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Vol. 14(37), pp. 2703-2714, 16 September, 2015
DOI: 10.5897/AJB2015.14417
Article Number: D9D6FF255467
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Nanoparticle (MPG)-mediated delivery of small RNAs into human mesenchymal stem cells

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Received 13 January, 2015; Accepted 14 September, 2015

The cellular membrane constitutes an effective barrier that protects the complex, yet highly ordered, intracellular compartment of the cell. Passage of molecules across this barrier is highly regulated and highly restricted. Cell penetrating peptides (CPPs) are a class of small cationic peptides that are able to defy the rules of membrane passage and gain access to the intracellular environment. MPG is one member of this class of cell penetrating peptides. In this study, MPG was used to deliver double-stranded siRNA into human multipotent stem (hMADS) cells. Confluent and differentiated hMADS cells were transfected for 4 h with 30 nM siRNA-MPG-complex (SMC) and the controls were overlaid with free siRNA (SO) and analysed after 24, 48 and 72 h incubation post-transfection for internalization of the siRNA complex using Zeiss Axioimager. There was no noticeable adverse morphological variation between the siRNA-MPG complex transfected groups and the 'free siRNA' and the 'cell only' control groups. There was also no observed cytotoxicity associated with transfection. The differentiation of the cells into adipocyte phenotype was observed normally in all the groups. Bright fluorescence speckles were detected in the SMC transfected cells at all the 3 time points in the cytoplasm as well as in the nucleus 24 and 48 h post-transfection. The efficiency of delivery 24 h after transfection in the confluent cells was about 90%, in cells differentiating three days toward adipocytes about 80, and 50 to 60% of the cells showed internalised siRNA-MPG complex even 48 and 72 h post- transfection. This study shows that MPG efficiently mediated cytoplasmic and nuclear delivery of double-stranded siRNA into human mesenchymal stem cells under mild conditions in proliferating and in differentiation stages. These results demonstrate that MPG is a very effective and robust non-viral based transfection agent, easy to apply to non-dividing adherent cells *in situ.* Thus, MPG is a valuable tool for transient gene and microRNA silencing *in vitro*.

Key words: Cell-penetrating peptides, microRNA, MPG, siRNA, stem cells, transfection, delivery, nanoparticles.

INTRODUCTION

Delivery of biomolecules holds tremendous promise in the treatment of many genetic and acquired diseases. This became more imperative with the availability of human genome sequence data as well as other organismal platforms that are continually added into databases fuelling the demands for validation of target prediction. However, identification of protein function and target validation require analytical tools to introduce or down-regulate genes. The main techniques used to "transiently silence" the expression of genes or proteins

include the classical antisense oligonucleotides (ONs) and small interfering RNAs (siRNAs) as well as miRNAs. Another approach is introducing full-length proteins or genes of interest. Expressing those constructs would provide a direct way to analyze protein function. However, the inability of those hydrophilic molecules to enter cells is a major obstacle (El Andaloussi et al., 2005). Gary and colleagues suggested that the future of gene therapy in humans is dependent upon the discovery of safe and efficient vector system for transport of bio active molecules (Gary et al., 2007). A lot of efforts have been made to address the permeability of the membrane to hydrophilic molecules. Methods like electroporation, viral and other non-viral based delivery systems like lipofection, calcium sulphate, had been used to deliver different cargos across membrane with various degrees of success. Several delivery vectors, viral and nonviral, have been developed to facilitate translocation of bioactive agents into cells. The viral vectors are by far the most effective delivery system but suffer from limited cargo carrying capacity, production problems, as well as possible viral recombination and immunogenicity *in vivo* (Gary et al., 2007)

The efficient protection against enzymatic or non-enzymatic degradation is particularly important for RNA molecules including siRNAs. In fact, while the therapeutic potential of siRNAs for the treatment of various diseases is in principle very promising, limitations of transfer vectors may turn out to be rate-limiting in the development of RNAi-based therapeutic strategies (Aigner, 2006). Attempts to improve RNA delivery have led to development of a number of delivery systems to circumvent observed limitations of current delivery tools. There has been an upsurge of interest in nanoparticle-mediated delivery by a number of groups in recent times (Chen et al., 2011; Kimura et al., 2009; Zhou et al., 2013; Wong et al., 2012; Rahim et al., 2009; Kolli et al., 2013; Yamanaka and Leong, 2008; Water et al., 2015). For instance, Kim and colleagues reported that nanoparticle-mediated RNA interference drugs represent a potential new approach in targeting lung cancer due to selective silencing effect of oncogenes and multidrug resistance related genes (Kim et al., 2015).

Peptides represent a class of vectors that can be extensively modified to meet the needs of a particular gene delivery system. Critical to successful delivery of biomolecules like RNA is the ability to traverse the cell membrane and to release the cargo molecule safe from degradation while maintaining the integrity of the cell. Cell penetrating peptides (CPPs) seem to be answers to efficient deliveries of biomolecules without the worries of degradation by endosomes. Cell-penetrating peptides (CPPs) equally referred to as protein transduction domains (PTDs), Trojan peptides or membrane translo-cating sequences (MTS), have shown significant potential in the field of drug delivery. CPPs can deliver a wide range of bioactive molecules such as proteins, peptides, oligonucleotides (ON), and nano-particles to several cell types and to different cellular compartments, both *in vivo* and *in vitro*. These peptides vary in size, amino acid sequence, and charge, but share the common feature that they have the ability to rapidly translocate the plasma membrane and enable delivery to the cytoplasm or nucleus (Lindgren et al., 2000).

According to Deshayes et al. (2005) CPPs are classified as follows with reference to peptides derived from protein transduction domains or their family for classification. Many CPPs were designed from sequences of membrane interacting proteins, such as fusion proteins, signal peptides, transmembrane domains and antimicrobial peptides. Within these sequences, short sequences called protein transduction domains or PTDs proved to efficiently cross biological membranes without the need of a carrier or of a receptor and to deliver peptides. The major obstacle for using small RNAs as drugs is to deliver them into the cytoplasm of cells. An exception may be mucosal tissues. In the lung and vagina, siRNA uptake is extremely efficient and occurs even in the absence of transfection reagents (Dykxhoorn et al., 2006). Early investigation of mechanism of action of CPPs supported non-endosomal pathway however recently studies led to the complete revision of the cellular uptake mechanism of CPPs, incorporating the involvement of the endosomal pathway (Richard et al., 2003). However, as for most CPPs, evidence for several cell-entry routes have been reported, some of which are independent of the endosomal pathway (Deshayes et al., 2005), so it is essential to identify the one leading to a biological response.

