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Original Article

Synergistic effects of enamel matrix derivatives and surface morphology of anodized titanium on osteogenic differentiation of bone marrow mesenchymal stem cells sheet

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Abstract: Objective: To explore the potential osteogenic induction mechanism of enamel matrix derivatives (EMDs) on bone marrow mesenchymal stem cell (BMSC) sheets with different titanium surface morphologies. Methods: The BMSCs were inoculated on the surfaces of titanium alloys with different morphologies: anodic oxidation (AO), sand-blasted, large grit and acid-etched, and no treatment (control). The proliferation and osteogenic differentiation of BMSCs on the different surface morphologies were observed with the same concentration of EMDs. To further understand the osteogenic mechanism of EMDs on BMSC sheets with different morphologies, a real-time RT-PCR and a western blot were used to detect the overall levels of osteogenic genes and osteogenic proteins. Finally, to verify the osteogenic effect of BMSC sheets stimulated by EMDs in vivo, BMSC sheets with different morphologies were implanted into the subcutaneous tissue of the back of nude mice, and the bone formation was detected by HE staining. Results: The EMDs and surface morphology in the AO group synergically increased the expression levels of osteogenic active factors (RUNX2, OSX and OCN) and enhanced the osteogenic differentiation effect of BMSCs. The in vivo experiments showed that the BMSC sheets in the AO group were rich in osteogenic active factors, and promoted the formation of ectopic bone tissue after implantation into the subcutaneous tissue of the back of nude mice. Conclusion: EMDs and AO morphology synergically enhance the secretion of bone osteogenic active factors of BMSCs and promote the formation of heterotopic bone.

Keywords: Enamel matrix derivative, anodic oxidation titanium surface, bone marrow mesenchymal stem cell, cell sheet

Introduction

Due to the advantages of excellent mechanical strength, chemical stability and biocompatibility, pure titanium and titanium alloys have been widely used in clinical practice as the main dental implant materials and became the gold standard materials for implants [1, 2]. In 1976, Branemark found that titanium implants could permanently fuse with bone, and the method of fusing living bone with an implant was called “osseointegration” [3]. The result of osseointegration is affected by many factors, such as the implant material itself and the patient’s nutritional status, age and diseases. Among them, the surface of the implant material is the most relevant factor due to its direct contact with the bone [4]. If the bone to implant contact area (BIC) is insufficient and the osseointegration is poor, the denture will not be able to provide stable biomechanics when the masticatory force is transferred between the implant and the jaw, which will lead to damage of the surrounding tissues and even inflammatory bone resorption, eventually leading to a possible failure of the implantation [5]. According to the statistics of the American Association of Oral and Maxillofacial Surgeons, there are more than 2

million dental implant surgeries in the world every year, among which about 5-11% of implants fail to achieve the required osseointegration in the maxillofacial bone [6].

To increase the quality of osseointegration, anodic oxidation (AO), sandblasting, and acid etching were developed as modifications applied to the titanium implant surface. The main purpose of the modifications is to better regulate the host/implant tissue response for better osseointegration. A study found that the percentage of BIC of the titanium implant surface after sandblasting, acid etching and AO treatment was significantly higher than that of untreated titanium implants [7]. The surface modifications achieved better mechanical coupling with living bone, and also promoted the adhesion, proliferation and differentiation of osteoblasts in osteogenesis [8]. However, there was still a certain degree of marginal bone loss around the implants, sometimes even progressive bone loss, and the surface of the implant material showed a certain impact on this marginal bone loss as well [2]. In addition to the surface modifications, researchers also added special bone induction materials to the surface of modified titanium implants to promote faster and better osseointegration [9-11]. Enamel matrix proteins (EMPs) are complex proteins synthesized and secreted by inner enamel epithelial cells of enamel organs and epithelial root sheath, which can regulate enamel mineralization and maturation during the development of dental crowns [12]. Artificially extracted EMPs are derived from pig teeth, and are called enamel matrix derivatives (EMDs), whose main components are amylogenin, which has been shown in vivo to significantly enhance the formation of new bone when combined with bone substitutes in the treatment of bone defects [13, 14]. EMDs showed high biocompatibility and osteogenic effects, which can be used as ideal active factors for bone induction. At present, the mechanism of EMDs on modified titanium surface materials is still unclear.

In clinical work, the implantation depth of a dental implant often needs to reach cancellous bone, which is connected with the bone marrow cavity. The bone marrow cavity is rich in bone marrow mesenchymal stem cells (BMSCs), osteogenic precursor cells, osteoblasts and osteoclasts, and others. These cells have a significant impact on the osseointegration of the implant. Among them, BMSCs are the first cells to colonize the implant surface after implantation and promote bone regeneration by differentiating into osteoblasts and producing growth factors [15]. In general, the functional activities of cells near the surface of the implant are highly influenced by the chemical, physical, mechanical and topographic characteristics of the material surface [16]. BMSCs were used as a research object to further explore the possible mechanisms of EMDs and different morphologies of the titanium surface for inducing osteogenesis. In 2004, Nishida et al. established a technique called “cell sheet”, which contained not only the cell itself, but also the extracellular matrix (ECM) and abundant active factors [17]. The cell sheet can be easily designed and processed in vitro and has strong adhesion, so it can easily be used for in vivo transplantation [18].

In this study, special BMSC sheets containing rich osteogenic factors were designed in vitro, in order to enhance the osteogenic differentiation of BMSC sheets and improve the osseointegration of titanium implants. First, EMDs with osteogenic induction were added to BMSC sheets on different titanium surfaces in vitro, and then the content of related osteogenic factors was detected by molecular experiments. Finally, special BMSC sheets containing rich osteogenic factors were implanted into subcutaneous tissue of nude mice to observe the osteogenic effect.

