Original Article
Impairment of mesenchymal stem cells derived from oral leukoplakia

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Abstract: Oral leukoplakia is one of the common precancerous lesions in oral mucosa. To compare the biological characteristics and regenerative capacities of mesenchymal stem cells (MSCs) from oral leukoplakia (epithelial hyperplasia and dysplasia) and normal oral mucosa, MSCs were isolated by enzyme digestion. Then these cells were identified by the expression of MSC related markers, STRO-1, CD105 and CD90, with the absent for the hematopoietic stem cell marker CD34 by flow cytometric detection. The self-renewal ability of MSCs from oral leukoplakia was enhanced, while the multipotent differentiation was descended, compared with MSCs from normal oral mucosa. Fibrin gel was used as a carrier for MSCs transplanted into immunocompromised mice to detect their regenerative capacity. The regenerative capacities of MSCs from oral leukoplakia became impaired partly. Collagen IV (Col IV) and matrix metalloproteinases-9 (MMP-9) were selected to analyze the potential mechanism for the functional changes of MSCs from oral leukoplakia by immunochemical and western blot analysis. The expression of Col IV was decreased and that of MMP-9 was increased by MSCs with the progression of oral leukoplakia, especially in MSCs from epithelial dysplasia. The imbalance between regenerative and metabolic self-regulatory functions of MSCs from oral leukoplakia may be related to the progression of this premalignant disorder.

Keywords: Mesenchymal stem cells, oral leukoplakia, epithelial hyperplasia, epithelial dysplasia, impairment

Introduction

Oral mucosa, with superficial epithelium and underlying mesenchyme, functions as a barrier against exogenous substances and pathogens. Interactions of epithelial and mesenchymal stem/progenitor cells are crucial to the morphogenesis of oral mucosa [1]. Once epithelial hyperplasia occurs, disease such as oral leukoplakia (epithelial hyperplasia and dysplasia) may emerge [2]. It is a common precancerous lesion of the oral cavity, which is defined as "a white patch or plaque that cannot be characterized as any other disease" clinically or pathologically (WHO) [2, 3]. Epithelial dysplasia often invades into the underlying mesenchyme and develops into oral squamous cell carcinoma (OSCC), one of the most common oral malignance. With epithelial dysplasia invasion, the basement membrane (BM) is destroyed [4-6].

BM is a key structure for the attachment between epithelium and mesenchyme. It pro-

vides a barrier to epithelial cell migration and supports tissue integrity in oral mucosa. Collagen IV (Col IV) is the main structure of the BM. The disruptions of Col IV can affect the integrity of BM and lead to functional impairment [5, 7]. In addition, matrix metalloproteinases-9 (MMP-9), a peptidase enzyme responsible for the degradation of extracellular matrix, can contribute to the damage of BM [8]. In previous studies, Col IV discontinuities and MMP-9 positive cells increasing adjacent to fragmented basement membranes have been shown to be similar in epithelial dysplasia and cancer [4, 9, 10]. Therefore, Col IV and MMP-9 play important roles in the maintenance and destruction of BM in oral mucosa.

Much attention has often been paid to changes of epithelial cells or fragmented BM in oral leukoplakia. Although the interactions between epithelial and mesenchymal stem cells (MSCs) are pivotal for oral mucosa [1], few reports have been focused on MSCs and their functional

Table 1. Summary of Individual Patient Information

No.	Gender	Age	Clinical diagnostic	Pathological diagnosis
Patient 1	Female	43	Oral leukoplakia	Epithelial hyperplasia
Patient 2	Female	53	Oral leukoplakia	Epithelial hyperplasia
Patient 3	Male	51	Oral leukoplakia	Epithelial hyperplasia
Patient 4	Male	48	Oral leukoplakia	Epithelial hyperplasia
Patient 5	Female	46	Oral leukoplakia	Epithelial dysplasia
Patient 6	Female	53	Oral leukoplakia	Epithelial dysplasia
Patient 7	Male	47	Oral leukoplakia	Epithelial dysplasia
Patient 8	Male	58	Oral leukoplakia	Epithelial dysplasia

Ages were recorded at the time of biopsy (year). Diagnoses were made in accordance with clinical criteria and hisopathological criteria.

change in oral leukoplakia. A variety of MSCs have been reported in human adult organs [11, 12] and some lesional tissues, such as irreversible pulpitis [13]. However, at present no study has reported on MSCs derived from oral precancerous lesions. We hypothesized that MSCs may be present in the white patch of oral leukoplakia. Therefore, the present study was designed to isolate these MSCs from oral leukoplakia and characterize their self-renewal and multi-differentiation capacities. Here, we are also interested in their collagenous regenerative function. Therefore, we tried to explore MSC functional role on the maintenance and destruction of BM by detecting the expression of Col IV and MMP-9.

Material and methods

Tissue sample collection

Lesional tissues were biopsied from patients with oral leukoplakia for definitive diagnosis. Normal tissues were obtained from oral mucosa adjacent to the mucous cyst. Volunteers were recruited at the Department of Oral Medicine, Peking University School of Stomatology (Beijing, China). Clinical and pathological diagnoses were made according to the criteria for oral leukoplakia (epithelial hyperplasia and dysplasia) (WHO) [2, 3]. A summary of information on individual patients is shown in **Table 1**. The study was approved by the Peking University Biomedical Institutional Review Board (IRB00001052-12007). Each participant has signed informed consent. Tissues were fixed with 10% formalin for 24 h and embedded in paraffin for hematoxylin and eosin (H&E) staining.

