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ORIGINAL ARTICLE

Effects of Aging on the Proliferation and Differentiation Capacity of Human Periodontal Ligament Stem Cells[△]

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Key words: periodontal ligament stem cells; aging; proliferation; osteogenic differentiation

Objective The aim of this study is to investigate the proliferation, differentiation and apoptosis of periodontal ligament stem cells (PDLSC) derived from different aged donors, and to evaluate the effects of aging on the biological characteristics of PDLSC.

Methods Periodontal ligament tissues were obtained from 24 surgically extracted human premolars during orthodontics therapy. The specimens were divided into three groups according to the donor's age. Group A: 18-20 years, group B: 30-35 years, group C: 45-50 years. PDLSC were isolated and cultured using a tissue-block-based enzymolytic method by limiting dilution assay. The colony forming efficiency of PDLSC for three experimental groups was determined. Senescence-Associated β -Galactosidase (SA- β -G) expression in the three groups was examined using β -galactosidase staining working solution. Cell cycle and apoptosis of the PDLSC were examined by the flow cytometry. Alkaline phosphatase (ALP) activity was evaluated by ALP staining. The expression of osteoplastic differentiation related genes Runt-related transcription factor-2 (Runx-2), Collagen Type 1 (col-1), and ALP of PDLSC were examined by quantitative real-time RT-PCR.

Results The colony forming efficiency of PDLSC in Group A, B and C was 36.67%, 22.67% and 9.33%, respectively, which decreased with donors' age ($P < 0.05$). SA- β -G expression of the senescent PDLSC in group A, B and C were 4.14%, 16.39%, 50.38%, respectively ($P < 0.05$). Cells in G2/S phase was 38.73%, 29.88%, 18.25% ($P < 0.05$), and the apoptosis rate was 1.57%, 4.56%, 5.84% ($P < 0.05$), in group A, B and C respectively. The ALP staining in the three groups decreased with the increase of donors' ages, and the expression of Runx-2, col-1 and ALP decreased gradually from group A to group C (all $P < 0.05$), which indicated the osteogenic differentiation capacity of PDLSC decreased while donor aging.

Conclusion Human PDLSC could be successfully isolated from periodontal ligament tissues of

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different aged donors. However, the proliferation and osteogenic differentiation capacity of PDLSC decreased while donor aging.

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PERIODONTAL disease is a common chronic disease in human oral infectious diseases, and is the major cause of periodontal tissue defects or tooth loss.¹ Studies have found that the severity and risk of periodontitis increase with age.² The ultimate objectives of the treatment for periodontal diseases are to eliminate local inflammation, reduce further loss of periodontal attachment, and promote the regeneration and functional restoration of periodontal tissues.³ This issue has been the concern of the foreign and domestic scholars. Although traditional periodontal treatment such as periodontal scaling and guided tissue regeneration can eliminate the pathogenic causes, control or slow the development of the disease,⁴ it is difficult to achieve ideal treatment effect because physiological and functional regeneration of periodontal tissue are hard to reach. Recent studies have found that adult stem cells participate in periodontal tissue regeneration, restoration, and function rebuilding, and play an important role in keeping dynamic balance of growth and decline of tissues and organs. It might be one of the key factors to restore the lost periodontal tissues.⁵

In 2004, Seo *et al.* successfully isolated periodontal ligament stem cells (PDLSC) from human impacted third molars for the first time.⁶ It was found that PDLSC had similar biological characteristics as bone marrow mesenchymal stem cells (BMSC). It could differentiate into cementum, periodontal ligaments, alveolar bone, peripheral nerves, and blood vessels under specific conditions, and help to maintain dynamic balance of periodontal tissue consequently.^{5,6} It became the most promising seed cell in periodontal tissue regeneration. In recent years, many studies have confirmed that PDLSC has strong proliferation and multi-differentiation capacity, and in special conditions it can form cementum/PDL-like structure *in vivo* in tissue engineering scaffolds.^{6,7} As a result, using adult stem cells for tissue and organ repair in the treatment of refractory diseases has a bright prospect. However, aging may lead to changes in the biological characteristics of stem cells, thereby affect the final transplant or therapeutic effect. As the elderly is the main population of stem cell therapy in the future, it is very important to understand the relationship of stem cells and aging of donors. The severity of periodontal disease was closely related to age. At present, there are few reports about the effects of aging on the biological characteristics of PDLSC. Therefore, it is nec-

essary and important to study aging induced changes in biological behavior of periodontal ligament stem cells. This study was conducted to investigate the impact of aging on isolation, culture, proliferation, and differentiation ability of PDLSC which was extracted freshly from healthy teeth of different aged donors.

