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# GFP labelling and epigenetic enzyme expression of bone marrow-derived mesenchymal stem cells from bovine foetuses

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ABSTRACT

Mesenchymal stem cells (MSC) are multipotent progenitor cells defined by their ability to self-renew and give rise to differentiated progeny. Since MSC from adult tissues represent a promising source of cells for a wide range of cellular therapies, there is high scientific interest in better understanding the potential for genetic modification and the mechanism underlying differentiation. The main objective of this study was to evaluate the potential for gene delivery using a GFP vector and lipofectamine, and to quantify the expression of epigenetic enzymes during foetal bMSC multilineage differentiation. Proportion of GFP-positive cells achieved (15.7% ± 3.5) indicated moderately low transfection efficiency. Analysis of DNA methyltransferase expression during MSC multilineage differentiation suggested no association with osteogenic and chondrogenic differentiation. However, up-regulation of KDM6B expression during osteogenic differentiation was associated with adoption of osteogenic lineage. Furthermore, increase in epigenetic enzyme expression suggested an intense epigenetic regulation during adipogenic differentiation.


## 1. Introduction

Mesenchymal stem cells (MSC) are multipotent progenitor cells defined by their ability to self-renew and give rise to differentiated progeny. According to the International Society for Cellular Therapy (ISCT), minimal criteria for defining MSC cultures include adherence to plastic under standard culture conditions, expression of surface antigens markers CD105 (endoglin), CD73 (ecto-5′-nucleotidase) and CD90 (Thy-1), lack of expression of hematopoietic markers CD45 (protein tyrosine phosphatase, receptor type, C), CD34 (CD34 molecule) and CD14 (CD14 molecule) and capacity for trilineage differentiation (Dominici et al., 2006). Several tissues have been explored with the aim to find an abundant source for MSC including bone marrow (BM), adipose, umbilical and placental. MSC are directly isolated from BM aspirates based on their ability to adhere to plastic when plated in monolayer culture. Isolated MSC replicate *ex vivo* and form a phenotypically homogeneous population of progenitor cells with different lineage commitments and organized in a complex hierarchy (Harichandan and Bühring, 2011; Russell et al., 2010). Thereafter, multipotent MSC are able to differentiate *in vitro* into mature cells of mesenchymal lineages including adipocytes, osteocytes and chondrocytes (Pittenger et al., 1999). Plasticity of MSC is not limited to mesenchymal derivatives, since MSC have also been induced to differentiate into ectodermal (neurons) and endodermal (hepatocytes) lineages (Dueñas et al., 2014; Safford et al., 2002). Since MSC from adult tissues represent a promising source of cells for a wide range of cellular therapies, there is high scientific interest in better understanding the mechanism underlying proliferation and differentiation of these cells.

In the recent years, the field of stem cell research has expanded from the traditional laboratory animal models to a broad variety of models including large animals (Calloni et al., 2014). Similarities in organ size and physiology with humans and a longer life span in comparison with laboratory animals support the use of large animal models for long-term experiments in regenerative medicine (Bosch et al., 2006; Patterson-Kane et al., 2012). Large animal models would be invaluable for testing the efficiency and safety of MSC for future cell therapies and for the creation of human disease models. In this context, the bovine experimental model can give advantages for clinical applications of MSC for human and veterinary medicine especially in musculoskeletal disorders (Aerssens et al., 1998; Bosnakovski et al., 2005; Bucher et al., 2013). We and others have reported the isolation and mesenchymal multilineage differentiation of bovine MSC (bMSC) derived from adult BM (Bosnakovski et al., 2005; Colleoni et al., 2005), foetal BM (Cortes et al., 2013), umbilical cord blood (Cardoso et al., 2012; Raoufi et al., 2010) and recently adipose tissue (Lu et al., 2014). Furthermore,

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the multipotency of foetal bMSC has also been demonstrated in *in vitro* differentiation experiments where bMSC were induced into neurogenic and hepatogenic lineages (Dueñas et al., 2014). The simplicity of isolation and the potential to differentiate into several cell types lays the foundation for bMSC from abattoir-derived bovine foetuses as an interesting source for investigation of stem cell biology.

A remarkable feature of MSC for regenerative medicine and gene therapy is the capacity to home to sites of injury after systemic delivery (Barry and Murphy, 2004). This mechanism, known as homing, is mediated by apparent evasion of normal immune response raised against transplanted allogenic cells (Devine et al., 2001). MSC may migrate to sites of injury to promote tissue repair and potentially engraft into damaged tissue. After homing, transduced MSC may induce gene delivery and express therapeutic genes for extensive periods of time. Considering that homing of MSC is inefficient and many MSC are trapped in the lung following systemic administration, it is imperative to trace the fate of injected MSC. Classical methods to label cells consists of viral or non-viral vectors that express fluorescent proteins such as green fluorescent protein (GFP), which has been helpful in gaining insights in homing and engraftment of MSC (Cheng et al., 2008). Despite viral-based transgenesis being reported as the most efficient system to generate stable transgenic MSC, viruses have limitations based on cytotoxicity, mutagenesis and immunological rejection (Jo and Tabata, 2008). In this respect, non-viral plasmid vectors are considered to induce a less efficient but also a safer gene delivery (McMahon et al., 2006).

Epigenetic regulation plays a crucial role in the promotion of appropriate transcriptional pathways during both embryonic development and adult tissue maintenance. Modification of chromatin architecture alters the accessibility of genes to transcription factors and other modulators and regulates gene expression at the epigenetic level. In recent years, epigenetic regulation has also emerged as an important modulator of stem cell differentiation (Wu and Sun, 2006). Despite investigation of the epigenetic regulation of cell fate determination largely focusing on embryonic stem (ES) cells, recent studies have indicated that epigenetic states are also responsible for lineage-specific differentiation of MSC. Major mechanisms underlying epigenetic regulation include DNA methylation and histone modifications. DNA methylation consists of the addition of a methyl group to position 5 of cytosine at cytosine-phosphate-guanine (CpG) dinucleotides and occurs symmetrically on both DNA strands. Enzyme DNA methyltransferase 1 (DNMT1) recognizes hemimethylated DNA and ensures methylation profile fidelity by catalyzing the methylation of its corresponding daughter strand (Jaenisch and Bird, 2003). Methyltransferases DNMT3a and DNMT3b are responsible for *de novo* DNA methylation during embryonic development and cell differentiation (Turek-Plewa and Jagodzinski, 2005). Histones contain unstructured N-terminal "tails" that can be covalently modified in different ways including acetylation and methylation to regulate gene expression (Kouzarides, 2007). Methylation of histone lysine can be catalysed by histone methyltransferases with either transcriptional activation or repression (Kouzarides, 2002). Histone methylase EZH2 catalyse addition of three methyl groups to lysine 27 of histone 3 (H3K27) resulting in chromatin condensation and repression of gene expression (Schwartz and Pirrotta, 2007). Conversely, histone demethylase KDM6B catalyses demethylation of H3K27 acting as a critical epigenetic regulator in BM-MSC fate commitment by regulation of osteogenic, adipogenic and neurogenic differentiation (Estaras et al., 2012; Ye et al., 2012).