Cell isolation and culture

Tissues were incubated with dispase (2 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) to strip off the epithelial and mesenchyme. Then the mesenchyme was minced into $1 \times 1 \times 1$ mm^3 fragments and digested in type I collagenase (3 mg/ml) and dispase (4 mg/ml) for 30 min-2 h at 37°C. The dissociated cell suspension was filtered through a cell strainer and plated on dishes containing α-modified Eagle's minimum essential medium (α-MEM; Gibco, Grand Island, NY, USA) and 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA). Cells at passages 2-4 were used in the experiments. MSCs from bone marrow (BMSCs) were selected as positive controls [14].

Cell proliferation assays

BMSCs as well as MSCs from oral mucosa and oral leukoplakia were seeded into 96-well plates at the density of 1×10^3/well. Proliferation of cells was assessed by Cell Counting Kit-8 (CCK8, Dojindo Laboratory, Japan) from day 1 to day 12. A_{450} value was determined and calculated using a plate reader (ELx808 Absorbance Microplate Reader, BioTek, USA) [14].

Colony-forming-unit assays

Single-cell suspensions (1×10^3 cells) were seeded in 10 cm dishes, incubated for 14 days and then fixed with 4% paraformaldehyde. Colonies were stained with 1 g/L toluidine blue (Sigma-Aldrich). A fibroblastic colony forming unit (CFU-F) was defined as a group of at least 50 cells. Colony forming efficiency (%) = colony number/number of seeded cells × 100% [15].

Flow cytometric analysis

More than 1×10^6 cells for each group were incubated with a STRO-1 antibody (R&D Systems, USA) or isotype-matched immunoglobulin control IgM (eBioscience, San Diego, CA, USA). Samples were then treated with a phycoerythrin (PE)-conjugated secondary antibody (R&D Systems). In addition, cells were treated with 20 μl of PE-conjugated human CD90, CD105 and FITC-conjugated human

CD34 (BD Biosciences Pharmingen, USA) or isotype-matched control IgG (Bioworld Technology, San Jose, CA, USA) for direct immunostaining. Thereafter, cells were washed and analyzed with a FACS Calibur Flow Cytometer (Becton Dickinson, Mountain View, CA, USA).

Multilineage differentiation in vitro

For osteogenic differentiation, MSCs were cultured in medium supplemented with osteogenic induction medium supplemented with 10 nM dexamethasone, 10 mM β-glycerophosphate, 0.1 mM $_L$-ascorbic acid-2-phosphate, 2 mM glutamine and 15% FBS. After 4 weeks, cells were fixed and evaluated by staining with 2% Alizarin Red S (Sigma-Aldrich) to detect calcium deposition by image analysis software (Image J, National Institutes of Health, Bethesda, MD, USA) [16]. Additionally, Alizarin-Red-S was quantified after elution from fixed cells with 10% cetylpyridinium chloride (Sigma-Aldrich), and the absorbance was measured at 570 nm using an ELx808 Absorbance Microplate Reader [17]. For adipogenic differentiation, MSCs were cultured in medium supplemented with adipogenic induction medium supplemented with 1 µM dexamethasone, 0.5 mM 3-isobutyl-l-methylxanthine, 60 mM indomethain, 10 mg/ml insulin, 2 mM glutamine and 15% FBS. After 21 days the cells were fixed, and lipid droplets were visualized by staining with 0.3% Oil Red O (Sigma-Aldrich). Then the quantification Oil Red O staining was then performed after absolute isopropanol treated by Microplate Reader at 490 nm [17, 18]. For neurogenic differentiation, MSCs were plated in 24-well plates and then induced with 100 µM CoCl$_2$ (Sigma-Aldrich) [19]. All groups were cultured for at least 3 days and fixed. They were incubated with primary antibodies, including rabbit polyclonal IgG for human neuron-specific enolase (NSE), followed by FITC-labeled secondary antibodies (Bioworld Technology, USA). Samples were observed using a confocal laser scanning microscope (LSM 5, Carl Zeiss, Oberkochen, Germany).

Quantitative real-time polymerase chain reaction

Total RNA was extracted using the TRIzol® reagent and cDNA was prepared by the RevertAid First Strand cDNA Synthesis Kit (Invitrogen Life Technologies, Grand Island, NY, USA). cDNA generated was used as a template for each PCR reaction using IQ SYBR Green and primers designed as follows: NSE forward, 5'-AGCCTCTACGGGCATCTATGA-3'; NSE reverse, 5'-TTCTCAGTCCCATCCAACTCC-3'; GAPDH (glyceraldehyde phosphate dehydrogenase, housekeeping gene) forward, 5'-AGCCTCTACGGGC-ATCTATGA-3'; GAPDH reverse: 5'-TTCTCAGTCC-CATCCAACTCC-3'. A relative quantitative analysis method was performed to quantify the gene expression compared with the level of GAPDH. PCR was performed using the 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA).

In vivo transplantation

To investigate the regenerative capacity, subcutaneous transplantation of MSCs from oral mucosa and oral leukoplakia was performed. All procedures were performed in accordance with the specifications of an approved animal protocol of the Health Science Center, Peking University (LA2012-13). Aggregates of approximate 2.0 × 10^6 MSCs with fibrin gel in each group were implanted into subcutaneous pockets of 6-week-old immunocompromised mice (CB-17/SCID; Vitalriver, Beijing, China). Meanwhile, pure fibrin gel with the same volume was transplanted into the opposite side of the same host as the control group. After 4 weeks of ectopic development, the transplants were harvested for H&E, immunochemical and Masson's trichrome staining studies [15, 20].