MPG is a bipartite amphipathic peptide derived from both the fusion peptide domain of HIV-1 gp41 protein and the NLS of SV40 large T antigen. It forms stable non-covalent complexes with nucleic acids and promotes their delivery into a large number of cell lines (Simeoni et al., 2003). The positively charged NLS most likely interacts electrostatically with DNA and condenses the cargo while the hydrophobic gp41 domain mediates membrane

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; **SMC,** siRNA-MPG complex; **SO,** siRNA only; **FAM,** 6-carboxy fluorescein; **DAPI,** 4'.6-diamidino-2-phenylindole; **CPPs,** cell penetrating peptides; **hMADS,** human multipotent stem cells; **PTDs,** protein transduction domains; **CO,** cell-only; **MIP,** maximum intensity projection.

fusion. Inside the cell, the NLS confers nuclear localization of cargo MPG, which stands for May, Pierre and Gilles, the names of the people that discovered the peptide. It is a 27-residue-long primary amphipathic peptide with the following sequences: GALFLGFLGAAGSTMGAWSQPKKKRKV. MPG consists of three domains: a variable N-terminal hydrophobic motif; a hydrophilic lysine-rich domain, which, is derived from the NLS (nuclear localization sequence) of SV40 (Simian virus 40) large T-antigen (KKKRKV), required for the actual interactions with nucleic acids and intracellular trafficking of the cargo (Crombez et al., 2007). The peptide contains three distinct domains: a N-terminal hydrophobic motif (GALFLGFLGAAGSTMGA) derived from the fusion sequence of the HIV-1 gp41 (glycoprotein 41), required for interaction with the lipid moiety of the cell membrane and cellular uptake, a hydrophilic domain derived from the nuclear localization sequence (NLS) of SV40 (Simian virus 40) large T-antigen (KKKRKV) involved in the interactions with nucleic acids and intracellular trafficking of the cargo, and a linker domain (WSQP), which improves the flexibility and integrity of the hydrophobic and the hydrophilic domains (Crombez et al., 2007). MPG carries a cysteamide group at its C terminus, which is essential for both cell entry and stabilization of the complexes with siRNA (Simeoni et al., 2003). MPG, originally designed to improve the cellular uptake of oligonucleotide and plasmid, was then optimized for the delivery of siRNA. A single mutation on the second lysine residue of the NLS to serine (MPG $^{\Delta}$NLS- GALFLGFLGAA-GSTMGAWSQPKSKRKV), was shown to abolish the nuclear translocation and facilitate a rapid release of the siRNA in the cytoplasm (Simeoni et al., 2003). MPG is important in targeting genes to the nucleus making this peptide an interesting vector for clinical trials.

The MPG peptide associates rapidly in solution with siRNA, initial contact occurs through electrostatic interactions involving the hydrophilic lysine-rich domain independently of specific sequences, followed by peptide–peptide interactions involving the gp41 hydrophobic domain, thus generating stable MPG–siRNA nanoparticles with size of approximately 200 nm diameter (Simeoni et al., 2003). Studies by Simeoni and colleagues suggest MPG exhibits high affinity in the nanomolar range for siRNA and that approximately 10 to 20 peptide molecules are required to form a highly stable nanoparticle (Simeoni et al., 2003). The presence of a peptide-based nanostructure associated to the siRNA dramatically improved its stability inside the cell and significantly protected it from degradation (Crombez et al., 2007). The internalization process of MPG according to Deshayes et al. (2004) is achieved through formation of a carrier/cargo complex and occurs through a non-endosomal pathway leading to a mainly nuclear final localization of the cargo. Divita's group showed in greater details that the outer part of the 'carrier-based nanoparticle' with the siRNA plays a key role in the interactions of MPG with the membrane. MPG strongly interacts with phospholipids, through their hydrophobic fusion sequence, which then adopted a *β*-structure required for insertion of the peptide into the membrane (Deshayes et al., 2004).

In another instance, Veldhoen and colleagues exploited the flexibility of a non-covalent strategy; by focusing on the characterisation of a novel carrier peptide termed MPGα, which spontaneously forms complexes with nucleic acids (Veldhoen et al., 2006). They used a luciferase-targeted siRNA cargo; to address the cellular uptake mechanism of MPGα/siRNA complexes in HeLa cells. They equally showed a significant reduction of the RNA interference with MPGα/siRNA complexes in the presence of several inhibitors of endocytosis. Using confocal laser microscopy they observed a punctual intracellular pattern rather than a diffuse distribution of fluorescently labelled RNA-cargo. Their data suggested strong evidence of an endocytotic pathway contributing significantly to the uptake of MPGα/siRNA complexes (Veldhoen et al., 2006).

Human multipotent adipose-derived stem (hMADS) cells provide a good model for studying cell fate decisions like adipogenesis (Rodriguez et al., 2004). The hMADS cells are mesenchymal stem cell derived from the stroma-vascular fraction of human adipose tissue. hMADS cell have shown great promise as model for studying adipogenesis, osteogenesis and chondrogenesis (Rodriguez et al., 2004; Zaragosi et al., 2007). However, hMADS cells are very difficult to transfect using most traditional non-viral methods like lipid based agents (Zaragosi et al., 2007). Hence, this study will investigate MPG for the internalization of FAM-labeled double-stranded siRNA into hMADS cells.

MATERIALS AND METHODS

Proliferation and differentiation of hMADS Cells

The hMADS cells were thawed, resuspended, and maintained in Dulbecco's modified Eagle's medium (DMEM) pre-warmed to 37°C containing 10% FBS, Penicillin and Streptomycin, Pen/Strep, 100 U+ug/mL, and 0.1 mg/mL Normocin (InvivoGen), 10 mM HEPES and L-Glutamine which was enriched every 4 weeks. hFGF-2 was added freshly to the medium for proliferation. The medium was changed every two days until cells reached confluence. After two days of confluence (designated Day 0), cells were then induced to differentiation using Medium II prepared with the reagents-DMEM/Ham's F12 medium supplemented with 10 ug/ml transferrin, 0.86 uM insulin, 0.2 nM of triiodothyronine, 1 uM dexamethasone, 100 uM isobutyl- methylxanthine, and 20 nM rosiglitazone. Three days later, the medium was changed to medium III (lacking DEX and IBMX). Only freshly prepared differentiation media were used. The cells were incubated in standard cell culture conditions, at a temperature of 37°C with air containing 20% oxygen, 5% carbon dioxide and nitrogen. The medium was sterile filtered to avoid contamination. The cells were grown in 10 mm cell culture dishes and sub-cultured into 35 mm dish (6-well plate) and 24 well plates during transfection.

Preparation of transfection complex and internalization

MPG peptide (N-ter Nanoparticle siRNA Transfection system, Sigma) was thawed at room temperature for 10 min and sonicated for 5 min in sonicating water bath (Elma, Transonic) at maximum output and continuous power. This step decreases aggregation tendency of the peptide and can reduce the variability of transfection efficiency. The peptide was then diluted with RNase-free water (DEPC water). To make a stock solution, 2 OD (10.52 nmole) 5´-6-FAM-labeled siRNA (Sigma) was solved in DEPC-water to form 50 µM stock solution, which was further diluted to 5 µM working solution. The 5 µM siRNA working solution was further diluted in MPG buffer. The tubes containing the peptide and the siRNA were mixed together, vortexed gently, pulse spurned and incubated at 37°C, for 40 min to form the siRNA-MPG complex (SMC), which from now will be referred to as SMC. This yielded a concentration of 650 nM SMC. The solution was further diluted to appropriate concentration of 30 nM with 0.5 × MPG. siRNA-only (SO) control was prepared by diluting 14 µl of 5 µM siRNA working solution with 436 µl of 0.5x MPG Buffer, while cell-only (CO) control was formed by mixing 75 µl of MPG buffer and 75 µl RNase free water and diluting with 300 µl of 0.5 × MPG Buffer.