Adult tissues represent a promising source of MSC with potential relevance in cellular and regenerative therapies in human and veterinary medicine. However, before MSC are considered for these applications, the feasibility of genetic modification and the key epigenetic pathways underlying the mechanism of cell differentiation should be investigated. The main objective of this study was to evaluate the potential for gene delivery using a GFP plasmid vector and lipofectamine, and to quantify the expression of epigenetic enzymes during foetal bMSC multilineage differentiation.

## 2. Materials and methods

### 2.1. Isolation and culture of bMSC from foetal bone marrow

All procedures have been approved by the Bioethical Committee of the National Commission for Scientific and Technology Research from Chile (Fondecyt). Bone marrow was aspirated from bovine foetuses ($n = 10$; 7–8 months of gestation) collected at a local abattoir. The marrow was drawn from femoral marrow cavity into syringes containing high glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Islands, NY, USA) supplemented with 10% foetal bovine serum (FBS), 1000 U heparin, 100 U/mL penicillin and 100 μg/mL streptomycin. Bone marrow samples were washed twice with phosphate-buffered saline (PBS) and twice with DMEM. Then cells were plated in DMEM (high glucose) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B. Cells were incubated at 38 °C in a humidified atmosphere containing 5% $CO_2$. After 2 days, non-adherent cells were removed by changing the culture medium. Following the initial 2 days, the medium was changed every 2–3 days. After three to four passages, cells were gently harvested when 90% confluent using 0.25% trypsin in 0.1% EDTA. Following determination of cell viability, cells were used to initiate transfection and differentiation experiments.

### 2.2. Transfection procedure

The day before transfection, bMSC at passage 3 were trypsinized, counted and plated in a 12-well plate at a concentration of $2 \times 10^5$ cells/mL until 70% confluency. On the day of transfection, vector DNA (pTracer-CMV/Bsd, Invitrogen, Carlsbad, CA, USA) and Lipofectamine LTX (Invitrogen) complexes were prepared as follow: (a) Diluted pTracer-CMV/Bsd (10 μg) with 2 mL of OptiMEM reduced serum medium (Invitrogen) and 20 μL of PLUS reagent (Invitrogen), mixed gently and incubated at room temperature for 10 min; (b) Mixed 600 μL of OpM in three dilutions with Lipofectamine LTX (6, 18 and 36 μL) and 600 μL of pTracer-CMV/Bsd-PLUS complex, mixed gently and incubated at room temperature for 30 min to allow the pTracer-CMV/Bsd-Lipofectamine LTX complexes to form. The medium was removed from each well and added 100, 200, 300 and 400 μL of pTracer-CMV/Bsd-Lipofectamine LTX complexes to each well containing cells and sufficient fresh medium to achieve a total volume of 500 μL. Twelve different concentrations of pTracer-CMV/Bsd (DNA) and Lipofectamine LTX were used. After transfection, cells were observed under fluorescent microscope and proportion of GFP-positive cells was determined by flow-cytometry. Determination of blasticidin sensitivity was performed by seeding $6 \times 10^4$ cells/mL in 12-well plates until 25% confluency in DMEM medium supplemented with 10% FBS. At the next day, medium was changed with fresh medium supplemented with 0, 2, 4, 6, 8 and 10 μg/mL of blasticidin. Cells were incubated at 38 °C under a 5% $CO_2$ atmosphere and medium was changed every 3 days until the minimum concentration of blasticidin necessary to kill all cells was determined (10 days). After determination of blasticidin sensitivity (2 μg/mL), selection of stable GFP-positive cells was performed. GFP-positive bMSC were trypsinized and suspended in DMEM with 10% FBS in 96-well plates for clonal culture. Stable GFP-positive cells were also fixed in RLT buffer (Qiagen, Incorporated, Valencia, CA, USA) for quantitative-PCR (Q-PCR) analysis.

### 2.3. Flow cytometry

Quantification of GFP-positive bMSC was performed using flow cytometry. Cells were removed from culture dishes using 0.25% trypsin in 0.1% EDTA for 10 min at 38 °C. Then cells were permeabilized using a Foxp3 kit (eBioscience, San Diego, CA, USA) by incubation at room temperature for 5 min with shaking. After three washes on PBS, the pellet was resuspended on cytometry buffer and analysed using a Gallios Flow Cytometer (Beckman Coulter, Brea, CA, USA) using a 488 nm (blue) laser light. The threshold for negative events was set on the first decade of fluorescence level. Negative procedural control corresponded to negative control bMSC.

### 2.4. Osteogenic differentiation and characterization

Cells ($5 \times 10^4/cm^2$) isolated separately from three foetuses were plated in T-25 culture dishes either in control or differentiation (three replicates) medium and cultured as described above for a 24-day experiment. Control medium consisted of DMEM (high glucose) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B. Differentiation medium consisted of control medium supplemented with 100 nM dexamethasone, 10 mM sodium β-glycerophosphate, 0.05 mM ascorbic acid (all from Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured for 24 days under 38 °C in a humidified atmosphere containing 5% $CO_2$, with the medium being changed every 2 days. Samples were obtained at Days 0, 8, 16 and 24 and analysed for gene expression by Q-PCR. Osteogenic differentiation was also analysed at Day 24 of differentiation by visualization of von Kossa staining of mineralized materials in the cell culture.

### 2.5. Chondrogenic differentiation and characterization

Cells ($1 \times 10^6$) isolated separately from three foetuses were resuspended into 1 mL of control or differentiation (three replicates) medium, transferred into 15-mL tubes and centrifuged at 500 *g* for 5 min. Control medium consisted of DMEM (high glucose) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B. Differentiation medium consisted in control medium supplemented with 10% ITS (6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 μg/mL selenious acid), 1 mM pyruvate, 50 μg/mL ascorbate 2-phosphate, 0.1 μM dexamethasone, and 8 ng/mL TGFβ1 (R&D Systems, Minneapolis, MN, USA). Pellets were cultured for 21 days under 38 °C in a humidified atmosphere containing 5% $CO_2$. Samples were obtained at 7-day intervals for a total of 21 days. Gene expression was analysed by Q-PCR. The level of chondrogenic differentiation was also analysed at Day 21 of differentiation by visualization of glycosaminoglycan formation using alcian blue staining in micromass histological sections.