Histological and immunochemical analyses

For histological analysis, explants samples retrieved from the dorsal skin of immunocompromised mice were embedded in paraffin, sliced into 4-µm sections and stained with H&E. For immunofluorescent study, sections were treated with heated antigen retrieval solution and incubated overnight with mouse anti-human Col IV (1:200, Abcam, Cambridge, MA, USA), which reacts with human Col IV and demonstrates no cross reactivity with other species, followed by Rhodamine-conjugated secondary antibodies (Bioworld Technology). Then samples were observed under a confocal laser scanning microscope. For quantification of the percentage of positive cells, at least three fields were randomly captured in each experiment. Positive staining was measured quantitatively with image J software. For immunocytochemi-
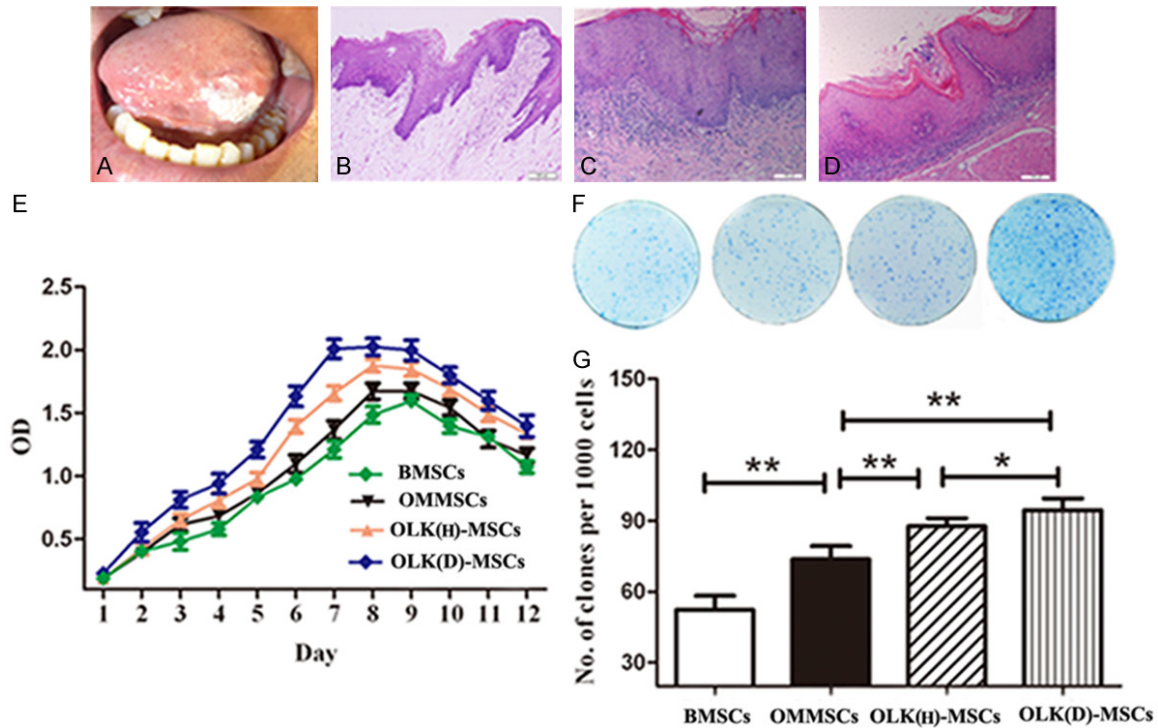
Figure 1. Clinical manifestations and histopathological features of oral leukoplakia. (A) Clinical manifestations of oral leukoplakia; Histopathologic features of normal oral mucosa (B), hyperplasia (C) and dysplasia (D) by H&E staining; (E) Proliferation capacity of BMSCs, OMMSCs, OLK(H) and OLK(D)-MSCs; (F) Macroscopic views of colonies of BMSCs, OMMSCs, OLK(H)-MSCs and OLK(D)-MSCs; (G) The calculated frequencies of colonies of BMSCs, OMMSCs, OLK(H)-MSCs and OLK(D)-MSCs. (n ≥ 3, *$P < 0.05$, **$P < 0.01$). Scale bar = 100 μm. Abbreviations: BMSCs: MSCs from bone marrow; OMMSCs: MSCs from oral mucosa; OLK(H)-MSCs: MSCs from hyperplasia; OLK(D)-MSCs: MSCs from dysplasia; H&E, hematoxylin and eosin.

cal analysis, cells were fixed with 4% polyoxymethylene and incubated with rabbit anti-human MMP-9 (1:100, Bioworld) overnight, followed by the Polymer Detection System (Zhongshan Goldenbridge Biotechnology, China), according to the manufacturer's instructions. Samples were visualized using 3,3-diaminobenzidine tetrahydrochloride (DAB, DAKO, Glostrup, Denmark) and counterstained with hematoxylin.

Western blot analysis

Samples were washed with PBS and harvested with RIPA buffer containing 1% proteinase and phosphatase inhibitors. Protein samples were separated by 10% SDS-PAGE and blocked in 5% nonfat milk. Thereafter, they were incubated with primary mouse anti-human Col IV (1:500, Abcam), rabbit antibodies to human MMP-9 (1:500) or tubulin-β (1:10,000, Bioworld Technology) at a dilution of 1:100 overnight at 4°C. Secondary antibodies (1:20,000) conju-

gated to horseradish peroxidase were then applied for 1 h at room temperature. The immunoblots were visualized and photographed using an enhanced chemiluminescence (ECL) detection and analysis system with Image Lab Software.