Transfection

Human multipotent adipose derived stem cells (hMADS) grown to 80%, 100% confluence, as well as cells in day 3 of differentiation into adipocytes (D 3), and cell in Day 10 after adipocytes differentiation (confirmed by Oil-Red-O staining) D10 on a glass coverslip (Corning) which was sterilized by dipping in 70% ethanol and in turn drying in bunsen burner flame, in a 6 well plate or 24 well plate were washed with PBS and then transfected with the diluted SMC, SO and CO to the respective wells, and allowed to incubate for 3 to 5 min at room temperature and thereafter equal amount of serum and antibiotic free DMEM medium was added and allowed to incubate for 4 h at standard cell culture condition at 37°C, 5% CO_2. After this incubation, complete medium was added and allowed to incubate for 24, 48, and 72 h. The cells were processed for fluorescence microscopy by fixing them in 3.7% formaldehyde for 10 min, counter-stained with DAPI and mounted on a slide as described below.

Slide preparation for fluorescence microscopy

The coverslip containing the transfected cells were carefully detached from the wells and rinsed in 2 changes of PBS, avoiding keeping the cells long in the PBS to prevent detaching of the adherent cells from the coverslip. The cells were fixed in 3.7% formaldehyde (prepared by diluting 10 x stock in PBS) for 10 min in a shaker at room temperature. The cells were washed in 2 changes of PBS for 5 min and counter stained with 0.05 ng/ uL 4',6-diamidino-2-phenylindole (DAPI) for 5 min. DAPI stains the nucleus blue. They were washed in PBS for 5 min, after which the coverslips were mounted on clean slides in PBS or vector-shield and sealed with sealant. PBS was mostly used since, mounting in vector-shield leads to the fading of the blue staining of DAPI after long exposure. The slides were then observed using Axioimager, Epifluorescence microscope (Zeiss), using FAM and DAPI and phase contrast channels.

Oil -Red-O staining

Oil-Red-O is a lipophilic diazo red dye that stains intracellular lipid droplets red. It was prepared as previously described (Massiera et al., 2003). 0.5 g Oil-Red-O was dissolved in 100 ml Isopropanol and stored at 4°C to make a stock solution. 18 ml stock solution was diluted in 12 ml milliq water (MQ) or deionized water giving a 30 ml final working solution. The resulting solution was filtered using Whatman filter paper. Cells were washed in PBS twice and fixed in 10% formaldehyde (in PBS) for 15 min at room temperature. The fixative was removed and replaced with filtered Oil-Red-O solution and incubated for 1 h at room temperature. After staining, the cells were washed thoroughly with PBS until excess stains were removed. Mature adipocytes containing lipid droplets stained red.

Fluorescent microscopy

Two fluorescent microscopes were used in this study. The Zeiss Axioimager and Olympus CKX 41 inverted microscopes with reflected fluorescence system. The later was used for routine cell culture microscopy. The Zeiss Axioimager is a wide-field epifluoresence microscope. Images were captured on a sensitive CCD camera. It was used for visualizing fluorescent probes in cells; images were taken in 4',6-diamidino-2-phenylindole (DAPI) channel which excites at 359 nm and emits at 461 nm. DAPI was used to detect the nuclei in blue channel. 6-carboxy fluorescein (FAM) excitation is at 494 nm and fluorescence emission is at 518 nm. FAM was used to detect the siRNA in green channel.

RESULTS

hMADS cells were successfully induced to adipocyte differentiation

The hMADS cells were grown to confluence and induced to adipocyte differentiation on day 0. The fibroblast-like morphology was visible before day 0, which was the day of induction (Figure 1A) and day 1 (B), but from Day 3 (C) there was remarkable change in morphology of the cells. The cell morphology gradually became oval with numerous globular shaped fat droplets. By day 8 (D), day 10 (E), and day 15 (F) differentiated fat cells were apparent with clusters of fat droplets. Oil-Red-O staining also confirmed the presence of fats cells as indicated by the red staining of the cells that developed fat droplets (G, H and I). The intensity of red staining which is a measure of degree of differentiation into fat cells increased from Day 8 through Day 10 and became much more intense by the fifteenth day (Day 15) as shown by Figure 1 (G, H, I, J).

Does the transfection of pre-confluent cells with SMC affect cell viability?

In order to investigate the above, hMADS cells were transfected with 15 nM of SMC for 4 h and examined with Axioimager after 48 h of incubation (Figure 1 upper panel: A,B,C). No cell mortality was observed in the SMC transfected cell. No evidence of cytotoxity was observed in the SMC SO, and CO. The cells overlaid with the free siRNA, SO (B) and the cells overlaid with cell only (CO) containing the MPG buffer alone (C, F, I) showed virtually similar cell morphology and structure with the cells transfected with SMC. There was no evidence to suggest

