Nanosecond Pulsed Electric Fields Enhance Mesenchymal Stem Cells Differentiation via DNMT1 regulated OCT4/NANOG gene expression

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Research

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Abstract

Background: Multiple strategies have been proposed to promote the differentiation potential of MSCs, which is the fundamental property in tissue formation and regeneration. However, these strategies are relatively inefficient that limit the application, thus more advanced methods are needed. In this study, we report a novel and efficient strategy, nanosecond pulsed electric fields (nsPEFs) stimulation, which can enhance the trilineage differentiation potential of MSCs; and further explained the mechanism behind.

Methods: We used histological staining to screen out the nsPEFs parameters that promoted the trilineage differentiation potential of MSCs, and further proved the effect of nsPEFs by detecting the functional gene. In order to explore the corresponding mechanism, we examined the expression of pluripotency genes and the methylation of their promoters. Finally, we targeted the DNA methyltransferase which was affected by nsPEFs.

Results: The trilineage differentiation of bone marrow derived MSCs was significantly enhanced in vitro by simply pre-treated with 5 pulses of nsPEFs stimulation (energy levels as 10 ns, 20 kV/cm; 100 ns, 10 kV/cm), and this was due to the nsPEFs demethylated the promoters of stem cell pluripotency genes OCT4 and NANOG through instantaneous downregulation of DNA Methylation Transferase 1 (DNMT1), thereby increased the expression of OCT4 and NANOG for up to 3 days, and created a treatment window period of stem cells.

Conclusions: In summary, nsPEFs can enhance MSCs differentiation via the epigenetic regulation, and could be a safe and effective strategy for future stem cell application.

Background

Mesenchymal stem cells (MSCs) have been used for cell-based therapies due to their significant contribution in tissue development and regeneration [1]. Two major characteristics of MSCs, i.e., the self-renewal ability and the differentiation ability, empowered the clinical application by enlargement of the cell population and contributing to the on-site neo-tissue formation. For example, bone marrow derived MSCs, which can be differentiated into osteo-, adipo-, and chondro-lineages [2], have brought positive clinical results in treating bone and cartilage defects [3, 4]. The differentiation capacity of MSCs, both *in vitro* and *in vivo*, directly relates to the therapeutic efficacy, depends on the tissue source (e.g., tissue derivation, health status of donor site), the cell isolation and culture conditions [5–7]. In order to maintain the therapeutic characteristics of MSCs, many attempts had been tried, such as treatments of growth factors [8, 9] or pre-conditioned with hypoxia [10]. However, these methods are functioning within an effective threshold that limited the application, more advanced methods are thus needed.

Pulsed electrical stimulation (PES) has been proven to have multiple biological effects on cells for the short-term permeabilization, which mostly depends on the parameters of electric field strength (from Millivolt/cm, mV/cm; to Megavolt/cm, MV/cm) and stimulation duration (from Nano Second, ns; to Second, s) [11]. PES based technologies have been applied for tumor therapy, because it can induce cell

apoptosis at the range of kV-MV/cm within ns-μs stimulation duration; and have been also used for electroporation for gene delivery at the range of V/cm, μs-s [11]. Nanosecond pulsed electric fields (nsPEFs), a novel technology with relatively short duration (nanoseconds, ns) and subsequent high voltages (up to kV/cm), are emerging in cell researches and have been reported to have the modulation effects on stem cells. nsPEFs can incur more comprehensive biological effects, compared with traditional pulsed electric fields (PEFs) which are above millisecond or microsecond. As the duration is shorter than the charging time of cell membrane, nsPEFs can further affect intracellular structures [12]. The biological responses of nsPEFs were previously reported in algae cells [13], and in human cancer cell lines with the mobilization of intracellular Ca^{2+} and activation of signaling pathways [14, 15]. The comprehensive and individualized reactions of cells are based on varied combinations of parameters (duration, voltage, frequency and number of pulses) of nsPEFs, and the physical and biological properties of cells [14, 16]. nsPEFs have been studied as a possible therapeutic intervention for cancer [17–19], but little is known about their effects on regulating cell phenotype and differentiation of stem cells.

Previously, our group have found that nsPEFs could affect chondrocyte phenotype through regulating Wnt/β-catenin signaling pathway [20]. Recently, we found that nsPEFs at the levels of 10 ns at 20 kV/cm, 60 ns at 5 kV/cm, 60 ns at 10 kV/cm, 60 ns at 20 kV/cm, and 100 ns, 10 kV/cm, separately, could upregulate chondrogenic gene expression of MSCs [21]. Notably, cells can respond to physical energy epigenetically [22], therefore it is possible that nsPEFs have a role in epigenetic regulation of MSCs. In this study, we find that nsPEFs with specific parameters can make MSCs more susceptible to induced differentiation. In addition, we reveal that nsPEFs can downregulate Methylation Transferase 1 (DNMT1) temporarily, and switch on the negative feedback loop between DNMT1 and OCT4 / NANOG. In contrast, overexpression of DNMT1 can block the effect of nsPEFs.

Methods

Harvest and culture of mesenchymal stem cells

Porcine bone marrow mesenchymal stem cells (pMSCs) were harvested from three Guizhou mini-pigs (Peking University, Laboratory Animal Center, PKU-LAC) at 6 to 10 months old (approved by IACUC, PKU-LAC). Femur and tibia of the pigs were drilled before bone marrow was washed out with phosphate-buffered saline (PBS) and collected in 50 ml centrifuge tubes. Human bone marrow mesenchymal stem cells (hMSCs) were obtained from bone marrow of three patients receiving total hip arthroplasty (male, 62 years old; male, 79 years old; female, 82 years old, from the People's Hospital, Beijing with IRB approval) and collected in 50 ml centrifuge tubes. After centrifuged at 1,000 rpm and supernatant removed, the collected cells were suspended with medium containing 90% Dulbecco's modified Eagle's medium (DMEM, Gibco), 10% fetal bovine serum (FBS, Gibico), and 1% penicillin/streptomycin (PS, Amresco) and cultured at 37 °C in humidified atmosphere with 5% CO_2. The cultured medium was changed every three days until the cells reached 85% of confluency. Then they were trypsinized with 0.25% trypsin (27250-018, Invitrogen), and MSCs at passage 5 were used for all subsequent experiments.

Application of nsPEFs

One million of MSCs were suspended in 1 mL of culture medium in gap cuvette (BTX, catalog number: 45–0125, 45–0126), and were subjected to 5 pulses of nsPEFs (10 ns at 20 kV/cm, 60 ns at 5 kV/cm, 60 ns at 10 kV/cm, 60 ns at 20 kV/cm, and 100 ns, 10 kV/cm, 1 Hz). And the time interval between two pulses is one second [21]. MSCs not subjected to nsPEFs served as control. The nsPEFs generator was applied as previously described [23]. The voltage waveform was monitored by a Digital phosphor oscilloscope (DPO4054, Tektronix) with a probe (P6015A, Tektronix).

qRT-PCR

Total RNA was extracted and isolated from MSCs in each stimulation condition with Trizol Reagent (New Industry) following the standard protocol, and quantified with Nanodrop spectrophotometer (ND-1000, Thermo). Then the reverse transcription reaction was performed on 500 ng of RNA with M-MLV reverse transcriptase (C28025, Sigma) and oligo (dT) (FSK-201, TOYOBO) in a PCR thermal. Quantitative real-time polymerase chain reactions (qRT-PCR) were performed in the PCR system (Pikoreal 96, Thermo) with RealMasterMix SYBR Green (FP202, Tiangen) following the manufacturer's procedures. The expression levels of stem cell pluripotency genes and trilineage differentiation related genes were analyzed. The primers were listed in **Supplementary Table 1**. The target genes of each sample were normalized to the values of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as internal control. Relative expression of each gene was expressed as fold changes by the $2^{-\Delta\Delta Ct}$ method. The experiment was repeated for three times, with five technological repeats for each assay.

Histology analysis of osteo-, adipo-, and chondrogenic differentiation

The treated MSCs were cultured with osteogenic induction medium, adipogenic induction medium, and chondrogenic induction medium [24] at 37 °C in humidified atmosphere with 5% CO_2, respectively. Media was changed every three days. After 14 days, cell cultures were stained with Alizarin Red (AR, for osteogenic induction) and Oil Red O (ORO, for adipogenic induction) and Alcian Blue(AB, for chondrogenic induction) staining, respectively, and following by extraction and measurement of O.D. values of AR stainning at 550 nm, ORO staining at 510 nm and AB staining at 620 nm. The experiment was repeated for three times, with five technological repeats for each assay.

Western Blotting

MSCs were lysed by RIPA lysis buffer (R0020, Solarbio) with fresh protease inhibitor of 0.1% phenylmethanesulfonyl fluoride 2 hours after nsPEFs, and mixed with 4 × SDS loading buffer (P1015, Solarbio). The western blotting was carried out according to the manufacturer's protocols. Rabbit polyclonal antibodies against DNMT1 (24206-1-AP, Proteintech), DNMT3a (3598, Cell Signaling), DNMT3b (orb372330, Biorbyt) and β-actin (4970S, Cell Signaling) were used in combination with secondary HRP-linked antibody of anti-rabbit IgG (7074S, Cell Signaling). The complex of the antigen and the antibody was detected with TANON 1600 Gel Imaging System (Tanon Science&Technology Co., Ltd,

Shanghai), and the expression level of protein is analyzed with Tanon Gis (Tanon Science&Technology Co., Ltd, Shanghai).