Statistical analysis

Statistical analysis was performed using a software SPSS 13.0. Data were expressed as the mean ± standard deviation (SD) at least three independent experiments. For analysis of three or more groups, data were analyzed by the analysis of variance (ANOVA). $P < 0.05$ was considered to be significant.

Results

Tissue sample collection

Clinical manifestation of oral leukoplakia is provided in **Figure 1A**, and histopathological images are shown in **Figure 1B** (normal oral muco-
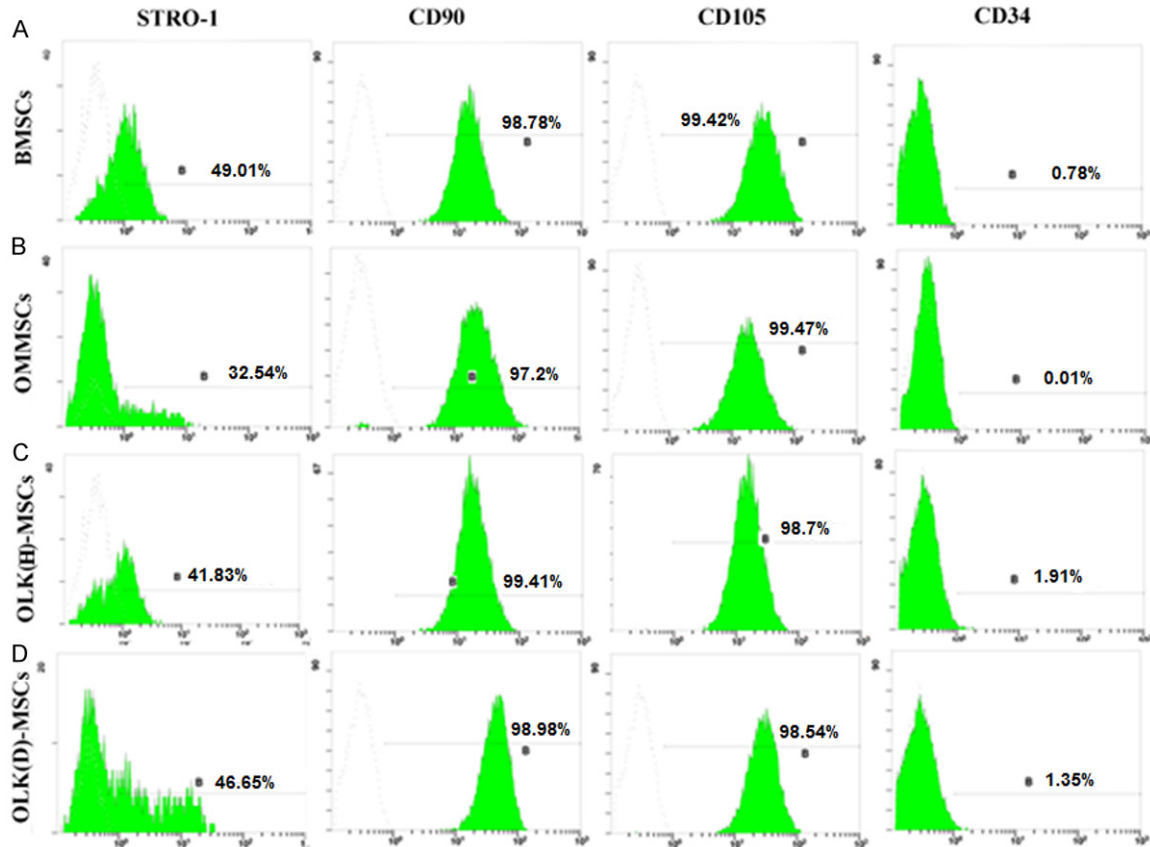
Figure 2. Identification of OMMSCs, OLK(H)-MSCs and OLK(D)-MSCs by flow cytometric analysis, compared to BMSCs. Expression of cell surface markers related to MSCs (STRO1, CD90 and CD105) or hematopoietic stem cells (CD34) in BMSCs (A), OMMSCs (B), OLK(H)-MSCs (C) and OLK(D)-MSCs (D). OMMSCs, OLK(H)-MSCs and OLK(D)-MSCs were positive for MSC related markers STRO1, CD90 and CD105, while they, as well as BMSCs, were negative for the hematopoietic stem cell marker CD34.

sa), **Figure 1C** (epithelial hyperplasia) and **Figure 1D** (epithelial dysplasia).

Cell proliferation assays

The proliferation of MSCs from oral leukoplakia was more rapid than that from oral mucosa. Between the different types of leukoplakia, MSCs from epithelial dysplasia grew more rapidly (**Figure 1E**).

Colony-forming-unit assays

Frequencies of colonies formed by BMSCs as well as MSCs from oral mucosa, epithelial hyperplasia and dysplasia were 52.33 ± 5.92∕1000 cells, 73.67 ± 5.65∕1000 cells, 87.67 ± 3.39∕1000 cells and 94.5 ± 4.89∕1000 cells, respectively. CFU formed by MSCs from oral mucosa was significantly lower in comparison to that by MSCs from epithelial hyperplasia ($P < 0.01$), while colony formation of MSCs from

epithelial dysplasia was the highest ($P < 0.05$). Macroscopic views of the colonies and their calculated frequencies are shown in **Figure 1F** and **1G**.

