solutions were prepared with Opti-MEM ® I (Gibco) and mixed together to form the DNA/lipid complexes. Culture medium was exchanged to DMEM (without serum or antibiotics), and the transfection mixture was then added to the cells. After 5 hours of incubation at 37°C, the medium was replaced by DMEM+10% FBS.

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An FSC-H/SSC-H gate was delineated to define the target cell population and a gate in the FL1-FL3 plot was set to exclude both PI-positive and GFP-negative cells. Non-transfected cells and cells treated with lipofectamine were used to determine nonspecific fluorescence.

GFP-expression was monitored on days 1, 2, 5 and 7 for cells transfected with GFP-enhanced plasmids. Cell recovery and viability were also determined using flow cytometric analysis.

VEGF quantification

A specific human VEGF ELISA kit was used to quantify VEGF production in culture supernatants of non-transfected and transfected cells 2 days after transfection (or 2 days of culture).

Multilineage differentiation assays

MSC’s differentiation ability into osteogenic, adipogenic and chondrogenic lineages after transfection was evaluated. The differentiation was only performed for a week, with the medium being changed once during that period.

Osteogenesis was induced using a StemPRO Osteogenesis differentiation medium (Gibco). After 7 days of differentiation, cells were tested for alkaline phosphatase (ALP) activity and von Kossa stainings for mineralization.

In order to induce adipogenesis, cells were cultured in a StemPRO Adipogenesis differentiation culture medium (Gibco). 7 days later, cells were tested with Oil Red-O (Sigma) staining to assess lipid accumulation in the vacuoles.
To test the ability of transfected cells to undergo chondrogenesis, cells were plated as small droplets on an ultra-low attachment culture plate and cultured in a StemPRO Chondrogenesis differentiation medium (Gibco). After a week, cells were tested with Alcian blue (Sigma) to detect sulfated glycosaminoglycans.

**Immunophenotypic profile**

Transfected cells were also analyzed by flow cytometry using four mouse anti-human monoclonal antibodies (PE-conjugated, since cells were expressing GFP) against: CD73 (Biolegend), CD90 (Biolegend), CD105 (Biolegend) and HLA-DR (BD Biosciences).

Cells were incubated 15 minutes in the dark with each antibody and then cells were washed in PBS and fixed in 2% PFA (paraformaldehyde). Controls with non-transfected cells and cells treated only with Lipofectamine (without pDNA) were also used as well as the appropriate isotype control.

Cells were analyzed by flow cytometry using the CellQuest software.

**Data analysis**

Results obtained from more than one donor are presented as mean ± standard error of mean (SEM).

**Results and discussion**

For simplification, pVAX-VEGF-IRES-GFP will be referred as p1, pVAX-VEGF-GFP as p2 and pVAX-VEGF as p3, while the plasmids with the synthetic poly A sequence will be referred as O2, and the original plasmids will be mentioned as C1.

**Plasmids production and purification**

Plasmids’ purification was performed with Endotoxin-free Plasmid DNA Purification Kit.

Purified plasmid solutions’ concentrations were measured by spectrophotometry at 260 nm: 2675 ng/µL for p2C1, 2683 ng/µL p2O2, 4448 ng/µL for p3C1 and 5212 ng/µL for p3O2.

pDNA integrity was verified by agarose gel electrophoresis stained with ethidium bromide (Figure 1).

**Figure 1 - Agarose gel electrophoresis of the purified pVAX-VEGF-IRES-GFP (4905 bp), pVAX-VEGF-GFP (4273 bp) and pVAX-VEGF (3681 bp).**

It is possible to see that the purified plasmids were successfully produced and purified, and only a minimal part of the plasmids became open circular (OC), while the bands corresponding to the supercoiled (SC) plasmids show higher intensities.

**Minicircles production and purification**

Purified minicircles solution concentrations were measured by spectrophotometry at 260 nm: 447 ng/µL and 134 ng/µL for MC-VEGF-GFP and MC-VEGF, respectively.

Minicircles integrity was verified by agarose gel electrophoresis stained with ethidium bromide (Figure 2 and Figure 3).
Figure 2 - Agarose gel electrophoresis of a) pMini-VEGF-GFP (5278 bp); b) after partial purification; and c) MC-VEGF-GFP (2457 bp) after the final separation.

Figure 3 - Agarose gel electrophoresis of a) MC-VEGF (1730 bp) + BB (2821 bp) + PP (4551 bp) purified with the purification kit; b) after partial purification; c) MC-VEGF (1730 bp) after the final separation.

It is possible to see that the minicircles were successfully separated from the contaminant species (parental plasmid (PP) and BB) and only SC and OC minicircles were obtained at the end of the purification. However, regarding the obtained MC-VEGF most of the minicircle seems to be in the OC conformation.