Materials and methods

Characteristic analysis of material surfaces

Three kinds of circular titanium slices with a diameter of 10 mm and a thickness of 1 mm provided by Xi’an Taijin Company were selected: (1) An untreated titanium surface was used for a control group; (2) A sand-blasted, large grit and acid-etched (SLA) titanium surface was used as experimental group 1; (3) A titanium surface after AO treatment was used as experimental group 2. The three titanium slices were sprayed with gold and observed under a scanning electron microscope (SEM).

Extraction and identification of BMSCs

Extraction of primary BMSCs: Specific-pathogen free SD rats of 1-2 weeks were selected, both male and female (provided by the Animal Experimental Center of Guizhou Medical Uni-

versity). This study was approved by the Experimental Animal Ethics Committee of Guizhou Medical University. The rats were euthanized by injecting pentobarbital sodium (200 mg/kg), followed by immersing them in a 75% ethanol solution for 10 min. The femurs of the bilateral hind limbs of the rats were removed, washed with phosphate-buffered saline (PBS), and the epiphyses at both ends were cut open with ophthalmic scissors to expose the bone marrow cavity. One end of the epiphyses was placed above the centrifuge tube. For the other end of the epiphyses, a complete medium containing DMEM basal medium (Gibco, Carlsbad, CA, USA), antibiotics (1% 10000 U/mL penicillin +10 mg/mL streptomycin) and 10% fetal bovine serum (BI Company, Israel) was extracted with a syringe, injected into the bone marrow cavity and rinsed repeatedly until the bone marrow cavity turned white. After centrifugation for 10 minutes, the supernatant was removed, then the complete culture medium was added and resuspended, and the culture was divided into T25 culture flasks and placed in an incubator containing 5% carbon dioxide (Thermo, USA) at 37°C.

Identification of primary BMSCs: The well-grown 3rd generation BMSCs were placed under an inverted phase contrast microscope to observe their cell morphology, and then photographed. Then, for the identification of BMSC surface markers, the cell density was adjusted to 1×10^6/L after trypsin digestion. Monoclonal antibodies including CD45, CD29, CD90, and CD11b (BioLegend, USA) were added to each tube successively. Also, a negative control was set for each tube. Then, the cells were incubated at 4°C in the dark for 30 min. Afterwards, the cells were washed with PBS, the unbound antibodies were removed, and the cells were suspended with 100 μL PBS and analyzed by flow cytometry. Finally, the cells were selected and operated according to the instructions of the osteogenic induction differentiation kit (Cyagen, USA) and adipogenic induction differentiation medium kit (Solarbio, China), and stained and photographed.

Proliferation assay of BMSCs and preparation of cell sheet

EMD toxicity test: The 3rd generation BMSCs were selected and cultured in a complete medium (Blank group) for 24 h, and then placed in a mixture of EMD and complete medium at concentrations of 0, 5, 25, 50, and 100 μg/mL, resulting in a control group (cells and complete medium) and experimental group (cells, complete medium and EMD). After treatment durations of 24 h, 48 h, and 72 h, 10 μL CCK8 reagent (Dojindo, Japan) was added into the well of each group and the cells were incubated for 2 h in the dark. Afterwards, the OD value at 450 nm was measured with a multi-purpose microplate analyzer (BioTek, USA). The survival rate of BMSCs was calculated as follows: Cell viability = $(OD_{dosed\ cells}-OD_{empty})/(OD_{control\ cells}-OD_{empty}) \times 100\%$.

Toxicity detection of BMSCs on titanium surface: BMSCs on the titanium surfaces of the control, SLA and AO groups were cultured with EMDs for 24 h and then stained with a Calcein-AM/PI live/dead cell double staining kit (Solarbio, China). Finally, the BMSCs were observed under a confocal laser scanning microscope (Leica, Germany).

Preparation of BMSC sheets: The 3rd generation BMSCs were inoculated on the different surfaces of titanium slices in three groups at 2×10^5 cells/mL, with 8 secondary wells in each group. 10% complete medium and 50 μg/mL ascorbic acid were added to each well. Afterwards, the cells were cultured in an incubator and the solution was changed every 3 days. After 10-14 days, the cell sheets were observed on the surface of the titanium slices, which were used for subsequent experiments.

Detection of osteogenic differentiation of BMSCs

Alkaline phosphatase activity detection: The BMSCs cultured on the titanium slices were selected and added with EMD stimulation on 7, 14, and 21 days. The cell sheets were scraped off with a cell scraper, and cell lysate (Solarbio, China) was added for treatment. The experimental operation was carried out according to the manual of an alkaline phosphatase test box (Nanjing Jiancheng, China). After 7 days of culture, 4% paraformaldehyde was added and fixed at room temperature for 30 min, then 5% bovine serum blocking solution was added and blocked at 37°C for 2 h, and the cultures were incubated with an ALP (Abcam, USA) for 12 h. The samples were incubated with goat anti-rabbit antibody Alexa Fluor488 (Abcam Cambridge, MA, USA) for 1 h and stained with 4', 6-diamino-2-phenylindole (DAPI; Solarbio,

Table 1. Primer sequence of the genes used in this study

Primer	Gene
GAPDH Forward Reverse	5'-AAGTTCAACGGCACAGTCAAGG-3'
	5'-ACGCCAGTAGACTCCACGACAT-3'
RUNX2 Forward Reverse	5'-GAACCAAGAAGGCACAGACAGAA-3'
	5'-GGCGGGACACCTACTCTCATACT-3'
Osterix Forward Reverse	5'-AGTGGTATTGTAGGTGCTGTGGTC-3'
	5'-AGTGGTATTGTAGGTGCTGTGGTC-3'
Osteocalcin Forward Reverse	5'-AGTGGTATTGTAGGTGCTGTGGTC-3'
	5'-AGTGGTATTGTAGGTGCTGTGGTC-3'

China). Fluorescence images were obtained under a confocal laser scanning microscope.

