Surface modification of titanium with collagen/hyaluronic acid and bone morphogenetic protein 2/7 heterodimer promotes osteoblastic differentiation

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The aim of this study was to evaluate the effects of a collagen/hyaluronic acid coating without or with incorporated heterodimeric bone morphogenetic protein 2/7 (BMP2/7) on in-vitro osteoblastic differentiation on titanium discs. The multilayer collagen/hyaluronic acid coatings without or without incorporated BMP2/7 were deposited on titanium discs via a layer-by-layer technique. The effects of the coatings were evaluated by assessing the alkaline phosphatase (ALP) activity (an early osteoblastic differentiation marker) and the osteocalcin expression (a late osteoblastic differentiation marker). The expression levels of the osteoblastic genes, such as alkaline phosphatase 2 (AKP2) and osteocalcin (OC) were detected using real-time RT-PCR. ALP activity and OC expression were significantly increased when cells were cultured with collagen/hyaluronic acid+BMP2/7 heterodimer (p<0.05). The same result was found in cells with the expression of a BMP2/7 fusion gene, OC and AKP2. These results indicated that collagen/hyaluronic acid+BMP2/7 heterodimer-coated discs might have the potential to greatly enhance osseointegration than a either BMP2 or BMP7 solution or a mixture of BMP2 and BMP7 BMP2/7.

**Keywords**: Bone morphogenetic protein 2/7 heterodimer, Alkaline phosphatase, Osteocalcin, Osteoblast

INTRODUCTION

Osseointegration, which is symbolized by a direct bone and implant contact, is considered to play a critical role in the long-term success of dental implants. A more rapid establishment of osseointegration is highly demanded in clinic to reduce the risk of inadequate osseointegration and implant loosening over time1). For this purpose, diverse strategies have been implemented to promote specific binding interactions between implant materials and host tissues, such as biomimetic surface engineering2), surface modification3) and layer-by-layer (LBL) self-assembly4). Among these approaches, LBL self-assembly, which involves adding cytokines to the surface of a titanium (Ti)-disc, is advantageous in terms of safety, feasibility, and the potential for clinical application compared to the other two approaches5).

LBL self-assembly, a technique used to create a thin polyelectrolyte membrane based on the electrostatic attraction between polyanions and polycations, has attracted increasing attentions in recent years6). Collagen type I (Col) is the main component of the extracellular matrix (ECM), and hyaluronic acid (HA) is another integral part of the ECM. Both components have excellent biodegradability and biocompatibility. When they are dissolved in a solution at pH 4, Col will be positively charged7) and HA may carry a negative charge8). Col and HA may function as two polyelectrolytes when they are dissolved in a solution or a mixture of BMP2 and BMP7 BMP2/7.

Bone morphogenetic proteins (BMPs), a group of dimeric disulfide-linked polypeptide growth factors in the transforming growth factor-β superfamily, have a strong effect on bone and cartilage growth. Consequently, BMPs bear a highly promising application potential in bone tissue engineering9). Although recombinant BMP (rBMP) homodimers have been used in experimental models and clinical trials to enhance bone formation, the effective doses are extremely high10,11), which results in not only high costs for patients but also several potential side-effects, such as the overstimulation of osteoclastic activity and ectopic bone formation in an unintended area12,13). To overcome these side-effects, one approach used to address potential issues in current BMP therapy is to use a more potent form of BMP, which may be effective at lower doses in inducing osteogenesis. Previous studies have found that BMP heterodimers, resulting from the coexpression of two different BMP genes in vitro, resulted in significantly higher effects on inducing alkaline phosphatase (ALP) activity and bone formation than the respective homodimers14-17). Zhao et al.17) used adenovirus-based gene therapy to express unique combinations of BMP2, BMP4, and/or BMP7 and found that the BMP2/7 and BMP4/7 combinations had significantly enhanced biological activity in stimulating both in-vitro osteoblastic differentiation and in vivo bone formation in comparison to homodimeric BMPs. In a subsequent study, enhanced cranial bone regeneration was observed using combinatorial gene therapy with BMP2/718). These findings suggested that BMP heterodimers were feasible and more promising substitutes than homodimers.