MATERIALS AND METHODS

Study subjects

The study was approved by the institutional Ethics Committee and carried out from 2015 to 2016 in the Department of Stomatology of Chinese PLA General Hospital. Primary cultures were obtained by culturing explants of healthy periodontal tissues from patients who underwent premolar extraction for orthodontic treatment or impacted wisdom tooth. All donors had no history of diabetes, which may affect the periodontal status. All donors have no smoking history. All of the teeth had no caries, inflammation, or periodontitis, and were extracted with integrated root. We assigned the extracted tooth into three groups according to the donor's age: group A for age of 18-20, group B for age of 30-35, and group C for age of 45-50. We collected 24 teeth in total, with 4 female and 4 male donors in each group ($n=8$). The median ages for group A, B and C were 19 years, 32.5 years and 47.5 years respectively. The Informed consent was obtained from each donor.

Isolation and culture of PDLSC

Human PDLSC were isolated and cultured from fresh PDL tissues using a tissue-block-based enzymolytic method.⁷ The extracted teeth were rinsed with sterile phosphate-buffered saline (PBS) repeatedly in the super-clean worktable, and for each tooth the PDL tissue was gently scraped from the surface of the middle part of the root surface using sterile surgical blades in a direction from crown to root. The PDL tissue was minced into 1 mm³ cubes, and then was digested with a solution of collagenase type I (Sigma Aldrich, St. Louis, MO, USA) in an incubator for 15 min. After centrifuging the tissues in the centrifuge tube at 800r / min, the supernatant fraction was discarded, and the tissues were resuspended and transferred into a six-well culture dish (Costar, Cambridge, MA) which contain a-MEM (Gibco BRL, Gaithersburg, MD, USA) complete medium supplemented with 10% fetal bovine serum (FBS,

Hangzhou Sijiqing Biological Engineering Materials Co, Ltd, Zhejiang, China) and 100 units/mL penicillin streptomycin, and then cultured in the 37°C, 5% CO₂ incubator. The medium was changed every 3-4 days till the primary cells migrated from the tissue and fused to 80%. Then we digested the cells with trypsin/EDTA (0.25/0.1, pH=6.4) and multiplied the number of cells through subculture. Cells at passages 2 or 3 (P2-P3) were used for comparison and study. Cells at passage 0 (P0) were cultured and single cell-derived colonies were obtained using the limited dilution method. The identification has been described previously.⁷

Colony-forming assay

Cells of the 2nd passage were made into single cell suspension. The cells were plated into a 10 cm-diameter culture dish (Costar, Cambridge, MA, USA) at a density of 1×10^3 per dish and cultured in standard culture medium, which was changed every 3 days. The clonogenic assays were performed in triplicate. Mediums were discarded completely when colony-forming units were observed (Aggregates of >50 cells were scored as colonies). After being washed with PBS for 3 times, the cells were fixed in 4% paraformaldehyde for 30 min, and stained with toluidine blue (Sigma-Aldrich) for 30 min at room temperature. After washing with PBS for 3 times, we calculated the colony forming efficiency with the formula $N = (\text{the number of colony-forming units} / \text{the number of cells implanted}) \times 100\%$. The experiment was repeated three times.

Senescence-associated β -galactosidase (SA- β -G) staining

When the 2nd passage cells fused to 50%-60%, cell aging was observed with β -galactosidase, which was the biological marker of cell senescence. Staining was done with the Senescence β -Galactosidase Staining Kit (Beyotime, Jiangsu, China). Cells of the 2nd passage were made into single cell suspension, and were plated into 6-well plates at a density of 5×10^4 per well and cultured in standard culturing medium till the cells reached 50% confluence. After being fixed by β -galactosidase fixative for 10 min and then washed 3 times with PBS (3min per time), the cells were stained with freshly prepared β -galactosidase staining working solution as indicated by the kit protocol, and incubated at 37 °C incubator in the closed-culture for 10-12h before observation. The positive staining cells were observed under inverted microscope and counted in 10 randomly selected microscopic fields. The percentage of β -galactosidase positive cells in each group was obtained. The experiment was repeated three times.

