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ARTICLE

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Mechanisms of unprimed and dexamethasone-primed nonviral gene delivery to human mesenchymal stem cells

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Abstract

Human mesenchymal stem cells (hMSCs) are under intense study for applications of cell and gene therapeutics because of their unique immunomodulatory and regenerative properties. Safe and efficient genetic modification of hMSCs could increase their clinical potential by allowing functional expression of therapeutic transgenes or control over behavior and differentiation. Viral gene delivery is efficient, but suffers from safety issues, while nonviral methods are safe, but highly inefficient, especially in hMSCs. Our lab previously demonstrated that priming cells before delivery of DNA complexes with dexamethasone (DEX), an anti-inflammatory glucocorticoid drug, significantly increases hMSC transfection success. This work systematically investigates the mechanisms of hMSC transfection and DEX-mediated enhancement of transfection. Our results show that hMSC transfection and its enhancement by DEX are decreased by inhibiting classical intracellular transport and nuclear import pathways, but DEX transfection priming does not increase cellular or nuclear internalization of plasmid DNA (pDNA). We also show that hMSC transgene expression is largely affected by pDNA promoter and enhancer sequence changes, but DEX-mediated enhancement of transfection is unaffected by any pDNA sequence changes. Furthermore, DEX-mediated transfection enhancement is not the result of increased transgene messenger RNA transcription or stability. However, DEX-priming increases total protein synthesis by preventing hMSC apoptosis induced by transfection, resulting in increased translation of transgenic protein. DEX may also promote further enhancement of transgenic reporter enzyme activity by other downstream mechanisms. Mechanistic studies of nonviral gene delivery will inform future rationally designed technologies for safe and efficient genetic modification of clinically relevant cell types.

KEYWORDS
dexamethasone, glucocorticoid, human mesenchymal stem cells, nonviral gene delivery, priming, transfection

1 | INTRODUCTION

Because of their unique roles in wound healing (Chamberlain, Fox, Ashton, & Middleton, 2007), trophic tissue support (Maxson, Lopez, Yoo, Danilkovitch-Miagkova, & Leroux, 2012; Singer & Caplan, 2011), and immunomodulation (Ren et al., 2008), along with their differentiation ability (Baksh, Song, & Tuan, 2004) and immune-privileged status (Jacobs, Roobrouck, Verfaillie, & Van Gool, 2013; Ryan, Barry, Murphy, & Mahon, 2005), human mesenchymal stem cells (hMSCs) are under study for applications of cell and gene

therapeutics (D'souza et al., 2015), as well as tissue engineering and regenerative medicine (Richardson et al., 2010). In addition to their natural healing and regenerative potentials, genetic modification of hMSCs could allow for targeted tissue tropism and delivery of recombinant factors in hMSC therapies.

Genetic modification by viral transduction is efficient, but suffers from safety issues related to immunogenicity and insertional mutagenesis (Nayerossadat, Maedeh, & Ali, 2012), as well as small transgene capacity and difficult design and scale-up (Yin et al., 2014). Nonviral methods that overcome many of the shortcomings of viral delivery suffer from low efficiency, especially in hMSCs. For example, optimized transfection of hMSCs with the commercially available Lipofectamine 2000 (Invitrogen, Carlsbad, CA) results in only 10–30% of cells successfully transfected (Hoare et al., 2010; Madeira et al., 2010; Peng, Gao, Xue, Huang, & Zhuo, 2013; Ribeiro et al., 2010), and the ubiquitously used 25 kDa branched polyethylenimine is only able to achieve about 20% transfection (Ahn et al., 2008; Peng et al., 2013; W. Wang et al., 2011). Furthermore, both lipid- and polymer-based transfection reagents are associated with significant toxicity in hMSCs (Corsi, Chellat, Yahia, & Fernandes, 2003; King, Kouris, Choi, Ogle, & Murphy, 2012). The low efficiency and high toxicity of nonviral gene delivery limits the translation of genetically modified hMSCs to clinical applications. Therefore, more efficient and less toxic nonviral gene delivery methods to hMSCs are needed to advance their potential clinical applications. To improve nonviral gene delivery, our group has focused on elucidating the biology of transfection to develop new strategies for improved efficiency.

Our previous work has sought to improve and better understand the biology of nonviral gene delivery by pharmacologically 'priming' cells for increased transgene expression (Nguyen, Beyersdorf, Riethoven, & Pannier, 2016) through modulation of relevant molecular pathways that are important to the biological processes involved in gene delivery (Martin, Plautz, & Pannier, 2015a; Martin, Plautz, & Pannier, 2015b). Specifically, we investigated anti-inflammatory glucocorticoid (Gc) drugs as priming candidates in hMSCs due to reports in other cell types that Gc can increase transfection efficiency by increasing plasmid DNA (pDNA) nuclear internalization or reducing the inflammatory response to transfection (Braun et al., 1999; Kim, Kim, Bae, Choi, & Lee, 2009). In hMSCs, derived from bone marrow stromal cells (BMSCs) of multiple donors, we showed that 90 to 360 nM dexamethasone (DEX), a Gc drug, delivered 0–30 min before delivery of pDNA lipoplexes increased the transgenic luciferase activity about 10-fold, increased transgenic enhanced green fluorescent protein (EGFP) mean fluorescence intensity of transfected cells about two-fold, increased transfection efficiency about three-fold (i.e., percent of EGFP + transfected cells), increased duration of transgene expression, and ameliorated transfection-induced metabolic decline, all while retaining differentiation capacity (Kelly, Plautz, Zempleni, & Pannier, 2016). Others have reported similar enhancement of transfection using steroids in other cell types. For example, priming of various cell types with steroids (i.e. glucocorticoids, estrogens, and progesterone) has generally shown moderate increases in gene delivery to a wide variety of cell types (i.e., 2–4-fold transgene expression increases; Bernasconi et al., 1997; Braun et al., 1999; Chen, Shank, Davis, & Ziady, 2011; Choi & Lee, 2005; Jain, Seth, & Gewirtz, 1999; Koster et al., 2002; Köster et al., 2006; Lin et al., 2003; Nair, Rodgers, & Schwarz, 2002), with a few exceptions (i.e., 10-fold increase in myoblasts; Braun et al., 1999) and 900-fold increase in bronchial epithelial cells (Wiseman, Goddard, & Colledge, 2001).

This work investigates, for the first time, the molecular mechanisms of transfection with and without DEX-priming in hMSCs derived from multiple donors of both BMSCs and adipose tissue derived (AMSCs). Since DEX-priming of hMSC transfection is mediated by binding of the glucocorticoid receptor (GR; Kelly et al., 2016), which makes use of similar intracellular transport mechanisms as transfected pDNA does (Davies, Ning, & Sánchez, 2002; Dhanoya, Wang, Keshavarz-Moore, Fassati, & Chain, 2013; Echeverria et al., 2009; Galigniana, Harrell, O'Hagen, Ljungman, & Pratt, 2004; Harrell et al., 2004; Lachish-Zalait et al., 2009), and GR modulates gene expression related to anti-inflammatory and stress pathways (Ratman et al., 2013), we analyze nonviral gene delivery barriers and pathways in hMSCs including internalization, cytoplasmic transport, nuclear translocation, transgene transcription, translation, and cellular stress response, along with the effects of DEX-priming on each of these barriers and pathways. We also explore the effects of different pDNA regulatory sequences on transgene expression and DEX-priming. Studying transfection, pharmacological priming, and the molecular mechanisms involved provides key insights into the gene delivery process to aid in the rational design of new gene delivery technologies and simple priming strategies for hMSCs and other clinically relevant cell types.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Bone marrow derived hMSCs (BMSCs) were purchased at passage 2 from Lonza (Walkersville, MD) or acquired at passage 1 from the Texas A&M Institute for Regenerative Medicine Health Science Center College of Medicine (Bryan, TX). All BMSCs were positive for CD29, CD44, CD105, and CD166 cell surface markers and negative for CD14, CD34, and CD45. Adipose derived hMSCs (AMSCs) were purchased at passage 1 from Lonza and were positive for CD13, CD29, CD44, CD73, CD90, CD105, CD166, and negative for CD14, CD31, CD45 cell surface markers. All human cells were acquired with informed consent using established ethical methods approved by appropriate authorities. All experiments and methods were performed in accordance with relevant guidelines and regulations. All experimental protocols were approved by the University of Nebraska-Lincoln Institutional Biosafety Committee. See Supporting Information Table S1 for BMSC and AMSC donor information. All cells were expanded and cultured in minimum essential medium α (MEMα; Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 6 mM L-glutamine (Gibco), and 1% penicillin–streptomycin (10,000 U/ml; Gibco) and incubated at 37°C with 5% CO_2. At 80% confluence cell media was removed and

cells were washed with 1× phosphate-buffered saline (PBS) and dissociated with 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA; Gibco), then an equal volume of growth medium was added and cells were pelleted to remove trypsin–EDTA, resuspended, and counted with trypan blue staining and a hemocytometer before diluting in growth medium (for transfection studies as described next) or medium with 5% dimethyl sulfoxide (DMSO) to 6×10^4 cells/ml for freezing in 1 ml aliquots stored in liquid nitrogen.

For transfection experiments, after dissociation and counting as described above, hMSCs were seeded into 48 or 96 well plates (Corning Life Sciences, Corning, NY) or T25 flasks (Corning), at passages 3 through 5, at 6,000 cells/cm^2 and allowed to adhere until about 80% confluence, then transfected as described below.