### 2.6. Adipogenic differentiation and characterization

Cells ($2–5 \times 10^3/cm^2$) isolated separately from three foetuses were seeded in control or differentiation (three replicates) medium. Control medium consisted of DMEM (high glucose) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B. Differentiation medium consisted of control medium supplemented with 10% FBS, 1 μM dexamethasone, 0.5 mM indomethacin, 10 μg/mL insulin and 100 mM 3-isobutyl-1-methylxanthine (all from Sigma). Cells were cultured in differentiation medium for 3 days and then in differentiation maintenance medium containing DMEM (high glucose), 10% FBS and 10 μg/mL insulin for 3 additional days in a total experimental period of 18 days. Samples were obtained at 6-day intervals for 18 days and analysed for gene expression by Q-PCR. The level of adipogenic differentiation was also analysed at Day 18 of differentiation by visualization of lipid vacuoles in cultured cells using Oil Red staining.

### 2.7. RNA extraction and cDNA synthesis

Approximately $3 \times 10^5$ bMSC were collected and immediately fixed in RLT buffer (Qiagen, Incorporated). Total RNA was extracted using RNeasy Mini kit (Qiagen) according to the manufacturing's instructions. The concentration and purity of the RNA in each sample were determined using spectrophotometry (BioRad Laboratories, Hercules, CA, USA). Samples were subjected to RT-PCR using a Brilliant II SYBR Green RT-PCR kit (Agilent Technologies, Santa Clara, CA, USA). The reaction protocol consisted of incubation for 5 min at 25 °C, 15 min at 42 °C, 5 min at 95 °C and hold at 4 °C using a DNA engine PCR thermocycler (Bio-Rad).

### 2.8. Quantitative-PCR

Samples were analysed for housekeeping (*GAPDH* and *β-ACTIN*), epigenetic enzyme (*DNMT1*, *DNMT3A*, *DNMT3B*, *KDM6A* and *EZH2*), bone extracellular matrix osteocalcin (*OC*, Osteogenic), cartilage extracellular marker (*ACAN*, Chondrogenic), and fatty acid binding protein (*AP2*, Adipogenic) gene expression by Q-PCR. Real-time PCR primers were designed using PrimerExpress software (Applied Biosystems Incorporated, Foster City, CA, USA) (Table 1). Each RT-PCR reaction (25 μL) contained the following: 2X Brilliant II SYBR Green QPCR master mix (12.5 μL), diluted reference dye (0.375 μL), target forward primer (200 nM), target reverse primer (200 nM), cDNA synthesis reaction (2 μL) and nuclease-free PCR-grade water

**Table 1**
Sequence of primers used for Q-PCR analysis.

| Gene | Sense | Antisense | Accession number |
|---|---|---|---|
| Housekeeping | | | |
| GAPDH | 5′CCTTCATTGACCTTCACTACATGGTCTA | 5′TGGAAGATGGTGATGGCCTTTCCATTG | NM 001034034.2 |
| βACTIN | 5′CGCACCACTGGCATTGTCAT | 5′TCCAAGGCGACGTAGCAGAG | K00622.1 |
| Epigenetic enzyme | | | |
| DNMT1 | 5′TGACTCCACCTACGAAGACC | 5′TCTCTACTTGCTCCACCACG | NM 182651.2 |
| DNMT3A | 5′CAACGGAGAAGCCTAAGGTCAA | 5′TTGAGGCTCCCACAAGAGATG | NM 001206502.1 |
| DNMT3B | 5′AGTATCAGGATGGGAAGGAGTTTG | 5′CCAGGAGAAACCCTTGATCTTTC | NM 181813.2 |
| KDM6A | 5′ ACAAAACTGGCAACATAATACAG | 5′AAAGTTGACCAAATAAAGACTTA | NM 001206575.1 |
| EZH2 | 5′ ACCCCCACCATCAACGTG | 5′ACCGGTGTTTCCTCTTCTTCTT | NM 001193024.1 |
| Lineage marker | | | |
| OC (Osteo) | 5′TGACAGACACACCATGAGAACCC | 5′AGCTCTAGACTGGGCCGTAGAAG | EF673278.1 |
| ACAN (Chondro) | 5′CACTGTTACCGCCACTTCCC | 5′GACATCGTTCCACTCGCCCT | NM 001099362.1 |
| AP2 (Adipo) | 5′CTGGCATGGCCAAACCCA | 5′GTACTTGTACCAGAGCACC | NM 001271626.1 |
| Reporter | | | |
| GFP | 5′GGTCCTTCTTGAGTTTGTAAC | 5′GTTGTCCCAATTCTTGTTGAATTAGATGG | |

to adjust final volume. The PCR amplification was carried out in StepOne Real Time PCR System (Applied Biosystems). Thermal cycling conditions were 95 °C for 10 min, followed by 40 repetitive cycles at 95 °C for 30 s and 60 °C for 1 min. The relative quantification of the target gene expression across treatment was evaluated using the comparative ΔΔCT method. The CT value was determined by subtracting the most stable endogenous gene CT value (*GAPDH*, osteogenesis and chondrogenesis; *β-ACTIN*, adipogenesis) from the target CT value of the sample. Calculation of ΔΔ CT involved using target gene expression on Day 0 (sample with the highest CT value or lowest target expression) as an arbitrary constant to subtract from all other CT sample values.

### 2.9. Immunofluorescence

Differentiated MSC were cultured in 35-mm dishes, fixed in a 4% paraformaldehyde (PAF) for 10 min and stored at 4 °C under PBS. Cells were then washed twice in PBS twice and blocked in donkey serum (Sigma-Aldrich) for 30 min at RT. Cells were incubated over-night at 4 °C with one of each primary rabbit polyclonal (DNMT1, Santa Cruz Biotechnology, Santa Cruz, CA, USA; EZH2, Sigma; KDM6A, Abcam, Cambridge, MA, USA) antibodies (1:50) diluted in donkey serum. After three washes with PBS, cells were incubated with goat anti-rabbit IgG conjugated to FITC (1:200 in donkey serum). Then cells were again washed three times in PBS and mounted under coverslips in a solution containing 4′, 6-diamidino-2-phenylindole (DAPI, Santa Cruz Biotechnology). Samples were examined under epifluorescence and the results captured by digital photomicroscopy (Olympus, Tokyo, Japan).