Overexpressing of DNMT1 in MSCs

For tet-on DNMT1 systems, we synthesized the coding sequence of p*DNMT1* gene from GENEWIZ by chemical method. The amplified sequence *pDNMT1* was then cloned into a pFU-tetO lentivirus backbone (19778, Addgene) linearizing with EcoR1 restriction enzyme. The FUdeltaGW-rtTA (19780) and third generation lentiviral helper plasmid (12253, 12252, 12251) were purchased from Addgene. pFU-tetO-pDNMT1 and FUdeltaGW-rtTA were co-transfected into MSCs. Plasmids with *GFP* genes were used as control. Because there was almost no significantly differences between nsPEFs with the two set parameters (10 ns at 20 kV/cm, and 100 ns at 10 kV/cm), nsPEFs of 100 ns at 10 kV/cm was used for studying the effects of downregulation of DNMT1. After stimulation by nsPEFs, doxycycline (Dox) were added to MSCs at 1 μg/ml for 2 hours. The expression levels of GFP and DNMT1 were evaluated by western blotting. The primers and annealing temperatures used for PCR of *GFP* and *DNMT1* are listed in **Supplementary Table 3**. The experiment was repeated for three times, with five technological repeats for each assay.

Statistical analysis

Results were presented as the mean ± SD/SEM, and was normalized to the control group defined as One-way ANOVA was carried out with the least significant difference (LSD) using Prism 5.03 software (GraphPad), depending on the group numbers. The statistical significance level was set as $p < 0.05$.

Results

Pre-conditioning with nsPEFs enhances trilineage differentiation potential of pMSCs

Stem cell properties of MSCs can be assessed by assaying the potential to differentiate along the osteogenic, adipogenic, and chondrogenic lineages [25]. In order to optimize the treatment conditions of nsPEFs, five sets of nsPEFs parameters (i.e., 10 ns at 20 kV/cm, 60 ns at 5 kV/cm, 60 ns at 10 kV/cm, 60 ns at 20 kV/cm, and 100 ns at 10 kV/cm) were firstly screened by the differentiation assays of pMSCs (**Fig. 1A**). We found that pre-conditioning of nsPEFs with the stimulation levels of 10 ns at 20 kV/cm or 100 ns at 10 kV/cm could significantly increase the subsequent trilineage differentiation of pMSCs, compared with the non-nsPEFs-preconditioned control group, but not the other three sets of parameters (**Table 1, Fig. 1B and C**).

The expression levels of differentiation genes were also evaluated at day 14. Osteogenic transcription factor *RUNX2* was upregulated by 10.53±1.91 and 9.03±1.77 fold by nsPEFs (10 ns at 20 kV/cm, and 100 ns at 10 kV/cm) (**Fig. 1D**); main regulating valves for adipogenic differentiation *PPARγ* was improved by 6.06±0.78 fold (10 ns at 20 kV/cm) and 9.93±1.42 fold (100 ns at 10 kV/cm) (**Fig. 1E**); chondrogenic transcription factor *SOX9* was increased by 10.50±1.95 fold (10 ns at 20 kV/cm) and 10.82±1.09 fold (100 ns at 10 kV/cm) (**Fig. 1F**). The expression of other related functional genes (*OCN*, *ALP*, *LPL*, *AP2*;

COLII, *AGG*) can be upregulated for 5-10 folds compared to the control group (**Fig.1D-F and Fig. S1A-C**). Taken together, these data suggest that the biological effects of nsPEFs depend on the time and energy levels of treatment. Only two sets of parameters, i.e., 10 ns at 20 kV/cm, and 100 ns at 10 kV/cm, could enhance the differentiation capacity of pMSCs.

Optimized nsPEFs do not influence the proliferation of pMSCs

Proliferation of pMSCs was evaluated with MTT assay over 7 days after pre-conditioning with nsPEFs, and nsPEFs treatments did not influence the proliferation of pMSCs (**Fig. S2A**). Moreover, cell cycle analysis and colony-forming units (CFU) assays were performed to evaluate the effects of nsPEFs. There were no significant differences in cell cycles (**Fig. S2B**) or CFU numbers (**Fig. S3C**) between nsPEFs treatments and control groups. These data indicate that our optimized nsPEFs parameters do not influence the clonogenicity and cell proliferation of MSCs.

nsPEFs enhance gene expression of OCT4 and NANOG via removing the methylation of their promoters

OCT4 and *NANOG* are critical transcriptional factors for stem cell pluripotency [26]. To further explore the cellular molecular mechanisms of the biological effects caused by nsPEFs, the expression levels of pluripotency genes *OCT4* and *NANOG* were examined. Interestingly, an instant elevation of *OCT4* and *NANOG* was found after 2 hours of nsPEFs treatment both in porcine MSCs (pMSCs) and human MSCs (hMSCs) (**Fig. 2A**). Expression of *OCT4* increased significantly 2 hours after nsPEFs, with 2.89±0.30 fold changes in pMSCs (*p=0.0029)*, and 4.82±0.97 fold in hMSCs (*p=0.0044*), in responsive of 10 ns at 20 kV/cm nsPEFs treatments; 3.56±0.30 fold in pMSCs (*p=0.001),* and 3.42±0.86 fold in hMSCs (*p=0.0476*), in responsive to 100 ns at 10 kV/cm of nsPEFs treatments (**Fig. 2A)**. The expression levels of *NANOG* gene was also upregulated significantly (pMSCs: 1.68±0.27 fold, *p=0.0396* and 1.7±0.16 fold, *p=0.0044 ;* hMSCs: 2.44±0.15 fold, *p=0.0005* and 1.96±0.21 fold, *p=0.0093*) in both nsPEFs treatment groups (10 ns at 20 kV/cm, and 100 ns at 10 kV/cm) (**Fig. 2A)**. We then tracked the gene expression levels of *OCT4* and *NANOG* of pMSCs after 3 days and 7 days of nsPEFs, and found that the upregulated *OCT4* subsequently decreased over 7 days (**Fig. S3A and C**), while the expression levels of NANOG remained the same after nsPEFs (**Fig. S3B and D**). In addition to the gene expression levels of *OCT4* and *NANOG*, we further examined the epigenetic modification by using bisulfite sequencing analysis. With the precondition of nsPEFs, a clearly drop was found in the methylation levels of CpG sites of *OCT4* and *NANOG* promoters, compared with non-treated pMSCs control group (**Fig. 2B and C)**. Therefore, these data suggest that nsPEFs can directly function on MSCs by demethylating the promoter region of *OCT4* and *NANOG.*

To further investigate if the instant upregulation of pluripotency genes was an universal effect for all stem cell types, we also evaluated the *OCT4* and *NANOG* changes in human embryonic stem cells (hESCs, details are in supplementary documents) after 2 hours of nsPEFs preconditioning. Interestingly,

we found that only nsPEFs with parameter of 100 ns at 10 kV/cm can enhance the gene expression of *OCT4* (4.92±1.00 fold, *p=0.0097)* and *NANOG* (4.63±1.16 fold, *p=0.0223)* of hESCs significantly, but not with 10 ns at 20 kV/cm (**Fig. S3C and D**).

nsPEFs temporally decrease DNMT1 expression

We next aimed to gain insights into how the hypomethylation of the *OCT4* and *NANOG* promoters was regulated by nsPEFs. DNA methylation of CpG dinucleotides is catalyzed by at least three different DNA methyltransferases (DNMTs), including DNMT1, DNMT3a and DNMT3b. And DNMT3a and DNMT3b function primarily as *de novo* methyltransferases that establish DNA methylation patterns, while DNMT1 is a key enzyme that maintains methylation patterns following DNA replication [27]. The DNMTs are essential for maintaining the methylation pattern in stem cells and for regulating their self-renewal and differentiation [24, 28]. The protein expression levels of DNMT1 substantially dropped by 0.58±0.11 and 0.27±0.05 fold respectively after 2 hours of nsPEFs treatment (10 ns at 20 kV/cm; 100 ns at 10 kV/cm) in pMSCs, while declined to 0.69±0.02 and 0.56±0.06 fold in hMSCs (**Fig. 3A**). Gene expression of *DNMT1* decreased significantly to 0.3±0.07 and 0.3±0.06 fold in pMSCs, and to 0.52±0.03 and 0.41±0.06 fold in hMSCs (**Fig. 3B**). However, the levels of DNMT3a and DNMT3b did not change in both pMSCs and hMSCs (**Fig. S4A and B**). To confirm the function of elevated DNMT1, the 5-methylcytosine contents which reflect global DNA methylation level were measured after 2 hours of nsPEFs. The global DNA methylation analysis revealed a 0.39±0.06 or 0.51±0.05 decrease in nsPEFs-preconditioned groups compared with control group (**Fig. 3C**).