2.2 | Priming reagents

Dexamethasone (DEX) was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in 100% ethanol (EtOH) and stored at −20°C. For transfection studies, DEX was diluted in EtOH and delivered to cell culture media at 150 nM, at <1% total media volume, 25 min before addition of DNA lipoplexes. EtOH was delivered as a vehicle control (VC) in place of DEX. The pharmacological inhibitors ivermectin (IV), importazole (IM), and nocodazole (Noco) were purchased from Sigma-Aldrich and ciliobrevin D (Cilio) was purchased from Merck Millipore (Billerica, MA). These inhibitors were dissolved in DMSO and stored at −20°C. Inhibitor stocks were diluted in EtOH and delivered to cell-culture media at designated concentrations in <1% total media volume, 1 hr before addition of DEX or EtOH VC. An appropriate corresponding amount of DMSO was diluted in EtOH and delivered as a VC in place of inhibitors where specified. In all inhibitor experiments, to remove inhibitors and manage toxicity, media was replaced 3 hr after addition of lipoplexes.

2.3 | Transfections

pDNA complexed with Lipofectamine transfection reagents (Invitrogen, Carlsbad, CA) was delivered to the cell media in well plates, after priming of cells as described above. pEGFP-Luc plasmid DNA was purchased from Clontech (Mountain View, CA), and the plasmid encodes a fusion protein of EGFP and firefly luciferase (Luc) under direction of a cytomegalovirus (CMV) promoter and containing simian virus 40 (SV40) enhancer. Where specified, modifications of the pEGFP-Luc plasmid were cloned and synthesized by Genscript (Piscataway, NJ). All plasmids are nonintegrating, producing transient transfection. Plasmids were purified from *Escherichia coli* bacteria using Qiagen (Valencia, CA) reagents and stored in Tris-EDTA (TE) buffer solution (10 mM Tris, 1 mM EDTA; pH 7.4) at −20°C. Lipoplexes were formed with Lipofectamine LTX (LF-LTX) or Lipofectamine 3000 (LF-3000; Invitrogen) in serum free Opti-MEM media (Invitrogen) following the manufacturer's instructions and as noted in the text. Amount of DNA and DNA:lipid ratios were optimized to allow for high transfection and low toxicity. All transfections were performed with 0.2 μg pDNA/cm^2 of cell growth area and DNA:lipid ratio of 1:2 complexed with LF-3000 following the manufacture's protocol. In inhibitor studies, BMSCs were transfected identically as above, but with LF-LTX.

2.4 | Transfection assessment

Fluorescence and phase microscopy was conducted 48 hr after lipoplex delivery to qualitatively assess cell health and EGFP expression using a Leica DMI 3000B fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). After microscopy, cells were washed with PBS and lysed with 200 μl per well of 1× reporter lysis buffer (Promega, Madison, WI) and stored at −80°C. Transgenic luciferase activity levels were quantified by measuring luciferase luminescence in relative light units (RLUs) with a luciferase assay kit (Promega) and a luminometer (Turner Designs, Sunnyvale, CA). RLUs were normalized to total protein amount determined with a Pierce BCA protein colorimetric assay (Pierce, Rockford, IL) using a DU730 UV-Vis spectrophotometer (Beckman-Colter, Brea, CA) to measure absorbance at 562 nm. Plotted fold changes for an experimental condition were calculated by dividing each treatment condition replicate value by each control replicate value.

2.5 | Luciferase quantitative western blot analysis

Forty-eight hours after BMSC and AMSC transfection with LF-3000 complexed with pEGFP-Luc, as described above, media was removed and cells were washed once with 1× PBS before dissociating with 0.25% Trypsin–EDTA and lysing in NP-40 buffer. Protein concentration was determined with the Pierce bicinchoninic acid protein colorimetric assay. Samples were denatured and reduced in NuPage® LDS sample buffer 4× and sample reducing agent (Invitrogen) at 70°C. Equal masses of protein were resolved on NuPAGE™ 10% Bis–Tris Protein Gels run in XCell SureLock™ Mini-Cell Electrophoresis System (Thermo Fisher Scientific). Protein was transferred to Immobilon-FL polyvinylidene fluoride membranes and total protein was stained with REVERT™ total protein stain (Li-Cor, Lincoln, NE) following the manufacturer's protocol. Membranes were washed, blocked, and probed for luciferase with rabbit polyclonal primary antibody (1:1000; Sigma-Aldrich) and goat antirabbit IgG (H + L) 800 CW secondary antibody (1:10,000; Li-Cor). Visualization and quantification was carried out with Odyssey CLx Scanner and software (Li-Cor) normalizing to total protein.

2.6 | Plasmid internalization studies

To quantify plasmid internalization into cells and nuclei, hMSCs were seeded into T-25 flasks in triplicate, then DEX-primed and transfected with 5.26 μg pEGFP-Luc complexed with LF-3000 as described above. After 48 hr, cells were washed with 1× PBS and dissociated as described above. Cells were washed again with 1× PBS and one-third of the cell suspension was frozen in 1× reporter lysis buffer for quantification of plasmids within whole cells. The remaining two-thirds of cells had their nuclei isolated by lysing cells in sucrose buffer I (0.32 M sucrose, 3 mM

$CaCl_2$, 2 mM MgCl, 0.1 mM EDTA, 10 mM Tris Cl, 1 mM dithiothreitol [DTT], 0.5% vol/vol Triton), passing lysate through a 100 μm cell strainer, layering lysate over the denser sucrose buffer II (2 M sucrose, 5 mM MgCl, 0.1 mM EDTA, 10 mM Tris Cl, 1 mM DTT), and centrifugation at 22,000*g* for 15 min. Nuclei were washed with PBS before freezing in 1× reporter lysis buffer. Nuclei isolation was confirmed by trypan blue stain and microscopy comparing cell and nuclei samples (data not shown). DNA was isolated from cells and nuclei using 25:24:1, phenol/chloroform/isoamyl alcohol (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocol and suspended in TE buffer. DNA quality was confirmed by spectrophotometric absorbance ratio of 260/280 nm from a NanoDrop (Thermo Fisher Scientific; data not shown). Quantification of nuclear plasmids was performed as previously described (Cohen, van der Aa, Macaraeg, Lee, & Szoka, 2009) using quantitative real-time polymerase chain reaction (qRT-PCR) on a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific) with Power SYBR Green Master Mix (Thermo Fisher Scientific) to determine the number of plasmid copies relative to a pDNA standard curve. Plasmid copy number was normalized to the number of cells relative to a synthetic ACTA1 sequence standard curve. ACTA1 was assumed to be a double copy gene, therefore every two copies of ACTA1 represent a single cell in the experiment. Synthetic ACTA1 sequence and primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA). See Supporting Information Table S2 for sequences of primers and synthetic ACTA1.

2.7 | Transgene mRNA quantification studies

To quantify relative mRNA transcript copy numbers, 48 hr after BMSC and AMSC transfection with LF-3000 as described above, cells were lysed and fractionated with TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Aqueous RNA was purified using a RNeasy micro kit (Qiagen, Hilden, Germany). RNA was treated with DNAse I (Thermo Fisher Scientific) and reverse-transcribed using iScript cDNA kit (Bio-Rad, Hercules, CA). qRT-PCR for relative EGFP mRNA transcript copy number was performed as described above and calculated by $\Delta\Delta C_t$ method normalizing to endogenous control RPL13A. See Supporting Information Table S2 for primer sequences (IDT).

2.8 | Total protein synthesis assay

Total protein synthesis was quantified with the protein synthesis assay kit (Cayman Chemical, Ann Arbor, MI) following the manufacturer's protocols. Twenty four hours after BMSC and AMSC transfection with LF-3000 complexed with pDNA expressing luciferase transgene, as described above, media was removed and cells were washed once with 1× PBS before addition of media containing cell-permeable, alkyne-containing, puromycin analog *O*-propargyl-puromycin (OPP) to be incorporated into translating proteins. Cells were incubated for 12 hr at 37°C with 5% CO_2 before fixing and labeling OPP with 5-FAM-Azide and staining DNA with 17.5 μg/ml Hoechst 33342 (Sigma-Aldrich). Five FAM-Azide and Hoechst fluorescence were measured with Synergy H1 plate reader (BioTek, Winooski, VT), with excitation/emission settings of 483/525 and 355/464 nm, respectively, and gain setting of 100. Nine measurements were taken per well in a 3 × 3 array equally spaced within the well, from which mean intensities were calculated. The 5 FAM-Azide relative fluorescent units (RFU) from each well was divided by the corresponding Hoechst RFU to normalize to cell number. Corrected values for each well were calculated by subtracting the average normalized values of cells not treated with OPP, but stained with 5 FAM-Azide and Hoechst.

2.9 | Apoptosis assay

Forty eight hours after BMSC and AMSC transfection with LF-3000 complexed with pDNA expressing luciferase transgene, as described above, media was removed and cells were washed once with 1× PBS before staining nuclei with 17.5 μg/ml Hoechst 33342 (Sigma-Aldrich) and using an apoptosis kit with annexin V Alexa Fluor 568 (Thermo Fisher Scientific) to stain apoptotic cells following the manufacturer's protocol. Alexa Fluor 568 and Hoechst fluorescence were measured with Synergy H1 plate reader (BioTek), with excitation/emission settings of 578/603 and 355/464 nm, respectively, and gain setting of 100. Nine measurements were taken per well in a 3 × 3 array equally spaced within the well, from which mean intensities were calculated. Alexa Fluor 568 RFU from stained apoptotic cells in each well were divided by the corresponding Hoechst RFU to normalize to cell number. Corrected "Relative Apoptosis" values for each well were calculated by subtracting the average normalized values of cells not treated with annexin V Alexa Fluor 568, but stained with Hoechst. Comparative analyses were made between conditions using one-way analysis of variance (ANOVA) with Tukey's posttest.