### 2.10. Data analysis

Values of gene expression and GFP-positive cells from three different replicates were transferred to a spreadsheet and then analysed using Infostat software. Data was normalized to logarithmic scale in base 10 for normality and mean values for each replicate were compared by one-way ANOVA. GFP-positive cells and gene expression values between days of culture and between treatments and controls were analysed using Duncan's multiple comparison test ($p < 0.05$).

## 3. Results

### 3.1. Isolation and plasmid-mediated GFP transfection of bMSC

bMSC were isolated from foetal BM based on the capacity for plastic attachment under standard culture conditions that included DMEM media supplemented with 10% FBS. After 5–6 days of culture, colonies of fibroblast-like cells were visualized attached to plastic culture flasks. Cells exhibited characteristic spindle

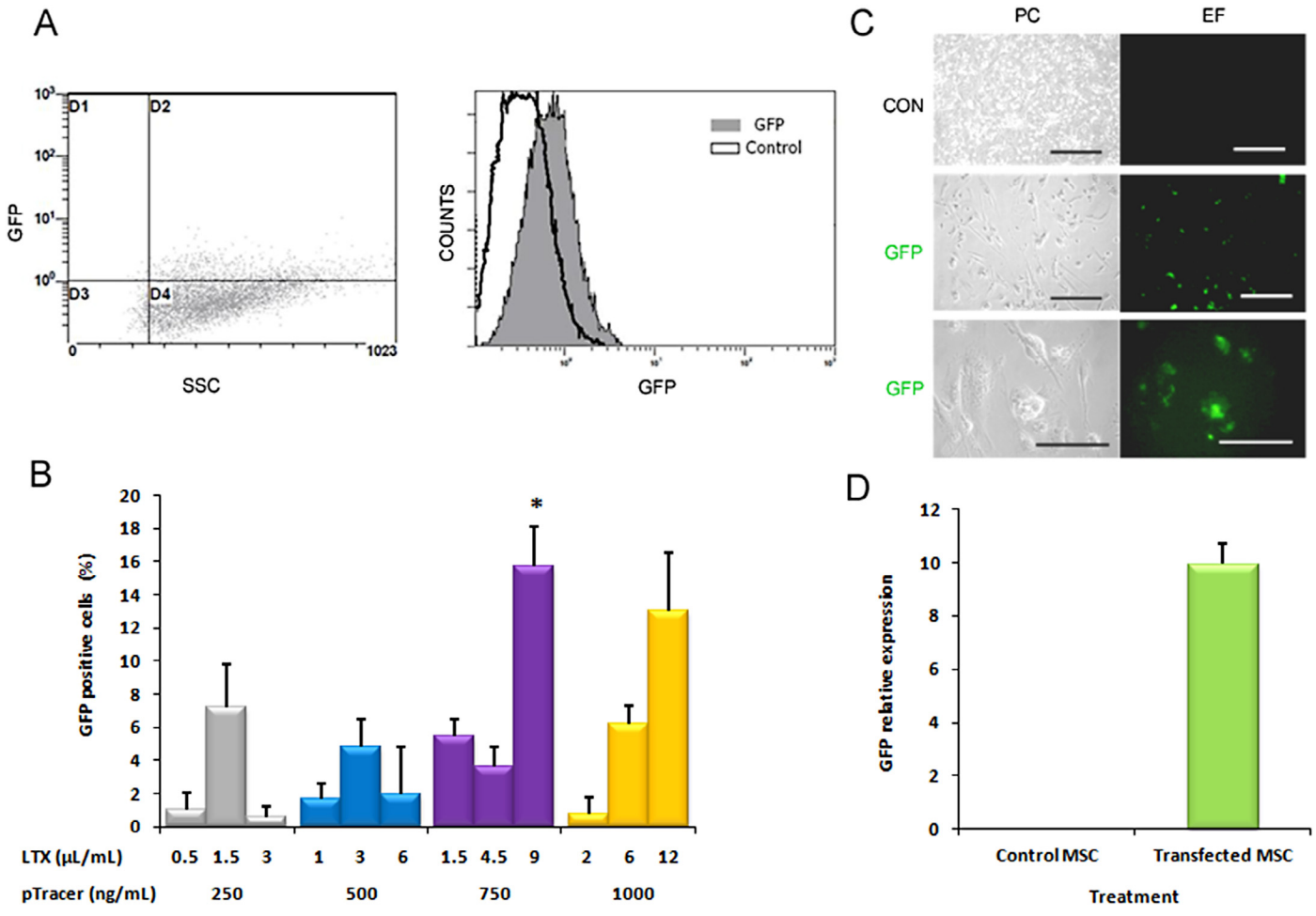


**Fig. 1.** Transfection efficiency for vector pTracer/CMV/Bsd in bMSC using lipofectamine LTX. (A) Representative flow-cytometry analysis of GFP-positive bMSC (B) Highest proportion (15.7% ± 3.5) of transfected cells were achieved using 9 µL/mL of Lipofectamine LTX and 750 ng/mL of pTracer/CMV/Bsd. (C) Few GFP-positive colonies of bMSC were observed after 10-Day blasticidin selection. (D) Expression of GFP mRNA was detected in bMSC stable-expressing GFP but not in control bMSC. (*) Indicate significant difference with other treatments ($p < 0.05$). Abbreviations: SSC, Side Scatter; CON, Control; PC, Phase Contrast; EF, Epifluorescence.

shape and polygonal morphology. Efficiency of GFP transfection was evaluated in bMSC using a pTracer/CMV/Bsd plasmid and lipofectamine LTX. Evaluation of different concentrations of lipofectamine LTX and pTracer/CMV/Bsd indicated that highest number of GFP positive cells (15.7% ± 3.5) were achieved using a combination of 9 µL/mL of Lipofectamine LTX and 750 ng/mL of pTracer (Fig. 1A and B). GFP-positive cells for other treatments ranged from 0.6% ± 0.7 (3 µL/mL of Lipofectamine LTX and 250 ng/mL of pTracer) to 13.1% ± 3.5 (12 µL/mL of Lipofectamine LTX and 1000 ng/mL of pTracer; Fig. 1B). Selection of stably-expressing bMSC using blasticidin for 10 days of culture allowed detection of GFP positive colonies (Fig. 1C). Expression of GFP mRNA was detected in bMSC stable-expressing GFP (average Ct = 27.2) but not in control bMSC (Fig. 1D).