Recently, some conclusions were drawn based on the use of conditioned medium from gene-transfected BMP producers rather than purified BMP heterodimers, which could not exclude the influence of other factors in the medium on cellular activities18,19). These studies mostly focused on in-vitro gene therapy, which could only suggest the clinical efficiency of BMP heterodimers, instead of
on the functional characteristics of BMP heterodimers. Zheng et al. indicated that rhBMP2/7 heterodimer was an osteoblastogenesis-inducer of not higher potency but lower effective concentration compared with rhBMP2 and rhBMP7 homodimers. In this article, we wished to develop a LBL self-assembled Col/HA layer with incorporated BMP2/7 on Ti discs and assess its effects on enhancing osteoblastic differentiation in comparison with BMP2 or BMP7 homodimers.

MATERIALS AND METHODS

Materials
Ti discs (purity>99.6%) with a thickness of 0.25 mm and a diameter of 20 mm were purchased from Zhejiang Guangci Biomedical Instrument (Zhejiang, China). Col, from calf skin, was supplied by Sigma-Aldrich (St. Louis, MO, USA). HA was obtained from the Furuida Biomedical (Shandong, China). BMP2, BMP7, and BMP2/7 heterodimers were purchased from R&D Systems (Minneapolis, MN, USA). All other reagents used in our experiments were used in the form in which they were received.

Surface modification
The Ti discs were divided into five groups (one control group and four experimental groups) according to the different treatment methods: the CHC-Ti (Col/HA coating) group, the Col/HA+BMP2 group, the Col/HA+BMP7 group, the Col/HA+BMP2+BMP7 group, and the Col/HA+BMP2/7 group.

1. Preparation of PEM films
The PEMs were built up LBL on the cleaned Ti discs using a dipping technique at room temperature. The Ti discs were polished with SiC paper to reduce the particle size (from #400 to #4000). The Ti discs were then washed by sonication with demineralized water, ethanol and acetone and then dried with blown air. For the preparation of the discs, the discs were pretreated with H2SO4/H2O2 (the volume ratio of 98% H2SO4 to 30% H2O2 was 1:1) at room temperature for 1 h. After being completely washed with deionized water, the Ti discs were dried in an N2 atmosphere. They were then stored completely washed with deionized water, the Ti discs were dried in an N2 atmosphere. They were then stored.

Col and HA were dissolved in 0.1% glacial acetic acid (HAc) solution and pure water, respectively. A 0.1 M HCl solution was used in our work to obtain a pH value of 4.0 in these two solutions. The bilayers were deposited by the LBL technique, beginning with the Col solution (1.0 mg/mL) and followed by the HA solution (0.5 mg/mL). After 15 min of deposition, each immersed disc was rinsed with deionized water three times. One assembly cycle consisted of a Col deposition procedure and an HA deposition procedure. The multilayers were generated by repeating this cycle. Herein, Col chains comprised the outermost layer, which was used for measurement or biological evaluation. The CHC-Ti substrates were dried with N2. All samples were sterilized via ultraviolet irradiation.

2. BMP immobilization
Solutions containing BMP2, BMP7, BMP2 and BMP7, and BMP2/7 heterodimers were separately generated in sterile 4 M HCl containing 0.1% bovine serum albumin (BSA) at a final concentration of 0.05 μg/μL in accordance with the manufacturer’s instructions.

The four CHC-Ti substrates with Col as the outermost layer were treated at room temperature with the BMP2, BMP7, BMP2 and BMP7, and BMP2/7 heterodimer solutions for 4 h and then washed with phosphate buffered saline (PBS; pH 7.4). The last group was the control group without BMP in the reaction medium and was subject to the same procedure. The above described procedure is likely to result in BMP being immobilized mainly on the outermost surface.

Cell culture
Pre-osteoblasts (MC3T3-E1 cell line, ATCC, Chinese Academy of Sciences, Shanghai, China), were cultured in Dulbecco’s modified Eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen). The cells were incubated at 37°C in an atmosphere of 5% CO2 and 95% air. The complete medium was replaced every 2–3 days, and confluent cells were subcultured through trypsinization.

To study the differentiation of the osteoblasts on the surfaces of the five groups of Ti discs, exponentially growing cells were plated at 4*10^4 cells/cm² in 6-well plates for the ALP activity assay and in 24-well plates for osteocalcin (OC) detection. To ensure the cells adhered to the titanium discs, they were allowed to adhere to the discs for half an hour before adding all of the medium to the cell culture plates. We refreshed the culture medium every 3 days and prepared samples for qualitative assessment on days 7 and 14.