2.10 | Statistical analysis

All experiments were performed in triplicate ($n = 3$) on duplicate days. Certain donors were used for certain experiments, such that at least two donors were tested for each experiment. Given limited availability of cells, it was not possible to test all donors in all experiments. All values are reported as mean ± standard error of the mean. Comparative analyses were completed using one-way ANOVA with Tukey's posttest or unpaired *t* test. Statistical difference was considered at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). Statistics and fold changes highlighted within figures are between treated versus control groups. All statistics were evaluated using Prism GraphPad software (GraphPad Software, Inc., La Jolla, CA).

3 | RESULTS

3.1 | DEX-priming increases both transgenic luciferase expression and activity in hMSCs

BMSCs and AMSCs derived from multiple human donors (denoted as Donors number or D#; Supporting Information Table S1) were

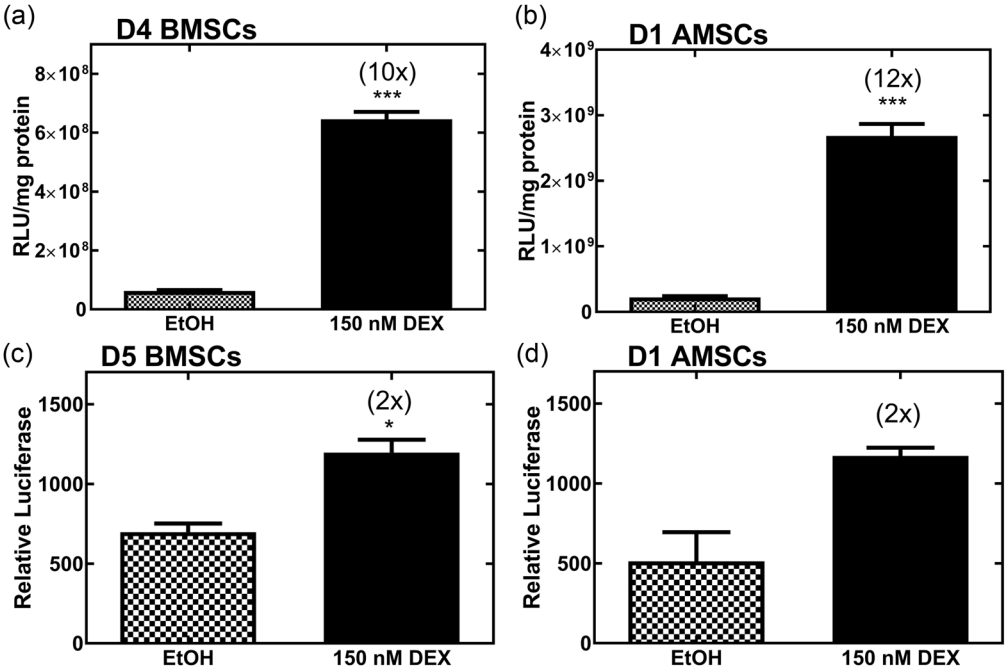

FIGURE 1 Dexamethasone (DEX)-priming significantly increases hMSC transgenic luciferase amount and further increases transgenic luciferase activity. hMSCs were primed with 150 nM DEX 25 min before transfection with Lipofectamine-3000 plasmid DNA complexes, and lysed for analysis after 48 hr. DEX-treated hMSCs displayed about 10-fold increases in the luciferase activity, normalized to total cellular protein, over ethanol (EtOH) treated cells in (a) Donor 4 (D4) BMSCs and (b) D1 AMSCs. In contrast, DEX only increased transgenic luciferase amount by about two-fold relative to EtOH, as quantified by western blot analysis, in transfected (c) D5 BMSCs, and (d) D1 AMSCs. Luciferase activity data plotted as mean ± *SEM* ($n = 3$) of luciferase luminescence relative light units per mg of total protein (RLU/mg protein). Relative luciferase amount data from western blot is normalized to total protein and plotted as mean ± *SEM* ($n = 2$). Asterisks (*) denote significance to EtOH conditions (*$p \leq 0.05$; ***$p \leq 0.001$). Fold change increase over EtOH conditions shown in parentheses. AMSCs: adipose tissue derived from mesenchymal stem cells; BMSCs: bone marrow derived from mesenchymal stem cells; hMSCs: human mesenchymal stem cells; *SEM*: standard error of mean

'primed' for transfection by treating with 150 nM DEX or EtOH VC 25 min before delivery of LF-3000 complexed with pEGFP-Luc plasmid (Supporting Information Figure S1) expressing a fusion protein of EGFP and luciferase. Treatment with DEX increased transgenic luciferase activity about 10-fold over (VC) depending on donor and tissue source, measured in luminescence relative light units normalized to total protein (RLU/mg protein; Figure 1). The increase in transgenic luciferase activity in DEX-primed cells compared to VC primed cells was statistically significant in all cells tested ($p \leq 0.001$; Figure 1a,b). In contrast, DEX-priming only increased transgenic luciferase amount by about two-fold over VC, as quantified by western blot analysis, normalized to total protein (Figure 1c,d). These DEX-induced transgene increases are consistent with our previous results in which the transgenic luciferase activity was increased 10-fold and EGFP mean fluorescent intensity (directly proportional to amount of EGFP amount) was increased two-fold in hMSCs transfected with the same pEGFP-Luc (Kelly et al., 2016), suggesting DEX-priming increases transgenic reporter amount whereas further increasing transgenic reporter enzymatic activity. While absolute transgene activity levels vary between hMSCs derived from different donors, DEX-priming consistently increased transgenic reporter amount and further increased transgenic reporter activity in all cells and donors tested. After validating our DEX-priming strategy for enhanced nonviral gene delivery to hMSCs, we next aimed to investigate the molecular mechanisms important to hMSC transfection and DEX-priming.

3.2 | DEX does not increase pDNA cellular OR nuclear internalization

To better understand the biology of nonviral gene delivery and DEX-priming in hMSCs, we studied classical intracellular barriers involved in transfection. To evaluate DEX-priming's effect on cellular and nuclear internalization of pDNA, hMSCs were pre-treated with 150 nM DEX or EtOH VC and transfected with pEGFP-Luc complexed with LF-3000. After 48 hr, isolated nuclei and whole cells were lysed and the number of internalized plasmids per cell was determined by qPCR against pDNA and endogenous genomic sequences (Figure 2). In all donors, the application of DEX did not increase the number of plasmids internalized within whole cells or nuclei, relative to EtOH VC (Figure 2). The mean number of plasmids internalized within DEX-treated whole cells and nuclei ranged between unaffected to about two-fold lower when compared to EtOH VC conditions, in all donors (Figure 2a–d). DEX significantly decreased pDNA internalized within cells in D4 BMSCs (Figure 2b; $p \leq 0.05$).

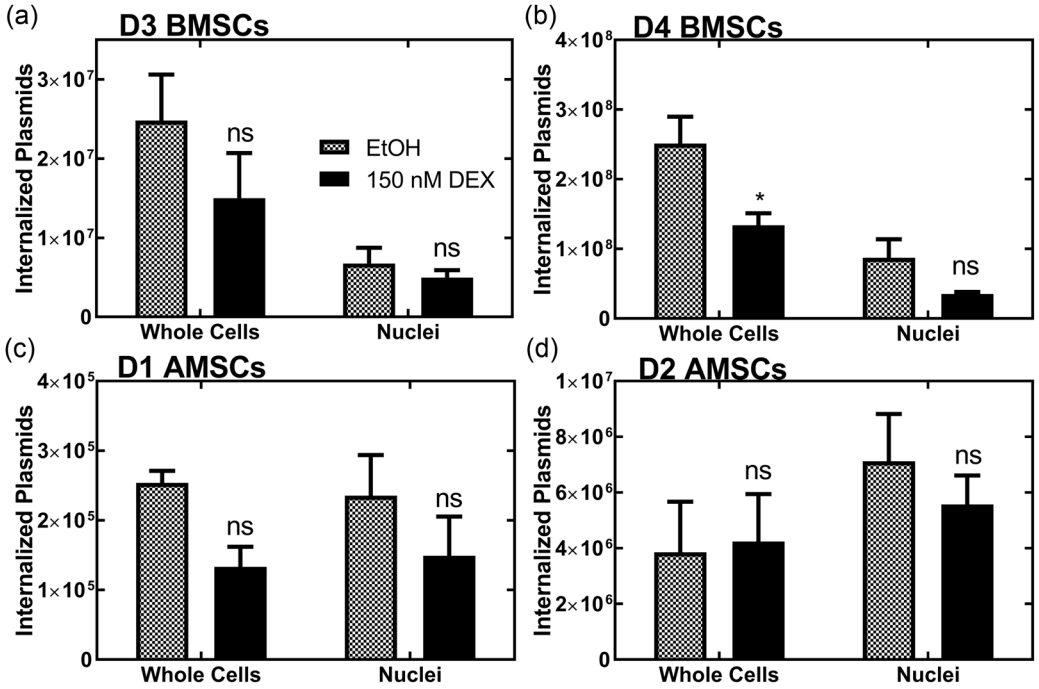

FIGURE 2 Dexamethasone (DEX)-priming does not increase plasmid DNA cellular or nuclear internalization in hMSCs. hMSCs were primed with 150 nM DEX or ethanol (EtOH) 25 min before transfection with Lipofectomine-3000 plasmid DNA complexes. After 48 hr, whole cells and isolated nuclei were lysed and the number of internalized plasmids per cell and per nuclei was determined by qPCR. DEX treatment did not increase the number of plasmids internalized by whole cells or nuclei in (a) Donor 3 (D3) BMSCs, (b) D4 BMSCs, (c) D1 AMSCs, (d) or D2 AMSCs. Data is plotted as means ± *SEM* ($n = 3$). Asterisks denote significance to EtOH conditions (*$p \leq 0.05$). AMSCs: adipose tissue derived from mesenchymal stem cells; BMSCs: bone marrow derived from mesenchymal stem cells; hMSCs: human mesenchymal stem cells; qPCR: quantitative polymerase chain reaction; *SEM*: standard error of mean