### 3.2. Multilineage in vitro differentiation of bone marrow MSC from abattoir-derived bovine foetuses

Cells isolated by plastic adherence were cultured for several weeks in monolayer and used for differentiation experiments after three to four passages. After culture under osteogenic conditions for a 24-day period, differentiated bMSC expressed higher ($p < 0.05$) levels of *OC* mRNA (67.4-fold relative to Day 0 vs. 2.4-fold in untreated control; Fig. 2). In addition, an intense matrix mineralization was detected in differentiated bMSC cultures using von Kossa staining. Culture of bMSC under chondrogenic conditions using a micromass culture system for 21 days induced cartilage formation (Fig. 3). Differentiated bMSC expressed higher ($p < 0.05$) levels of *ACAN* mRNA (82.3-fold relative to Day 0 vs. 5.3-fold in untreated control) at Day-21 of chondrogenic differentiation. Staining of histological section of micromass with alcian blue indicated presence of glycosaminoglycans. Culture of bMSC under adipogenic conditions induced up-regulation of *AP2* mRNA levels (14.3-fold relative to Day 0 vs. 4.6-fold in untreated control; Fig. 4). In addition, lipid vacuoles were stained using oil red in differentiated bMSC cultures at Day 18 of adipogenic differentiation.

### 3.3. Epigenetic enzyme expression in multilineage differentiating bMSC

Expression of epigenetic enzymes involved in DNA methylation (DNMT1, DNMT3 and DNMT3B), histone methylation (EZH2) and histone demethylation (KDM6A) were evaluated using Q-PCR in bMSC during osteogenic, chondrogenic and adipogenic differentiation. Levels of *DNMT1*, *DNMT3A*, *DNMT3B* and *EZH2* mRNA were not different ($p > 0.05$) between treatments or days of osteogenic culture (Fig. 2). However, differentiated bMSC expressed higher levels of *KDM6A* mRNA at Day 24 of osteogenic differentiation (4.1- versus 3.7-fold in control relative to Day 0). At this stage of osteogenic differentiation, immunofluorescent staining for KDM6A indicated nuclear localization in differentiated bMSC (Fig. 5A). During chondrogenic differentiation, levels of *DNMT1*, *DNMT3A*, *DNMT3B*, *EZH2* and *KDM6A* mRNA were not different ($p > 0.05$) between treatments or days of chondrogenic culture (Fig. 3). During adipogenic differentiation, levels of *DNMT1* and *EZH2* were down-regulated ($p < 0.05$) at Day 6 of culture (0.2- and 0.2-fold versus 0.5- and 0.5-fold in control bMSC, respectively relative to Day 0). Thereafter, levels of mRNA of *DNMT1*, *DNMT3A*, *DNMT3B*, *EZH2* and *KDM6A* were up-regulated ($p < 0.05$) at Days 12 (1.2-, 3.6-, 1.2-, 1.9-, 4.2-fold versus 0.3-, 0.5-, 0.3-, 0.3- and 0.7-fold in control bMSC, respectively relative

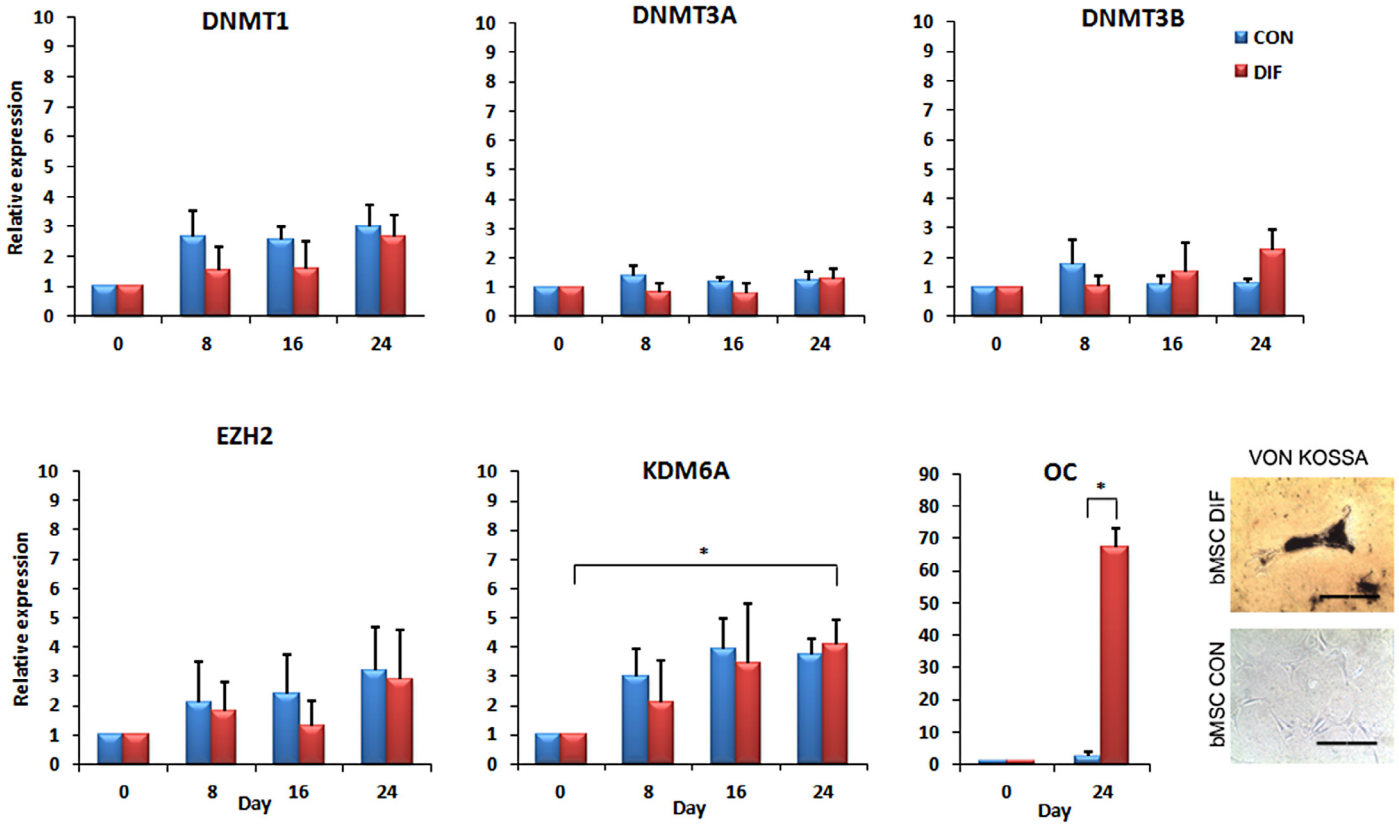


**Fig. 2.** Epigenetic enzyme expression during *in vitro* osteogenic differentiation of bMSC. No significant differences in mRNA levels of *DNMT1*, *DNMT3A*, *DNM3B* and *EZH2* were detected between bMSC treatments or day of culture. However, differentiated bMSC expressed higher levels of *KDM6A* mRNA at Day 24 of osteogenic differentiation. Moreover, differentiated bMSC expressed higher ($p < 0.05$) levels of *OC* mRNA and showed intense von Kossa staining (matrix mineralization) indicating osteogenic differentiation (inserted picture). Scale bars: 500 µm. Abbreviations: CON, Control; DIF, Differentiation.