To investigate how long the effects can last, protein expression levels of DNMT1 in pMSCs at 2, 12, 24 and 72 hours after nsPEFs were examined. After nsPEFs treatment, the expression of DNMT1 gradually increased from a lower level after 2 hours, and peaked at 24 hours, which was greatly higher than control groups, and then entered the end point of a dynamic equilibrium to the levels of control groups after 72 hours (**Fig. 3D**).

Overexpression of DNMT1 blocks the upregulation of OCT4 and NANOG induced by nsPEFs

To further justify if nsPEFs-reduced DNMT1 directly affect the expression of *OCT4* and *NANOG*, as well as the subsequent differentiation of pMSCs, we established a tet-on system to drive the DNMT1 expression in pMSCs (GFP as system control) (**Fig. 4A**). Because there were no significantly differences between the two sets of nsPEFs parameters (10 ns at 20 kV/cm *v.s.* 100 ns at 10kV/cm) in terms of the biological effects in earlier experiments, nsPEFs at the levels of 100 ns at 10 kV/cm were used in this section. Overexpression of DNMT1 by the tet-on system increased the protein expression of DNMT1 by 1.33±0.09 fold (*p=0.0138),* which indicated that we successfully established the DNMT1 overexpression model. Treatment of nsPEFs lowered the protein expression of DNMT1 by 0.34±0.06 fold in GFP control

group (**Fig. 4B**), which matched with the earlier results in pMSCs and hMSCs (**Fig. 3A, 3C**). Notably, the enhanced expression levels of *OCT4* (3.50±0.77 fold, p=0.0309, nsPEFs$^+$ group) and *NANOG* (1.95±0.22 fold, *p=0.0121,* nsPEFs$^+$ group) were blocked by overexpression of DNMT1, and the gene expression levels of *OCT4* and *NANOG* stayed unchanged after 2 hours of nsPEFs treatment (**Fig 4C,** DNMT1$^+$/nsPEFs$^+$ group). We then evaluated the percentage of CpG demethylation of *OCT4* and *NANOG* promoters with bisulfite sequencing analysis in this DNMT1 overexpression model (**Fig. 4D**), and the results were consistent with the genes expression levels (**Fig. 4C**). Taken together, these data show that overexpression of DNMT1 can block the effects of nsPEFs on gene expression of *OCT4* and *NANOG* in pMSCs.

Overexpression of DNMT1 blocks the subsequent effects of nsPEFs on stem cell differentiation

To further investigate if DNMT1 erased the subsequent differentiation performance of MSCs enhanced by nsPEFs, both trilineage differentiation and related functional genes were evaluated with DNMT1 overexpression (**Fig. 5A**). Osteogenic differentiation, which was indicated by the quantification of alizarin red staining intensity (**Fig. 5B**), was increased by 1.37±0.09 fold (*p=0.0071,* GFP$^+$/nsPEFs$^+$ group) by nsPEFs (100 ns, 10 kV/cm), and decreased by 0.78±0.06 fold by overexpression of DNMT1 (*p=0.0068,* DNMT1$^+$ group). Meanwhile, there was a significant difference between control group (GFP$^+$ group) and nsPEFs stimulated DNMT1 overexpression group (p=0.4912, DNMT1$^+$/nsPEFs$^+$ group). The differentiation performance of pMSCs into adipogenic lineage (oil-red O staining, **Fig. 5B**) and chondrogenic lineage (Alcian blue staining, **Fig. 5B**) shared the same trends as osteogenic differentiation. The expression levels of trilineage differentiation related key genes (osteogenic: *RUNX2*, *OCN*; adipogenic: *PPARγ*, *LPL*; chondrogenic: *SOX9*, *COLII*) showed similar trends with the differentiation assays, that all functional genes of trilineage differentiation were upregulated in GFP$^+$/nsPEFs$^+$ groups, and had no significant change in DNMT1$^+$/nsPEFs$^+$ groups (**Fig. 5C**).

Discussion

In this study, we discovered that a simple pre-condition of MSCs with nsPEFs (5 pulses in 10 seconds) can enhance the differentiation capacity of cultured stem cells. We then investigated the cellular and molecular mechanism of this phenomenon and found that nsPEFs can remove the methylation of promoters of the pluripotency genes *OCT4* and *NANOG* temporally *via* downregulating the DNA methyltransferases, in particularly, the DNMT1, where the higher expression of *OCT4* and *NANOG* was seen **(Fig. 6)**. These nsPEFs induced epigenetic responses probably can further establish a hypersensitive phase for cell differentiation, thus performed better in all trilineage differentiation assays.

Early mechanistic studies on the biological effects of nsPEFs indicated that the short-term high energy stimulation can influence the intracellular membranes by electroporation and permeabilization [11], while their effects on epigenetic regulation have not been reported. Treatment of nsPEFs with sufficient short

pulse durations and rapid rise times can induce supra-electroporation in all membranes of a cell, and extensively penetrate all membranes of organellars, supported by patch-clamp and fluorescent imaging results [11]. We here further explored the biological effects of nsPEFs, and found that nsPEFs can regulate DNA modification and gene expression epigenetically.

Notably, the biological effects on cells depend on the energy level and duration of nsPEFs. For example, higher energy and longer duration (300 ns, 1.8 kV/cm) can cause cell apoptosis [29]. It of course raises the safety concerns in the further application in stem cells. Our group has narrowed down a safety and effective range of nsPEFs, and have adopted two sets of parameters, the 10 ns at 20 kV/cm, and 100 ns at 10 kV/cm, for studying the biological effects of nsPEFs on MSCs [21]. In our previous study, we have checked the cell viability of MSCs by flow cytometry after one hour of nsPEFs stimulation at the levels of 10 ns at 20 kV/cm and 100 ns at 10 kV/cm, which showed no difference with regular cultured cells [21]. In addition, the current study also showed that the cell proliferation, cell cycle and colony-forming capacity of MSCs are not affected by nsPEFs at the defined levels. Our results indicated that these two sets of nsPEFs parameter (10 ns at 20 kV/cm; 100 ns at 10 kV/cm) are safe for MSCs.

Here we found that nsPEFs can enhance stem cell differentiation through temporally fine-tuning gene expressions of *OCT4* and *NANOG*. *OCT4*, *SOX2* and *NANOG* comprise a core transcriptional network that regulates self-renewal and pluripotency of stem cells, and are key elements for somatic cell to reprogram into iPSCs [30, 31]. These pluripotency genes are related to the differentiation potential of MSCs and can be seen as early-stage indicators and regulators of stem cell potencies [32, 33]. Many biophysical approaches can regulate the expression of these genes and the differentiation abilities of stem cells. For instance, low-intensity pulsed ultrasound stimulation could upregulate *NANOG* expression and the subsequent osteogenic differentiation of MSCs [34]; continuous hypoxia (1% oxygen concentration) has been used to enhance and accelerate the osteogenic ability of MSCs with the upregulation of *OCT4* [35]. In the study, we reported a similar effect of nsPEFs in regulating stem cell behaviors, that 5 pulses in 10 seconds of nsPEFs (10 ns at 20 kV/cm; 100 ns at 10 kV/cm) can efficiently upregulate *OCT4* and *NANOG* for 2-4 folds in human and porcine MSCs. Our nsPEFs treatment method provides a simpler and more effective way in regulating stem cells with the similar effect compared with the reported physical, chemical or biotechnological methods, most of which request full-time consuming and complicated operation procedures.

We also find a feedback regulation between DNMT1 and *OCT4*, *NANOG* in MSCs. Previous studies have proposed that partial DNA demethylation in the *OCT4* and *NANOG* promoter regions are required for gene activation in ESCs, iPSCs and other cell types [36-39]. *OCT4* and *NANOG* are hypomethylated in human ESCs and induced pluripotent stem cells (iPSCs), but are hypermethylated in their fibroblast derivatives [40]. DNMT1 have been shown to contribute to the methylation of *OCT4* and *NANOG* during mouse embryonic cell differentiation *in vivo* [41]. These research results indicated that DNMT1 plays an important role in tissue development, and it can block the expression of pluripotency genes and maintain a fully-differentiated stage of cells after embryo development and terminal differentiation. Here we found that DNMT1 protein in MSCs was immediately downregulated by nsPEFs, meanwhile *OCT4* and *NANOG*

gene expression was significantly upregulated with demethylation of their promoters. These results suggested that MSCs firstly respond to nsPEFs epigenetically and genetically, and then reconstruct to a hypersensitive phase for differentiation. In addition, when DNMT1 was overexpressed, *OCT4* and *NANOG* genes remained low and unchanged, and this suggested that there was a threshold of DNMT1 in regulating *OCT4* and *NANOG* gene expression, and certain level of DNMT1 is enough to keep the gate for *OCT4* and *NANOG* in MSCs. We also illustrated that the effects of nsPEFs on the expression of DNMT1 were a dynamic equilibrium procedure, that DNMT1 dropped after 2 hours, and gradually increased and peaked to ~2 folds at 24 hours compared to untreated control, and back to the levels of control groups after 72 hours. This phenomena probably can be explained by the regulating effects of *OCT4* and *NANOG* on DNMT1, which has been reported that *OCT4* and *NANOG* can directly bind to the promoter region of DNMT1 to promote the DNMT1 expression in MSCs and fibroblasts [24, 42]. Therefore, together with our findings, we believe that there should be a feedback loop between DNMT1 and *OCT4/NANOG* in MSCs, and the regulation effect of nsPEFs on MSCs can last for 3 days.