3.3 | Cytoplasmic transport inhibition moderately decreases enhancement by DEX

Since both pDNA and the glucocorticoid receptor (GR), the receptor bound by DEX and other Gcs to induce their effects by genomic and nongenomic mechanisms (Strehl et al., 2011), are known to utilize similar cytoplasmic transport mechanisms (i.e., dynein motor protein on microtubules; Davies et al., 2002; Dhanoya et al., 2013; Echeverria et al., 2009; Galigniana et al., 2004; Harrell et al., 2004; Lachish-Zalait et al., 2009) and GR binding is required for DEX-mediated transfection enhancement in hMSCs (Kelly et al., 2016), the role of cytoplasmic transport mechanisms in hMSC transfection and priming by DEX was investigated by performing transfection studies with specific inhibitors of cytoplasmic transport along with DEX-priming (Figure3). To inhibit polymerization of microtubules, hMSCs were treated with 1 μM nocodazole (Noco; Lindberg, Fernandez, Ropp, & Hamm-Alvarez, 2001). To inhibit dynein motion, hMSCs were treated with 12 μM Cilio (Cardarelli et al., 2016). One hour after inhibitor treatment, hMSCs were primed with 150 nM DEX, 25 min before transfection with pDNA complexed with either LF-LTX or LF-3000. After 3 hr, media was replaced with fresh media containing no drug and transgenic luciferase activity was assayed 48 hr after transfection. Control cells were treated with EtOH in lieu of inhibitors and/or DEX. Fold change for each condition was calculated relative to the transgenic luciferase activity of cells treated with no inhibitor and no DEX (−/−/−). Both Noco and Cilio in the absence of DEX (+/−/− and −/+/−) had little effect on luciferase activity compared to cells treated with no inhibitor and no DEX (−/−/−) in BMSCs (Figure 3a,b) and AMSCS (Figure 3c,d). Noco treatment with DEX (+/−/+) decreased DEX fold-change enhancement to about 70% of the fold-change enhancement observed in cells treated with DEX and no inhibitor (−/−/+) in all hMSCs, and this decrease was statistically significant in D4 BMSCs, D2 AMSCs, and D3 AMSCs (Figure 3b–d; $p \leq 0.01$). Cilio treatment with DEX (−/+/+) decreased DEX fold-change enhancement to about 50–70% of the fold-change enhancement observed in hMSCs treated with DEX without inhibitor (−/−/+) in D1 and D4 BMSCs (Figure 3a,b), as well as in D2 and D3 AMSCs (Figure 3c,d), and this decrease was statistically significant in D4 BMSCs, D2 AMSCs, and D3 AMSCs (Figure 3b–d; $p \leq 0.01$). In summary, inhibition of cytoplasmic transport had little effect on transfection in the absence of DEX, but significantly decreased the enhancement induced by DEX-priming, possibly by preventing GR trafficking.

3.4 | Nuclear import inhibition reduces transfection, but does not prevent enhancement by DEX

Given that both the GR and pDNA have been shown to utilize similar highly-conserved nuclear import mechanisms (i.e., importin α/β- and importin β-mediated pathways; Davies et al., 2002; Dhanoya et al., 2013;

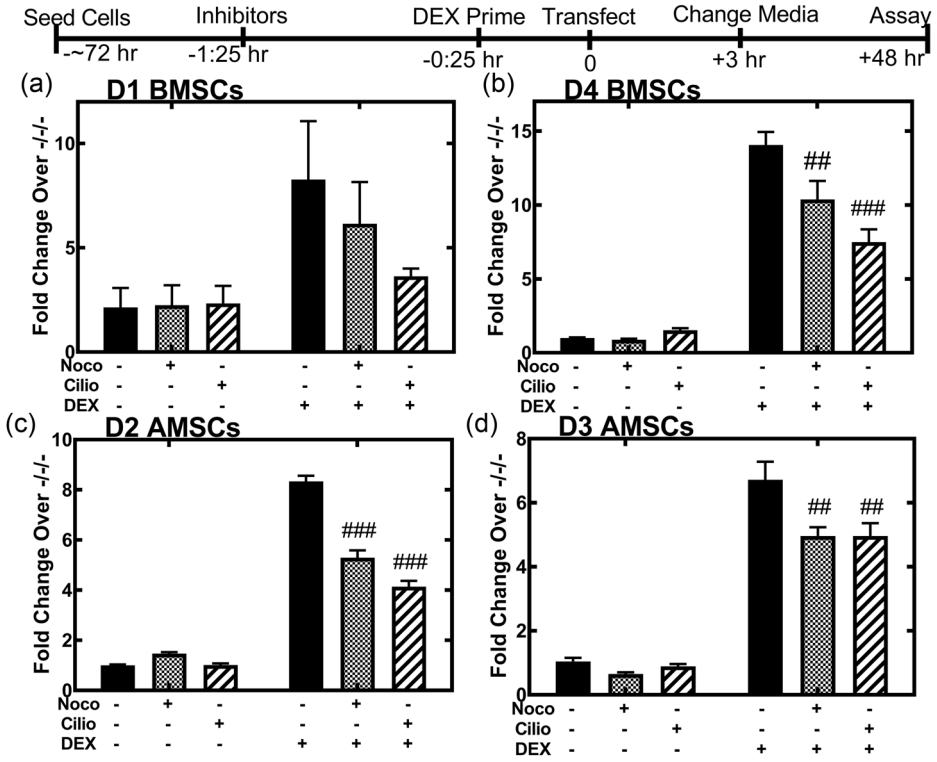

FIGURE 3 Cytoplasmic transport inhibition moderately decreases the hMSC transgenic luciferase activity enhancement induced by dexamethasone (DEX)-priming. hMSCs were treated with 1 μM nocodazole (Noco) or 12 μM ciliobrevin (Cilio), specific inhibitors of microtubule polymerization or dynein motor protein motion, respectively, 1 hr before priming with 150 nM DEX 25 min before transfection with Lipofectamine plasmid DNA complexes. Noco and Cilio in the absence of DEX (+/−/− and −/+/−) had little effect on luciferase transgene activity compared to control cells treated with no inhibitor or DEX (−/−/−), but decreased fold-change enhancement by DEX-priming (+/−/+ and −/+/+) relative to control cells treated with DEX, but no inhibitor (−/−/−) in (a) Donor 1 (D1) BMSCs, (b) D4 BMSCs, (c) D2 AMSCs, and (d) D3 AMSCs. Data is plotted as mean of fold changes relative to control cells not treated with inhibitors or DEX (−/−/−) ± *SEM* ($n = 3$). Asterisks (*) denote significance to −/−/− cells and pounds (#) denote significance to control cells treated with DEX, but no inhibitor (−/−/+; $^{\#\#}p \le 0.01$; $^{\#\#\#}p \le 0.001$). One-way ANOVA was performed, as comparisons were made between a control condition and conditions in which one independent variable was manipulated (i.e. inhibitor vs. no inhibitor, whereas not varying whether or not DEX was present). Appropriate controls were treated with ethanol in lieu of inhibitor or DEX. AMSC: adipose tissue derived from mesenchymal stem cells; BMSC: bone marrow derived from mesenchymal stem cells; hMSCs: human mesenchymal stem cells; *SEM:* standard error of mean

Echeverria et al., 2009; Galigniana et al., 2004; Harrell et al., 2004; Lachish-Zalait et al., 2009), we next investigated the role of nuclear import mechanisms in hMSC transfection and enhancement of transgene expression by DEX-priming. Transfection studies using inhibitors of specific nuclear import pathways were performed in hMSCs along with DEX-priming (Figure 4). To inhibit the importin α/β-mediated pathway, hMSCs were treated with 1 μM ivermectin (IV; Kosyna, Nagel, Kluxen, Kraushaar, & Depping, 2015; Wagstaff, Sivakumaran, Heaton, Harrich, & Jans, 2012). To inhibit the importin β-mediated pathway, hMSCs were treated with 25 μM importazole (IM; Soderholm et al., 2011). One hour after inhibitor treatment, hMSCs were primed with 150 nM DEX 25 min before cells were transfected with pEGFP-Luc complexed with either LF-LTX or LF-3000. Control cells were treated with EtOH in lieu of inhibitors and/or DEX. After 3 hr, media was replaced with fresh media containing no drug or complexes. Transgenic luciferase activity was assayed 48 hr later and fold-change was calculated for each condition relative to the transgenic luciferase activity of cells treated with no inhibitor and no DEX (−/−/−). In all BMSC (Figure 4a,b) and AMSC (Figure 4c,d) donors, IV treatment in the absence of DEX (+/−/−) resulted in small to moderate decreases in transgenic luciferase activity compared to transfected cells not treated with inhibitor or DEX (−/−/−). This decrease was statistically significant in D4 BMSCs and D2 AMSCs (Figure 4b and d; $p \le 0.05$ and 0.001, respectively). IV treatment when applied with DEX (+/−/+) did not significantly affect the fold-change enhancement induced by DEX when compared to fold-change in cells not treated with inhibitors, but primed with DEX (−/−/+) in all donors (Figure 4). IM treatment in the absence of DEX (−/+/−) resulted in large and significant decreases in transgenic luciferase activity relative to transfected cells not treated with inhibitor or DEX (−/−/−; <0.25 fold-change; $p \le 0.001$) in all donors, but when IM was applied with DEX (−/+/+) the fold-change enhancement induced by DEX was not reduced compared to cells not treated with inhibitors, but primed with DEX (−/−/+; Figure 4), and even significantly ($p \le 0.001$) increased DEX-mediated fold-change enhancement in D3 and D4 BMSCs (Figure 4a,b). In summary, inhibiting classical nuclear import pathways significantly decreases hMSC transgene expression in the absence of DEX, but does not decrease fold-change enhancement of transfection induced by DEX-priming, and may even increase its effect.