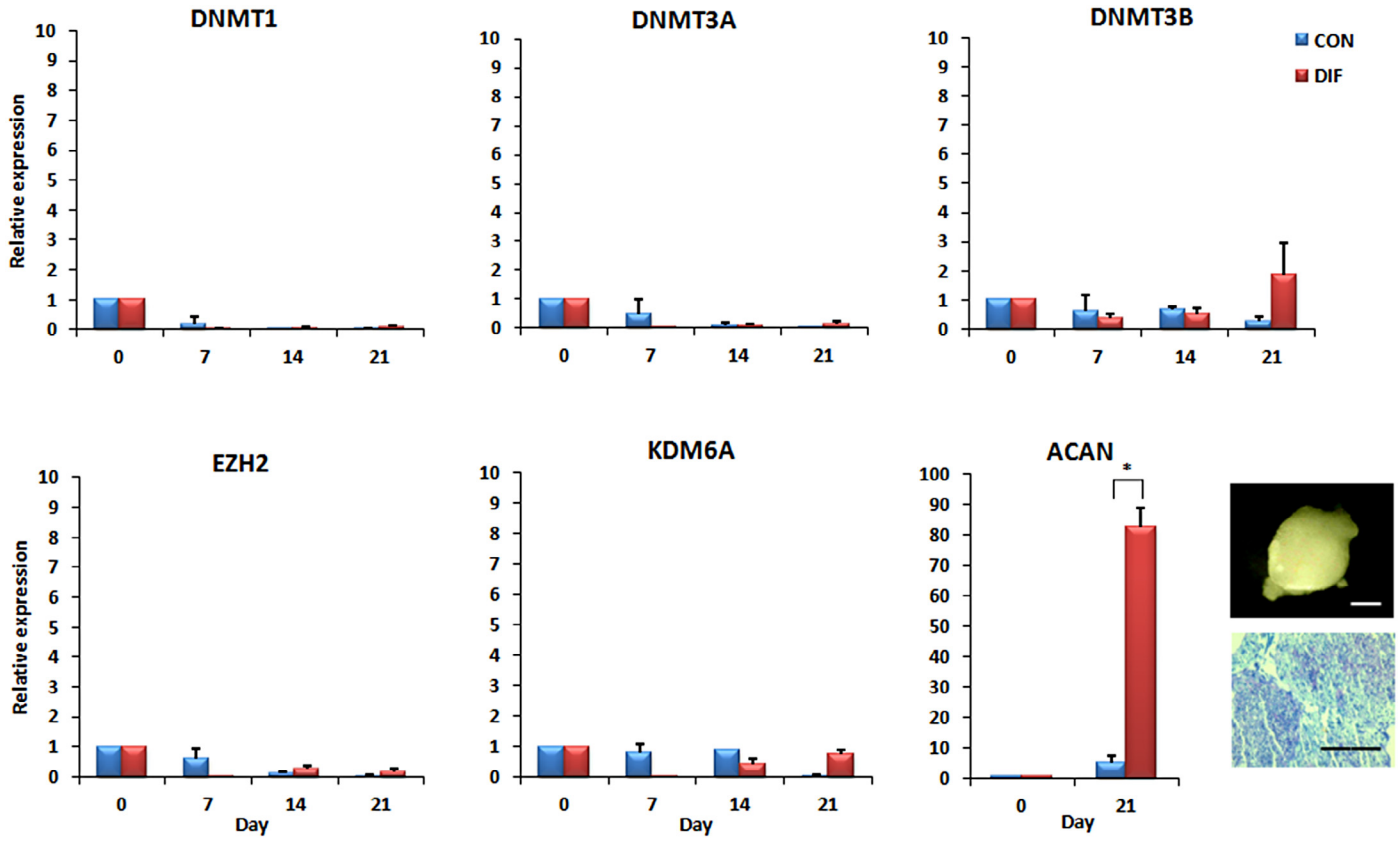


**Fig. 3.** Epigenetic enzyme expression during *in vitro* chondrogenic differentiation of bMSC. Levels of epigenetic enzymes evaluated were not different ($p > 0.05$) between treatments or days of chondrogenic culture. Culture of bMSC under chondrogenic conditions using a micromass culture system for 21 days induced cartilage formation (inserted picture, upper micromass; lower, micromass histological section). Differentiated bMSC expressed higher ($p < 0.05$) levels of *ACAN* mRNA and histological section of micromass stained with alcian blue (glycosaminoglycans) indicating chondrogenic differentiation. Scale bars: upper 1 mm; lower 500 μm. Abbreviations: CON, Control; DIF, Differentiation.

to Day 0) and 18 of culture (2.3-, 4.9-, 1.5-, 5- and 6.1-fold versus 0.2-, 0.9-, 0.2-, 0.5- and 1.2-fold in control bMSC, respectively relative to Day 0). DNMT1, KDM6A and EZH2 were immunolocalized in bMSC adipogenic cultures at Day 18 of differentiation (Fig. 5B, C and D). Immunofluorescent staining for KDM6A and EZH2 indicated nuclear localization in differentiated bMSC (Fig. 5C and D).

## 4. Discussion

In the present study, bMSC were isolated from BM collected from abattoir-derived foetuses based on the capacity to adhere to plastic substrate under monolayer culture conditions. In previous studies, we reported the expression of lineage-specific markers in bMSC after treatment with osteogenic, chondrogenic, adipogenic, neurogenic and hepatogenic differentiation media, demonstrating the multipotency of these cells (Cortes et al., 2013; Dueñas et al., 2014).