Quantitative analysis by Alizarin Red staining: The BMSC sheets cultured on different titanium surfaces were stimulated with EMDs for 2 weeks. Afterwards, they were fixed with 2 mL 4% neutral formaldehyde solution at room temperature for 30 min, rinsed twice with PBS and then treated with 1 mL Alizarin Red dye solution for 5 min. Calcium nodules were dissolved by adding cetylpyridine 10% chloride (Aladdin, China) after the alizarin red staining solution was aspirated, and a semi-quantitative detection was performed at 540 nm using a multifunctional microplate reader.

RT-PCR assay

The BMSC sheets on the different titanium surfaces were collected at days 7, 14 and 21, respectively, and the total RNA was extracted using the AxyPrep Total RNA kit (Axygen, USA). A reverse transcription reaction was performed according to the reverse transcription kit (TaKaRa, Japan). The coding sequences of the target and internal reference genes were retrieved from NCBI Genbank database. Primers were provided by Sangon Biotech (Shanghai) Co., Ltd. The primer sequences are shown in **Table 1**. GAPDH was used as internal reference gene, and RUNX-2, OSX and OCN were used as target genes. A SYBR™ Green Master Mix kit was used and the reaction mixture was prepared as required. The total reaction system volume was 10 μL, in which the 2 X PowerUp SYBR Green Master Mix was 5 μL, forward primer was 0.5 μL, reverse primer was 0.5 μL and the cDNA template and ddH2O were 4 μL. The chosen amplification conditions were UDG enzyme activation at 50° for 2 min, predenaturation at 95° for 2 min, denaturation at 95° for 15 sec and annealing/extension at 60° for 1 min. Finally, quantitative polymerase chain reaction was performed on the ViiA 7RT-PCR system using the mixed solution of the SYBR™ Green Master Mix kit. The relative quantification was performed by $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

The expression of RUNX-2, OSX and OCN proteins was detected after the 3rd generation BMSCs were cultured into cell membranes on titanium slices and stimulated by EMDs for 7, 14 and 21 days. After the addition of PMSF and RIPA lysates, the BMSCs were subjected to ultrasonic treatment and centrifugation. The proteins in the supernatant were transferred to a PVDF membrane and the membrane was sealed. The samples were incubated with primary antibodies including RUNX-2 (1:1500, CST, USA), OSX (1:1500, Abcam Cambridge, MA, USA), OCN (1:1500, Abcam Cambridge, MA, USA) and GAPDH (1:1000, Beyotime, China) overnight. The next day, they were incubated with goat anti-rabbit secondary antibodies (1:6000, Beyotime, China) for 1 h, and the membrane was exposed using an ECL kit (SOLARBIO, China) to show the bands of immunoreactive protein. Finally, ImageJ analysis software (NIH, USA) was used to detect the gray value of the strip.

In vivo implantation of BMSC sheets

The 3rd generation BMSCs were inoculated on the surface of the different titanium slices in three groups at a density of 2×10^5 cells/mL, and EMDs were added at a concentration of 25 μg/mL for stimulation. Two weeks later, the titanium slices with formed BMSC sheets on the surface were implanted into the subcutaneous tissues of the back of three nude mice (purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd), respectively. After 6 weeks, the nude mice were euthanized by injecting pentobarbital sodium (200 mg/kg), and tissue samples at the implantation site were taken for pathological staining. The animal experiment was approved by the Experi-

mental Animal Ethics Committee of Guizhou Medical University.

Statistical analysis

SPSS 25.0 (IBM, USA) was used for statistical analysis of all experimental data. If the test features showed a normal distribution and homogeneity of variance, their mean ± standard deviation was used for expression. One-way ANOVA was used for comparison among the groups. Between the two groups, the comparison was analyzed by using an LSD-t test. The statistical software GraphPad Prism 8.0 was used for mapping, and $P<0.05$ was chosen as the significance threshold.

Results

Characteristic analysis of titanium surfaces and identification of BMSCs

The three different titanium surfaces were sprayed with gold and scanned with SEM to explore their surface morphology. Compared with the SLA and AO groups, the surface morphology of the slice in untreated Control group was relatively flat. In the SLA group, the surface was treated with large particle acid etching and sandblasting, and obvious uneven granular morphology was observed. However, the surface of AO group showed scaly distribution morphology (**Figure 1A**). Under the light microscope (**Figure 1Bb1**), the extracted primary cells showed adherent growth in a long spindle shape, which was consistent with the morphological performance of BMSCs. After cells were cultured with osteogenic induction medium, Alizarin Red staining results showed a large amount of extracellular calcium salt deposition, suggesting that the extracted primary cells had osteogenic mineralization ability (**Figure 1Bb2**). After oil Red-O staining of the extracted cells (**Figure 1Bb3**), a large number of scattered lipid droplets were formed, indicating that the cells had adipogenic differentiation characteristics. Finally, flow cytometry (**Figure 1C**) showed that isolated and extracted cells expressed high level of stem cell surface antigens CD29 and CD90, with positive rates of 97.0% and 99.7%, respectively, while the levels of expression of non-stem cell surface antigens CD45 and CD11b were lower, with positive rates of 0.82% and 0.86%, respectively. It was confirmed that the extracted primary cells had high expression of stem cell surface markers. In conclusion, the extracted primary cells showed adherent growth in long spindle shape. The cells had adipogenic and osteogenic mineralization characteristics, and the cell surface was rich in CD29 and CD90 antigens, which basically conformed to the characteristics of BMSCs.

Proliferation assay and BMSC sheets

To explore the effect of different titanium surfaces and different concentrations of EMD, the proliferation rate of BMSCs was evaluated by propidium iodide/Calcein AM immunocytochemistry and CCK8 methods. Propidium iodide/Calcein AM immunocytochemical assay showed that strong green fluorescence staining was observed on different titanium surfaces in the Control, SLA and AO groups, while the red fluorescence staining was much weaker (**Figure 2A**). Image J semi-quantitative analysis showed that the mortality of BMSCs growing on the three slice surfaces was below 5% (**Figure 2B**). BMSCs on different titanium surfaces formed cell sheets after 14 days of EMD stimulation (**Figure 2C**). The effects of different concentrations of EMD on the proliferation of BMSCs were determined by CCK8 experiment (**Figure 2D**). EMD concentrations of 5 μg/mL, 25 μg/mL, and 50 μg/mL showed different promotion rates of BMSCs proliferation within 72 h (proliferation rates >1). EMD at 25 μg/mL had a better effect on BMSC proliferation than other concentration groups at 24 h and 48 h ($P<0.05$), but when the concentration of EMD reached 100 μg/mL, the proliferation of BMSCs was inhibited (proliferation rate <1).