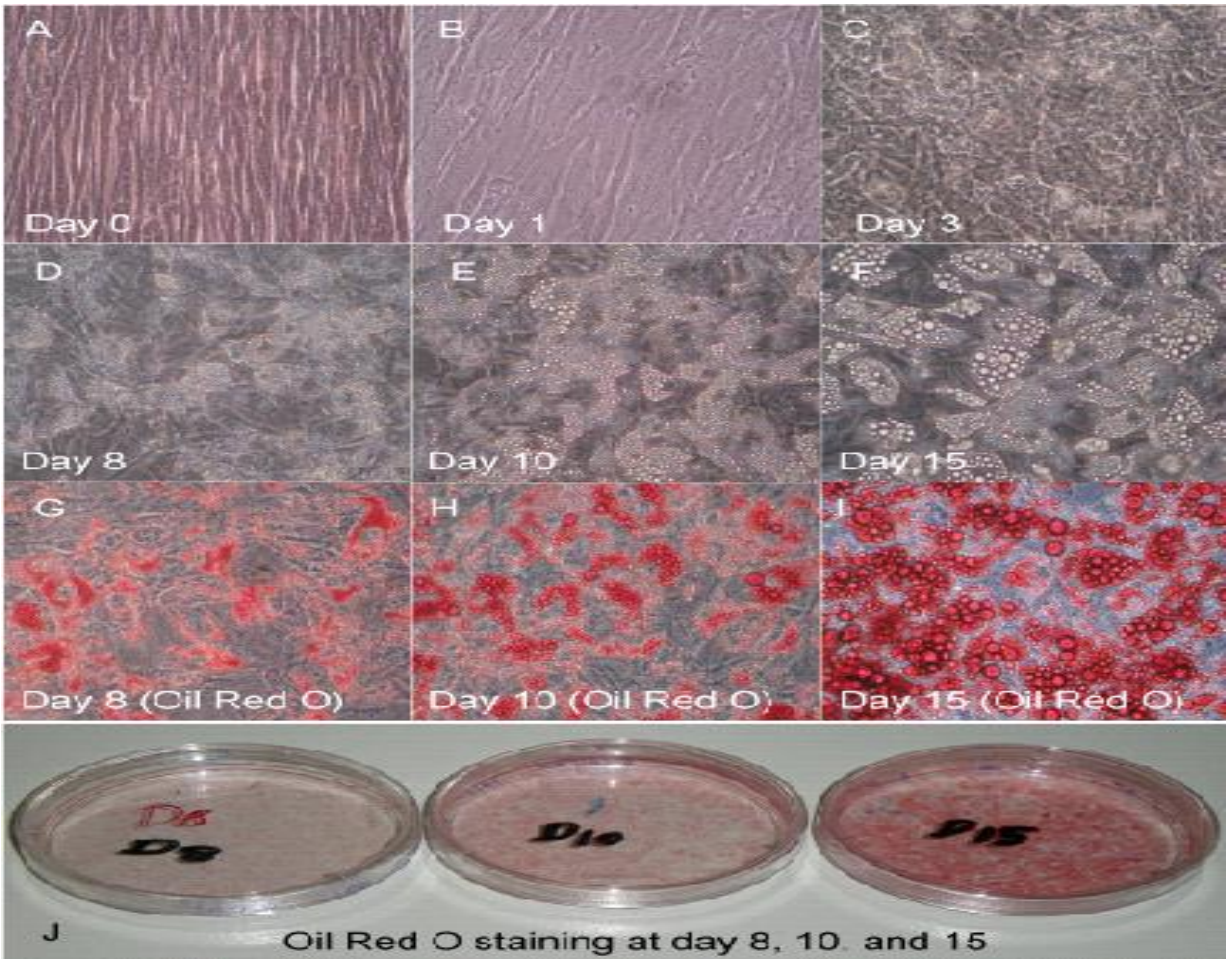

Figure 1. Differentiation of hMADS cells. hMADS cells were grown to confluence and induced to adipocyte differentiation on Day 0. The fibroblast-like morphology seen day 0 (A) and day 1 (B), from day 3 (C) cell morphology gradually became oval with numerous globular shaped fat droplets that increased in size and in number as differentiation progressed from day 3, day 8 (D) through day 10 (E) and to become fully matured adipocytes in day 15 (F). Oil-Red-O staining of the cells at time points day 8 (G), day 10 (H) and day 15 (I) with pictures of dishes showing increasing staining intensity from day 8 to day 15 (J).

that there was any morphological variation in cells attributable to the complex. These results suggest that the MPG was not toxic to hMADS cells within the investigated concentration.

Transfection of confluent and differentiating hMADS cells with siRNA-MPG complex (SMC) does not affect cell viability

Confluent (Figure 2 middle panel: D, E, F) and adipocyte differentiating hMADS cells (Figure 2 lower panel, G, H, I) were transfected with 30 nM of SMC for 4 h and examined with Axioimager after 48 h of incubation. There was no evidence to suggest. There was no morphological variation in cells attributable to the complex. There was no mortality of the cells. The cells overlaid with the free siRNA (E, H) and the cells overlaid with cell only (CO) (right panel, F, I) showed virtually similar cell morphology and structure with SMC transfected cells (D, G). The cell morphology of the SMC and SO and CO were virtually similar in cell content. There was no distortion or detachment of the adherent cells that could be directly linked to SMC. There was no evidence of cell mortality associated to the MPG transfection. There was comparable morphology and cell architecture in the SMC, SO and CO as indicated by Phase contrast images of the confluent and the differentiated cells. There was also no indication that the MPG interfered with the differentiation process. MPG showed no adverse effect on hMADS cells viability. This result suggests that MPG was mild to the hMADS cell, and as such does not adversely affect the viability of hMADS cells within the investigated concentration.

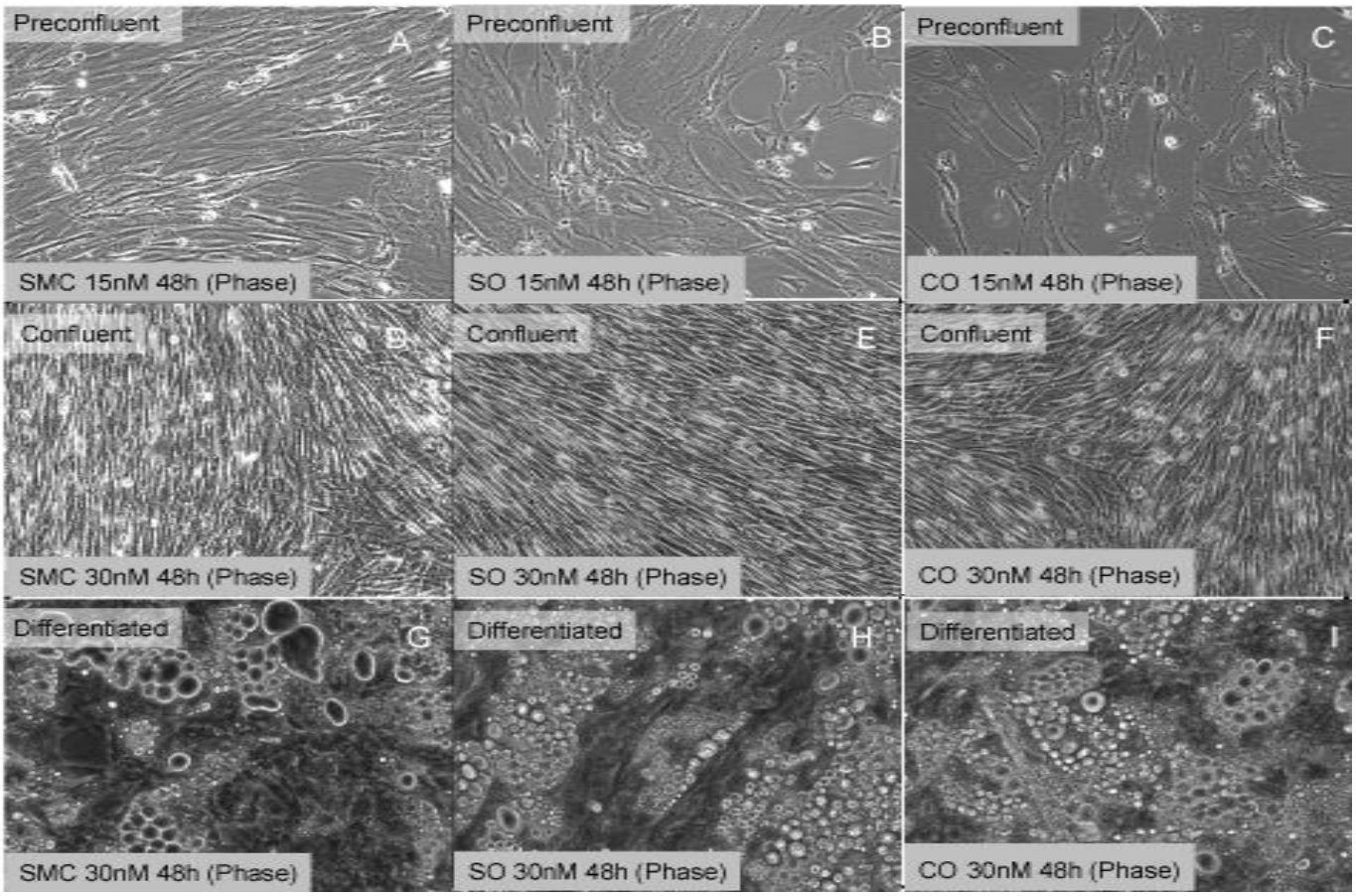

Figure 2. Cell viability test. Phase contrast images of hMADS cells transfected with SMC and overlaid with free siRNA, SO and cell only, CO at concentration of 15 nM for the pre-confluent time point (A,B,C), 30 nM for the confluent (D,E,F) and differentiated stages (G,F,I), 48 h post-transfection. There was no visible adverse morphological variation between the peptide treated cells (left panel: A, D, G), free siRNA, SO (middle Pane, B, E, H) and the cell only (CO) (right panel: C, F, I), in both the differentiated and undifferentiated stages.