Evaluation of the potential gene transfer in MSC is crucial in order to investigate the effect of genetic modification in cell differentiation and immunocompetence. In addition, labelling of MSC using a reporter gene expression such as GFP is essential for tracking of MSC following *in vivo* transplantation. Although homing of bMSC has not been demonstrated, in the present study we sought to evaluate the efficiency for gene transfer in bMSC using lipofectamine and a pTracer/CMV/Bsd vector. Highest efficiency of transient GFP-transfection achieved was of 15.7% ± 3.5, indicating that use of non-viral vector and lipofectamine display moderately low efficiency in bMSC. Similarly, previous reports indicated that transfection of non-viral vector using lipofectamine in MSC isolated from rat, porcine and primates yielded less than 25% of transgene-positive cells (Ke et al., 2009; McMahon et al., 2006; Stiehler et al., 2006). Although higher transfection efficiencies have been reported using non-viral vector in MSC derived from bovine (58%) and porcine (67%), these transfection experiments were performed using electroporation instead of lipofectamine (Colleoni et al., 2005). In contrast, higher transfection efficiencies have been reported in MSC using viral-vectors including lentivirus (95.5% rat; 44.1% porcine; 44.6 primate), adenovirus (70.6%, rat), and adeno-associated virus (30.4%, rabbit; 90%, porcine) (Ke et al., 2009; McMahon et al., 2006; Stiehler et al., 2006). Although low efficiency has been achieved by non-viral vectors in MSC, other reports have demonstrated the transfer and stable expression of functional genes using these systems, including human bone morphogenetic protein-2 (rhBMP-2) in human MSC (Moutsatsos et al., 2001) and human vascular endothelial growth factor (VEGF) in ovine MSC (Locatelli et al., 2013). In the present study, few GFP-positive bMSC colonies were observed after a 10-Day selection for stable transfection using blasticidin. Thereafter, we were unable to expand these cell colonies using clonal culture. Thus, data presented in this and previous studies suggest that transfection of using non-viral vector and lipofectamine achieve moderately low efficiency in MSC.

Given the therapeutic potential of MSC, it is crucial to continue elucidating the precise mechanisms that direct MSC fate. Although mechanism for stem cell differentiation are largely mediated by DNA sequence, other regulatory processes including post-transcriptional, translational, post-translational and epigenetic are responsible for multiple levels of regulation. In general, promoter DNA methylation is associated with repression of the corresponding gene; however, genes associated with methylation-free CpG

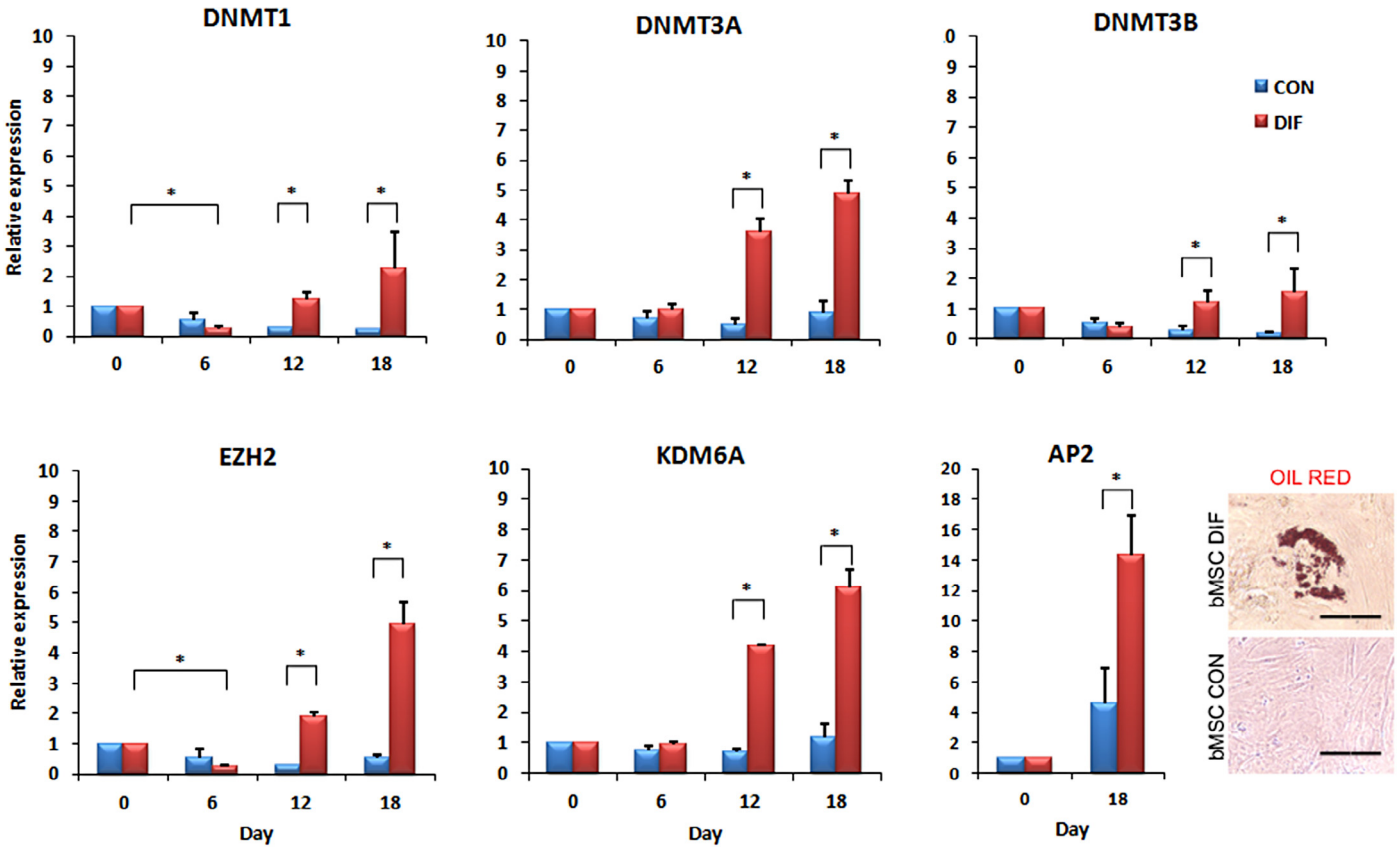


**Fig. 4.** Epigenetic enzyme expression during *in vitro* adipogenic differentiation of bMSC. Levels of *DNMT1* and *EZH2* mRNA were down-regulated ($p < 0.05$) at Day 6 of culture. Thereafter, levels of *DNMT1*, *DNMT3A*, *DNMT3B*, *EZH2* and *KDM6A* mRNA were up-regulated ($p < 0.05$) at Days 12 and 18 of culture. Culture of bMSC under adipogenic conditions induced up-regulation of *AP2* mRNA levels ($p < 0.05$). In addition, lipid vacuoles were stained using oil red in differentiated bMSC cultures (inserted picture). Scale bars: 500 μm. Abbreviations: CON, Control; DIF, Differentiation.