nsPEFs can downregulate DNMT1 temporally and enhance gene expression of *OCT4* and *NANOG*, as well as subsequent osteo-, chondro- and adipo-genetic differentiation of MSCs, which provides us a novel and precise tool for future stem cell research. nsPEFs induced demethylation of the promoter regions of specific genes is able to achieve reversible epigenetic regulation within a treating window of 3 days. Therefore, nsPEFs-based technologies have the potentials to be applied in iPSCs research to enhance the yield rate of iPSCs during reprogramming, as both the inhibition of DNMT1 [43-45] and electromagnetic fields [22] have been used to improve reprogramming efficiency, as reported previously. Despite all this, the parameters applying in iPSCs could be different from those in MSCs, because we found that only one set paraments of nsPEFs, 100 ns at 10 kV/cm, can regulate *OCT4/NANOG* in hESCs, which suggested that different cell types may need more detailed parameter segmentations. Another potential future application scenario of nsPEFs is in disease treatment, since the downregulation of DNMT1 has been reported to be able to promote the relief of the osteoarthritic symptoms in chondrocyte [46]. We have analyzed the proteomics of MSCs and found that among 3808 proteins, 59 were increased (Fold change > 1.33) and 44 were downregulated (Fold change < 0.75) after 2 hours of nsPEFs treatment, among which 6 proteins were related to epigenetic regulation (data not shown). This result suggested more genes can be regulated *via* nsPEFs epigenetically. Given the unlimited parameter combinations, nsPEFs could identify multiple epigenetic targets, and regulate them either temporally or persistently, which have great potential in many bio-applications, such as in development, aging and regeneration.

Conclusions

This study demonstrates that nsPEFs (as levels in 10 ns at 20 kV/cm, and 100 ns at 10 kV/cm) can enhance differentiation potential of both human and porcine mesenchymal stem cells. As to the molecular mechanism, nsPEFs could temporally unlock the stabilizer of DNA methylation with downregulation of DNMT1, which lead to the upregulation of *OCT4* and *NANOG*. Taken together, nsPEFs pre-conditioning provides a simple and effective method to improve the differentiation potential of MSCs. And we propose that nsPEFs can further establish a hypersensitive phase for cell differentiation.

Abbreviations

MSCs: Mesenchymal stem cells

nsPEFs: Nanosecond pulsed electric fields

DMEM: Dulbecco’s modified Eagle’s medium

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

RUNX2: Runt-related transcription factor 2

OCN: Osteocalcin

ALP: Alkaline phosphatase

PPARγ: peroxisome proliferators-activated receptor-γ

LPL: Lipoprotein lipase

SOX9: SRY-related high mobility group-box gene9

COLII: Type II Collagen

AGG: Aggrecan

MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide, Thiazolyl Blue Tetrazolium Bromide

OCT4: Octamer-binding transcription factor 4

DNMT: DNA methyltransferase

Declarations

Ethics approval and consent to participate

Animal experiments were approved by the Institutional Animal Care and Use Committee of Peking University, while human samples were harvested with approval of People's Hospital of Peking University.

Consent for publication

All authors agree to publish this manuscript in current format.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Kejia Li: conception and design, collection and assembly data, data analysis and interpretation, manuscript writing; Tong Ning: conception and design, collection and assembly data, data analysis and interpretation; Hao Wang: DNMT1 over-expression; Yangzi Jiang: data analysis and interpretation, manuscript writing; Jue Zhang: conception and design, final approval of manuscript; Zigang Ge: conception and design, data analysis and interpretation, manuscript writing.

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References

1. Squillaro T, Peluso G, Galderisi U. Clinical Trials With Mesenchymal Stem Cells: An Update. Cell Transplant. 2015;25:829–48.
2. Yan L, Munzer SR, Richardson JL. What is stemness? *Studies in History & Philosophy of Science Part C Studies in*. History Philosophy of Biological Biomedical Sciences. 2009;40:312–20.
3. Shinya Y, Hisashi M, Maki I, Yusuke H, Shigeyuki W. Cartilage Repair With Autologous Bone Marrow Mesenchymal Stem Cell Transplantation: Review of Preclinical and Clinical Studies. Cartilage. 2014;5:196–202.
4. Hashimoto Y, Nishida Y, Takahashi S, Nakamura H, Mera H, Kashiwa K, Yoshiya S, Inagaki Y, Uematsu K, Tanaka Y. Transplantation of autologous bone marrow-derived mesenchymal stem cells under arthroscopic surgery with microfracture versus microfracture alone for articular cartilage lesions in the knee: A multicenter prospective randomized control clinical trial. Regenerative therapy. 2019;11:106–13.
5. Erin C, Fei G, Maosong Q, Ivan M, Phillip R, Lingyun S, Gilkeson GS. Differential efficacy of human mesenchymal stem cells based on source of origin. J Immunol. 2014;193:4381–90.

6. Tuli R, Seghatoleslami MR, Tuli S, Wang ML, Hozack WJ, Manner PA, Danielson KG, Tuan RS. A simple, high-yield method for obtaining multipotential mesenchymal progenitor cells from trabecular bone. Molecular biotechnology. 2003;23:37–49.
7. Jiang Y, Cai Y, Zhang W, Yin Z, Hu C, Tong T, Lu P, Zhang S, Neculai D, Tuan RS. Human Cartilage-Derived Progenitor Cells From Committed Chondrocytes for Efficient Cartilage Repair and Regeneration. Stem Cells Translational Medicine. 2016;5:733–44.
8. Sotiropoulou PA, Perez SA, Maria S, Baxevanis CN, Michael P. Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. Stem Cells. 2010;24:462–71.
9. Li C, Wu X, Tong J, Yang X, Zhao J, Zheng Q, Zhao G, Ma Z. Comparative analysis of human mesenchymal stem cells from bone marrow and adipose tissue under xeno-free conditions for cell therapy. Stem Cell Res Ther. 2015;6:55.
10. Antebi B, Rodriguez LA, Walker KP, Asher AM, Kamucheka RM, Alvarado L, Mohammadipoor A, Cancio LC. Short-term physiological hypoxia potentiates the therapeutic function of mesenchymal stem cells. Stem Cell Res Ther. 2018;9:265.
11. Ning T, Zhang K, Heng BC, Ge ZG: Diverse Effects of Pulsed Electrical Stimulation on Cells - with a Focus on Chondrocytes and Cartilage Regeneration. *European Cells & Materials* 2019.
12. Yao C, Hu X, Mi Y, Li C, Sun C. Window effect of pulsed electric field on biological cells. IEEE Transactions on dielectrics electrical insulation. 2009;16:1259–66.
13. Bai F, Gusbeth C, Frey W, Nick P. Nanosecond pulsed electric fields trigger cell differentiation in Chlamydomonas reinhardtii. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2017;1859:651–61.
14. Morotomi-Yano K, Uemura Y, Katsuki S, Akiyama H, Yano K. -i: Activation of the JNK pathway by nanosecond pulsed electric fields. Biochem Biophys Res Commun. 2011;408:471–6.
15. Semenov I, Xiao S, Pakhomov AG. Primary pathways of intracellular Ca2 + mobilization by nanosecond pulsed electric field. Biochimica et Biophysica Acta (BBA) - Biomembranes. 2013;1828:981–9.
16. Chopinet L, Rols M-P. Nanosecond electric pulses: a mini-review of the present state of the art. Bioelectrochemistry. 2015;103:2–6.
17. He L, Xiao D, Feng J, Yao C, Tang L. Induction of apoptosis of liver cancer cells by nanosecond pulsed electric fields (nsPEFs). Med Oncol. 2017;34:24.
18. Wu S, Wang Y, Guo J, Chen Q, Zhang J, Fang J. Nanosecond pulsed electric fields as a novel drug free therapy for breast cancer: An in vivo study. Cancer Lett. 2013;343:268–74.
19. Zou H,., Gan XL, Linghu LJ, Chen C, Hu, Zhang LN. Y,. Intense nanosecond pulsed electric fields promote cancer cell apoptosis through centrosome-dependent pathway involving reduced level of PLK1. International Journal of Gynecology Obstetrics. 2012;119:658–8.
20. Zhang K, Guo J, Ge Z, Zhang J. Nanosecond Pulsed Electric Fields (nsPEFs) Regulate Phenotypes of Chondrocytes through Wnt/β-catenin Signaling Pathway. Sci Rep. 2014;4:5836.