Transfection of pre-confluent cells

Since hMADS cells are hard to transfect (Zaragosi et al., 2007), transfection of hMADS cells were undertaken to find out whether SMC could penetrate hMADS cells to deliver labeled double stranded siRNA cargo. Figures 3, 4, and 5 illustrate the results obtained. To investigate whether MPG could deliver labeled double-stranded siRNA into hMADS cells, 50% confluent hMADS cells were transfected for 4 h with 15 nM of SMC and overlying the controls with free FAM labeled-siRNA and cell only control consisting of MPG buffer (which served as control for cell viability and toxicity evaluation) after 24 and 48 h showed that MPG efficiently delivered the FAM-labeled siRNA across the membrane into the cytoplasm and in some cases into the nucleus or at the nuclear membrane of the treated hMADs cells. But MPG here showed dotted or punctuated fluorescent speckle within the cells transfected with SMC (Figure 3A, B and C). The control group overlaid with free siRNA did not show these green fluorescent speckles. The same observation was true for the cell only (CO) control group which did not have the speckle. However, there was background green fluorescence in the control sometimes highlighting the cell inside, which was instructive in determining whether the fluorescence was inside or outside the cell. In Figure 3, pre-confluent hMADS cells were transfected for 4 h with 15 nM, 5'-6-FAM labeled siRNA-MPG complex (C, D). Control cells were overlaid for 4 h with cell only control composed of MPG buffer (A, B). In (A) blue staining nuclei were apparent but with no fluorescent speckles as seen in C and D. Image B in FAM channel, contrary to C did not show the bright speckles but highlighted the background outlines of the cells. Therefore in C and D the bright green speckles showed the labeled siRNA construct. In D the MIP showed the speckle at the nuclear membrane as well as within the cytoplasm. The punctuate speckles were exclusively detected in the SMC cells indicating internalization of the siRNA by MPG. The image A shows bright green

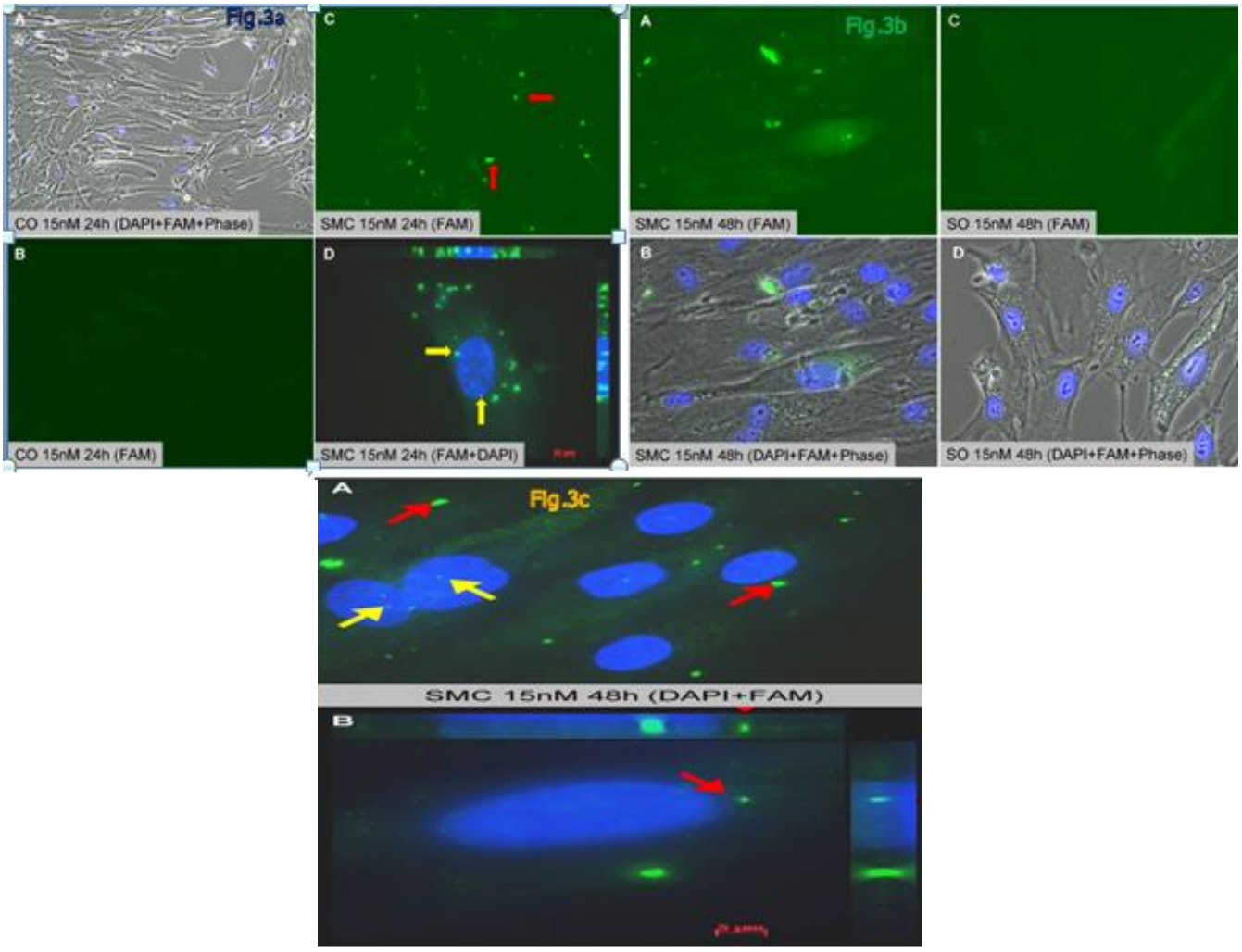

Figure 3. Fluorescence microscopy analysis 48 h after transfection of pre-confluent hMADS cells.

punctuate speckles or spots in 90% of the cells in the shown field of view. B) Maximum intensity projection MIP, of the fluorescent speckles (red arrow) lie at the plane of the nucleus within the cytoplasm.

Transfection of confluent cells (24 h post-transfection)

The hMADs cells are known to be hard-to–transfect (Zaragosi et al., 2006). This prompted the evaluation of transfection of confluent hMADS cells using MPG. The transfection of confluent hMADS was performed for 4 h and examined with Axioimager 24, 48 and 72 h post-transfection using concentration of 30 nM SMC, while the control groups were overlaid with free FAM-labeled siRNA (SO) and cell only (CO). Figure 4 shows transfection after 24 h. There were strong punctuated fluorescent speckles detected in SMC transfected cells in about 80 and 95% of the cells (Figure 4 A, B). The pattern of distribution of fluorescence was similar to the pattern observed in the pre-confluent cell stag which presented spotted or punctuate, non- homogenously distributed fluorescent appearance.

Intra-nuclear delivery

To show that the fluorescence speckles were localized in the nucleus of the cell, the maximum intensity projection image at the plane of the nucleus showed localization of the fluorescence within the nucleus (yellow arrow). The speckles were high-lighted by the yellow arrow in the nucleus, indicating that the speckles lie within the nucleus which might suggest nuclear delivery of the labeled siRNA (Figure 4, right panel).