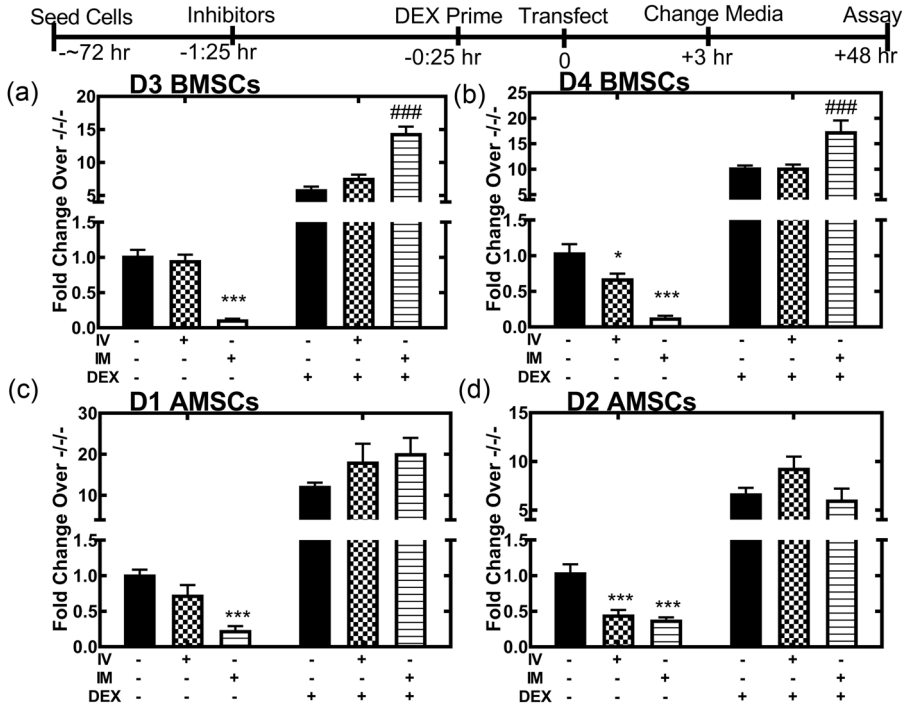

FIGURE 4 Nuclear import inhibition does not reduce transgenic luciferase activity enhancement induced by dexamethasone (DEX)-priming. hMSCs were treated with either 1 μM ivermectin (IV) or 25 μM importazole (IM), specific inhibitors of nuclear import mediated by either the importin α/β pathway or the Importin β pathway, respectively, 1 hr before priming with 150 nM DEX 25 min before transfection with Lipofectamine plasmid DNA complexes. IV in the absence of DEX (+/−/−) showed moderate decreases in transgenic luciferase activity, wh IM in the absence of DEX (−/+/−) dramatically decreased transgenic luciferase activity relative to control cells not treated with inhibitor or DEX (−/−/−), but neither inhibitor decreased fold-change enhancement induced by DEX (+/−/+ and −/+/+) relative to control cells treated with DEX, but no inhibitor (−/−/+) in (a) Donor 3 (D3) BMSCs, (b) D4 BMSCs, (c) D1 AMSCs, and (d) D2 AMSCs. Data are plotted as mean of fold changes relative to control cells not treated with inhibitors or DEX (−/−/−) ± *SEM* ($n = 3$). Asterisks denote significance to −/−/− cells (*$p \leq 0.05$; *** $p \leq 0.001$) and pounds (#) denote significance to control cells treated with DEX, but no inhibitor (−/−/+; ###$p \leq 0.001$). One-way analysis of variance was performed, as comparisons were made between a control condition and conditions in which one independent variable was manipulated (i.e., inhibitor vs. no inhibitor, whereas not varying whether or not DEX was present). Appropriate controls were treated with ethanol in lieu of inhibitor or DEX. AMSCs: adipose tissue derived from mesenchymal stem cells; BMSCs: bone marrow derived from mesenchymal stem cells; *SEM*: standard error of mean

3.5 | Enhancement by DEX is pDNA sequence element independent

Given that the above studies may not fully explain the large DEX-mediated enhancement of hMSC transgene expression, we next explored the importance of specific pDNA sequence elements on hMSC transfection and priming by DEX. hMSCs were DEX-primed and transfected with various modifications of pEGFP-Luc plasmid (Supporting Information Figure S1) complexed with LF-3000. Plasmids used included: unmodified pEGFP-Luc which is driven by a CMV promoter, pEGFP-Luc with the SV40 enhancer removed (denoted CMV-NoSV), pEGFP-Luc with consensus glucocorticoid response elements (GREs) inserted upstream of CMV promoter (denoted CMV-GRE), pEGFP-Luc with CMV promoter replaced with the endogenous elongation factor 1 α promoter (denoted EF1α), and pEGFP-Luc with the CMV promoter replaced by rous sarcoma virus promoter (denoted RSV; Supporting Information Figure S1). Removal of the SV40 enhancer (CMV-NoSV) or addition of consensus GRE elements (CMV-GRE) resulted in variable decreases in transgenic luciferase activity in the absence of DEX-priming, compared to unmodified pEGFP-Luc (CMV), which contains the SV40 enhancer and lacks consensus GRE sequences (i.e. 0–50% less transgenic luciferase activity with CMV-GRE plasmid than with unmodified pEGFP-Luc (CMV)) in D2 and D4 BMSCs, and D2 AMSC (Figure 5a–c). To simplify interpretation of results, we only tested the effect of SV40 and GRE sequences within pDNA constructs with a CMV promoter, given that all other experiments in this paper and in our previous work used CMV promoter (Kelly et al., 2016). Replacing the CMV promoter with the mammalian EF1α or the rous sarcoma virus promoter (RSV) resulted in large decreases in transgenic luciferase activity in the absence of DEX (i.e., 90–95% less transgenic luciferase activity than unmodified pEGFP-Luc [CMV]) in D2 and D4 BMSCs, and D2 AMSC (Figure 5a–c). In contrast, DEX-priming resulted in similar relative fold-change luciferase activity enhancement compared to EtOH VC (i.e., about 6–10-fold), regardless of any plasmid sequence modifications tested in D2 and D4 BMSCs, and D2 AMSCs (Figure 5a–c). DEX even induced an increase of about 40-fold compared to EtOH VC in D4 BMSCs when transfected with RSV plasmid (Figure 5b). In summary,

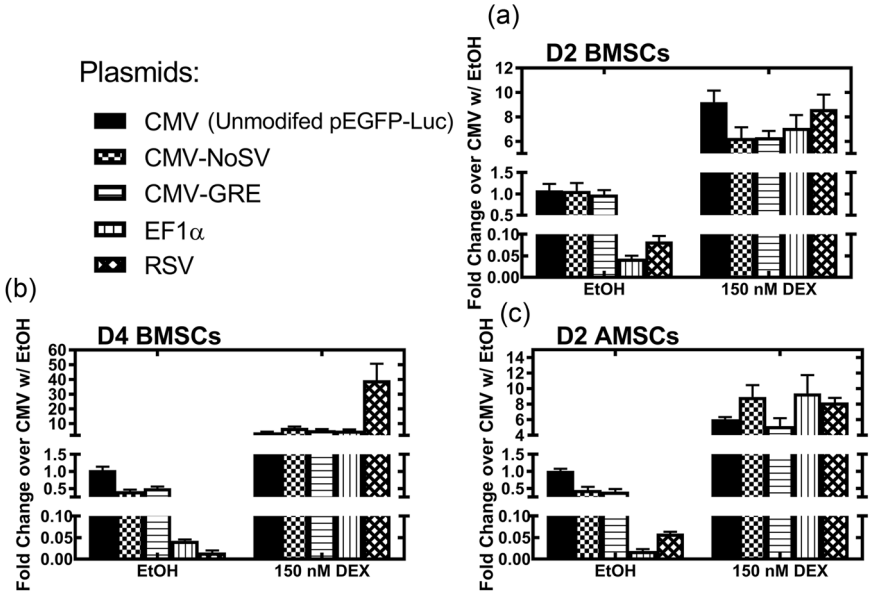

FIGURE 5 Plasmid sequence modifications do not affect hMSC transgenic luciferase activity enhancement by dexamethasone (DEX)-priming. hMSCs were transfected with Lipofectomine-3000 complexed with modifications of pEGFP-Luc expressing luciferase transgene driven by cytomegalovirus promoter with SV40 enhancer (*CMV* [*unmodified pEGFP-Luc*]) or without SV40 enhancer (*CMV-NoSV*), with consensus glucocorticoid response element added upstream of CMV promoter (*CMV-GRE*), or with CMV promoter replaced by elongation factor 1 α promoter (*EF1α)* or rous sarcoma virus promoter (*RSV*). Cells were treated with 150 nM DEX or ethanol (EtOH) 25 min before transfection. Sequence modifications had variable effect on transgenic luciferase activity levels, but did not prevent enhancement by DEX-priming in (a) Donor 2 (D2) BMSCs, (b) D4 BMSCs, or (c) D2 AMSCs. Data is plotted as mean of fold changes relative to cells transfected with CMV plasmid and not primed with DEX ± *SEM* ($n = 3$). AMSC: adipose tissue derived from mesenchymal stem cell; BMSC: bone marrow derived from mesenchymal stem cell; CMV: cytomegalovirus; hMSCs: human mesenchymal stem cells; *SEM*: standard error of mean

pDNA regulatory sequences can have dramatic effects on transgene expression in hMSCs, but no sequence modifications tested reduce enhancement induced by DEX.