Cell cycle detection

Cells of the 3rd passage were made into single cell suspension. The cells were plated into a 10 cm-diameter culture dish (Costar, Cambridge, MA, USA), digested by trypsin after reaching 80% confluence. The cells were washed with PBS repeatedly and then fixed in 4 °C by 70% alcohol for 16h. After centrifugalization (1000 r/ min) for 6 minutes, the supernatant was discarded and the cells were washed for 2 times with precooling PBS, centrifugalized at a speed of 10 000 r/ min for 6 minutes. Propidium iodide staining solution (100 g/L, 400 μ L) was added and fully mixed after the cells were gently triturated with 100 μ L PBS. The cells were stained at room temperature away from light for 30 min. Cell cycle distribution was analyzed using Guava easyCyte™ flow cytometer (FACSaria III, BD bioscience, USA), and the percentage of cells in G1, S, and G2 phase was analyzed with post-analysis using FLOWJO version 10 software (TreeStar, San Carlos, CA). The experiment was repeated three times.

Apoptosis detection

Cells of the 3rd passage were made into single cell suspension. The cells were plated into a 6 cm-diameter culture dish (Costar, Cambridge, MA, USA), and were digested by trypsin after they reached 80% confluence. Apoptosis was detected by Annexin V-FITC apoptosis detecting kit and flow cytometry (FACSaria III, BD Bioscience, USA). The experiment was repeated three times.

Activity of alkaline phosphatase

Cells of the 3rd passage were made into single cell suspension. The cells were plated into 6-well plates at a density of 5×10^4 cells per well and cultured in standard culture medium till reaching 80% confluence. The medium was then switched to osteoinductive medium (supplemented with 5 mmol/L β -sodium phosphate, 50 mg/L vitamin C, and 1×10^{-8} mol/L dexamethasone (all from Sigma-Aldrich, USA); the medium was changed every 3 days. After 1-week osteogenic induction, the osteoinductive medium was discarded, and the cells were washed with PBS repeatedly and then were fixed in 4% paraformaldehyde for 30 min. Activity of ALP was examined using alkaline phosphatase staining kit (Beyotime, Jiangsu, China). After washing with PBS for 3 times, cells were observed under inverted microscope and photographed for record. The experiment was repeated three times.

RT-PCR to detect the expression of bone related genes

Real-time RT-PCR was used to examine the expression of

genes that were related to osteoblastic differentiation, including alkaline phosphatase (ALP), collagen type 1 (col-1), and Runt-related transcription factor-2 (Runx-2). The total RNA of PDLSC cultures was extracted using Trizol reagent. The reverse transcription kit (Fermentas, USA) was used for synthesis of cDNA template. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the internal control. Each cell source was examined in triplicate. The PCR primers were designed and synthesized by The Beijing Genomics Institute (BGI). The specific sequences of each primer were shown in Table 1.

Statistical analysis

Each cell line was tested in triplicate independently. Continuous variables with normal distribution were expressed as means and standard deviation (SD). The data was analyzed by repeated measurement analysis of variance. Linear regression were performed to examine the trend of changes.

Comparisons among/between groups were performed by one-way analysis of variance (ANOVA) using SPSS software (version 17.0). A *P* value of < 0.05 was accepted to be statistically significant.

RESULTS

Extraction of human PDLSC

Under reverse microscopy, we found that cells successfully grew out from PDL tissue after culturing for 5-14 d. The time when it reached confluence in group A, B and C were 7 d, 13 d and 20 d, respectively, which indicated that the growing rate of initial cells was influenced by donors' age. PDLSC presented an elongated morphology, and they showed a fibroblast-like appearance. After ten days of culture, PDLSC obtained from young subjects (group A) showed more typical spindle type morphology than those from relatively aged donors (group B and group C) (Fig. 1).