Flow cytometric analysis

MSCs from oral mucosa and oral leukoplakia were positive for MSC related markers STRO-1, CD90 and CD105, while they, as well as BMSCs, were negative for the hematopoietic stem cell marker CD34 (**Figure 2A-D**). STRO-1 expression in BMSCs, MSCs from oral mucosa, epithelial hyperplasia and dysplasia was 49.01%, 32.54%, 41.83% and 46.65%, respectively. Meanwhile, high expression levels of CD90 and CD105 (more than 90%) were observed in all groups.

Multilineage differentiation in vitro

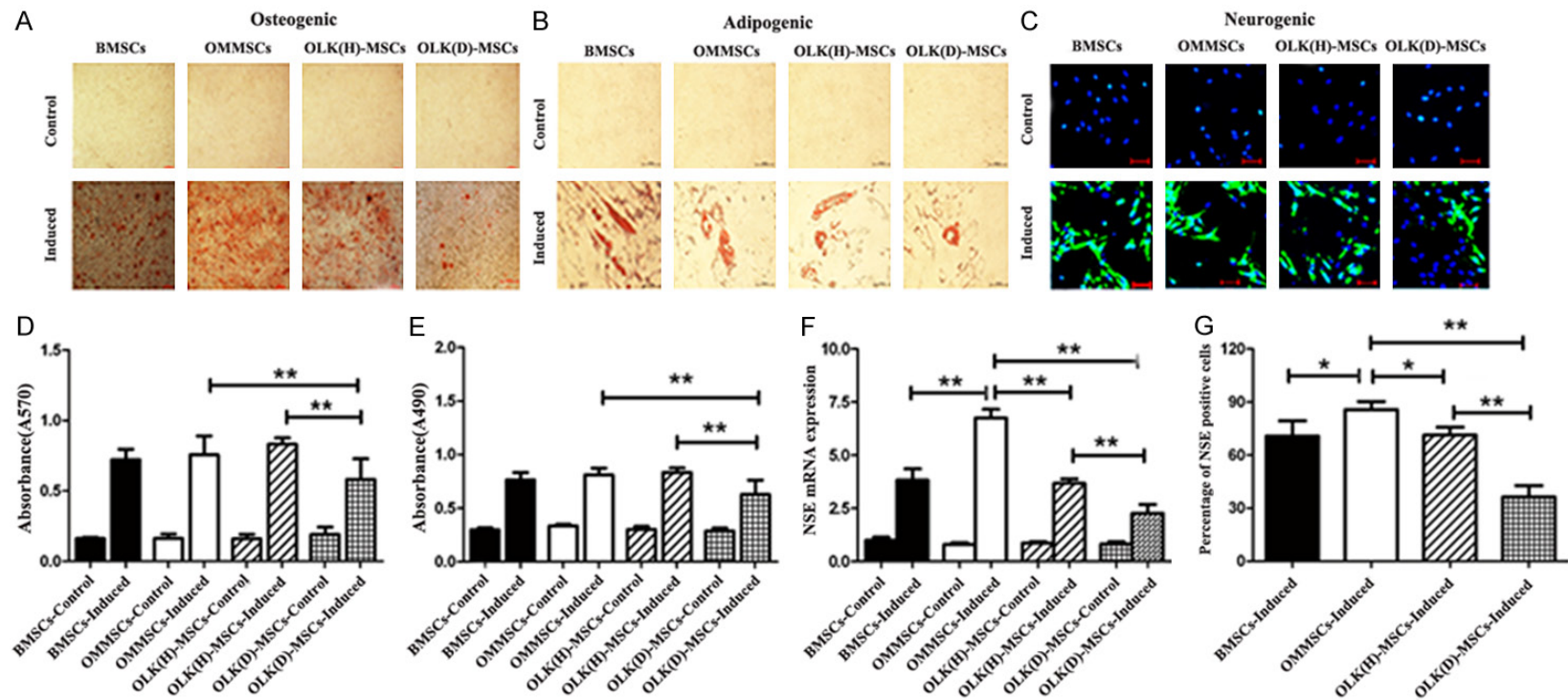MSCs from oral mucosa and oral leukoplakia maintain multi-differentiation capacities like