Effect of EMD on osteogenic differentiation of BMSC sheets on different titanium surfaces

To explore the effects of EMD induction stimulation on osteogenic differentiation of BMSC sheets on different titanium surfaces, immune cell fluorescence technology, alkaline phosphatase detection kit and Alizarin Red staining were used to measure BMSCs (**Figure 3A**). Semi-quantitative analysis of immunofluorescence map showed that after 7 days of EMD stimulation, the expression of ALP in BMSCs in the Control group was significantly lower than that in the SLA and AO groups ($P<0.05$), while in the experimental groups, the expression of ALP in BMSCs on the surface of the AO group was

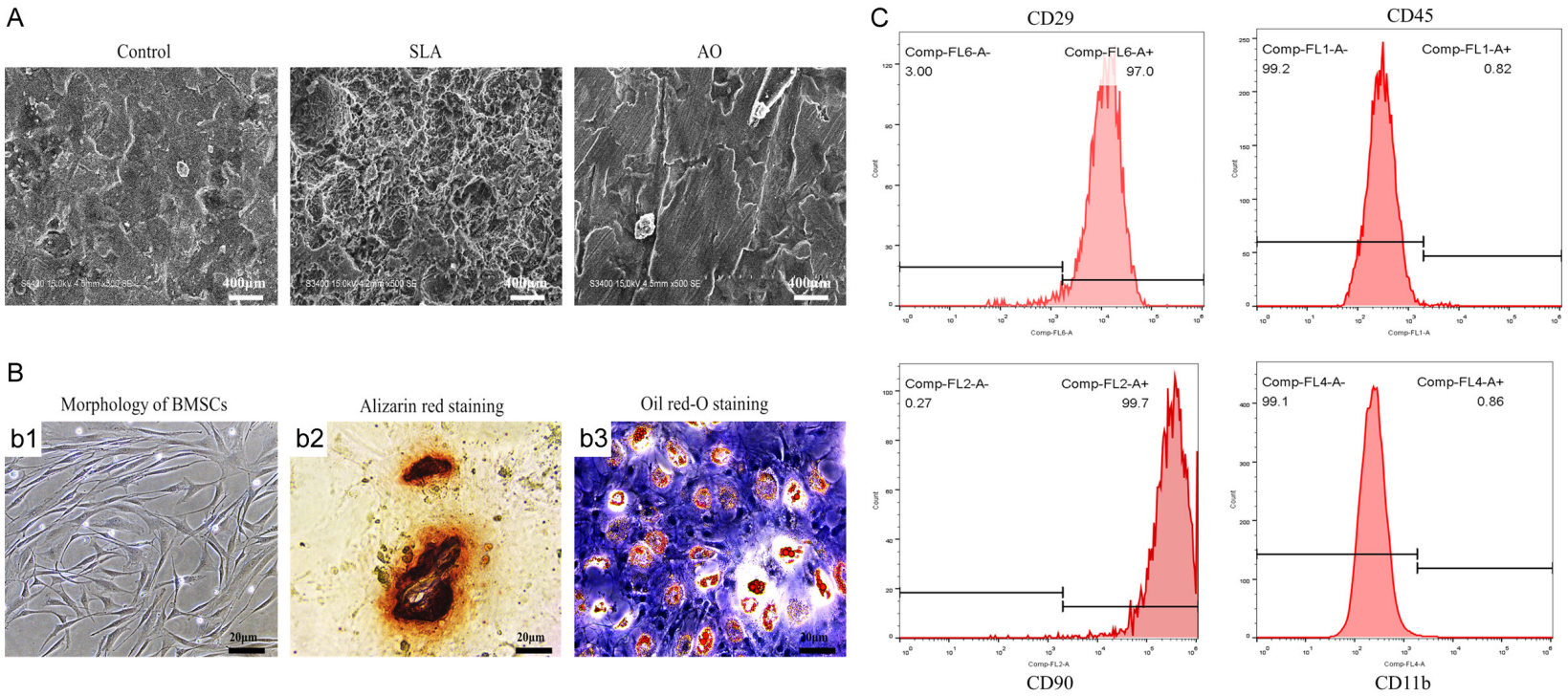

Figure 1. Electron microscope scans of different titanium surface morphologies and identification of BMSCs. A: Electron microscope image of different titanium surfaces in the control, SLA and AO groups. Scale bar: 400 μm, ×500. B: b1: Light microscopic observation of the morphology of BMSCs. Scale bar: 20 μm, ×20. b2: BMSCs were osteogenically induced and the resulting calcium precipitates were stained red using Alizarin Red staining. Scale bar: 20 μm, ×20. b3: BMSCs were adipogenically induced and red circular lipid droplets were seen using Oil Red O staining. Scale bar: 20 μm, ×20. C: Based on flow cytometry results, the surface markers of BMSCs were positive. SLA: Sand-blasted, Large grit, Acid-etched; AO: Anodic Oxidation; BMSCs: Bone Marrow Mesenchymal Stem Cells.