Surface morphology
The comparison of the surface morphology of the CHC-Ti with that of the AE-Ti substrates was observed by a field emission scanning electronic microscope (FESEM; SIRION-100, Philips, Amsterdam, The Netherlands) with an accelerated voltage of 80 kV obtained with E-1020 ion sputtering.

ALP activity assay
To evaluate the early differentiation of osteoblasts stimulated by BMPs in different solutions (BMP2, BMP7, BMP2 and BMP7, and BMP2/7 heterodimer), ALP activity and protein content were measured after BMP treatment on days 7 and 14. The ALP activity was measured by a LabAssay™ ALP colorimetric assay kit (Wako Pure Chemicals, Osaka, Japan) using cell lysates (Sigma Aldrich) We used a commercial BCA Protein Assay kit (Beyotime, Shanghai, China) to determine the protein content, which was measured at 570 nm and used to determine the ALP activity as mmol p-NP/mg total protein.

OC expression assay
To assess the later differentiation of osteoblasts
stimulated by BMPs (BMP2, BMP7, BMP2 and BMP7, BMP2/7 heterodimer), OC secreted into the cell culture medium were measured. Before detection, the cell supernatants were collected on days 14, and were centrifuged (10,000 rpm, 0°C, 5 min). The OC concentrations of the supernatants were measured by ELISA using a mouse OC EIA kit (Biomedical Technologies, Stoughton, MA, USA).16-20

Real-time RT-PCR
According to the manufacturer’s protocol, we collected total RNA from the osteoblasts on the titanium discs with an RNeasy Mini kit (Qiagen, Valencia, CA, USA). The concentration of RNA was determined by a spectrophotometer, and cDNA was synthesized from the RNA (1.5 μg) with the SuperScript II reverse transcriptase (Thermo Fisher, Shanghai, China). The ABI Prism 7,500 sequence detection system (Perkin Elmer) was used for quantitative real-time RT-PCR. The sequences of primers used for the ALP and OC genes in humans are listed in Table 1. The amplification reactions were performed according to the protocol included in the SYBR PrimeScript RT-PCR kit (Takara, Dalian, China). We added 10 μL SYBR Premix EX Taq, 0.4 μL of both forward and reverse primer, 7.8 μL dH2O and 2 μL of cDNA sample to each well of a 96-well plate. The plate was covered and centrifuged briefly to remove air bubbles, and then the PCR quantification was performed using the following cycling parameters: 40 cycles of 95°C for 10 s and 60°C for 34 s. All samples were analyzed in triplicate. The relative quantities of alkaline phosphatase 2 (AKP2) and OC were calculated using the comparative 2^(-DDCt) method. The expression of the GAPDH gene in the blank control group was used as an internal parameter. The expression change of each gene in each group was expressed in the form of the relative fold change compared to the control group.

Statistical analysis
Statistical analysis was carried out using a SPSS statistical software package (V22, SPSS, Chicago, IL, USA). Data is in normal distribution. Mean value±standard deviation (SD) was adopted to calculate each parameter and differences among all groups were analyzed by one-way analysis of variance (ANOVA). Post hoc comparisons were made using Bonferroni corrections. p<0.05, was considered a statistically significant difference. For comparison between two groups, unpaired t test was conducted.

RESULTS
Characterization of surface modifications
Upon observation of the Ti discs by microscopy, no significant difference in the surface morphology was found among the five groups, e.g. the Col/HA group, the Col/HA+BMP2 group, the Col/HA+BMP7 group, the Col/HA+BMP2/7 group and the Col/HA+BMP2+BMP7 group. Their surface roughness’s were about Ra=0.25±0.05 μm, and no significant difference was found among the roughness’s of the four surfaces. According to the surface wettability, the contact angles of the four surfaces were about 23°. No difference was found among the wettability’s of the four surfaces (p>0.05). SEM micrographs of the different Ti surfaces are shown in Fig. 1. In Figs. 1a and b, we can see many fine granules