3.6 | DEX does not increase transgene mRNA levels

Since DEX does not increase internalization of pDNA into cells or nuclei (Figure 2), nor does its enhancement of transgene expression depend on the sequence of the plasmid (Figure 5), we sought to determine if DEX increased transgene expression in hMSCs at the transcriptional level. Transgene (i.e. EGFP) messenger RNA (mRNA) was quantified in 150 nM DEX-treated hMSCs by qRT-PCR relative to hMSCs treated with EtOH VC, 48 hr after transfection with pEGFP-Luc complexed with LF-3000. DEX-priming did not significantly affect the relative amount of transgene mRNA in D4 and D5 BMSCs or D1 and D2 AMSCs (Figure 6). These results suggest that DEX-priming does not increase transgene transcription or mRNA stability.

3.7 | DEX ameliorates hMSC protein synthesis inhibition induced by transfection by preventing apoptosis

After finding that DEX-priming results in no significant increases in the amount of pDNA internalized into either cells or nuclei (Figure 4) and does not increase the amount of transgenic mRNA (Figure 6), we explored the effect of DEX-priming and transfection on total protein translation in hMSCs. hMSCs were either untreated and untransfected (Ngtv) or primed with 150 nM DEX or EtOH VC 25 min before transfection with pDNA complexed with LF-3000. After 24 hr, cells were cultured in media containing OPP, which is incorporated into translated proteins over the next 24 hr and subsequently fluorescently labeled. Translation was measured in relative fluorescence units (RFUs) normalized to total cellular DNA fluorescently stained with Hoechst. Transfection in the absence of DEX (condition EtOH) resulted in decreased total cell protein synthesis by 2–4-fold relative to untreated and untransfected hMSCs, and this decrease was statistically significant in D1 and D5 BMSCs as well as D2 AMSCs (Figure 7a,b, and d; $p \leq 0.001$, ≤0.001, and ≤0.01 respectively). DEX-priming ameliorated the inhibition of hMSC protein synthesis induced by transfection, resulting in about a 2–3-fold increase in total protein synthesis in cells transfected and DEX treated, compared to cells transfected without DEX (condition EtOH) and increases were statistically significant in all donors (i.e. D1 and D5 BMSCs, and D1 and D2 AMSCs; Figure 7a, b, c, and d; $p \leq 0.001$, ≤0.001, ≤0.01, and ≤0.001, respectively).

Given that transfection significantly reduces hMSC protein synthesis and DEX-priming ameliorates this transfection-induced reduction in protein synthesis, and we previously demonstrated that DEX rescues transfection-induced hMSC viability decrease (Kelly et al., 2016), we hypothesized that DEX may enhance transgene

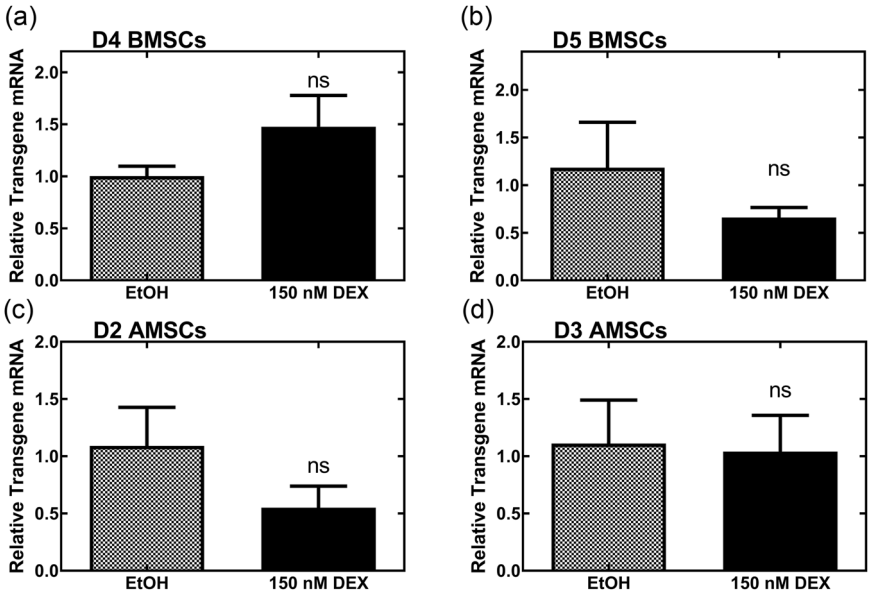

FIGURE 6 Dexamethasone (DEX)-priming does not increase transgene (i.e., EGFP) messenger RNA (mRNA) levels. hMSCs were primed with 150 nM DEX or ethanol (EtOH) 25 min before transfection with plasmid DNA Lipofectomine-3000 complexes. After 48 hr, total RNA was extracted and relative copies of transgene mRNA was determined by qRT-PCR. DEX-priming did not significantly affect relative transgene mRNA transcript amount in (a) Donor 4 (D4) BMSCs, (b) D5 BMSCs, (c) D2 AMSCs, or (d) D3 AMSCs. Data is plotted as means ± *SEM* ($n = 3$). Statistical significance was considered at $p < 0.05$. AMSC: adipose tissue derived from mesenchymal stem cell; BMSC: bone marrow derived from mesenchymal stem cell; hMSC: human mesenchymal stem cell; qRT-PCR: quantitative real-time polymerase chain reaction; *SEM*: standard error of mean

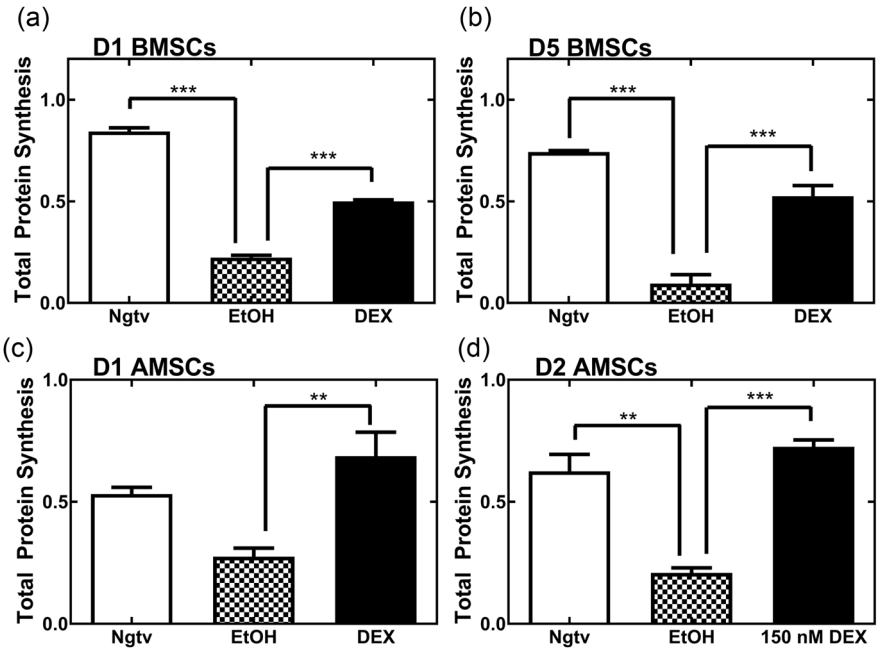

FIGURE 7 DEX-priming ameliorates the reduction in protein synthesis induced by transfection in hMSCs. hMSCs were either untreated and untransfected (Ngtv), transfected with 150 nM DEX-priming, or transfected with ethanol (EtOH) control priming. After 24 hr, cells were fed media containing *O*-propargyl-puromycin, which is incorporated into translated proteins over the next 24 hr and subsequently fluorescently labeled. Translation was measured in relative fluorescence units normalized to total cellular DNA fluorescently stained with Hoechst in (a) Donor 3 (D3) BMSCs, (b) D5 BMSCs, (c) D1 AMSCs, and (d) D3 AMSCs. Data is plotted as means ± *SEM* ($n = 3$). Asterisks denote significance compared to EtOH conditions (**$p \leq 0.01$; ***$p \leq 0.001$). AMSC: adipose tissue derived from mesenchymal stem cell; BMSC: bone marrow derived from mesenchymal stem cell; DEX: dexamethasone; hMSC: human mesenchymal stem cells; RFU: relative fluorescence unit; *SEM*: standard error of mean