21. Ning T, Guo J, Zhang K, Li K, Zhang J, Yang Z, Ge Z. Nanosecond pulsed electric fields enhanced chondrogenic potential of mesenchymal stem cells via JNK/CREB-STAT3 signaling pathway. Stem Cell Res Ther. 2019;10:45.
22. Baek S, Quan X, Kim S, Lengner C, Park JK, Kim J. Electromagnetic fields mediate efficient cell reprogramming into a pluripotent state. ACS Nano. 2014;8:10125–38.
23. Wang J, Guo J, Wu S, Feng H, Sun S, Pan J, Zhang J, Beebe SJ. Synergistic effects of nanosecond pulsed electric fields combined with low concentration of gemcitabine on human oral squamous cell carcinoma in vitro. PLoS One. 2012;7:e43213.
24. Tsai C-C, Su P-F, Huang Y-F, Yew T-L, Hung S-C. Oct4 and Nanog Directly Regulate Dnmt1 to Maintain Self-Renewal and Undifferentiated State in Mesenchymal Stem Cells. Mol Cell. 2012;47:169–82.
25. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. 8:315–317.
26. Yuin-Han L, Qiang W, Joon-Lin C, Vega VB, Weiwei Z, Xi C, Guillaume B, Joshy G, Bernard L, Jun L. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet. 2006;38:431–40.
27. Dor Y, Cedar H. Principles of DNA methylation and their implications for biology and medicine. Lancet. 2018;392:777–86.
28. Bhutani N, Brady JJ, Damian M, Sacco A, Corbel SY, Blau HM. Reprogramming towards pluripotency requires AID-dependent DNA demethylation. Nature. 2010;463:1042–7.
29. Gianulis EC, Labib C, Saulis G, Novickij V, Pakhomov AG. Selective susceptibility to nanosecond pulsed electric field (nsPEF) across different human cell types. Cellular Molecular Life Sciences CMLS. 2016;74:1–14.
30. Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Schöler H, Smith A. Formation of Pluripotent Stem Cells in the Mammalian Embryo Depends on the POU Transcription Factor Oct4. Cell. 1998;95:379–91.
31. Looijenga LHJ, Hans S, Leeuw HPJC, De, Brazao CA, De Gouveia, Gillis AJM, Roozendaal KEP, Van, Zoelen EJJ Van, Weber RFA, Wolffenbuttel KP, Herman VD. POU5F1 (OCT3/4) identifies cells with pluripotent potential in human germ cell tumors. Can Res. 2003;63:2244.
32. Liu TM, Wu YN, Guo XM, Hui JH, Lee EH, Lim B. Effects of ectopic Nanog and Oct4 overexpression on mesenchymal stem cells. Stem Cells Dev. 2009;18:1013–22.
33. Hyslop L, Stojkovic M, Armstrong L, Walter T, Stojkovic P, Przyborski S, Herbert M, Murdoch A, Strachan T, Lako M. Downregulation of NANOG induces differentiation of human embryonic stem cells to extraembryonic lineages. Stem Cells. 2005;23:1035–43.
34. Kusuyama J, Hwan SC, Ohnishi T, Bandow K, Matsuguchi T. 10. Low-Intensity Pulsed Ultrasound (LIPUS) Stimulation Helps to Maintain the Differentiation Potency of Mesenchymal Stem Cells by Induction in Nanog Protein Transcript Levels and Phosphorylation. J Orthop Trauma. 2016;30:4.

35. Ding H, Chen S, Yin JH, Xie XT, Zhu ZH, Gao YS, Zhang CQ. Continuous hypoxia regulates the osteogenic potential of mesenchymal stem cells in a time-dependent manner. Mol Med Rep. 2014;10:2184–90.
36. Marta G, Jochen U. From skin to the treatment of diseases–the possibilities of iPS cell research in dermatology. Exp Dermatol. 2011;20:523–8.
37. Cowan CA, Jocelyn A, Melton DA, Kevin E. Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science. 2005;309:1369–73.
38. Freberg C, Dahl J, Collas S. P: Epigenetic reprogramming of OCT4 and NANOG regulatory regions by embryonal carcinoma cell extract. Mol Biol Cell. 2007;18:1543–53.
39. Simonsson S, Gurdon J. DNA demethylation is necessary for the epigenetic reprogramming of somatic cell nuclei. Nat Cell Biol. 2004;6:984–90.
40. Zhao L, Yu Y, Deng C. Protein and mRNA expression of Shh, Smo and Gli1 and inhibition by cyclopamine in hepatocytes of rats with chronic fluorosis. *Toxicology Letters*, 225:318–324.
41. Jing-Yu L, Min-Tie P, Ryutaro H, Bin-Zhong L, Yan-Nv H, Rong Z, Nai-He J, Taiping C, En L, Hiroyuki S. Synergistic function of DNA methyltransferases Dnmt3a and Dnmt3b in the methylation of Oct4 and Nanog. Molecular Cellular Biology. 2007;27:8748–59.
42. Liu Y, Mukhopadhyay P, Pisano MM, Lu X, Huang L, Lu Q, Dean DC. Repression of Zeb1 and hypoxia cause sequential mesenchymal-to-epithelial transition and induction of aid, Oct4, and Dnmt1, leading to immortalization and multipotential reprogramming of fibroblasts in spheres. Stem Cells. 2013;31:1350–62.
43. Mikkelsen TS, Jacob H, Xiaolan Z, Manching K, Marius W, Patrick S, Bernstein BE, Rudolf J, Lander ES, Alexander M. Dissecting direct reprogramming through integrative genomic analysis. Nature. 2008;454:49.
44. Danwei H, Kenji O, René M, Wenjun G, Astrid E, Shuibing C, Whitney M, Melton DA. Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. Nat Biotechnol. 2008;26:1269–75.
45. Shi Y, Desponts C, Do JT, Hahm HS, Schöler HR, Ding S. Induction of Pluripotent Stem Cells from Mouse Embryonic Fibroblasts by Oct4 and Klf4 with Small-Molecule Compounds. Cell Stem Cell. 2008;3:568–74.
46. Zhu X, Chen F, Lu K, Wei A, Jiang Q, Cao W. PPARgamma preservation via promoter demethylation alleviates osteoarthritis in mice. *Ann Rheum Dis* 2019.

Table 1

Table 1. Quantification of histological staining intensity of MSCs pre-conditioned with five parameters of nsPEFs and differentiated to osteogenic, adipogenic and chondrogenic lineage for 14 days.

10ns 20kV/cm	60ns 5kV/cm	60ns 10kV/cm	60ns 20kV/cm	100ns 10kV/cm
1.58± 0.05	1.34± 0.07	1.15± 0.07	1.20± 0.09	1.48± 0.06
****	*	NS	NS	***
1.72± 0.03	1.21± 0.10	1.34± 0.10	1.46± 0.10	1.83± 0.03
****	NS	**	***	****
1.46± 0.05	1.96 ± 0.17	1.30 ± 0.08	1.76 ± 0.10	1.45± 0.03
***	****	NS	***	****

(3 batches of studies were tested with 3 biological donors, values are mean ± SEM from one representative batch with 5 technical repeats, one-way ANOVA, *p < 0.05, **p < 0.01, ***p<0.001, ****<0.0001 and NS, p>0.05)