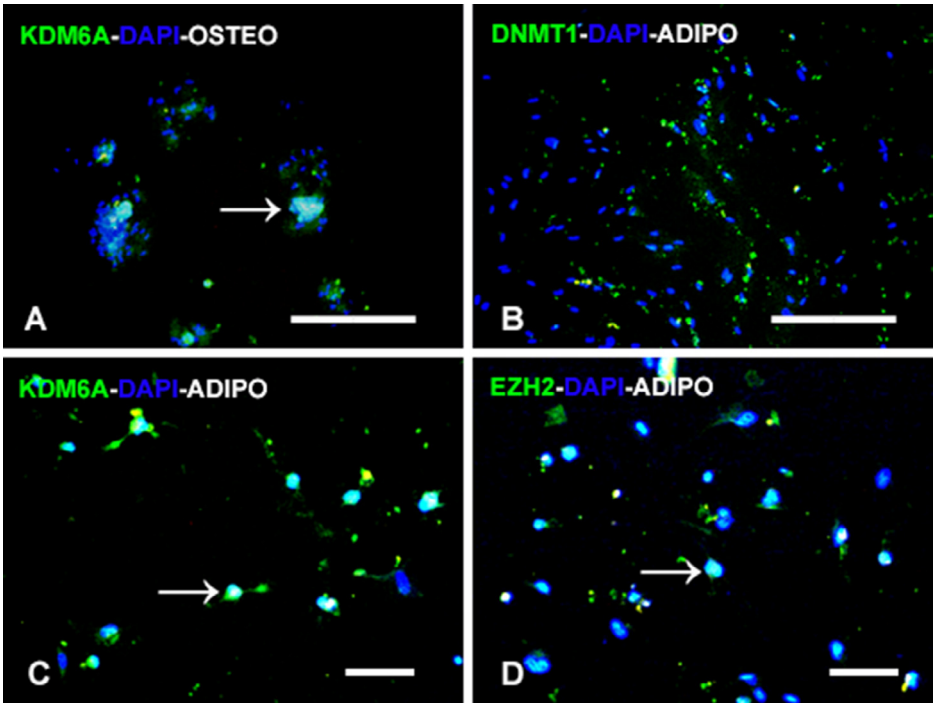


**Fig. 5.** Immunolocalization of epigenetic enzyme during osteogenic and adipogenic differentiation of bMSC. (A) Immunofluorescence staining associated with histone demethylase KDM6A was detected in bMSC at 24 days of osteogenic (OSTEO) differentiation. (B, C, D) DNA methyltransferase DNMT1, KDM6A and histone methylase EZH2 were immunolocalized in differentiated bMSC at Day 18 of adipogenic (ADIPO) culture. Immunofluorescence associated with KDM6A in osteogenic, and KDM6A and EZH2 in adipogenic cultures showed nuclear localization in differentiated bMSC (white arrows). Scale bars: A–B, 500 μm; C–D, 100 μm.

islands often remain silent while genes that correspond to methylated promoters occasionally undergo transcription (Song et al., 2005). This relation may depend on the content of promoter CpG dinucleotides, where methylation of high content CpG promoters usually repress transcription, while methylation of low content CpG promoters can either activate or repress transcription (Weber et al., 2007). In the present study, patterns of DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) expression varied among bMSC differentiating lineages, suggesting differential roles for these enzymes depending on MSC fate. While DNA methyltransferase mRNA levels showed no association with osteogenic and chondrogenic differentiation; these enzymes were up-regulated in bMSC at latter stages of adipogenic differentiation suggesting that DNA methylation may be required for adoption of the adipogenic lineage. Many coregulators and transcription factors central to adipogenesis have chromatin-modifying activities, supporting the role of epigenetic regulation during the differentiation of MSC to adipocytes (Guo et al., 2009). Commitment of MSC to the adipogenic lineage may be reflected by a particular epigenetic signature in which adipogenic gene promoters are hypomethylated while nonadipogenic promoters are methylated. *In vitro* analyses have correlated the demethylation of various adipogenic promoters including that of peroxisome proliferator-activated receptor γ (PPARγ), with adipogenic differentiation (Fujiki et al., 2009). Conversely, DNA methyltransferase may play a significant methylating activity of nonadipogenic promoters allowing expression of genes that direct adipogenic lineage during latter stages of bMSC adipogenic differentiation.

Histone modifications may also serve a larger role in MSC differentiation capacity (Collas, 2010). In the present study, histone demethylase KDM6B mRNA levels were up-regulated and immunolocalized in bMSC cultures at latest stages of osteogenic differentiation. These results are consistent with previous reports where KDM6A has been involved as a critical epigenetic regulator in BM-MSC fate commitment by promoting osteogenic differentiation (Ye et al., 2012). KDM6B epigenetically regulate osteogenic differentiation of MSC by removing H3K27, a repressive epigenetic mark, and subsequently activating genes associated with osteogenesis (Ye et al., 2012). Moreover, during osteogenic differentiation of BM-MSC, KDM6B demethylases histones in the promoters of bone morphogenetic protein 2 and 4 (BMP2 and BMP4) and homeobox protein C6 (HOXC6) associated with regulation of Runt-related transcription factor 2 (RUNx2) (Ye et al., 2012). Furthermore, our data showed that histone demethylase KDM6A and methylase EZH2 mRNA levels increased at latter stages of adipogenic differentiation. Histone-mediated chromatin architecture modifications have been previously documented as multipotent MSC become preadipocytes during adipogenic determination (Musri et al., 2007). H3K4, an active mark of transcription has been identified in promoters of adipogenic genes including adiponectin (APM1), glucose transporter type 4 (GLUT4), and leptin (LEP) during determination (Musri et al., 2007). As cells progress towards committed adipocyte precursors during differentiation, further characteristic epigenetic marks have been described. In addition to promoter DNA demethylation at GLUT4 and LEP (Melzner et al., 2002), these promoters also undergo H3K9 demethylation, H3 acetylation, and H3K4 trimethylation (Musri et al., 2007).

In conclusion, transfection of non-viral system pTracer/CMV/Bsd using lipofectamine in bMSC achieved moderately low efficiency. Analysis of DNA methyltransferase expression during MSC multilineage differentiation indicated no association with osteogenic and chondrogenic differentiation. However, up-regulation of KDM6B expression during osteogenic differentiation suggested a role for histone demethylase during osteogenesis. Furthermore, increase in epigenetic enzyme expression at latest stages of bMSC adipogenic differentiation suggested an intense epigenetic regulation during adoption of the adipogenic lineage.

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