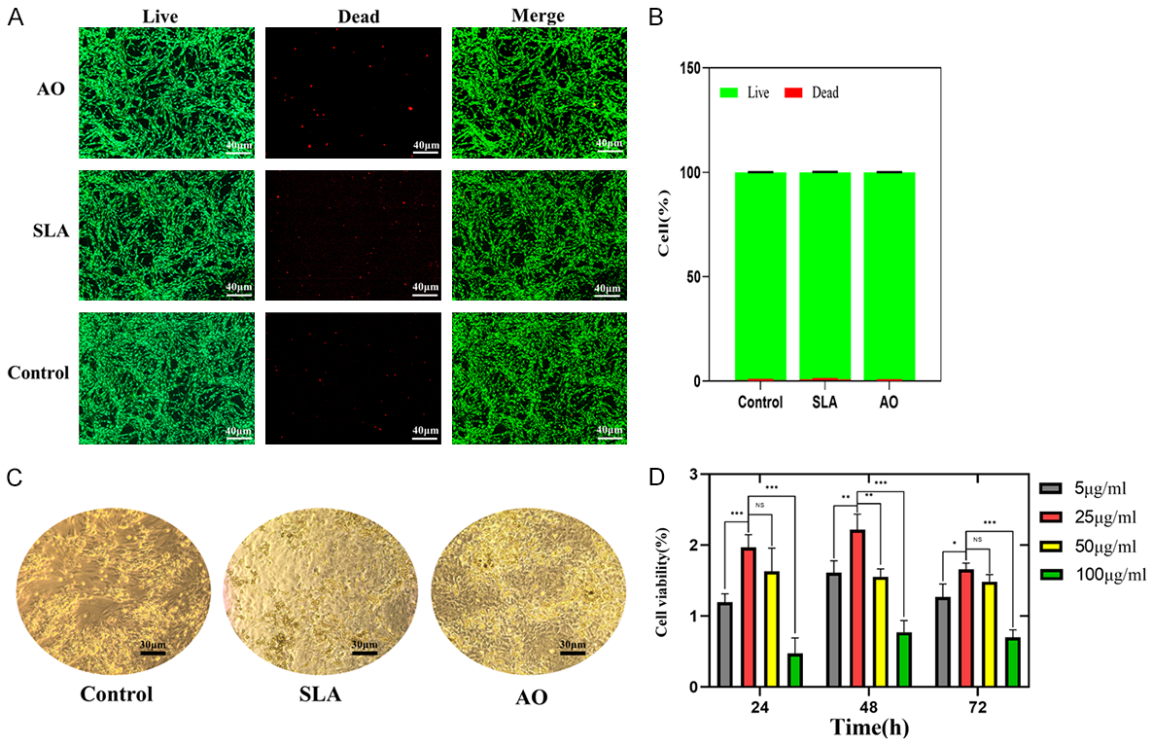

Figure 2. Cell activity assay and morphology observation of BMSC sheets. A: By staining with Calcein-AM and propidium iodide, both surviving and dead cells can be observed. Green: living cells, red: dead cells. Scale bar: 40 μm, ×10. B: Quantitative statistical analysis by staining with calcein-AM and propidium iodide. C: BMSCs on different titanium surfaces form cell sheets after 14 days of stimulation by adding EMD. Scale bar: 30 μm, ×10. D: The proliferation rate of BMSCs was assessed using the CCK8 method at 24 h, 48 h and 72 h respectively. *P<0.05, **P<0.01. SLA: Sand-blasted, Large grit, Acid-etched; AO: Anodic Oxidation; BMSCs: Bone Marrow Mesenchymal Stem Cells; EMD: Enamel Matrix Derivative.

higher than that in the SLA group (P<0.05) (**Figure 3B**). The alkaline phosphatase quantitative kit assay found that the titanium slices with surface modification (the experimental groups) significantly promoted the expression of ALP in BMSCs compared with the slice without surface modification (the Control group; P<0.05) (**Figure 3C**). In the experimental groups, it was found that the AO group expressed higher ALP activity at the early stage (7 d) than the SLA group, but there was no significant difference in ALP expression between the AO and SLA groups at day 14 (P>0.05). Moreover, quantitative statistical analysis by Alizarin Red staining showed that compared with the Control group, the modified titanium surfaces had better osteogenic mineralization effect (P<0.05) (**Figure 3D**). In the experimental groups, the osteogenic mineralization effects of the AO group on BMSCs at 7 and 14 days were better than those of the SLA group (P<0.05). However, there was no significant difference in the osteogenic mineralization effect between the AO and SLA groups at 21 days (P>0.05).

Effect of EMD on the expression of osteogenesis-related genes of BMSC sheets on different titanium surfaces

To explore the effect of EMD on the expression changes of RUNX-2, OSX and OCN genes during osteogenic differentiation of BMSC sheets on different titanium surfaces, real-time PCR was used to test BMSCs on the three different surfaces at 7, 14 and 21 days (**Figure 4**). At 7, 14 and 21 days, BMSCs osteogenesis related genes RUNX-2, OSX and OCN were significantly up-regulated in the experimental groups (SLA and AO groups) compared with those in the Control group (P<0.05). The expression levels of osteogenesis related genes Runx-2, OSX and OCN of BMSCs in the AO group were significantly higher than those in the SLA and Control groups (P<0.05).