Transfection of differentiating cells at day 3

Having observed punctuate fluorescent speckles of

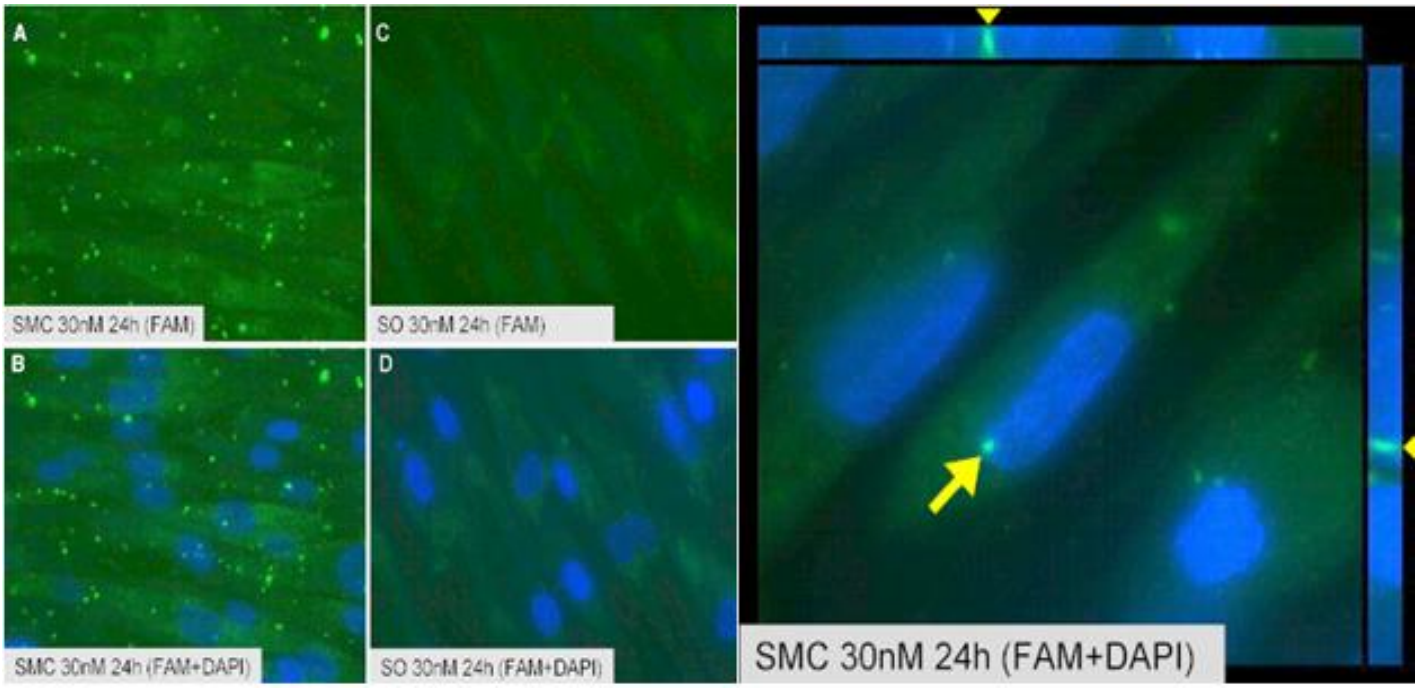

Figure 4. Fluorescence microscopy analysis 24 h after transfection of confluent hMADS cells. Cells were transfected for 4 h with 30 nM siRNA-MPG complex (SMC) (A and B). There were numerous punctuate fluorescent speckles seen in A (FAM channel) and B (Merged DAPI and FAM channels) in more than 95% of the cells. Control experiment: cells were overlaid for 4 h with free siRNA only (C, D). The control showed no fluorescent speckle. FAM channel (C) and merged DAPI and FAM channels (D). The nucleus was counter-stained with DAPI (blue). SMC, siRNA-MPG complex; SO, siRNA only; FAM, 6- carboxy fluorescein; DAPI, 4',6-diamidino-2-phenylindole.

siRNA-MPG complex in the cytoplasm and the nucleus in confluent cells, hMADs cells were differentiated and transfected on day 3 after induction to see whether a reproducible result will be obtained in the differentiating cells. Transfection was done with 30 nM SMC, and overlaid with SO and CO, for 4 h. The result was consistent with that observed in the pre-confluent and confluent stages, as indicated below. After 24 h post-transfection of differentiating hMADS cells at Day 3, there was evidence of punctuate speckles of the 5´-labeled siRNA by the MPG complex at the concentration of 30 nM SMC.

The maximum intensity projection MIP (Figure 5), of the fluorescent speckle of the labeled SMC were seen localized in the cytoplasm (red arrow) indicating that the speckles localized in the cytoplasm and the yellow arrows point at the speckle in the nucleus. This demonstrated that MPG was able to deliver siRNA into the cytoplasm and nucleus of differentiating hMADS after 24 h Post transfection.

siRNA-MPG complex is able to deliver double-stranded siRNA into differentiating hMADS cells (48 h post-transfection analysis)

To assess the ability of MPG to deliver siRNA into day 3 adipocyte differentiating cells, the cells were transfected for 4 h with 30 nM SMC and control overlaid with SO for 4 h. They were examined 48 h post-transfection using Axioimager. Figure 5A depicts the SMC in FAM channel (A) and the merged image of FAM, DAPI and Phase contrast (B).

Punctuated fluorescence speckles were observed in the SMC within the cytoplasm. In the control (Figure 5C and D), no fluorescence was observed in the control SO. Maximum intensity projection MIP in Figure 5 (left and down panels) demonstrated the presence of fluorescent speckles within the nucleus. In summary, there was both cytoplasmic and nuclear localization of the fluorescent speckles within the plane of the nucleus shown by the MIP image (Figure 5). The results show that MPG could deliver labeled siRNA into the cytoplasm and nucleus of Day 3 adipocyte differentiating cells.

Transfection of differentiating cells at Day 10

To investigate whether SMC was delivered into Day 10 adipocyte differentiating cells, the cells were transfected for 4 h with 30 nM SMC and control overlaid with SO for 4 h. They were examined 48 h post-transfection using Axioimager. Figure 6A and B depict the SMC in FAM channel (A) and the merged image of FAM, DAPI, and

