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Mesenchymal stem cells participate in oral mucosa carcinogenesis by regulating T cell proliferation

Yichen Chen[a], Xi Wang[a], Juan Fang[a], Jingjing Song[a], Da Ma[a], Liqun Luo[b], Bailin He[b], Juan Xia[a], Vivian Wai Yan Lui[c], Bin Cheng[a,*,1], Zhi Wang[a,*,1]

[a] Guangdong Provincial Key Laboratory of Stomatology, Guanghua School of Stomatology, Stomatological Hospital, Sun Yat-Sen University, No. 56, Lingyuanxi Road, Guangzhou, CN 510000, China
[b] Zhongshan School of Medicine, Sun Yat-Sen University, No. 74, Zhongshan 2nd Road, Guangzhou, CN 510275, China
[c] School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong. Lo Kwee-Seong Integrated Biomedical Sciences Building, Area 39, CN 999077, Hong Kong Special Administrative Region

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ABSTRACT

Recent evidences suggested that Mesenchymal stem cells (MSCs) may be involved in tumor formation by modulating of the tumor microenvironment, but it is still unclear the potential of MSCs in the malignant transformation of oral mucosa. Using a chemically-induced oral carcinogenesis model by 4-nitroquinoline-1-oxide (4NQO), we generated precancerous lesions and cancerous lesions in the oral cavity of rats. Flow cytometric analysis on lesions derived single cell suspension revealed an increase in the proportion of MSCs and a decreased proportion of T cell during oral mucosa malignancy. Moreover, MSCs showed increased immunosuppression capacity on T cell proliferation during mucosa malignancy. At last, we demonstrated that higher frequency of lesions resident MSCs was correlated with more Ki67 expression in the lesion, which indicated higher cellular proliferative status in the lesions. Our study demonstrated that MSCs may play an important role in oral mucosa malignant transformation through regulating T cell proliferation.

1. Introduction

Oral squamous cell carcinoma (OSCC), with the world incidence of 3.5 per 100,000, is an aggressive head and neck cancer with a five year survival rate of ~50% [1]. The molecular biology of oral cancer formation is not fully understood. According to the statistics, many OSCC developed from oral pre-cancerous lesion (condition), which has a greater risk of transforming into malignancy [2,3]. Clinically, pre-cancerous lesions include various lesions and conditions like leukoplakia, erythroplakia, oral lichen planus etc. And histopathologically, pre-cancerous lesions were defined from atrophy to cancer in-situ in the oral epithelium. Although its clinical presentation and definition are well described [4,5], meaningful evidence-based malignant transformation potential features in pathophysiological insights is still difficult to draw from retrospective studies [6]. In recent years, several studies indicated that the immune cells play an important role in the pre-cancerous lesion malignancy [7], which may lead to future prevention of oral cancer formation in patients with pre-cancerous lesions.

As a stromal component, mesenchymal stem cells (MSCs) have been identified in many oral tissues including dental pulp, dental follicle, apical papilla, periodontal ligament and palatine tonsil [8–10]. MSCs from these perivascular niches, along with MSCs in the bone marrow, migrate to the sites of injury and inflammation like tumor site [11]. Recent evidences suggested that MSCs may be involved in tumor formation by modulating of the tumor microenvironment, including angiogenesis, lymph-angiogenesis, formation of tumor associated fibroblasts, as well as modulation of the immune system in the microenvironment [12]. But it is unclear the potential of MSCs in the malignant transformation of oral mucosa.

2. Materials and methods

2.1. Animals

Eighteen six-week-old female Sprague-Dawley rats (180–220 g) were purchased from the laboratory animal center of Sun Yat-sen University, and rats were kept in a specific pathogen-free animal facility. The chemically-induced model was established as previously

* Corresponding authors.
E-mail addresses: chengbin@mail.sysu.edu.cn (B. Cheng), wangzh75@mail.sysu.edu.cn (Z. Wang).
[1] Lead contact.

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described [13,14]. Briefly, twelve animals were given 0.05 g/L 4-nitroquinoline-1-oxide (4NQO) (TCI, Japan) in the drinking water for 22 weeks for the development of oral lesions, while six were untreated controls. All experimental protocols were performed in compliance with the Guidelines for Animal Experiments approved by the Institutional Animal Care and Use Committee (IACUC) at the Sun Yat-sen University.

2.2. Regents, antibodies and flow cytometry

The medium for T-cell culture was RPMI 1640 (Corning, USA), supplemented with 2 mM L-glutamine, 1% sodium pyruvate, 2×10^{-5}M 2-mercaptoethanol and 10% FBS (all from Thermo Fisher Scientific, USA). Fluorchrome-conjugated anti-rat CD29 (HMB1-1), CD31 (TLD-3A12), CD45 (OX-1), CD90 (HIS-51) and functional grade anti-CD3 (G4.18) antibodies were purchased from eBioscience (Affymetrix, USA). All the flow cytometric acquisition was performed using the BD FACSVerse (BD Bioscience, USA) and data were analyzed using FlowJo 10 (Tree Star, USA).

2.3. Generation MSCs from oral lesions

Single cell suspensions were obtained from lesions as previously described [15]. Briefly, oral lesions were carefully excised without surrounding tissue, digested with collagenase IV (500 U/mL, MP bio, USA) and Dnase (50 U/mL, Sigma, USA). After passed through a 70 μm cell strainer, cell suspensions were cultured for clone forming assay.