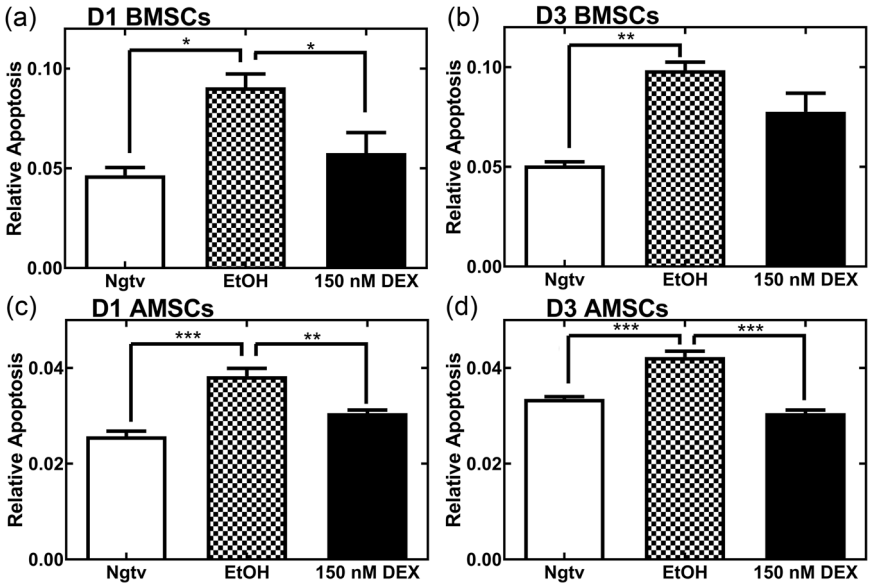

FIGURE 8 DEX-priming decreases apoptosis induced by transfection in hMSCs. hMSCs were either untreated and untransfected (Ngtv), transfected with 150 nM DEX-priming, or transfected with ethanol (EtOH) control priming. After 48 hr, apoptotic cells stained with fluorescently labeled annexin were measured in relative fluorescent units normalized to total cellular DNA fluorescently stained with Hoechst to calculate plotted relative apoptosis in (a) D1 BMSCs, (b) D3 BMSCs, (c) D1 AMSCs, and (d) D3 AMSCs. Data is plotted as means ± *SEM* ($n = 3$). Asterisks denote significance compared to EtOH conditions (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$). AMSC: adipose tissue derived from mesenchymal stem cell; BMSC: bone marrow derived from mesenchymal stem cell; DEX: dexamethasone; hMSCs: human mesenchymal stem cells; *SEM*: standard error of mean

expression by preventing transfection-related toxicity resulting in apoptosis. hMSCs were either untreated and untransfected (Ngtv) or primed with 150 nM DEX or EtOH VC 25 min before transfection with pDNA complexed with LF-3000. After 48 hr, hMSCs were stained with fluorescently labeled annexin and relative apoptosis was measured in RFUs normalized to total cellular DNA fluorescently stained with Hoechst. Transfection in the absence of DEX (EtOH condition) resulted in significantly increased apoptosis over untreated and untransfected hMSCs in D1 and D5 BMSCs, and D1 and D2 AMSCs (Figure 8; $p \leq 0.05$, ≤0.01, ≤0.001, and ≤0.001, respectively). However, DEX-priming ameliorated the induction of hMSC apoptosis by transfection, resulting in statistically significant decreases in apoptosis relative to transfection without DEX (EtOH condition) in D1 BMSCs, and D1 and D3 AMSCs (Figure 8a, c, and d; $p \leq 0.05$, ≤0.01, and ≤0.001, respectively). In summary, transfection toxicity induces apoptosis in hMSCs, resulting in inhibition of protein synthesis. DEX-priming reduces apoptosis induced by transfection, preventing reduction of protein synthesis in transfected hMSCs.

4 | DISCUSSION

This work explored the mechanisms of hMSC nonviral transfection and DEX-induced enhancement of transgene expression. This DEX-priming effect on transfection is robust and consistent as evidenced by large increases in transgenic luciferase activity levels relative to hMSCs transfected in the absence of the DEX (i.e., about 10-fold luciferase activity; Figure 1a,b); the effect is seen in both BMSCs and AMSCs derived from multiple human donors. While DEX and other steroids have been used to increase nonviral gene delivery efficiency in several cell types before this study, other cell types generally show only moderate transgene expression increases (i.e. 2–4-fold increases relative to VC; Bernasconi et al., 1997; Braun et al., 1999; Chen et al., 2011; Choi & Lee, 2005; Jain et al., 1999; Koster et al., 2002; Köster et al., 2006; Lin et al., 2003; Nair et al., 2002) compared to those seen here in hMSCs. Although DEX increased transgenic luciferase activity normalized to total protein by about 10-fold in transfected hMSCs, DEX only increased the amount of transgenic luciferase protein by about two-fold, as quantified by western blot analysis normalized to total protein (Figure 1c,d), suggesting that DEX-priming somehow increases transgenic enzyme activity downstream of transgenic protein synthesis. Our results showing increased transgenic enzyme activity in addition to increased transgenic protein production (all relative to VCs) are probably not specific to only luciferase transgenes because we previously reported DEX-priming yielded 4–7-fold increases in transgenic β-galactosidase enzyme activity, whereas also increasing transgenic EGFP mean fluorescence intensity by about two-fold, all relative to VC (Kelly et al., 2016). It is likely that hMSC transfection could be primed with many other Gc drugs in addition to DEX, as our previous work demonstrated that priming with cortisol similarly resulted in significant enhancement of transgene expression in transfected hMSCs (Kelly et al., 2016). In addition to replicating our previous reports of DEX-priming transfection in BMSCs derived from multiple human donors (Kelly et al., 2016), here we also extend our simple protocol to demonstrate similar enhancement in AMSCs from multiple human donors.

Furthermore, this work represents the first report in which classical nonviral gene delivery barriers were systematically investigated with and without DEX-priming in hMSCs, including internalization, cytoplasmic transport, nuclear translocation, transgene transcription, translation, transgene activity, and cellular stress response. We also explored the effect of different pDNA promoter and enhancer sequences on transgene expression and DEX-priming. Mechanistic studies of nonviral gene delivery in primary cells, like hMSCs, are important because the physiology of therapeutically relevant cells differs from transformed cell lines, presenting unique challenges to the design of efficient delivery protocols.

Most Gc effects within cells are mediated by binding and activation of the GR and we previously demonstrated that binding of the GR is required for DEX-mediated enhancement of transgene expression in hMSCs (Kelly et al., 2016). Since DEX has been shown to alter nuclear membrane permeability (Kastrup, Oberleithner, Ludwig, Schafer, & Shahin, 2006; Shahin, 2006; Shahin et al., 2005), and the GR has been shown to interact with similar transport and nuclear import pathways as pDNA, we quantified the number of plasmid copies in transfected hMSCs and isolated nuclei, with and without DEX-priming. DEX-priming did not increase cellular or nuclear internalization of pDNA, but actually resulted in slightly less internalized plasmid copies per cell and nuclei 48 hr after transfection (Figure 2), possibly due to increased proliferation of DEX-treated hMSCs diluting pDNA number within dividing cells, as we previously showed DEX ameliorates the decrease in hMSC viability brought on by transfection toxicity, as quantified by WST-1 proliferation assays (Kelly et al., 2016). It should be noted that these pDNA internalization results are not consistent with our previous data where we showed increased plasmid internalization induced by DEX in hMSCs (Kelly et al., 2016), but those previous experiments were performed with only one biological replicate ($n = 1$), used cells from different human donors than used in the current work, and also used the LF-LTX transfection reagent and iodoxanol gradient nuclear isolation methods. In contrast, the LF-3000 transfection reagent, which was used in this current study due to its improved transfection in AMSCs over LF-LTX, as well as sucrose gradient nuclear isolation methods were used in this current study. Thus, the different results reported here can be attributed to changes in donors and nuclear isolation methods, but also the results reported greatly improve the statistical power, given the use of hMSCs from four human donors. Furthermore, these differing results may indicate that the mechanism of enhancement could be specific to different transfection reagents.

Since both pDNA and the GR are known to utilize similar cytoplasmic transport mechanisms involving microtubules and dynein (Davies et al., 2002; Dhanoya et al., 2013; Echeverria et al., 2009; Galigniana et al., 2004; Harrell et al., 2004; Lachish-Zalait et al., 2009; Vaughan, DeGiulio, & Dean, 2006), the role of cytoplasmic transport mechanisms in hMSC transfection and priming by DEX was investigated by performing transfection studies with specific inhibitors of cytoplasmic transport. Inhibition of microtubule polymerization with Noco or inhibition of dynein motion with Cilio had little effect on transfection of hMSCs in the absence of DEX (Figure 3). Previous studies in other cell types have shown lipofection is enhanced by treatment with inhibitors of microtubule-based transport by preventing transport of complexes to lysosomes (Cardarelli et al., 2016; Hasegawa, Hirashima, & Nakanishi, 2001; Lindberg et al., 2001; L. Wang & MacDonald, 2004), but our studies used significantly lower Noco concentration due to its toxicity in hMSCs. However, even using a lower concentration, Noco treatments did reduce fold-change enhancement of transgene expression mediated by DEX-priming, suggesting transport was inhibited to attenuate DEX-mediated priming of transfection. pDNA transport to nuclei on microtubules has been shown to involve pDNA binding of transcription factors (Badding, Vaughan & Dean, 2012), so others have suggested activated GR can directly transport pDNA via dynein on microtubules through the cytoplasm to nuclei, resulting in increased nuclear pDNA and thus increased transfection (Chen et al., 2011). However, it appears this is not the mechanism by which DEX increases transgene expression in hMSCS, since DEX did not increase pDNA nuclear internalization (Figure 2). Furthermore, while inhibiting cytoplasmic transport reduced the fold-change increase in transgene expression by DEX, it is likely that inhibition of the transport of GR or other factors on microtubules resulted in downstream effects that modulate nonviral gene delivery success as opposed to modulating pDNA transport. To better understand these results, we next explored the role of nuclear import pathways.

In addition to cytoplasmic transport, pDNA and GR also employ similar mechanisms in their nuclear translocation in that they both interact with importins and the nuclear pore complex during internalization (Dhanoya et al., 2013; Echeverria et al., 2009; Lachish-Zalait et al., 2009). Since pDNA nuclear translocation has been shown to be a limiting barrier to transfection success in other cell types (Ludtke, Sebestyén, & Wolff, 2002; Young, Benoit, & Dean, 2003), we aimed to study transfection and DEX-priming of hMSC transfection in response to inhibition of classical nuclear import mechanisms (Figure 4). Large transgenic luciferase activity decreases by IM treatment in the absence of DEX reiterates the importance of the importin β-mediated nuclear import pathway to successful transfection, which has been reported in other cell types (Badding, Lapek, Friedman, & Dean, 2013), and confirms its importance in hMSCs. However, observing no decrease in DEX-mediated fold-change enhancement by nuclear import inhibitors (Figure 4), along with our data showing DEX does not increase pDNA nuclear internalization (Figure 2), suggests that mechanisms by which DEX-priming enhances transfection are independent of nuclear import pathways.