Table 1. Primer sequences of genes related to osteoblastic differentiation

Genes name	Sequences of the primers	bp
GAPDH	F: 5'-TCTGCATCATCCAGGAGCTTATT-3' R: 5'-TGATACAGAAGGCAGGTTACAA-3'	238
ALP	F: 5'-AGCTTTCGAAGAACAACGGA-3' R: 5'-TCTTGAAATGCTTGGGTCC-3'	239
Runx-2	F: 5'-CCCGTGGCCTTCAAGGT-3' R: 5'-CGTTACCCGCCATGACAGTA-3'	92
Col-1	F: 5'-GCAAGGTGTGTGCGATGA-3' R: 5'-TGGTCGGTGGGTGACTCTG-3'	235

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; ALP: alkaline phosphatase; Runx-2: Runt-related transcription-2; Col-1: collagen type-1.

Figure 1. Cell morphology of periodontal ligament stem cell (PDLSC) in culture (Original magnification: $\times 10$).

Primary cells of PDLSC in group A (A), group B (B) and group C (C). Cells successfully grew out from PDL tissue. P1 cells of PDLSC (arrows) in group A (D) showed more typical spindle type morphology than those in group B (E) and group C (F).

Reproductive ability of PDLSC in each group

The clonogenic assay of 3 experimental groups all showed colony-like growth and the ability of mono-clonal formation (Fig. 2). The colony forming efficiency in group A, B and C were 36.67%, 22.67% and 9.33%, respectively, which decreased significantly with increasing of donors' age ($F=26.294$, $P=0.001$). Trend test confirmed that the ability of PDLSC clone formation rate significantly decreased with the increase of donors' age ($P=0.007$).

SA- β -G staining of PDLSC

Under the microscope, the second passage cells in all 3 experimental groups were stained. The stained cells in group C excelled in numbers and intensity, while very few PDLSC in group A were positively stained (Fig. 3). The counts of senescent cells of PDLSC was remarkable in group C (50.38%), moderate in group B (16.39%), and mild in group A (4.14%) ($F=142.895$, $P=0.000$). Trend test confirmed that the SA- β -G expression of PDLSC increased with the increase of donors' age ($P=0.007$).

Figure 2. Gross observations of PDLSC clone formation of the experimental groups.

A. group A; B. group B; C. group C. The colony forming efficiency decreased with the increase of donors' age.

Figure 3. Inverted microscopic graphs of senescence-associated β -galactosidase (SA- β -G) staining (blue) of PDLSC in the three experimental groups.

A. group A; B. group B; C. group C. The stained senescent cells in group C excelled in numbers and intensity when compared to group A and B. (Scale: 200 μ m)

Figure 4. Flow cytometry results of cell cycles of PDLSC in the experimental groups.

A. group A; B. group B; C. group C. Percentages of cell in the S + G2/M phase decreased with the increase of donors' age.

Cell cycle alterations of PDLSC

The percentage of G1 phase cells in group A, B and C were 61.27%, 70.12% and 81.75% respectively, which increased significantly with increasing of donors' age ($F=53.077$, $P=0.000$). The percentage of cells in S+G2/M phase in group C was significantly lower than those of other two groups (Fig. 4). Trend test confirmed that the proliferation ability of PDLSC decreased with the increase of donors' age ($P=0.006$).

Cell apoptosis of PDLSC

Cell apoptosis analysis showed that the early apoptosis rate of group A, group B and group C were 0.46%, 1.18% and 2.31%, respectively, and the late apoptosis rate was 1.11%, 3.38%, 3.53%, respectively. Compared with the group A, PDLSC of group B and group C had higher apoptosis rates ($F=62.571$, $P=0.000$) (Fig. 5). Trend test confirmed that the overall apoptosis rate of PDLSC increased with increasing of age ($P=0.007$).

The osteogenic differentiation of PDLSC

After induction in osteoinductive medium for a week, the three experimental groups exhibited significant osteogenic differentiation potential. The gross and microscopic ob-

servation of PDLSC showed that all three experimental groups had stained cells, and those in group A showed the highest intensity and largest area of staining, while cells in group C showed the least intensity and the smallest area of staining. This result indicated that ALP staining decreased with the increase of donors' age (Fig. 6).