Figures

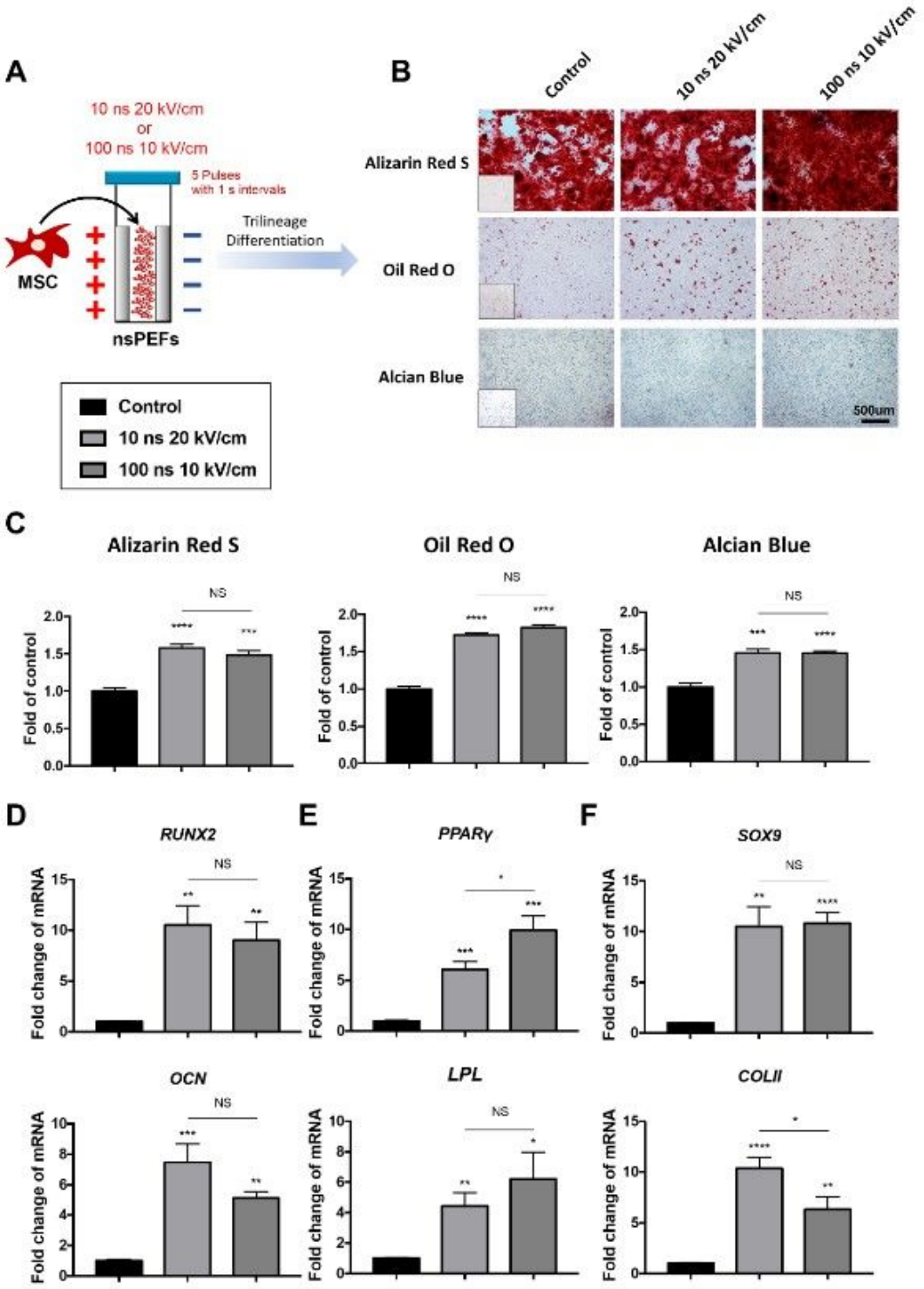

Figure 1

A single nsPEFs treatment (5 pulses, less than 10 seconds) can enhance the differ-entiation of MSCs. (A) Schematic of MSCs stimulated by nsPEFs. (B) Alizarin Red S, Oil red O staining and Alcian blue staining for osteogenic, adipo-genic and chondrogenic differentiation at days 14, insets show the no-staining counter-parts. (C) Quantification of differentiation into osteogenic, adipogenic, and chondrogenic lin-eages. (3 batches of studies were tested with 3 biological donors, values are mean ± SEM from one

representative batch with 5 technical repeats, one-way ANOVA, *p≤0.05; **p≤0.01, ***p≤0.001, ****p≤0.0001, NS, p>0.05) (D-F) qRT-PCR for the mRNA levels of genes associated with trilineages differentia-tion (osteogenic: RUNX2, OCN; adipogenic: PPARγ, LPL; chondrogenic: SOX9, COLII) respectively at days 14. (3 batches of studies were tested with 3 biological donors, val-ues are mean ± SEM from one representative batch with 5 technical repeats, one-way ANOVA, *p≤0.05; **p≤0.01, ***p≤0.001, ****p≤0.0001, NS, p>0.05)

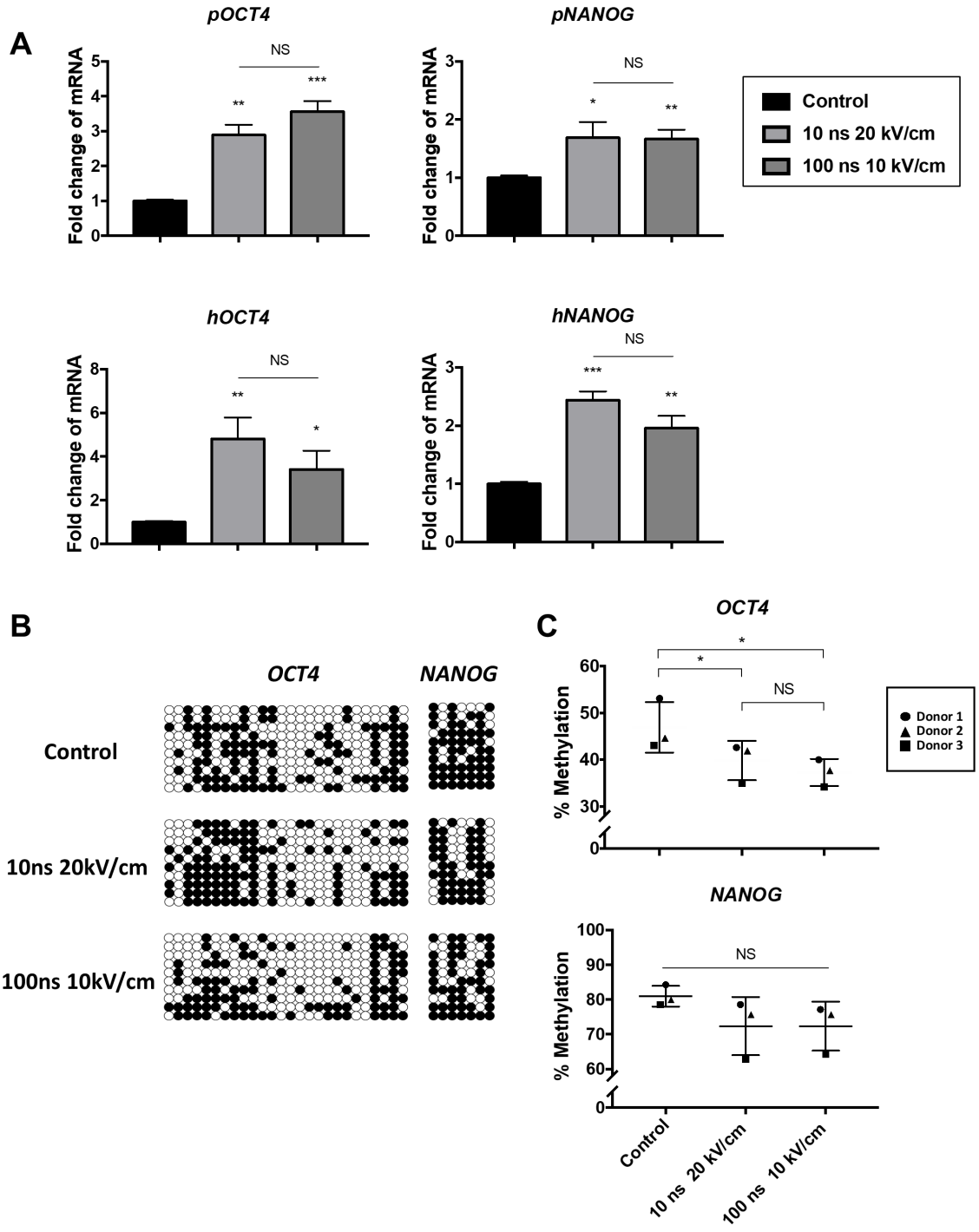

Figure 2

nsPEFs promote OCT4 and NANOG expression with increasing demethylation level of promoter. (A) qRT-PCR for the expression of OCT4 and NANOG of pMSCs and hMSCs after 2 hours stimulation by nsPEFs. (3 batches of studies were tested with 3 biological donors, values are mean ± SEM from one representative batch with 5 technical repeats, one-way ANOVA. *p≤0.05; **p≤0.01, ***p≤0.001, ****p≤0.0001, NS, p>0.05) (B) Bisulfite sequencing analysis of OCT4 and NANOG promoter of pMSCs after 2 hours stimulation by nsPEFs. Each CpG is represented by a circle in the 50–30 orienta-tion; each row represents the methylation state of each CpG in one bacterial clone of PCR product. ●, unmethylated CpG; ●, methylated CpG. (C) Percentage of CpG demethylation for each promoter. (Values are mean ± SD, n=3, one-way ANOVA, *p=0.0329, *p=0.0171)