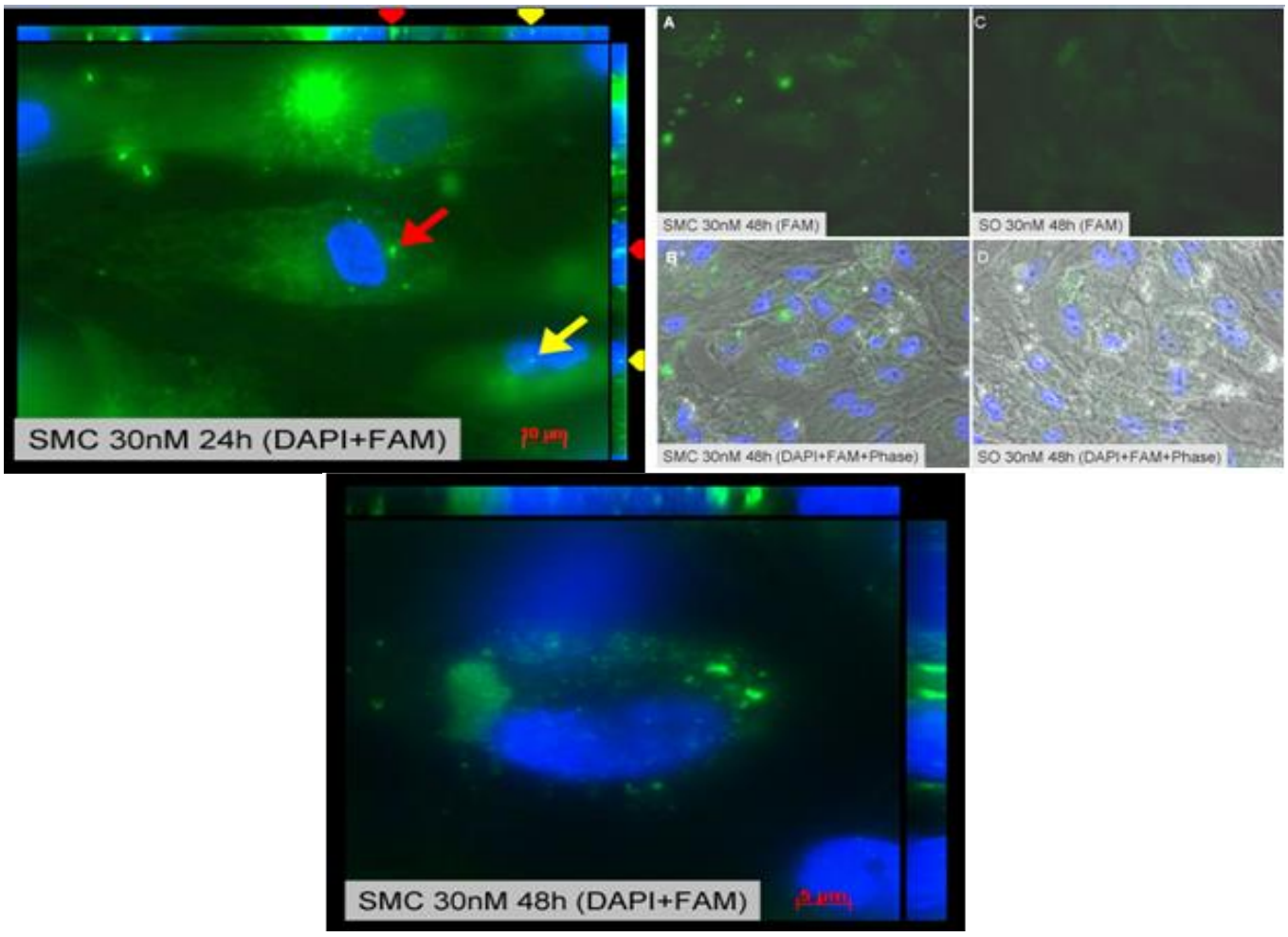

Figure 5. Fluorescence microscopy analysis 24 h after transfection of differentiating hMADS cells at day 3.

Phase contrast (B). Stronger fluorescence speckles were observed in the SMC within the cytoplasm that showed a more condensed pattern. The speckles were also detected within some fat droplets. Although, some background fluorescence was observed in SO, there was clear difference between the control and the SMC transfected cells. Maximum intensity projection (MIP) equally demonstrated the presence of the fluorescent speckle within the nucleus which suggests nuclear localization of the siRNA construct. In summary, there was both cytoplasmic and nuclear localization of the fluorescent speckle within the plane of the nucleus shown by the MIP image (Figure 6).

DISCUSSION

Divita and co-workers reported that MPG delivers active macromolecules permitting the control of the release of the cargo in the appropriate target subcellular compartment. Therefore, by tampering with the NLS sequence of MPG, delivery between the nucleus and the cytoplasm can be discriminated and MPG containing the NLS efficiently was reported to deliver promoter-directed siRNA into the nucleus to inhibit transcription. MPG has also been successfully applied for delivery of siRNA *in vivo* into mouse blastocytes (Crombez et al., 2007). The results presented here are in agreement with the earlier report by Simeoni et al. (2003) showing that MPG mediates cytoplasmic and nuclear delivery of siRNA in many cell types.

Viability

Effect of MPG on the viability of hMADS cells transfected with 15 and 30 nM of SMC and during proliferation and in adipocyte differentiation show that there was no observed cytotoxicity associated with the complex. There was no cell mortality or distortion of cell morphology due to the MPG complex. These results demonstrate that MPG while efficiently delivering siRNA into hMADS cells; it

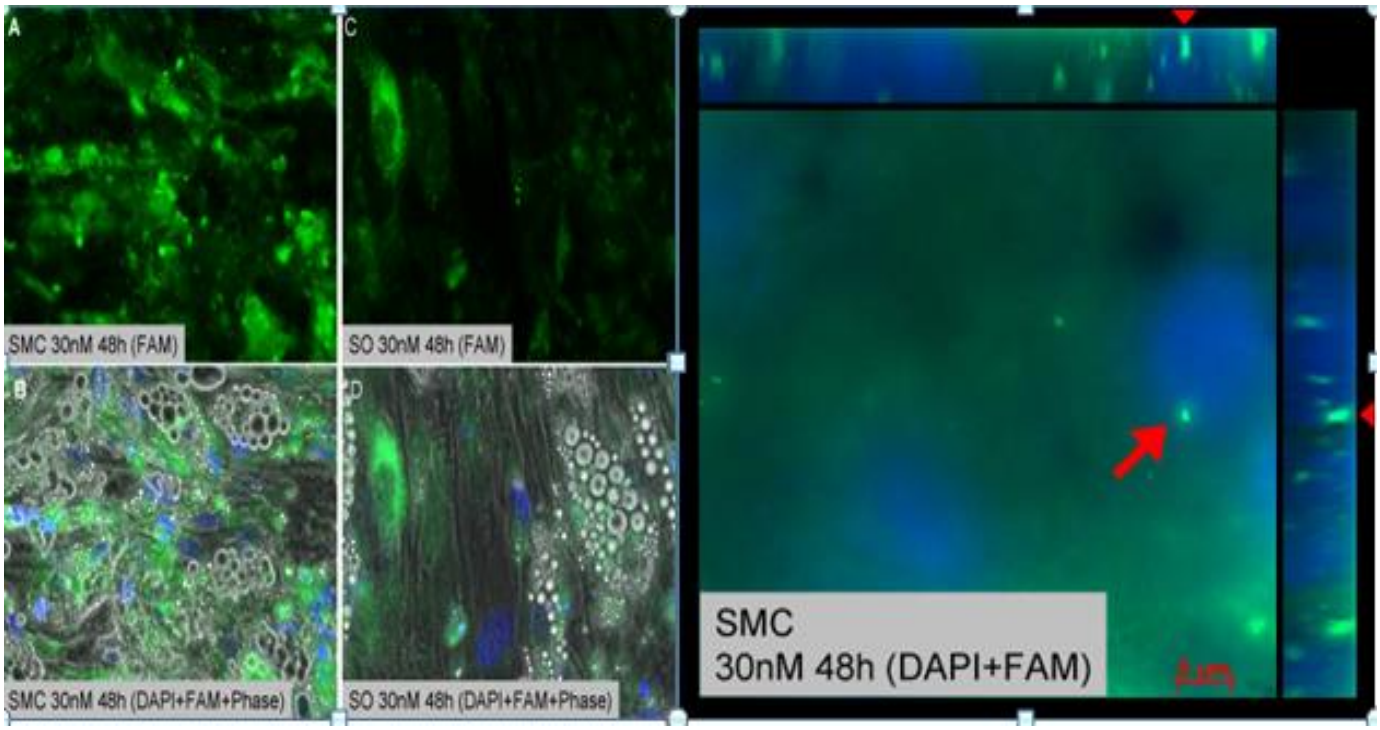

Figure 6. Fluorescence microscopy analysis 48 h post-transfection of adipocyte differentiating hMADS cells at day 10. Cells were transfected for 4 h with 30 nM siRNA-MPG complex (SMC). A (FAM) B (DAPI, FAM, Phase contrast); stronger fluorescence speckles were observed in the SMC that showed more condensed pattern in FAM channel. Control experiment (C, D): cells were overlaid for 4 h with free siRNA only. C: FAM. D: (merged image of DAPI, FAM, Phase contrast). The observed fluorescence was due to back-ground which were weaker than those of the SMC.

preserves the cell structural integrity. The complex had a mild effect on the cells. Most lipid based transfection agents according to Zaragosi lead to low cell viability or induce cell toxicity in the course of transfection. Unpublished report on hMADS also showed that electroporation of hMADS cell also led to high cell mortality. In view of these, MPG presents a mild transfection possibility in hMADS cells.