Genetic expression of ALP, col-1 and Runx-2

The results of real-time reverse polymerase chain reaction (RT-PCR) showed the expression of osteogenesis-related genes, ALP, col-2 and Runx-2 decreased gradually from group A to group C. The differences among groups were statistical significantly for ALP ($F=42.684$, $P=0.000$), col-1 ($F=31.354$, $P=0.01$) and Runx-2 ($F=30.794$, $P=0.01$) (Fig. 7). The group A had the highest genetic expressions compared to group B and group C. The differences between any two groups were also significantly (all $P<0.05$). Trend test confirmed that the expression of ALP ($P=0.007$), col-1 ($P=0.01$) and Runx-2 ($P=0.007$) decreased with the increase of age. These results suggest that osteogenic differentiation of PDLSC is age-dependent, and PDLSC from young donors show relatively stronger ability of differentiation than those from older donors.

Figure 5. Cell apoptosis of PDLSC in the experimental groups.

A. group A; B. group B; C. group C. The overall apoptosis rate increased with increasing of donors' age.

Figure 6. Inverted microscopic photographs of alkaline phosphatase (ALP) staining of PDLSC in the experimental groups. (Original magnification: $\times 10$).

A. group A; B. group B; C. group C. ALP staining decreased with the increase of donors' age.

Figure 7. Bar graphs of Real-time RT-PCR measurements for osteogenesis-related genes ALP, col-1 and Runx-2 in the experiment groups.

The values are ratios relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and fold changes are expressed as the mean relative quantity (RQ) $\pm$ square deviation (SD). * $P < 0.05$.

DISCUSSION

Aging, as an unavoidable physiological course of every kind of organism, is a major cause of many chronic diseases, characterized by gradual reduction in the function of organs in human body.⁸ Stem cells are generally in a balanced state in the body. With the increase of age, cell senescence can inhibit the proliferation of damaged cells. With accumulation of senescent cells in various tissues and organs, its secretions may damage the tissue structure and function, which damage the function of stem cells, and destroy the balance.⁹ In the process of aging, the self-regulating mechanism of stem cells changes, so that the biological characters become unstable, mainly manifest as the reduction of resistance and adaptation to changes in the internal and external environment, thereby inducing the disease and eventually causing death.^{10,11} There is a close relationship between aging and oral diseases. The anti-inflammatory, anti-infective, and healing abilities of the gingival tissues decrease during the aging process.

Periodontitis is a chronic inflammatory disease with a high incidence rate. It can cause injury of alveolar bone and soft tissue in periodontal tissue, which is the main cause of tooth loss. The treatment of periodontitis has been one of the most important and difficult issue in oral clinical researches. Whether the defected periodontal tissue could regenerate is the key to the treatment of periodontal diseases. The essential aspects for periodontal regeneration are to raise and activate sufficient amount of functional cells, to migrate them to the defected tissue area, and to ensure them to proliferate, differentiate, and reconstruct the periodontal tissue.¹² Stem cell is an important foundation to maintain tissue and organ regeneration. Oral tissue contains a variety of adult stem cells. Bone marrow mesenchymal stem cells and periodontal ligament stem cells are considered as ideal seed cells. Regenerative medicine research showed that, compared with bone

marrow mesenchymal stem cells, the periodontal ligament stem cells were more easily expanded in vitro, which reflects the advantage of strong cell reserve.¹³ In addition, studies found that periodontal ligament stem cells were a potential source of stem cell-mediated treatment of periodontitis.^{14,15} Periodontal tissue regeneration and repair are closely related to the heterogeneity of periodontal ligament stem cells. Periodontal ligament stem cells have the potential of multi-directional differentiation into bone, cartilage, muscle, tendon, and adipose tissue at specific conditions. When transplanted to the subcutaneous area of athymic mouse, cementum/PDL like tissues were observed.^{16,17} Animal experiments also suggested that periodontal ligament stem cells played an important role in the biological root regeneration and treatment of periodontal disease.¹⁷ A case report described that periodontitis was successfully treated by periodontal ligament stem cells.¹⁸ In addition, periodontal ligament stem cells can be obtained and isolated from the extracted teeth without much pain or discomfort of patients. The convenience in acquisition makes using PDLSC in the treatment of periodontitis less constrained by moral and ethical requirement. In this study, periodontal ligament stem cells collected from donors in different ages were used as the research subjects.