**BM and UCM MSC transfection**

MSC were plated at a cell density previously optimized by Boura and co-workers\(^\text{20}\) (3000 cells/cm\(^2\)) and the transfection was performed 72 hours after plating, using a DNA mass equivalent to 1 µg of pVAX-GFP and 1 µL of lipofectamine, as previously optimized by Madeira and co-workers\(^\text{10}\).

MSC transfection with the several VEGF-encoding plasmids and minicircles was evaluated in terms of cell recovery and viability at different time-points (days 1, 2, 5 and 7) after transfection. MSC lipofection using VEGF-GFP-encoding vectors was also assessed in terms of GFP\(^+\) cells and transfection yield at the same time-points. Supernatants harvested 2 days after transfection were also used for VEGF quantification using a specific human VEGF ELISA kit.

The viability of transfected cells allows evaluating how cells handled the transfection procedure (given by the viability of the cells treated with lipofectamine (LF)) and the DNA-uptake. This was determined from the equation:

\[
\text{Viability} (\%) = \left( \frac{\text{alive cells}}{\text{total cells}} \right) \times 100.
\]

Transfected UCM MSC (Figure 5) show higher viabilities than transfected BM MSC (Figure 4), which suggests that UCM MSC handle better and recover faster from the transfection than BM MSC. Moreover, it is possible to see that the lipofection process per si does not affect cell viability.

**Figure 4 - Cell viabilities of BM MSC:** control cells, cells treated with lipofectamine (LF) and cells transfected with the VEGF-GFP-encoding vectors. Data obtained from experiments with 3 different donors (n=3) ± SEM. Day 7 data obtained from experiments with 2 different donors (n=2) ± SEM.
The viabilities of UCM cells transfected with VEGFencoding vectors were similar to the ones obtained with the VEGF-GFP-encoding vectors.

Cell recovery offers an estimative of the amount of cells that remained adherent to the culture plate. Cell recovery was determined by the expression: $CR(\%) = [(\text{Transfected cells alive}) / (\text{Non-transfected cells alive})] \times 100$.

Cell recoveries from transfected UCM MSC (Figure 7) were higher (reached 30%) than BM MSC (Figure 6) recoveries, which were below 10%. Moreover, UCM MSC transfected with the minicircle have higher cell recoveries, while BM cells did not show significant differences between each vector.

The recoveries of UCM cells transfected with VEGF-encoding vectors were similar to the ones obtained with the VEGF-GFP-encoding vectors.

The amount of GFP$^+$ cells allows the estimation of the number of cells that expressed the transgene after transfection, while the quantity of GFP$^+$PI$^-$ cells provides information about the number of viable cells expressing GFP.

Comparing GFP$^+$ and GFP$^+$PI$^-$ percentages from the BM cells transfection (Figure 8), it is possible to see that most of the GFP-expressing cells were non-viable, especially the cells transfected with conventional plasmids. This way, transfection with minicircles might lead to a lower viability loss.

Thus, UCM transfection (Figure 9) achieved higher GFP$^+$PI$^-$ cells percentage (13%) than BM cells (9.8%), despite BM reaching considerably higher total GFP$^+$ cells (34%) than UCM cells (23%). This result suggests that UCM cells can withstand better DNA-uptake than BM cells.
Although UCM cells transfection attained the highest GFP\(^{+}\)PI\(^{-}\) cells percentage, differences compared to BM cells results are not substantial.

**Figure 8 -** GFP\(^{+}\)PI\(^{-}\) and GFP\(^{+}\) total cells (%) of BM MSC transfected with the VEGF-GFP-encoding vectors. Data obtained from experiments with 3 different donors (n=3) ± SEM. Day 2 and 7 data obtained from experiments with 2 different donors (n=2) ± SEM. GFP\(^{+}\)PI\(^{-}\) cells are represented as bars and GFP\(^{+}\) total (T) cells correspond to the squares.

**Figure 9 -** GFP\(^{+}\)PI\(^{-}\) and GFP\(^{+}\) total cells (%) of UCM MSC transfected with the VEGF-GFP-encoding vectors. Data obtained from experiments with 3 different donors (n=3) ± SEM. Day 7 data obtained from experiments with 2 different donors (n=2) ± SEM. GFP\(^{+}\)PI\(^{-}\) cells are represented as bars and GFP\(^{+}\) total (T) cells correspond to the squares.

The transfection yield assesses the transfection efficacy through the evaluation of the recovered GFP-expressing cells: Yield of transfection (%) = [(Transfected cells alive × GFP\(^{+}\) cells) / (Non-transfected cells alive)] × 100.

UCM cells reached higher yields of transfection than BM cells (4 fold). Both for UCM and BM cells, although minicircles did not led to the highest values during the first days, these vectors led to the highest yields on days 5 and 7, exhibiting a more persistent expression than conventional plasmids (about 2 fold).