Table 1  Forward (F) and Reverse (R) primers for target genes (human)

| Gene name | Amplicon length (bp) | 5′-3′primer sequence |
|-----------|----------------------|----------------------|
| AKP2      | 164                  | F 5′-TGCTACTTTGTTGGCCGTGAA-3′ |
|           |                      | R 5′-TCACCGAGTGATCTAATG-3′ |
| OC        | 317                  | F 5′-AAGCCCAAGCCACAGTCTGAGTCT-3′ |
|           |                      | R 5′-GGGCAAGAACAGGAAACAG-3′ |
| GAPDH     | 150                  | F 5′-TGCTGCAGTGAATGCGACAGG-3′ |
|           |                      | R 5′-TTGCTGTTAGCAGGACAG-3′ |

Fig. 1  SEM images of Ti surfaces after treated by H2SO4/H2O2 for 1 h.
(a) AE-Ti, (b) CHC-Ti, (c) BMP2 incorporated Col/HA coating (BMP2-Col/HA-Ti), Scale bar=500 nm
with diameters of 20–30 nm on the acid-etched (AE-Ti) surfaces. On the other hand, compared with the AE-Ti and CHC-Ti surfaces, as shown in Fig. 1c, the depth of the parallel grooves was decreased on the Col/HA+BMP2-Ti surfaces, and the fine granules were almost completely invisible.

**ALP activity and OC expression**

In this study, we found that ALP activity and OC expression were significantly higher in the different Col/HA+BMPs groups than the Col/HA group ($p<0.05$). The expression of OC and the ALP activity were increased in a dose-dependent and time-dependent manner. Additionally, BMP2/7-containing supernatants were more potent than any other. The OC expression level at the optimal dose (between 150–200 ng/mL) of BMP2/7 in the supernatant was 2-fold or 3-fold higher than the maximal level induced by BMP7 or BMP2 ($p<0.05$ for both comparisons). In Fig. 2, it was shown that the ALP activity induced by this dose of BMP2/7-containing supernatant was significantly higher than the maximal level induced by BMP7 and BMP2 ($p<0.05$).

In our study, we also found that in addition to the significantly increased ALP activity and OC expression, BMP2/7-containing supernatants accelerated the onset of ALP activity in osteoblast cells versus single BMP-containing supernatants. ALP activity was detected as early as 4 days after stimulation for supernatants containing 200 ng/mL BMP2/7 and continued to increase for 14 days. In contrast, supernatants containing higher concentrations of BMP2 or BMP7 did not induce detectable ALP activity until day 7, and the level of ALP activity induced by BMP2/7-containing supernatants at the day 14 endpoint was still significantly higher than that induced by BMP2 or BMP7 ($p<0.05$ for both comparisons). Pre-osteoblast cells stimulated with a mixture of BMP2 and BMP7 showed a similar pattern of ALP activity as those stimulated with BMP2 alone.

OC expression was also induced in BMP2/7-containing supernatants by day 4, with further increases detected at days 7 and 14. Although supernatants containing BMP7 and BMP2 increased OC expression in osteoblast cells in a similar time-dependent manner, the levels were lower than those induced by BMP2/7 supernatants throughout the time course. In Fig. 3, it is shown that the OC expression induced by BMP2/7-containing supernatant was significantly greater than the maximal level induced by BMP7 and BMP2 at day 14 ($p<0.05$). In addition, ALP activity and OC expression at day 14 were significantly higher than those at day 7 for all groups.

**The expression of related genes**

We used RT-PCR of OC and AKP2, which are important osteogenic phenotype genes for monitoring the progress...
of osteogenic differentiation, to detect the effect of BMP2/7 on osteogenic differentiation. As is shown in Fig. 4, the expression levels of OC and AKP2 increased with culture time, and the expression levels of OC and AKP2 in the Col/HA+BMP2/7 group were significantly higher than those in the BMP group at all the time points \((p<0.05)\). Additionally, the expression of these genes were also significantly higher in the BMP group than in the Col/HA group \((p<0.05)\).