Figure 3. Osteogenic, adipogenic and neurogenic differentiation potential of BMSCs, OMMSCs, OLK(H)-MSCs and OLK(D)-MSCs and unstimulated controls *in vitro*. A. Unstimulated controls and mineralized nodules by Alizarin Red staining for osteogenic differentiation. B. Unstimulated controls and lipid droplets in cells by Oil Red O staining for adipogenic differentiation. C. Unstimulated controls and morphology of neuron-like changes by Immunofluorescent cell staining for neurogenic differentiation. D. Quantification of Alizarin Red S staining after osteogenic differentiation. E. Quantification of Oil Red O staining after adipogenic differentiation. F. Semiquantification of NSE-positive cells by real-time PCR after neurogenic differentiation. G. Quantification of induced MSCs stained for NSE by Immunofluorescence detection after neurogenic differentiation. (n ≥ 3, *P < 0.05, **P < 0.01). Scale bar = 500 µm in A. Scale bar = 50 µm in B and C.
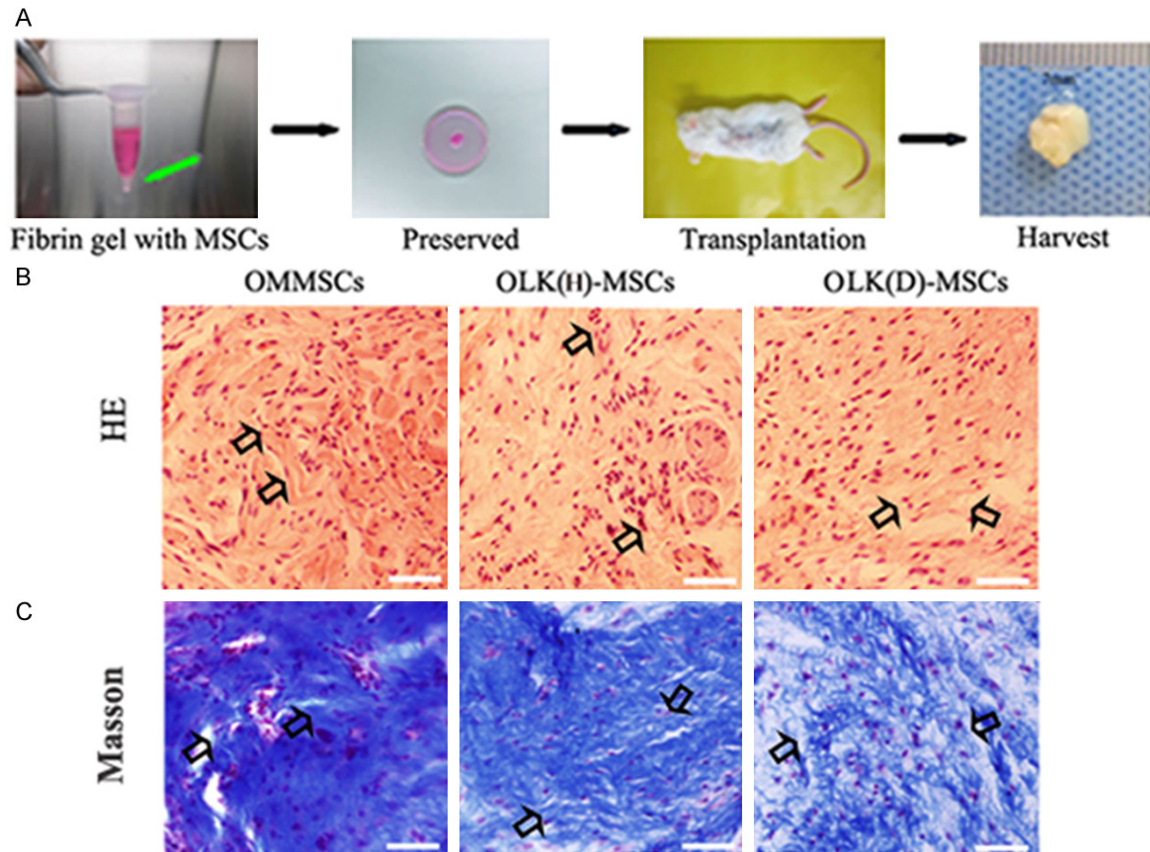
Figure 4. *In vivo* regenerative capability of OMMSCs, OLK(H)-MSCs and OLK(D)-MSCs. A. Schematic for serial transplantation strategy of MSCs. Then explants were retrieved from immunocompromised mice after implantation for 4 weeks. B. The substantial amount of collagen fibres (arrows) were shown in the explants formed by MSCs OMMSCs, OLK(H)-MSCs and OLK(D)-MSCs. C. Masson's trichrome staining showed blue collagen fibres formed by MSCs (arrows). Scale bar = 50 μm.

BMSCs. In osteogenic differentiation studies, there was no difference between induced MSCs from oral mucosa and epithelial hyperplasia by the detection of calcium depositions and quantification of Alizarin Red staining (**Figure 3A** and **3D**). Osteogenesis was decreased in MSCs from epithelial dysplasia (*P* < 0.01). For the adipogenic differentiation analysis, induced MSCs from oral mucosa and epithelial hyperplasia formed more lipid droplets (**Figure 3B** and **3E**) and higher absorbance of Oil Red O staining compared with the induced group of epithelial dysplasia (*P* < 0.01). After neurogenic differentiation, the morphology of MSCs from all induced groups presented neural changes. Expression of the NSE antigen was used to identify the formation of neuronal cells (**Figure 3C**). NSE expression of MSCs from epithelial hyperplasia and dysplasia became lower and lower gradually than MSCs from oral mucosa (**Figure 3F** and **3G**) by indirect immunofluorescence and real-time RT-PCR (*P* < 0.05).

Regenerative collagenous tissue in vivo

To explore MSC regenerative capacity, MSCs from oral mucosa, epithelial hyperplasia and dysplasia were transplanted subcutaneously with fibrin gel into immunocompromised mice (**Figure 4A**). The control group with fibrin gel alone was absorbed without any immunological rejection. Fibrin gels containing MSCs from three different groups regenerated mesenchymal tissue-like transplants, including the presence of fibroblast-like cells. Robust collagen fibers were formed by MSCs from oral mucosa. However, the regenerative capacity was down-regulated in MSCs from epithelial hyperplasia and dysplasia (**Figure 4B** and **4C**).