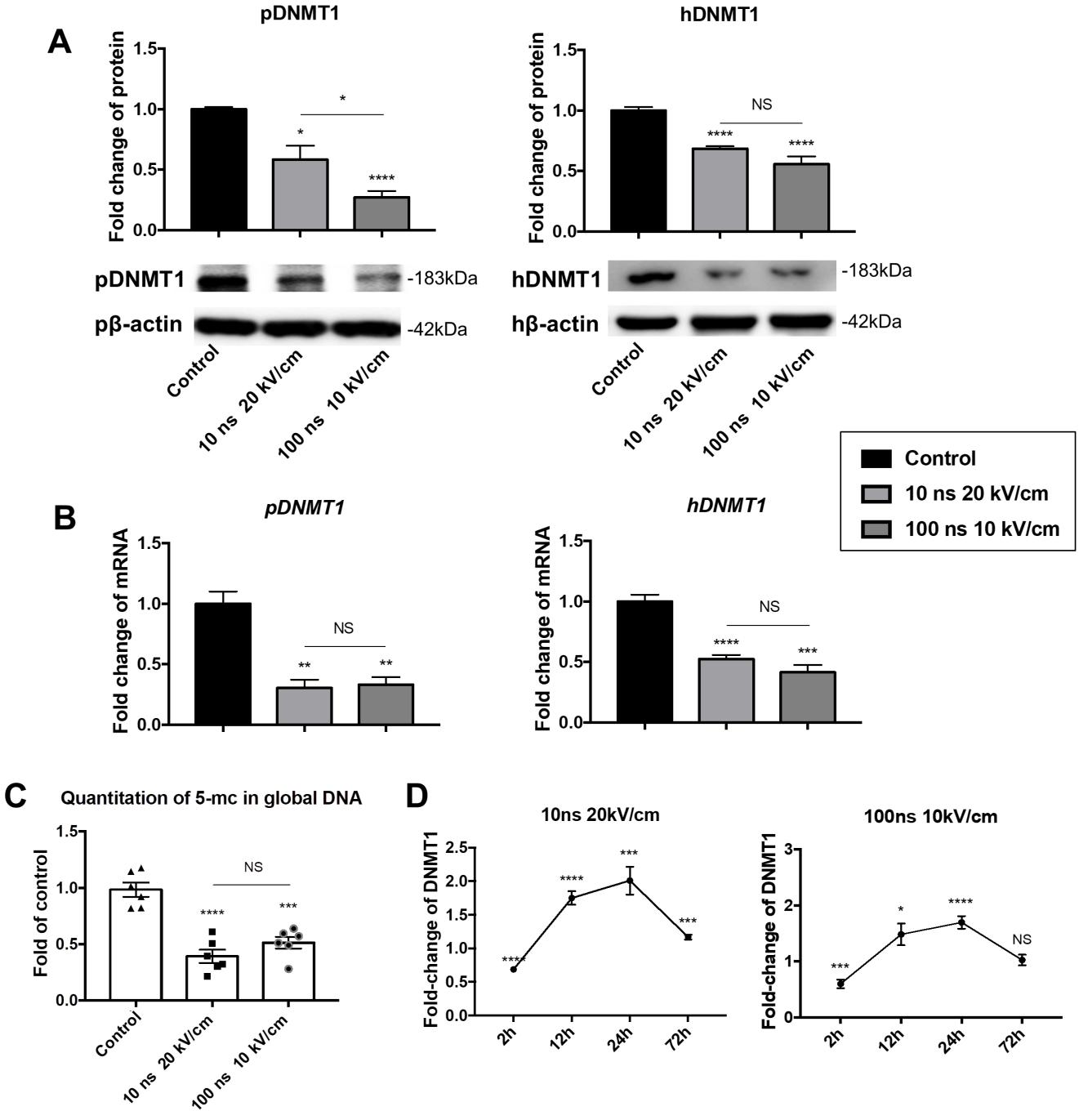

Figure 3

DNMT1 responds for nsPEFs and opens a short window of 3 days. (A) Western blot for DNMT1 protein expression level of pMSCs and hMSCs after 2 hours stimulated by nsPEFs. (3 batches of studies were tested with 3 biological donors, values are mean ± SEM from one representative batch with 5 technical repeats, one-way ANOVA, *p≤0.05; **p≤0.01, ***p≤0.001, ****p≤0.0001, NS, p>0.05) (B) qRT-PCR for the expression of DNMT1 of pMSCs and hMSCs after 2 hours stimu-lated by nsPEFs. (3 batches of studies were tested with 3 biological donors, values are mean ± SEM from one representative batch with 5 technical repeats, one-way ANOVA, *p≤0.05; **p≤0.01, ***p≤0.001, ****p≤0.0001, NS, p>0.05) (C) The global DNA methylation level after 2 hours stimulated by nsPEFs. (n=6, one-way ANOVA, ****p<0.0001, NS=0.1515, ***p=0.0002) (D) Protein quantification for the expression of DNMT1 at 2, 12, 24, and 72 hours after stimulated by nsPEFs. (3 batches of studies were tested with 3 biological donors, values are mean ± SEM from one representative batch with 5 technical repeats, one-way ANOVA, *p≤0.05; **p≤0.01, ***p≤0.001, ****p≤0.0001, NS, p>0.05)

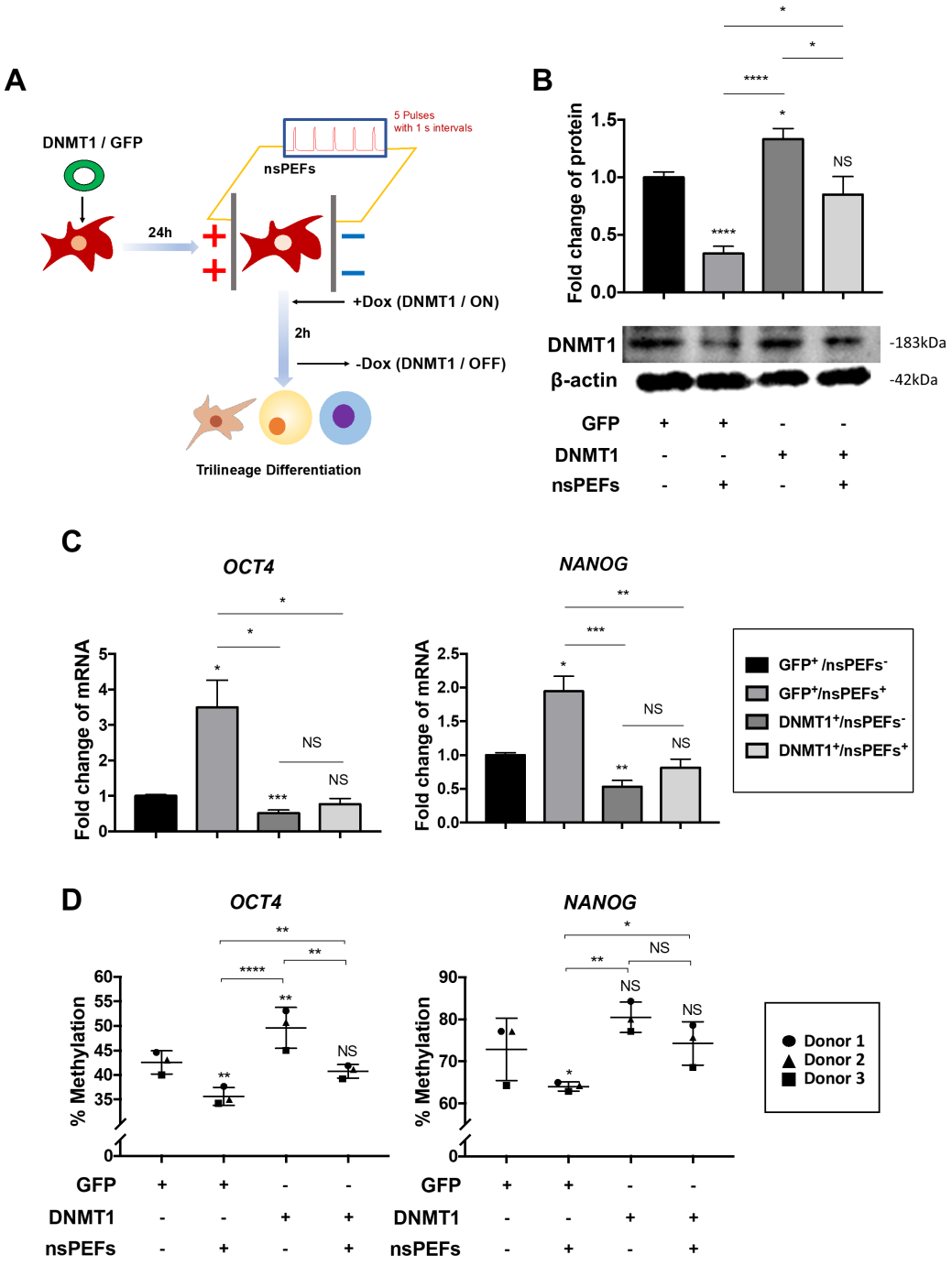

Figure 4

Overexpression of DNMT1 blocks demethylation caused by nsPEFs. (A) Schematic of MSCs stimulated by nsPEFs with overexpression of DNMT1. (B) Western blot for DNMT1 after pre-treated with nsPEFs with overexpression of GFP or DNMT1. (3 batches of studies were tested with 3 biological donors, values are mean ± SEM from one representative batch with 5 technical repeats, one-way ANOVA, *p≤0.05; **p≤0.01, ***p≤0.001, ****p≤0.0001, NS, p>0.05) (C) Quantitative RT-PCR for the expression of OCT4 and NANOG

after 2 hours stimula-tion by nsPEFs. (3 batches of studies were tested with 3 biological donors, values are mean ± SEM from one representative batch with 5 technical repeats, one-way ANOVA, *p≤0.05; **p≤0.01, ***p≤0.001, ****p≤0.0001, NS, p>0.05) (D) Percentage of CpG demethylation for OCT4 and NANOG promoter. (Values are mean ± SD, n=3, one-way ANOVA, *p≤0.05; **p≤0.01, ***p≤0.001, ****p≤0.0001, NS, p>0.05)