The microenvironment has a certain effect on the self-renewal and multi-differentiation ability of stem cells. A recent study found that the proliferation ability of bone marrow mesenchymal stem cells decreased with increases of age; the number of cell division, the proliferation and anti-apoptotic ability of mesenchymal stem cells derived from aged donors were less than that from young individual donors.¹⁹ In addition, studies have pointed out that after long-term culture *in vitro*, the ability of proliferation, differentiation, immune phenotype and gene expression of mesenchymal stem cells may change, even to a malignant transformation.^{20,21} Previous studies have reported that expression of genes that is related to cell replication, cell cycle and mitosis, specifically DNA damage repair de-

creased in aging cells.^{21,22}

Proliferation, aging and apoptosis are three basic life activities of cells, which reflect the different functions of cells. Therefore, the proliferation, senescence and apoptosis of the three groups of donors were examined in this study. Proliferation of periodontal ligament stem cells form/make up tissue structures; maintain their quantity *in vivo*; and accordingly achieve its self-renewal capacity. Primary culture cells in different age groups were purified by limited dilution, and the effects of aging factors on the biological characteristics were studied. The results showed that PDLSC in three different aged groups had a strong ability to proliferate, but the colony formation rate decreased as donors' age increased. Cell cycle is one of the important indicators of cell proliferation. Flow cytometry showed that the percentage of PDLSC in the S phase (synthetic status) decreased with age, while the percentage of PDLSC in the G0/G1 phase (quiescent status) increased with age. The decreased proportion of S phase PDLSC in aged group may be the consequence of cycle arrest, which slows down the cell development from G1 phase to S phase. This may be one of the reasons for the slow growth of PDLSC of the elderly donors. Our results further suggested that the proliferation of PDLSC decreased with the increase of age.

Apoptosis of cells is an active death process under certain physiological or pathological conditions in accordance with their own procedures. In this study, the physiological apoptosis of PDLSCs caused by aging was detected by flow cytometry. The results showed that the percentage of apoptotic cells increased with age. This result may be related to the aging microenvironment, where the number of stem cells and their regeneration ability decrease.^{10,11}

In addition, we examined the expression of alkaline phosphatase after a week of osteogenic induction and the expression of genes that may be involved in osteoblastic differentiation at the level of RNA. Positive alkaline phosphatase staining was detected in all three experimental groups, and expression levels of Runx-2, col-1 and ALP in the young group were significantly higher than those in the aged group ($P < 0.05$). Our result demonstrated that the PDLSC has strong proliferation and differentiation potential of osteogenesis. A study has shown that after transplanting sheets of PDLSCs with osteogenic induction on rat's back, cementum like tissue and periodontal ligament like tissue were observed,⁵ which indicated that PDLSCs, as seed cells, have promising role in the reconstruction and regeneration of periodontal tissues in the future.

Regarding differentiation ability of PDLSC, literatures have shown that human PDLSCs can differentiate into

Schwann cells. A study found that injecting human PDLSC into mental nerve injured rats promoted axonal regeneration and sensory function recovery,²³ which indicated that transplantation of PDLSC has a potential value in repairing mental nerve injury. In addition, scholars have demonstrated that primary human PDLSC could be directed to retinal progenitors with competence for photoreceptor differentiation, which is retinal progenitor cells of Pax6 (nuclear) Rx(+).²⁴ These results confirm that the PDLSC has strong proliferation and differentiation ability to a variety of tissues.

Consequently, stem cell therapy may not only play an important role in periodontal regeneration therapy, but also be very valuable in other aspects of treatments. The elderly may be the main population receiving the treatment of stem cell regeneration in the future, considering the high incidence of periodontal disease, as well as degradation of stem cells in other organs. Stem cell therapy may not only postpone aging, but also promote tissue reconstruction and regeneration of aging organs. Further researches are needed to discover its mechanism and to translate it into the clinical treatment for periodontal disease as well as other degenerated diseases.

Conflicts of Interest Statement

The authors have no conflicts of interest to disclose.

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