Immunocytochemical staining and Western blot analysis

Col IV was expressed by MSCs within fibrin gels of three groups (**Figure 5A-C**). The expression of Col IV showed an apparent decrease by
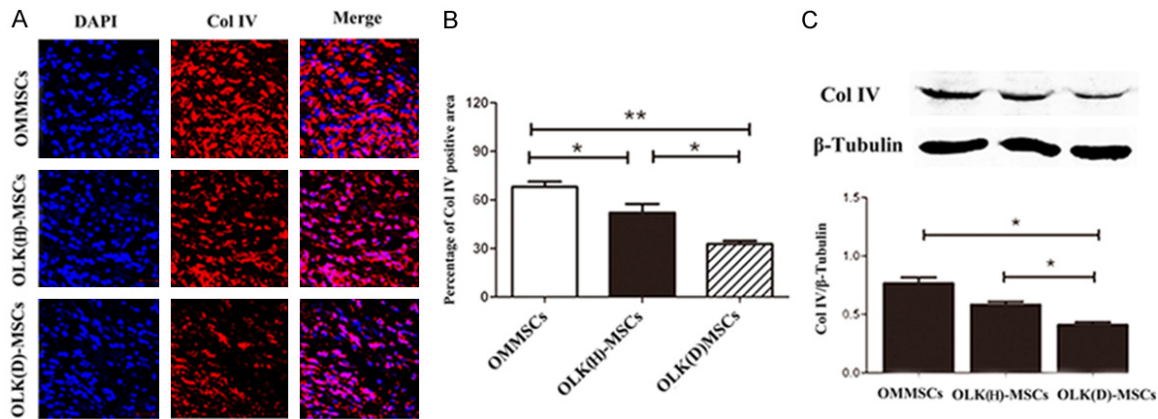
Figure 5. Expression and analysis of Col IV in MSCs. (A) Representative images of immunofluorescence staining of Col IV formed by OMMSCs, OLK(H)-MSCs and OLK(D)-MSCs. Quantification for the expression of Col IV of OMMSCs, OLK(H)-MSCs and OLK(D)-MSCs in fibrin gel by immunofluorescent cell staining (B) and Western blot (C). (n ≥ 3, *P< 0.05). Scale bar = 50 μm. Abbreviations: Col IV, type IV collagen.
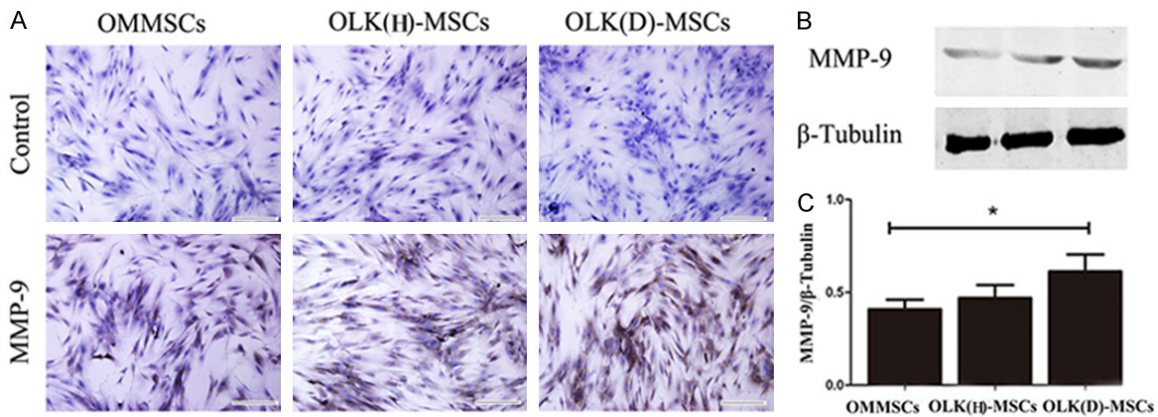

Figure 6. Expression and analysis of MMP-9 protein in OMMSCs, OLK(H)-MSCs and OLK(D)-MSCs by immunocyto-chemical staining (A) and Western blot analysis (B and C). Values are expressed as the mean ± SD. (n ≥ 3, *P < 0.05). Scale bar = 100 μm. Abbreviations: MMP-9, matrix metalloproteinases-9.

MSCs from oral mucosa, epithelial hyperplasia and dysplasia, corresponding to the progression of oral leukoplakia (*P* < 0.05). MMP-9 expression in MSCs from oral mucosa and epithelial hyperplasia were found to be significantly lower than that in epithelial dysplasia by immunocytochemical and western blot (*P* < 0.05) (**Figure 6A** and **6B**).

Discussion

Stem/progenitor cells play an important role in the generation of the epithelium by terminal differentiation and epithelial-mesenchymal interactions in oral mucosa [1, 21, 22]. As some cases of epithelial dysplasia undergo malignant transformation into oral squamous cell carci-

noma (OSCC) [2, 3, 23], much attention has been paid to the change of epithelial cells in oral leukoplakia at the histological and genetic level. Reports about the functional change of mesenchyme at the cellular level are not available. MSCs from inflamed human dental pulp and hyperplastic gingival have been reported to reflect the condition of diseases partly [13, 15], we presumed that characteristics of MSCs from oral leukoplakia may reflect changes of the mesenchyme and implicate the progression of this disease in some aspect.

Within past few years, MSCs have been isolated from various healthy nonhematopoietic organs and lesional tissues [11-13, 24, 25]. In our study, new populations of progenitor cells

from oral mucosa, epithelial hyperplasia and dysplasia were isolated and characterized. Phenotypic characteristics used to identify putative MSCs include markers, such as STRO-1, SH2 (CD105), SH4 (CD73), CD90, CD146 and CD29, and the typical lack of hematopoietic stem cell markers CD34 [26, 27]. The heterogeneity of STRO-1/CD90/CD105 positive and CD34 negative MSCs in all groups of our study implies that these cells represented as a stem-cell enriched population. Thus, these results offered definitive proof for the existence of stem/progenitor cells in oral mucosa and oral leukoplakia.