**DISCUSSION**

Ti-based implant materials have specific complications associated with their application, such as the loosening of the implant-host interface due to inadequate cell adhesion. Hence, a surface that exhibits selective biointeractivity and enhances host cell responses would be highly desirable\(^{21}\). Zhang \textit{et al.} has already proved that the attachment, spreading, proliferation and differentiation of preosteoblasts (MC3T3-E1) is significantly improved when cultured on the surface of HA/Col PEM coating compared to uncoated Ti surfaces. Ti discs treated with a mixture of H\(_2\)O\(_2\) and H\(_2\)SO\(_4\) for 4 h at room temperature could create a unique contaminant-free, nanostructured surface with a reproducible oxide layer on the surface. Moreover, H\(_2\)SO\(_4\)/H\(_2\)O\(_2\)-treated Ti may facilitate osseointegration by up-regulating the expression of bone sialoprotein and osteopontin and enhancing bone osteogenesis. In that study, they showed that Col/HA PEM is successfully conjugated with Ti substrate by FESEM and XPS examinations; they also measured the thickness of the Col/HA PEM coating and its degradation behavior. The conjugation of BMPs with the Col/HA PEM coating was also mentioned in that study\(^{22,23}\). Consequently, in this study, we focused on the functionalization of HA/Col PEM coating with different BMPs. To improve biocompatibility, we coated HA and Col on Ti-discs in the form of PEMs. BMP2/7 heterodimers showed significantly higher biological activity than the corresponding homodimers at many sites in a peri-implant bone defects model\(^{24}\). To evaluate whether BMP2/7 heterodimers would also enhance osteoblast cell differentiation on Ti discs, we generated Ti discs coated with Col/HA coupled with surface-immobilized cell adhesive BMPs. Before adsorption, the discs were treated with H\(_2\)SO\(_4\)/H\(_2\)O\(_2\) at room temperature, and the H\(_2\)SO\(_4\)/H\(_2\)O\(_2\)-treated Ti discs could upregulate the expression of bone sialoprotein and osteopontin to enhance bone osteogenesis and facilitate osseointegration\(^{25-27}\).

Moreover, a uniformly amorphous TiO\(_2\) gel layer covered with hydroxyl groups (Ti-OH) was produced after the acid treatment. These abundant hydroxyls could increase the surface polarity of the Ti discs. The negatively charged surface made it possible to alternatively adsorb positively charged collagen and thereafter the negatively charged HA to prepare the PEM coating. There were only slight changes in the topography when the LBL technique was used to modify the surface. For instance, the CHC-Ti discs mixed with BMP2 had a similar surface topography in comparison to those of the AE-Ti and CHC-Ti discs at the micro scale, but significant differences were found in these three types of discs at higher magnification. (Figs. 1a–c).
BMPs play pivotal roles in many morphogenetic events in the body, from the earliest embryonic patterning to organ development. Lutz et al. was convinced that BMPs could enhance the osseointegration of titanium (Ti) implants. The infiltration of bone-forming cells is an important event during osseointegration. Exogenous BMPs may be one of the dominant modulators of cell migration, especially when they are used at an unphysiologically high amount for bone regeneration. Different BMPs differed in their chemotactic effects in different targeting cell types. Hidaka et al. reported that BMP 2/7 heterodimers had a significantly higher activity in inducing bone regeneration than BMP2/7 homodimers. In this study, we used purified BMP heterodimers and single BMPs that were commercially available to compare their actions on osteoblasts by evaluating ALP activity, OC expression and the osteoblastic phenotype genes OC and AKP2.

Salvi et al. indicated that Different BMP-2 release profiles were obtained for each pH condition and all pH conditions exhibited a sustained release profile for at least 25 days. They also proved that BMP-2 was shown to maintain bioactivity after release from a PEM and the presence of a PEM was shown to preserve BMP-2 structure.

Osteoblasts are derived from the mesenchymal mesoderm, which secretes bone matrix, and the main component of the mesenchymal mesoderm is type I collagen. After the formation of collagen fibers, vesicle-like structures that contain calcium and phosphorus in osteoblasts will be released and then deposited on collagen fibers, and hydroxyl apatite crystallization will promote bone matrix mineralization. Furthermore, the osteoblasts are surrounded and turned into bone cells, producing new bone tissue. Thus, osteoblastic activity has a direct effect on bone tissue formation. In addition, many factors can affect osteoblast activity, and polypeptide growth factors are the most important, as shown by the effects of the BMPs in this study.