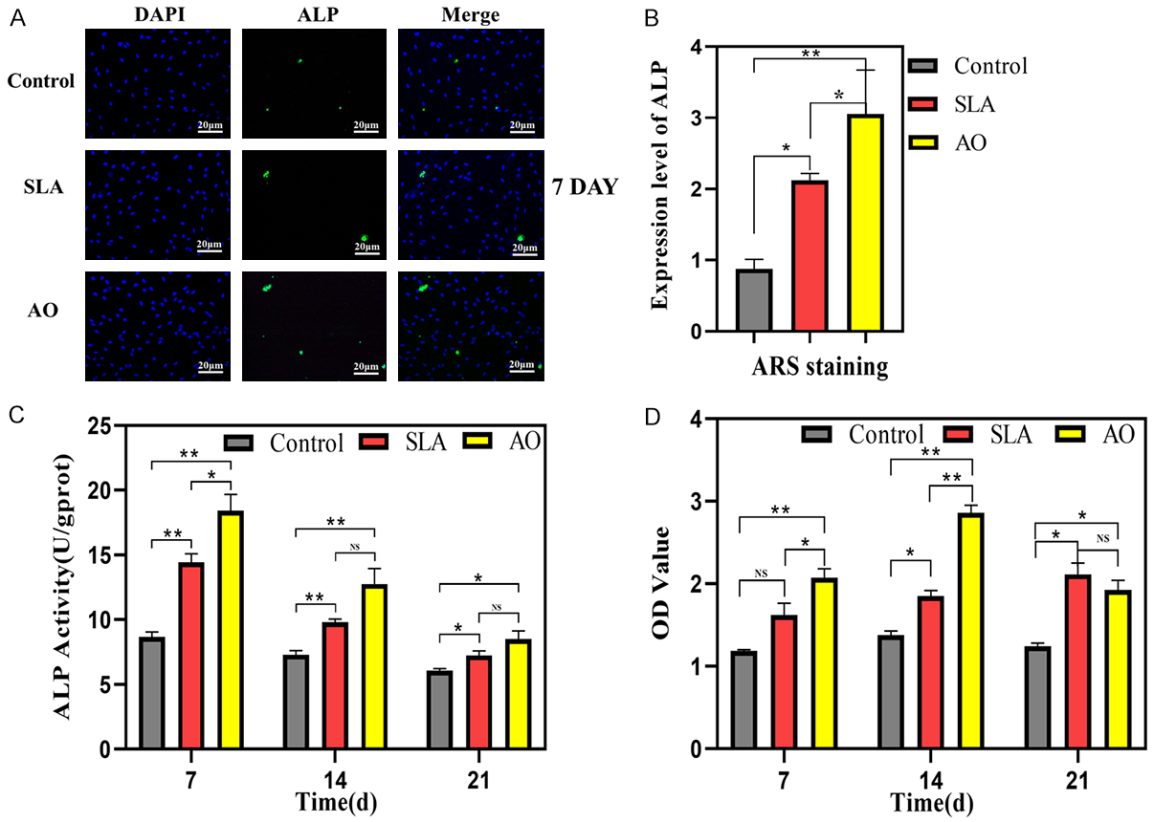

Figure 3. Detection of ALP and calcium nodule. A: The expression of alkaline phosphatase in BMSCs was detected by immunofluorescence. Green: alkaline phosphatase, blue: cell nucleus. Scale bar: 20 μm, ×20. B: Semi-quantitative analysis using ImageJ software. C: Detection of alkaline phosphatase expression during osteogenic differentiation of BMSCs using an alkaline phosphatase kit. D: Semi-quantitative statistical analysis of calcium nodules secreted by bone marrow mesenchymal stem cells after staining them red with Alizarin Red. $^{*}P<0.05$, $^{**}P<0.01$. SLA: Sand-blasted, Large grit Acid-etched; AO: Anodic Oxidation; BMSCs: Bone Marrow Mesenchymal Stem Cells; ALP: Alkaline Phosphatase.

Effect of EMD on osteogenic related protein expressions in BMSC sheets on different titanium surfaces

Western blot was used to detect the expressions of osteoblast-related proteins RUNX-2, OSX and OCN in the Control, SLA and AO groups at 7, 14 and 21 days (**Figure 5A**). Then quantitative statistical analysis was performed using ImageJ software (**Figure 5B**). The results showed that at the same time period, EMD significantly up-regulated the expressions of RUNX-2, OSX and OCN proteins in BMSCs in the experimental groups compared with those in the Control group ($P<0.05$), and EMD significantly up-regulated the expression of the above osteogenesis related proteins in the AO group ($P<0.05$). After transplantation of BMSC sheets cultured on three different titanium surfaces under EMD induction stimulation into the subcutaneous tissue of nude mice and pathological staining with HE, the formation of ectopic bone tissue was observed on the cell sheets of the AO group, while no obvious ectopic bone tissue was observed on the BMSC sheets of the Control and SLA groups (**Figure 5C**).

Discussion

Among the factors affecting osseointegration, bone tissue-implant surface factors are crucial to the success of implant implantation [3]. Under the influence of specific microenvironment and active factors, BMSCs in the bone marrow cavity can be differentiated into osteogenic, cartilage and adipose tissues [19]. Mesenchymal stem cells derived from bone marrow have higher osteogenic differentiation ability compared with mesenchymal stem cells derived from other tissues [20]. With the extensive application of titanium alloy implants in clinical practice, the surface topography of tita-