Self-renewal is one of the basic characteristics of stem cells [24, 28]. The proliferation rates and colony-forming efficiency of MSCs from oral mucosa, epithelial hyperplasia and dysplasia in our study were increased gradually, concurrent to the progression of oral leukoplakia. This phenomenon may be related to the epithelial-to-mesenchymal transition (EMT), the main morphogenetic event accounting for mesenchymal cell formation from epithelial cells [29]. During EMT, epithelial cells lose apical/basolateral polarity, sever intercellular adhesive junctions and become migratory [30]. The presence of epithelial dysplasia is generally accepted as one of the most important predictors of malignant development in premalignant lesions [3, 31], and EMT is fundamental to both development and the progression of epithelial dysplasia to tumors [30, 32]. MSCs from epithelial dysplasia may be related to epithelial cells invading into the mesenchyme during EMT, which may be also an early predictor for the progression of the disease. It might be helpful for us to understand the EMT-mediated development of malignancy at the cellular level. However, further studied are still required to confirm.

Stem cells also have the capacity for multipotent differentiation [26, 27]. In the current study, the osteogenic, adipogenic and neurogenic differentiation capacity was greatly decreased in MSCs from epithelial dysplasia, compared with MSCs from oral mucosa and epithelial hyperplasia. Interactions of stem/progenitor cells of epithelium and mesenchyme are crucial for the morphogenesis and regeneration of oral mucosa [33, 34]. Thus epithelial dysplasia may interfere the regulation of epithelial-mesenchymal interactions and then

interfere the function of MSCs indirectly. In addition, inflammation has been reported to reduce the differentiation capacity of MSCs from dental pulp and apical papilla [13, 35]. And high levels of inflammatory factors such as TNF-α, were reported in oral leukoplakia [36, 37], which may also contribute to the functional impairment of MSCs from epithelial dysplasia.

The identification of different MSCs expands the scope of potential clinical benefits of MSCs to help regenerate connective tissues such as dentin, cementum and periodontal ligaments [26, 38]. Biomaterial scaffolds may provide a conducive environment for oral mucosal regeneration [33, 34]. Fibrin gel is an assembled fiber with a three-dimensional structure, with demonstrated properties of natural and controllable degradation and low immunogenicity [15, 39]. It has been applied widely in operations, tissue engineering and regenerative medicine. To further examine the regenerative capacity of MSCs from oral mucosa and oral leukoplakia *in vivo*, they were transplanted subcutaneously with fibrin gel into immunocompromised mice. MSCs from oral mucosa maintained their regenerative capacity in fibrin gel and resembled like fibrous mesenchymal tissues in oral mucosa after subcutaneous transplantation. The results suggest that these MSCs, an easily obtained from tissues in the oral discarded tissues following surgical procedures, represent a unique population with potential implications in cell-based therapeutic applications for tissue engineering. However, this regenerative capacity was gradually down-regulated in MSCs from epithelial hyperplasia and epithelial dysplasia, implying the impairment of some MSCs from oral leukoplakia.

Basement membrane (BM), a key structure of oral mucosa, provides a barrier to epithelial cell migration and supports tissue integrity. To detect the potential mechanism for the regenerative change of MSCs from oral leukoplakia, the maintenance and destruction of BM was selected to study by detecting the expression of Col IV and MMP-9. On the one hand, Col IV is one of the main components of the basement membrane and an important constituent of the extracellular matrix. The reduction in quantity or dysfunction of Col IV may influence the physiological functions and the integrity of BM [5, 7]. BM maintains its integrity in normal mucosa and epithelial hyperplasia, discontinuities in

epithelial dysplasia and cancer in by staining patterns of Col IV [4, 9, 10]. On the other hand, MMP-9, the most abundant of MMP molecules, can degenerate collagen of types IV, V, VII, X and XI, fiber fibrinogen, glass laminin and entactin. MMP-9 can destroy BM and extracellular matrix by degrading Col IV, facilitate cells through the histological barrier into surrounding tissues and shape the local microenvironment. MMP-9 positive cells could pass through fragmented BM from tissues of dysplasia to carcinoma, where Col IV and MMP-9 are co-localized [4, 10]. Therefore, Col IV and MMP-9 play an important role in the maintenance and destruction of BM and were selected to study MSC functional change.

In our study, the Col IV generative capacity of MSCs from epithelial dysplasia was decreased apparently and MMP-9 expression in this group was increased significantly. In previous research, MSCs are equipped with motor proteins and a proteolytic arsenal which enables them to migrate throughout embryonic regions, they can also interact with and respond to signals from the extracellular matrix [24, 38, 40]. Whether the difference of MSCs is the result of an intracellular signaling disruption caused by epithelial dysplasia or extracellular changes in surrounding environments, including the local immune system, remains elusive and requires further mechanistic study.

In conclusion, our study demonstrates that MSCs can be isolated from oral leukoplakia. MSCs from epithelial dysplasia become partially impaired by the down-regulation of their differentiation and regenerative capacities. The expression of Col IV and MMP-9 of MSCs may be potentially important for the maintenance and destruction of the oral mucosal basement membrane. Thus, there may be a balance between the regenerative and metabolic self-regulatory functions of MSCs from oral mucosa. The disturbance of which may be related to the progression of oral leukoplakia, however, further in-depth studies on the related mechanism is still warranted.

Acknowledgements

Disclosure of conflict of interest

None.

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