In this study, we have shown that BMP2/7 heterodimers accelerate osteoblastic differentiation in vitro compared with BMP2 or BMP7 alone or a combined solution of BMP2 and BMP7 because heterodimers might be a pivotal effector at sites with fewer BMP producers, where they could provide a lower concentration of BMP homodimers. It is also important to note that the ALP activity induced by BMP2/7 heterodimers was significantly higher than that induced in the other four groups on days 7 and 14 (Fig. 2, p<0.05). The differences in OC expression between the BMP2/7 group and the other BMP groups were significantly higher on days 7 and 14. The inconsistency with the results obtained by Wei et al. was indicated that BMP activity induced by the dose of the BMP2/7-containing supernatant was 27-fold greater than the maximal levels induced by rBMP7 and 33-fold greater than the maximal level induced by rBMP2, may be due to the usage of a mammalian producer, conditioned medium instead of purified protein, and a single selected concentration.

These results indicated that the BMP2/7 heterodimer showed more rapid effects and accelerated the process of differentiation and mineralization, suggesting that the BMP heterodimer plays a pivotal role in the initial stage of osteogenic activity.

The process of the differentiation of multifunctional precursor cells into osteoblast phenotype cells is often accompanied by the expression of many osteoblast phenotype-associated genes, such as AKP2 and OC. This study shows that the gene expression of AKP2 and OC on days 7 and 14 stimulated by the BMP2/7 heterodimer were significantly higher than those stimulated by other BMPs, as determined by real-time reverse transcription (RT)–polymerase chain reaction. However, the results also indicated that the final maximum levels stimulated by the BMP2/7 heterodimer were similar to those stimulated by BMP2. Therefore, within the suitable concentration range, the BMP2/7 heterodimer was shown to be more potent than the other BMPs in stimulating osteogenic differentiation. This is consistent with the conclusion of a previous study.

The mechanism accounting for the enhancement of osteoblastic differentiation by the BMP2/7 heterodimer remains unclear. One possible mechanism may rely on the structural specificity of the BMP2/7 heterodimer, which is different from that of the BMP2 or BMP7 homodimer. BMP signaling can be activated by various receptor complexes, and different BMPs exhibit different binding affinities for different receptors. The BMP2/7 heterodimer, which contains both the BMP2 and BMP7 moieties in the same ligand, may have a higher probability of combining different types of receptors and thus be able to form more active signaling complexes.

Another possible mechanism would be a complicated BMP antagonism network. Noggin is one of the predominant BMP-induced competitive antagonists. It was reported that the BMP2/7 heterodimer induced a significantly lower level of Noggin and showed a lower affinity for Noggin than the respective BMP homodimers. This may contribute to the increased osteogenic potency of the BMP2/7 heterodimer in vitro and in vivo. Similarly, another antagonist of BMPs, called Thrombospondin-1 (TSP-1), behaved similarly to Noggin when stimulated by the BMP2/7 heterodimer.

In the present study, we have shown that BMP2/7 heterodimers could accelerate osteoblastic differentiation in vitro with better performance and in larger quantities compared with BMP2 or BMP7 alone at a very low dose. Its induction of side effects might also be similar to that of the homodimers in the same concentration range. Consequently, further study should clarify this point prior to the clinical use of BMP heterodimers. One limitation of this study was that we only used one concentration of BMPs and two time points; broader concentration and time ranges could be used to completely investigate the functional characteristics of both the heterodimer and other BMPs. The cells used in the present study was the other limitation. The primary cells would be tested in our coming research.
CONCLUSION

In conclusion, Col/HA PEMs with BMP coatings were fabricated on smooth Ti discs via acid etching combined with the LBL technique. SEM observation indicated that acid etching and Col/HA and BMP coating did not significantly change the surface topology of the Ti discs at the macro- and micro-scales and demonstrated the successful coating of Col/HA multilayers and BMPs onto Ti discs; however, differences were found at the nanoscale. Osteoblasts were cultured to evaluate the biological properties of Col/HA compared with those of the BMP2/7 heterodimer. It was found that the Col/HA+BMP2/7 heterodimer coating greatly promoted the cell differentiation of osteoblasts in contrast with that observed in the other four groups. The results suggested that the BMP2/7 heterodimer could improve the quality and quantity of bone by enhancing the osseointegration of titanium (Ti) implants and ensuring the long-term success of endosseous dental implants.

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