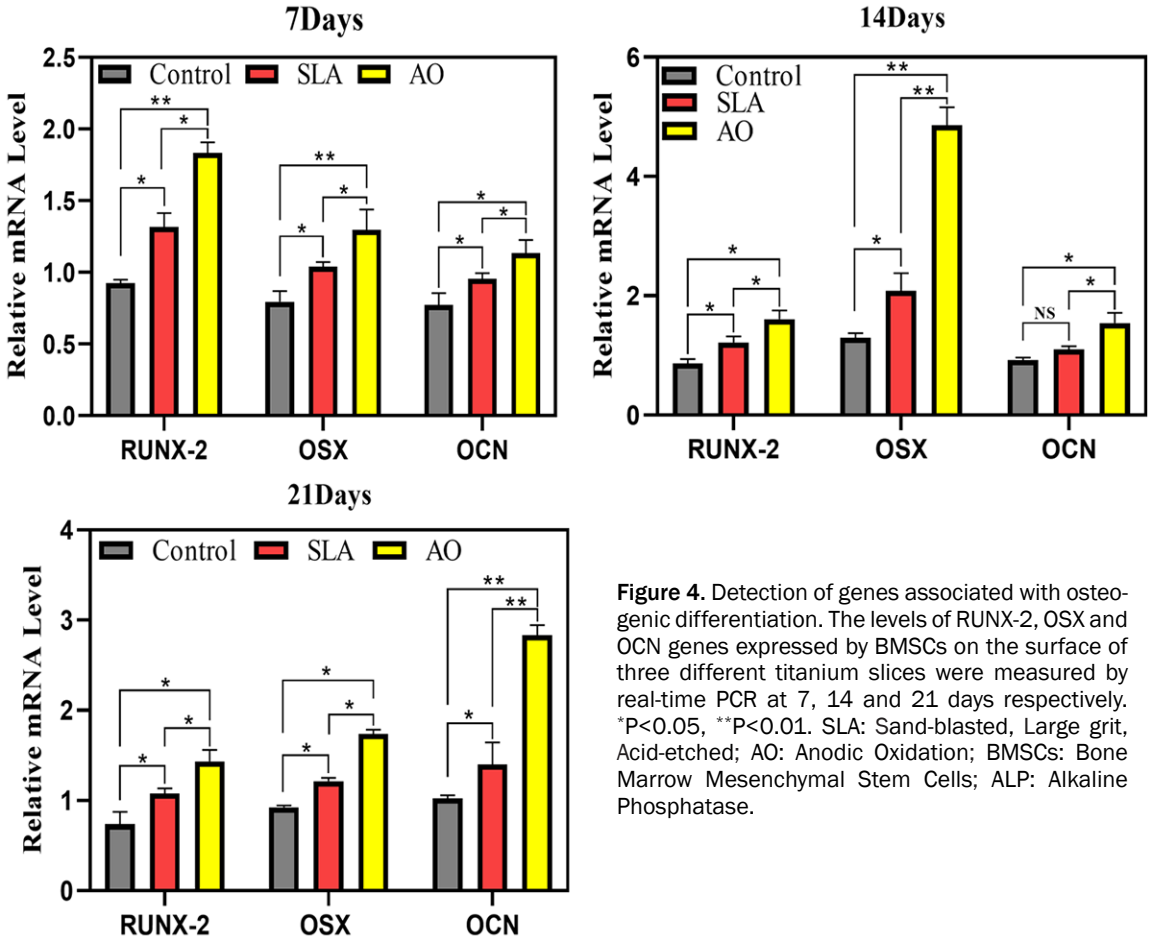

Figure 4. Detection of genes associated with osteogenic differentiation. The levels of RUNX-2, OSX and OCN genes expressed by BMSCs on the surface of three different titanium slices were measured by real-time PCR at 7, 14 and 21 days respectively. *P<0.05, **P<0.01. SLA: Sand-blasted, Large grit, Acid-etched; AO: Anodic Oxidation; BMSCs: Bone Marrow Mesenchymal Stem Cells; ALP: Alkaline Phosphatase.

nium alloys also plays a role in regulating the microenvironment of bone tissue repair. Previous studies have shown that EMD could enhance the proliferation and differentiation of periodontal ligament cells and osteoblasts [21]. Our CCK8 experiments showed that EMD at a concentration of 25 μg/ml could significantly promote BMSC proliferation (**Figure 2**). However, previous study showed that rough titanium surface might impair the activity of some cells [22]. However, in the living/dead cell staining experiments, it was observed that the activity of BMSCs was not significantly inhibited by the addition of EMD culture on rough titanium surfaces (the AO and SLA groups) or surface of untreated flat titanium (the Control group; **Figure 2**), and they could grow well on the surface of titanium slices. This may be because the effect of EMD on promoting BMSC proliferation counteracts the damaging effect of rough titanium surface.

Under specific conditions, activated transcription factors (mainly RUNX2) can initiate and promote the differentiation of BMSCs into osteoblasts and the development of activated cells into osteoblasts [23]. BMSCs can be differentiated into osteogenic progenitors under the action of RUNX2, which will further proliferate and mature to form osteoblasts and eventually differentiate into osteocytes [23]. Results of real-time PCR and Western blot indicated that EMD significantly up-regulated the expression level of RUNX2 mRNA and promoted the translation of RUNX2 protein in BMSCs in the AO group compared with those in the SLA and Control groups (**Figures 4** and **5**). In other words, EMD accelerated the differentiation of BMSCs into osteoblasts on AO titanium surface. Hao et al. reported that mesenchymal stem cells might perceive the terrain, pattern and other mechanical information on the material surfaces through their own contractile force, so as to guide mesenchymal stem cells to achieve specific functions [24]. This contractile ability of BMSCs to sense material surface features and promote osteogenic differentiation may be mediated by integrins, because