**Figure 10 -** BM MSC transfection yields (%) for transfection with VEGF-GFP-encoding vectors, 1, 2, 5 and 7 days after transfection. Data obtained from experiments with 3 different donors (n=3) ± SEM. Day 7 data obtained from experiments with 2 different donors (n=2) ± SEM.

**Figure 11 -** UCM MSC transfection yields (%) for transfection with VEGF-GFP-encoding vectors, 1, 2, 5 and 7 days after transfection. Data obtained from experiments with 3 different donors (n=3) ± SEM. Day 7 data obtained from experiments with 2 different donors (n=2) ± SEM.

**VEGF quantification**

Since MSC express VEGF\(^{3}\), and lipofectamine could have some effect on VEGF production, non-transfected cells and cells treated with lipofectamine were also evaluated in terms of VEGF expression. The quantification was performed with supernatant samples.
harvested two days upon transfection (or just culture, for non-transfected cells).

As expected, ELISA results showed that engineered-MSC achieved higher VEGF expression than non-engineered cells. Moreover, since BM cells supernatants presented higher VEGF concentration than UCM cells supernatants, it was considered to be more accurate to evaluate VEGF production increase considering the production per cell (Figure 12).

![Figure 12 - VEGF production increase in engineered-MSC two days after transfection. Values presented as a ratio between VEGF concentration’s average in transfected-cells supernatant and in the supernatant of non-transfected-cells treated with lipofectamine.](image)

Regarding VEGF concentration, it is possible to see that engineered-BM cells reached higher increases than engineered-UCM cells. BM control cells supernatant had 1931 pg/mL, while engineered-BM cells attained 8704 pg/mL of VEGF concentration. Concerning VEGF concentration in UCM cells supernatant: control cells showed 500 pg/mL while engineered-UCM cells showed 3013 pg/mL and 5942 pg/mL of VEGF concentration with pVAX-VEGF-GFP and pVAX-VEGF, respectively. These results show that not only transfected BM cells supernatant had higher VEGF concentrations than UCM cells, but also presented a higher increase in VEGF production than UCM cells (54 vs 33 times).

One of the most noticeable results is the difference in the supernatant VEGF production increase when transfecting with p2C1 and p3C1: p3C1 increased VEGF’s concentration in the medium twice comparing to p2C1 increase (pVAX-VEGF increased 66 times while pVAX-VEGF-GFP attained a 33-times increase for UCM cells transfection).

Regarding the poor results obtained by MC-VEGF, the amount of MC in the OC conformation (which is less efficient in terms of gene expression than the SC conformation) might explain the low increase in VEGF concentration compared to the other vectors’ results.

**Conclusions**

This work has shown that MSC have great potential for the treatment of CVD through angiogenesis’ promotion. Although low transfection efficiencies were obtained (4.2 ± 0.7% maximum for UCM cells), engineered-MSC have increased VEGF production up to 66-fold, compared to non-engineered-MSC. Furthermore, engineered-MSC retained their immunophenotypic profile and multilineage differentiation capacity towards adipogenic and osteogenic lineages.

Regarding the different vectors used, no major differences between them were observed. Minicircles achieved the best results mostly in the last days of experiment, showing a previously described more persistent expression.
Concerning the different MSC sources evaluated, UCM cells showed lower viability loss, a quicker recovery and higher cell recoveries than BM cells. However, non-engineered and engineered-BM cells showed higher VEGF expression than non-engineered and engineered-UCM cells. In terms of clinical use, allogeneic and autologous BM cells can be used while nowadays UCM cells can only be used for allogeneic transplantation. Since MSC are immunoprivileged, both allogenic and autologous transplantations can be performed. However, to guarantee that transplanted cells will not induce an immune response an autologous transplantation is needed and therefore by now only BM MSC can be used.

If we were able to reach higher transfection yields and cell recoveries, we could be capable of obtaining even higher VEGF production by the engineered-MSC, which would let us closer to their therapeutic use. Thus, improvements in non-viral gene delivery methods and vectors are needed in order to overcome the difficulties we currently encounter.

Since MSC VEGF expression is up-regulated in hypoxia\textsuperscript{13,24,25}, and MSC expansion is more effective in such conditions\textsuperscript{25}, it would be important to study engineered-MSC VEGF expression under hypoxic conditions.

As xenogeneic serum-containing medium presents safety concerns and MSC cultured in xeno-free conditions attain higher cell densities\textsuperscript{26}, it would be pertinent to further study MSC’ transfection results in xeno-free conditions.

Overall, this work shows that gene delivery to MSC can improve their therapeutic potential, particularly for CVD treatment.

References