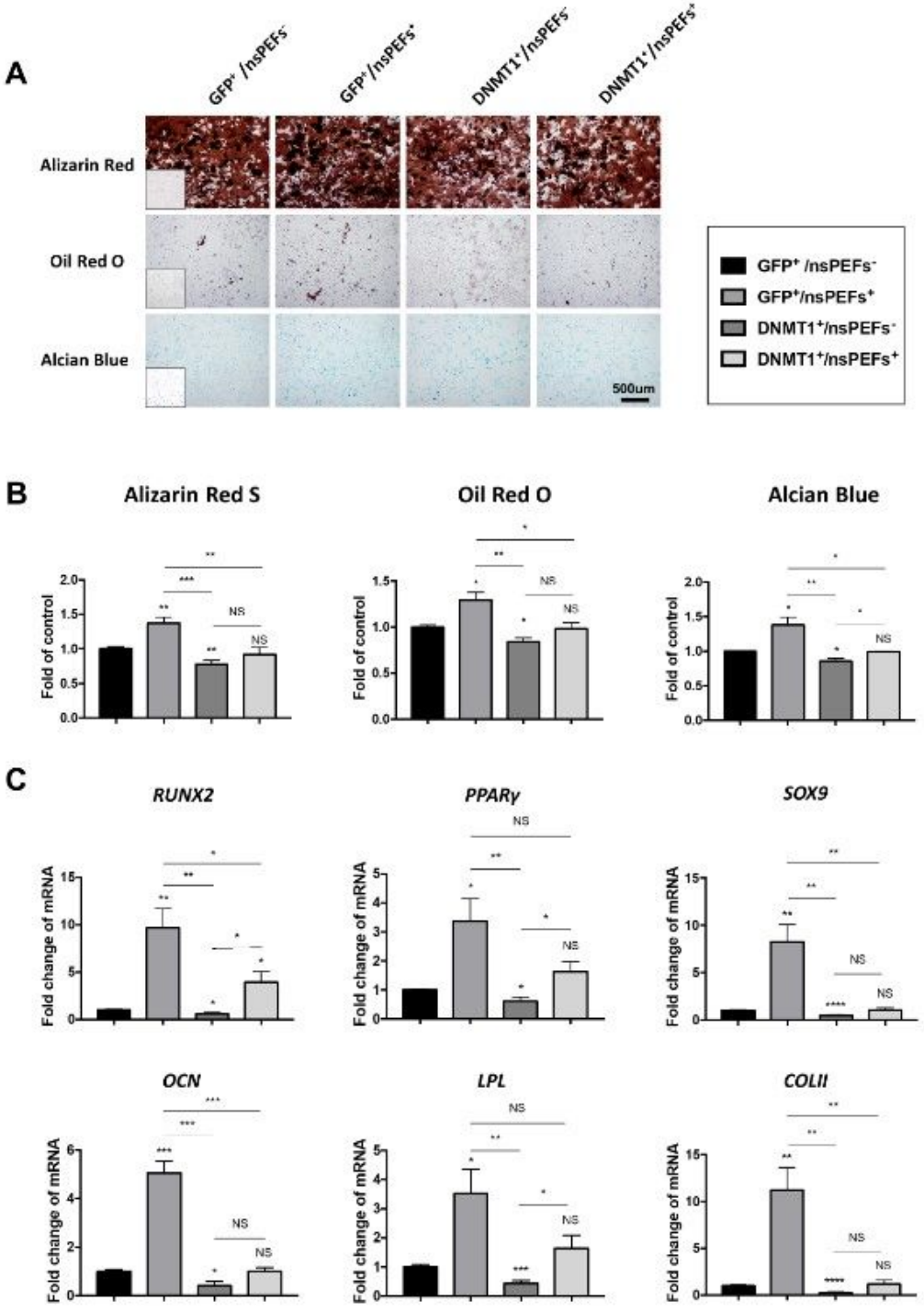

Figure 5

Overexpression of DNMT1 hinders the differentiation potential of MSCs caused by nsPEFs. (A) Alizarin Red S, Oil red O staining and Alcian blue staining for osteogenic differen-tiation, adipogenic and chondrogenic differentiation at days 14, insets show the no-staining counterparts. (B) Quantification of differentiation into osteogenic, adipogenic, and chondrogenic lin-eages. (3 batches of studies were tested with 3 biological donors, values are mean ± SEM from one representative batch with 5 technical repeats, one-way ANOVA, *p≤0.05; **p≤0.01, ***p≤0.001, ****p≤0.0001, NS, p>0.05) (C) MSCs were induced to undergo osteogenic, adipogenic, and chondrogenic differen-tiation for 14 days, and qRT-PCR was performed. Trilineage differentiation related key genes, osteogenic: RUNX2, OCN; adipogenic: PPARγ, LPL; chondrogenic: SOX9, COLII (3 batches of studies were tested with 3 biological donors, values are mean ± SEM from one representative batch with 5 technical repeats, one-way ANOVA, *p≤0.05; **p≤0.01, ***p≤0.001, ****p≤0.0001, NS, p>0.05)

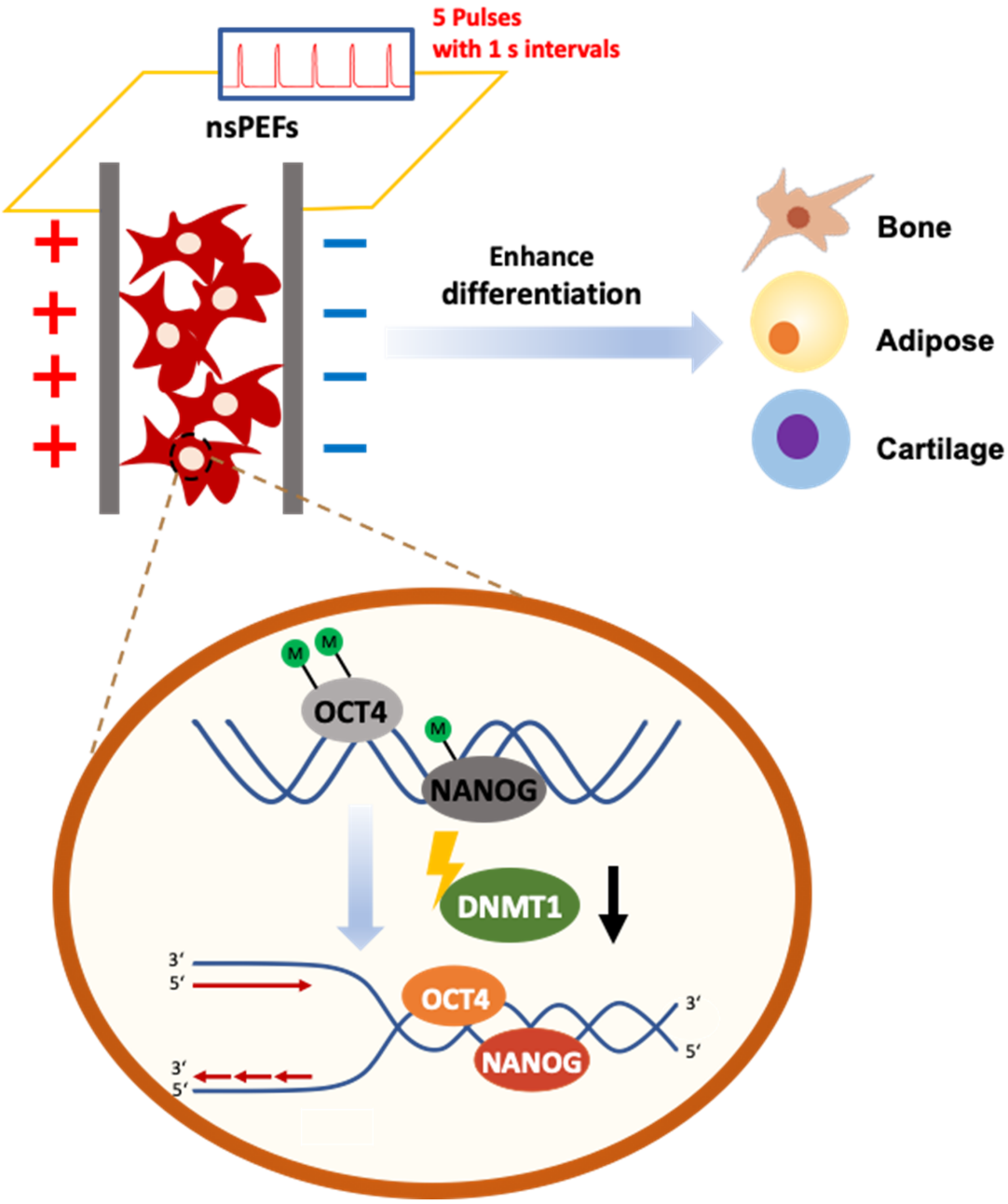

Figure 6

Schematic illustration of the possible molecular mechanisms induced by nsPEFs in MSCs.

Supplementary Files

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