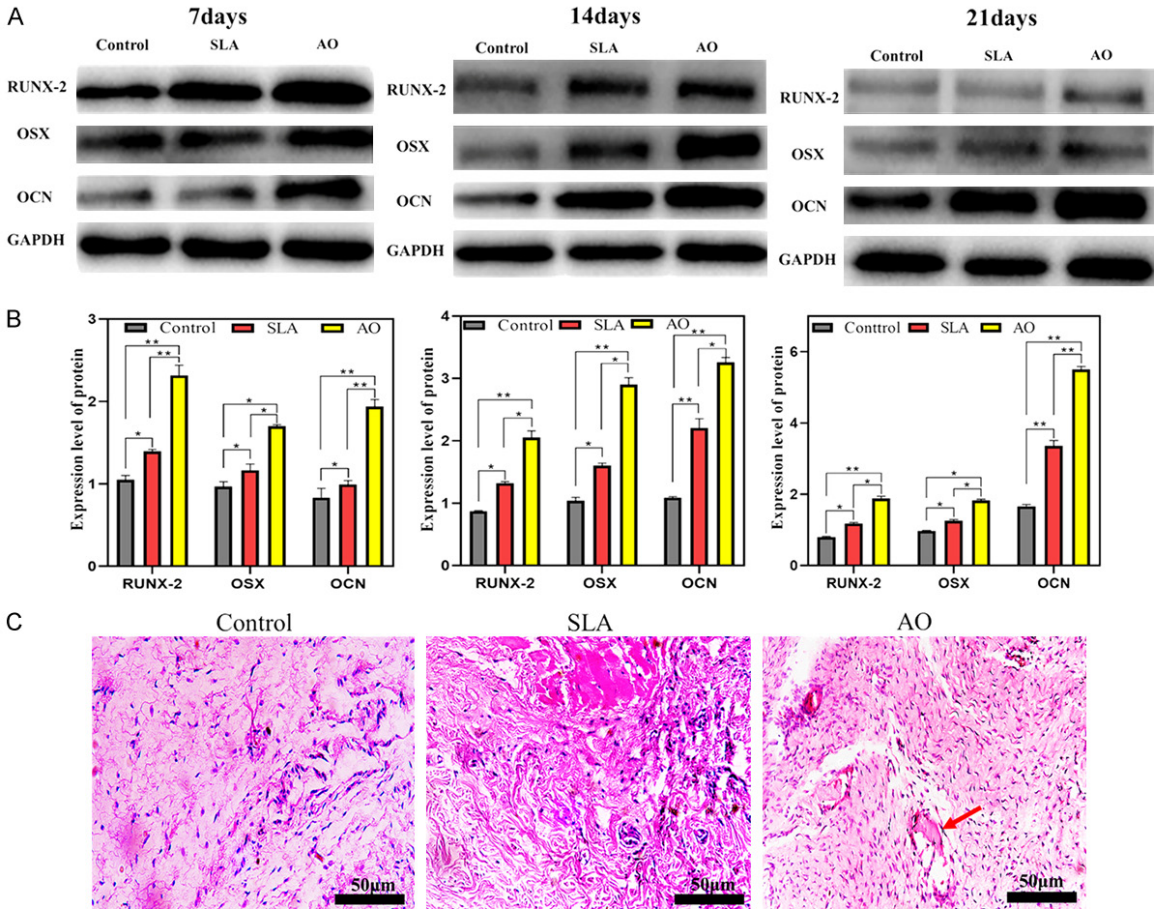

Figure 5. Detection of osteogenic differentiation related proteins and HE pathological staining test. A, B: The osteogenesis-related proteins RUNX-2, OSX and OCN expressed by BMSCs on the surface of titanium slices in the Control, SLA and AO groups were detected at 7, 14 and 21 days respectively using Western blot technique, followed by quantitative statistical analysis using ImageJ. C: Bone marrow mesenchymal stem cell sheets formed on the surface of different titanium slices were implanted into the subcutaneous tissue of mice and the specimens were removed after 6 weeks for HE staining. Scale bar: 50 μm, ×10. $^{**}P<0.01$. SLA: Sand-blasted, Large grit, Acid-etched; AO: Anodic Oxidation; BMSCs: Bone Marrow Mesenchymal Stem Cells; ALP: Alkaline Phosphatase.

surface properties of materials interfere with integrins and affect the interaction between integrins and their ligands [25]. Further analysis of the experimental results showed that compared with the flat titanium surface in the Control group, the surface in the AO group did not achieve the same BMSCs differentiation effect as that of the AO group after the addition of EMD stimulation, indicating that the AO surface morphology may also be involved in the differentiation process of BMSCs into osteoblasts. BMSCs may also perceive the mechanical information of AO titanium with special morphology in the above way and convert it into cytochemical signals, and cooperate with EMD to promote the osteogenic differentiation of BMSCs.

RUNX2 regulates the expressions of downstream OSX and OCN osteogenic genes, which play a key role in osteoblast differentiation and bone formation [26]. Our results showed that, compared with the SLA and Control groups, the titanium surface of the AO group significantly promoted the transcription of OSX and OCN genes downstream of RUNX2 under the same concentration of EMD (**Figure 4**), which may be related to the high expression of RUNX2 factor upstream. OSX and OCN are important transcription factors for osteoblast differentiation and bone mineralization, and OSX also plays an important role in intramembranous and endochondral ossification [27]. In the early differentiation stage of osteoblasts, the expression of alkaline phosphatase increases, which

is regarded as one of the markers of early differentiation of osteoblasts, while osteocalcin and mineralization are specific markers of late differentiation and maturation of osteoblasts, which are the characteristics of the beginning of osteogenesis mineralization [28]. Therefore, the Alizarin Red staining and alkaline phosphatase quantitative analysis of BMSCs in the experimental groups found that the expression and mineralization degree of BMSCs alkaline phosphatase in the AO group were significantly higher than those in the SLA and Control groups (**Figure 3**). These results indicated that EMD could induce osteogenic mineralization of BMSCs on surface of AO titanium.

In order to compare the osteogenic effect of AO and SLA surfaces in vivo, several researchers implanted SLA and AO titanium materials, respectively, into the femur of New Zealand white rabbits. After histological and histomorphometric analysis, it was found that there was no significant difference in osteogenic effect between the two implant surfaces [8]. However, in this experiment, the BMSC sheets in the AO and SLA groups under the same addition of EMD and in the Control group were implanted into the subcutaneous tissue of nude mice for ectopic bone experiment. After HE staining, only ectopic bone-like tissue formation was observed in the AO group (**Figure 5**). This may be because cell sheet of AO group promoted the secretion of osteogenesis related factors (RUNX2, OSX and OCN) of BMSCs on titanium surface to ECM under the dual induction of EMD and titanium surface topography of the AO group in vitro. Therefore, when transplanted into the body, BMSC sheets enriched with osteogenic factors stimulated the formation of surrounding bone tissue.

This study still has the following limitations. First, in this study, we did not explore the interaction mechanism between EMD and BMSC sheets on the AO surface of titanium, which is necessary for further studies. In addition, the in vivo experiments in this study need to be verified by different detection methods. We will continue to conduct in-depth research to strengthen our findings in the future.

Conclusion

Our study found that the effect of EMD on the BMSC sheets on the AO surface of titanium promoted the differentiation of osteogenic factors. We hope to provide some experimental evidence for the treatment of bone defects around implants.

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Disclosure of conflict of interest

None.

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