After demonstrating that inhibition of cytoplasmic transport pathways significantly decreased transfection enhancement mediated by DEX-priming (Figure 3), and that inhibition of importin β-mediated nuclear import in absence of DEX dramatically decreased hMSC transgenic luciferase activity (Figure 4), we next explored how specific pDNA sequences, known to modulate transport, nuclear localization, and transcription, affect hMSC transfection and DEX-priming by modifying the plasmid pEGFP-Luc (Supporting Information Figure S1) used in all previous experiments. The SV40 enhancer,

found in unmodified pEGFP-Luc, is a known DNA targeting sequence (DTS; Badding, Vaughan & Dean, 2012; Breuzard et al., 2008; Cramer et al., 2012; D. A. Dean, Strong, & Zimmer, 2005; D. Dean, 1997; Gonçalves et al., 2009; Miller & Dean, 2009; Van Gaal et al., 2011; Wilson, Dean, Wang, & Dean, 1999; Young et al., 2003), which is bound by several transcription factors that can increase transport and nuclear internalization of pDNA in some cell types (Badding et al., 2013). Removal of the SV40 DTS inhibited hMSC transfection in the absence of DEX as expected, but priming by DEX seems to be independent of mechanisms related to DTS binding, as there was no significant difference in fold-change enhancement by DEX relative to DEX fold-change enhancement of transgene expression from unmodified pEGFP-Luc (Figure 5), suggesting again that DEX does not increase transfection by modulating pDNA intracellular transport. Another sequence element of interest is consensus GREs, well-defined short palindromic repeats found within Gc-induced genes' promoters, which are bound by GR dimers to recruit transcriptional machinery (i.e. transactivation; Ratman et al., 2013). Unmodified pEGFP-Luc does not contain consensus GREs, so GREs were added directly upstream of the CMV promoter on the pEGFP-Luc plasmid to determine if GREs could facilitate binding of DEX-activated GR to enhance transgene expression. Addition of GREs resulted in small to moderate decreases in transgenic luciferase activity in the absence of DEX, and did not impact the fold-change enhancement by DEX (Figure 5), meaning DEX-priming of hMSC transfection cannot be further enhanced by addition of GREs within delivered pDNA. While there are several studies in other cell types in which GRE-containing pDNA facilitated higher Gc-induced transgene expression fold-changes compared to pDNA not containing GREs (Dames, Laner, Maucksch, Aneja & Rudolph, 2007; Mader & White, 1993; Y. Wang et al., 2012), our differing results here suggest DEX does not enhance hMSC transgene expression through direct GR-pDNA binding.

In addition to promoting transcription from constructs containing GRE sequences (Dames, Laner, Maucksch, Aneja & Rudolph, 2007; Mader & White, 1993) DEX has also been shown to induce GR-dependent transcriptional activation of transgenes driven by CMV promoter in some cell types (Inoue-Toyoda, Kato, Nagata, & Yoshikawa, 2015; Van Damme et al., 2015). Replacing the CMV promoter with the mammalian EF1α or the rous sarcoma virus promoter (RSV) resulted in dramatic decreases in absolute transgenic luciferase activity in the absence of DEX, but large and significant fold-change enhancement by DEX-priming was still demonstrated (Figure 5). This result distinguishes the DEX-priming effect in hMSCs from previous reports of sequence-dependent direct activation of CMV promoter by Gc. Our experiments conducted with modified plasmids indicate that hMSC transfection enhancement by DEX-priming is independent of plasmid sequence elements and indicates that the priming effect may not be mediated by direct association of pDNA with the GR to promote transcription. These results also show that modifications to plasmid sequence like the removal of the SV40 enhancer can significantly affect transgene expression levels in unprimed and DEX-primed hMSCs, similar to reports that demonstrate the addition of the SV40 enhancer increases nuclear internalization of pDNA by about 10-fold and more than doubles plasmid average rate of movement in A459 cells relative to pDNA lacking the SV40 enhancer (Badding et al., 2013). Furthermore, our results reiterate the importance of promoter choice in hMSCs, as replacing the strong CMV promoter with the endogenous mammalian EF1α promoter or viral RSV promoter dramatically reduced transgenic luciferase activity in the absence of DEX. To our knowledge, this is the first comparison of promoter performance in hMSCs, but studies of transgene expression in rats MSCs have shown the EF1α promoter to outperform a CMV promoter (Ferreira et al., 2012; McGinley et al., 2011; Qin et al., 2010), suggesting hMSC physiology differs significantly from MSCs obtained from other species.

Our results reported here show DEX-priming does not modulate pDNA internalization, intracellular transport, or nuclear import mechanisms, but the increase in hMSC transfection may be the result of other downstream mechanisms, so we next sought to determine if DEX increases hMSC transgene expression at the transcriptional level. However, DEX did not significantly increase the amount of transgene mRNA present in hMSCs 48 hr after transfection with pDNA (Figure 6). This result suggests that DEX-priming does not increase transgene transcription rate or mRNA stability, but may enhance transgene expression at the posttranscriptional level. We next aimed to explore pathways related to transfection and DEX-priming of transfection that could modulate posttranscriptional mechanisms of transgene expression.

Since nonviral gene delivery has been shown to induce cellular stresses (Martin et al., 2015a; Martin, Plautz, & Pannier, 2013), like reactive oxygen species, proinflammatory factors, and apoptotic mediators (Lonez, Vandenbranden, & Ruysschaert, 2012), and it is well known that many cellular stresses result in inhibited protein synthesis, we tested the effect of transfection and DEX-priming on hMSC total cellular protein synthesis. hMSC total protein synthesis was significantly decreased by transfection, but was significantly rescued by DEX-priming (Figure 7), which may be partly responsible for increased transgenic protein production (Figure 1). Since it is well known that protein synthesis is inhibited during apoptosis (Jeffrey, Bushell, Tilleray, Morley, & Clemens, 2002) and we have previously shown that DEX rescues transfection-induced hMSC viability decreases, we next demonstrated, by annexin staining, that transfection induces hMSC apoptosis and that DEX-priming significantly reduces this apoptotic response (Figure 8), which presumably allows for the increased translation of transgenic protein found in DEX-primed hMSCs (Figure 1a,b). Our results are consistent with reports that DEX treatment ameliorates transfection-induced hMSC viability decrease as measured by WST-1 cell proliferation assay (Kelly et al., 2016). Furthermore, DEX has also been shown to rescue cytotoxicity and apoptosis induced in transfected HC92 rat cardiomyocytes (Kim et al., 2009), and reduced death of chinese hamster ovary (CHO) cells producing recombinant protein by modulating apoptosis-related gene expression (Jing et al., 2012). In addition to increasing transgenic protein synthesis, attenuation of apoptosis by DEX-priming could also promote the observed increase in relative transgenic enzyme

activity by modulating posttranslational events like folding or active-site stabilization, as Gc has been shown to improve CHO recombinant protein production by reducing protein aggregation through upregulation of genes that modulate redox conditions (Qian, Jing, & Li, 2010). DEX has also been shown to modulate endoplasmic reticulum (ER) stress responses by promoting correct protein folding (Das et al., 2013), trafficking (Fujii et al., 2006), and by preventing apoptosis (Mihailidou, Panagiotou, Kiaris, Kassi, & Moutsatsou, 2016). Further study of posttranslational mechanisms like transgenic protein folding as well as the effect of transfection and DEX-priming on inflammatory and stress pathways, like oxidative and ER stress, are needed to elucidate details that may be key to understanding unprimed and DEX-primed nonviral gene delivery to hMSCs. In addition to future mechanistic studies, we will also apply our DEX-priming protocol to express therapeutic transgenes in hMSCs, as safe and efficient nonviral delivery of therapeutic genes to hMSCs is needed to translate to clinical applications.

5 | CONCLUSIONS

In summary, these studies systematically investigated the mechanisms of hMSC nonivral transfection and DEX-priming of transfection. DEX-priming of hMSCs presents a simple protocol to significantly enhance nonviral gene delivery success in therapeutically relevant cells, and results in increased production of transgenic protein, as well as increased transgenic enzyme activity. We show that hMSC transgene expression is largely affected by pDNA promoter and enhancer sequence changes, but DEX-mediated enhancement is unaffected by any pDNA sequence changes. DEX-mediated enhancement is not the result of increased pDNA cellular or nuclear internalization, or transgene mRNA transcription or stability. Our studies demonstrate that DEX-priming inhibits hMSC apoptosis induced by transfection to prevent subsequent protein synthesis inhibition, which allows increased translation of transgenic protein. Our findings highlight the importance of understanding downstream molecular biological mechanisms of nonviral gene delivery like translational and posttranslational events that limit transfection success. Further exploration of these pathways will be key in the rational design of new technologies to advance the many clinical applications of safe and efficient genetic modification of hMSCs and other therapeutically relevant primary cells.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHORS CONTRIBUTION

The study was conceptualized by A. H. and A. K. P and the methodology was given by A. H. and A. N. The study was investigated by A. H. and K. B. whereas A. H. has written the original draft. A. H. and A. K. P helped in writing, reviewing and editing the article. The funding acquisition was provided by A. K. P.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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