Differentiation

Data from this study also showed that differentiation of cells into adipocyte was not hindered by the transfection of hMADS cells with MPG-siRNA complex. There was no evidence to suggest that MPG adversely affected differentiation of hMADS cells to adipocytes as there were differentiated cells with oval shaped lipid droplets in both SMC treated and the cell only control (Figure 2 lower panel). The siRNA used in the study was a scrambled control not targeting any specific messenger RNA, so it was not meant to affect differentiation process.

siRNA delivery in proliferation and in adipocyte differentiation

This study was designed to evaluate the MPG mediated delivery of Double stranded siRNA into hMADS cells. hMADS cells have been shown to be difficult to transfect with plethora of conventional lipid based transfection system (Crombez et al., 2007). Zaragosi and colleagues reported very low transfection efficiency coupled with high cell mortality in respect of transfection with lipid based reagents but they reported high efficiency of delivery with nucleofection for both stable and transient integration of transgenes into hMADS cells. The results of this study show that MPG successfully delivered double-stranded 5' 6-FAM labeled siRNA into hMADS cells in pre-confluent, confluent and adipocyte differentiated hMADS cells. Delivery efficiency as monitored by detection of fluorescence in the MPG transfected cells was significantly higher than in the control groups. The transfection efficiency of the delivered siRNA was above 90 to 95% in the confluent hMADS cells 24 h post-transfection, the retention of fluorescence 48 and 72 h were however weak and was between 50 to 60% efficiency. Using Olympus CKX41 microscope, evidence of siRNA delivery was apparent as indicated by punctuate fluorescence speckles after 4 h post-transfection and this indicates that the internalization was rapid. Fluorescence was detectable up to 6 days post-transfection in confluent stage. However, the exact localization of the observed fluorescence in this case could not be resolved, as such could not be said to be

inside or outside the cell due to the limitation of the microscope used. Fluorescence in the pre-confluent stage was detected 24 and 48 h post-transfection, at a concentration of 15 nM of SMC. In the confluent and differentiated stages, detection was after 24 to 72 h at a concentration of 30 nM of SMC.

In the Day 10 adipocyte differentiated cells, stronger fluorescence speckles were detected in the SMC within the cytoplasm that showed a more condensed pattern. The speckles were also observed within some fat droplets. However, some background fluorescence was present in SO that could not be readily explained. This background fluorescence might be due to auto-fluorescence of the lipid droplets that were well developed in the day 10. However, there was clear difference between the control and the SMC transfected cells. Maximum intensity projection MIP (Figure 6) equally demonstrated the presence of the fluorescent speckle within the nucleus which suggests nuclear localization of the siRNA construct. In summary, there was both cytoplasmic and nuclear localization of the fluorescent speckle within the plane of the nucleus shown by the MIP image. The results show that MPG could deliver labeled siRNA into the cytoplasm and nucleus of Day 10 adipocyte differentiated cells. The MIP images showed the fluorescence speckles within the nucleus as well as within the cytoplasm at the plane of the nucleus, demonstrating internalization of double-stranded siRNA mediated by MPG.

Pattern of delivery

The delivered siRNA was detected as brilliant punctuated fluorescent speckles in the green channel that was not homogenously distributed in the cells. This observation was in line with the observation of Veldhoen et al. (2006) who worked on similar peptide but with a mutation at the nuclear localization sequence, called MPGα. The bright speckles are thought to mark points of entry of the labelled probes into the cells. But contrary to their observation, MPG efficiently delivered siRNA into the cytoplasm as well as nucleus of hMADS cells. Gregory et al. (2007) also observed punctuate and non-homogenously distributed pattern of delivery of cargo with TAT peptide. It then follows that the punctuate spotted fluorescent speckle observed in the SMC transfected cells were indicative of successful internalization of siRNA in the cytoplasm, nuclear membrane and in the nucleus as observed in the results presented in this study. The pattern of internalization was contrary to most convectional transfection reagents which usually follow homogenous distribution pattern in most cell types but MPG here showed dotted or punctuated fluorescent speckles within the cells transfected with SMC. The control group overlaid with free siRNA did not show these green fluorescent speckles. The same observation was true for the cell only (CO) control group which did not have the speckle. However, there was background green fluorescence in the control sometimes highlighting the cell inside, which was instructive in determining whether the fluorescence was inside or outside the cell.

The result of this work demonstrated that Nucleofection is not the only non-viral based vector that has the ability of efficiently transfecting hMADS cells as observed by Zaragosi and colleagues (Zaragosi et al., 2007). The benefit of nucleofection was the achievement of stable expression of a transgene in hMADS cells. However, the internalization of siRNA observed with MPG is transient as fluorescence intensity weakened with time but was detectable at 6 days post-transfection.

In summary, this study could demonstrate that MPG efficiently internalized labeled double-stranded siRNA cargo into the difficult transfect hMADS cells in proliferation, confluency and adipocyte differentiating hMADS cells. That MPG mediated nuclear localization of siRNA constructs supports previous observation (Crombez et al., 2007; Simeoni et al., 2005). More interesting is that transduction of non-dividing cell has been the preserve of the lentiviral system which is limited by their high bio safety requirement and as such are kept out of routine use of many laboratory. However, lentiviral systems are used for stable integration into the genome of cells while CPPs can only be used for transient internalization. MPG did not lead to cell mortality as indicated by the normal cell morphology exhibited by the SMC transfected cells, which was comparable to the morphology of the siRNA only (SO) and cell only (CO) overlaid control groups. This observation makes MPG an interesting vector system for translocation of small peptides and nucleic acids into the cell combining robust efficiency of delivery with mildness to the cell structure integrity.

Conclusion

In conclusion, this study shows that MPG efficiently delivered double-stranded siRNA into the nucleus and cytoplasm of hMADS cells. The efficiency of delivery was between 90 to 95% in the pre-confluent and confluent stages 24 h post-transfection, 84% in Day 3 differentiating cells 24 h post-transfection. The efficiency of the delivery 48 and 72 h post-transfection was between 50 to 60%. The cell viability was not adversely affected while differentiation of hMADS cells proceeded normally. These results show that MPG is a very effective and robust non-viral based transfection agent that efficiently translocates hMADS cells to transiently deliver siRNA. The integration was transient as it lasted for about six days.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

This work was done at the Institute for Genomics and Bioinformatics, TU Graz, Austria under the guidance of Dr. Scheideler and Dr. Dietmar helped with microscopy. Thanks to the Afro-asian institute, Austria for the support.

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