2.4. MSCs differentiation assay

Adipocytic differentiation was achieved in adipogenic medium containing 10% FBS DMEM, 1 μM dexamethasone, 0.5 μM IBMX, 10 μg/mL insulin, and 100 μM indomethacin. Two weeks later, cells were fixed and stained with 0.5% Oil Red O solution. Osteoblastic differentiation was achieved in osteoblastic medium containing 10% FBS DMEM, 1×10^{-7}M dexamethasone, 50 μg/mL ascobic acid, and 10 mM of β-glycerophosphate. Three weeks later, cells were fixed and stained with 2% Alizarin Red solution. Specific markers of osteoblasts and adipoblasts were analyzed by real-time quantitative PCR at day 7 after differentiation.

2.5. Histology and immunohistochemistry analysis

Formalin-fixed lesions were embedded in paraffin. Serial sections of 4-μm thickness were stained with H&E or immunostained with anti-CD3 mAb (SP7), anti-ki67 pAb, and isotype controls (eBioscience, USA). Positively-stained cells were pictured under a light microscope and cells in five representative areas were counted using ImageJ (NIH, USA).

2.6. Co-culture of splenocytes with MSCs

Different lesion derived MSCs (1×10^5) were seeded into a 24-well plate and incubated for 24 h. Immobilized anti-CD3 (1 μg/mL) pre-stimulated rat splenocytes (1×10^6) were directly loaded onto MSCs at a ratio of 1:10 and co-cultured for additional 48–96 h. Proliferated T-cells was detected by CFDA SE (CFSE, Life technologies, USA) and apoptotic T-cells were detected using the Annexin V / Pi kit (BD bioscience, USA), according to the manufacturer's instruction.

2.7. T-cell migration assays

A transwell system (Corning, USA) were used in this assay. MSCs (1×10^5) were seeded into the lower chamber and incubated for 24 h. The splenocytes labeled with CFSE and pre-stimulated for 48 h with anti-rat CD3 mAb were loaded into the upper chamber and cultured for another 48 h. Migrated CFSE labeled cells were observed under fluorescent microscope and cells in five representative areas were counted using ImageJ (NIH, USA).

2.8. qPCR tissue

RNA was extracted with RNAiso Plus (Takara) as manufacturer's instruction. And mRNA expression was analyzed by qPCR according to the manufacturer's instructions using the Lightcycler 480 sequence detection system (Roche, USA). Expression was normalized to the expression of GAPDH. Primers-probes were purchased from life technology as: CCL2 (F: 5′-gctgctactcattcactggc-3′; R: 5′-ggtgctgaagtcct-tagggt-3′); CCL3 (F: 5′-caccctctgttacctgctca-3′; R: 5′-atctgccggtttctcttggt-3′); CCL 21 (F: 5′-acaggcaaagagggagctag-3′; R: 5′-ctccttcttccttccctggg-3′); CXCL8 (F: 5′-acacactccacacctttcca-3′; R: 5′-acggatcttgtttctcagcc-3′); CXCL11 (F: 5′-gccctcaaattcgagctctg-3′; R: 5′-cttctgaatccaaccagcgg-3′); CXCL12 (F: 5′-ctttcactctccgtccacct-3′; R: 5′-gctctcaaagaatcggcagg-3′).

2.9 Statistical analysis An unpaired two-tailed Student's *t*-test and one-way ANOVA were used for statistical analyses with Prism 7.0a (Graphpad, USA). The correlation was evaluated on the basis of R^2. Results with a P value $< .05$ were considered statistically significant. Data are presented as mean ± SEM.

3. Results

3.1. Mesenchymal stem cells are enriched in carcinogen induced dysplasia and cancers

The previous report on the role of MSCs in head and neck cancer [16] prompted us to investigate the possible involvement of MSCs in the oral mucosa carcinogenesis progress. After 22 weeks of chemical induction with 0.05% 4NQO solution in drinking water, 100% of rats developed tongue lesions or lesions in the oral mucosa. Animals were sacrificed, and the lesions were carefully collected by excision. Part of the lesions were used for histological analysis to classify pre-cancerous lesions and OSCC. Our results showed that 50% of the animals displaying dysplastic change (n = 6), while the remaining 50% developed cancerous lesions (n = 6) (Fig. 1A). None of the control rats (no treatment with 4NQO solution) developed any observable oral lesions.

The remaining part of the lesions were gently digested into single cell suspensions for detection of MSCs. Four representative MSCs surface markers ($CD90^+$, $CD29^+$, $CD45^-$, $CD34^-$ [16,17]) were used for the identification of MSCs by flow cytometry (Fig. 1B). As shown in Fig. 1C–D, there were more MSCs in cancerous lesions (4.053 ± 0.506%, 446 ± 121 per mg tissue, n = 6) than that in dysplasia (2.503 ± 0.2712%, 230 ± 127 per mg tissue, n = 6). In addition, both the frequencies of MSCs in the cancer and dysplasia groups were significantly higher than that of the normal control group (0.4967 ± 0.1150%, 86 ± 37 per mg tissue, n = 6). To investigate the cause of increased MSCs in lesions, the proliferative capacity of MSCs in different environments were analyzed. As Fig. 1E shown, the frequency of $Ki67^+$ MSCs were not significantly changed among MSCs derived from different stages of malignant progress. Since it is possible that inflammatory and histological changes in the lesions would induce MSCs to migrate from bone marrow and perivascular niches to the lesion site, we evaluated the MSCs proportion in bone marrow. But there were no significant differences were observed between groups (Supplemental Fig. 1A). As it is known that inflammatory chemokines are involved in MSCs migration [18], we further evaluated the mRNA expression of several related chemokines. As shown in Fig. 1F, CCL21 and CXCL12 expression were significant elevated in dysplasia and cancer compared to normal control. Yet, there is no difference in the level of these chemokines between dysplasia and cancerous lesions.

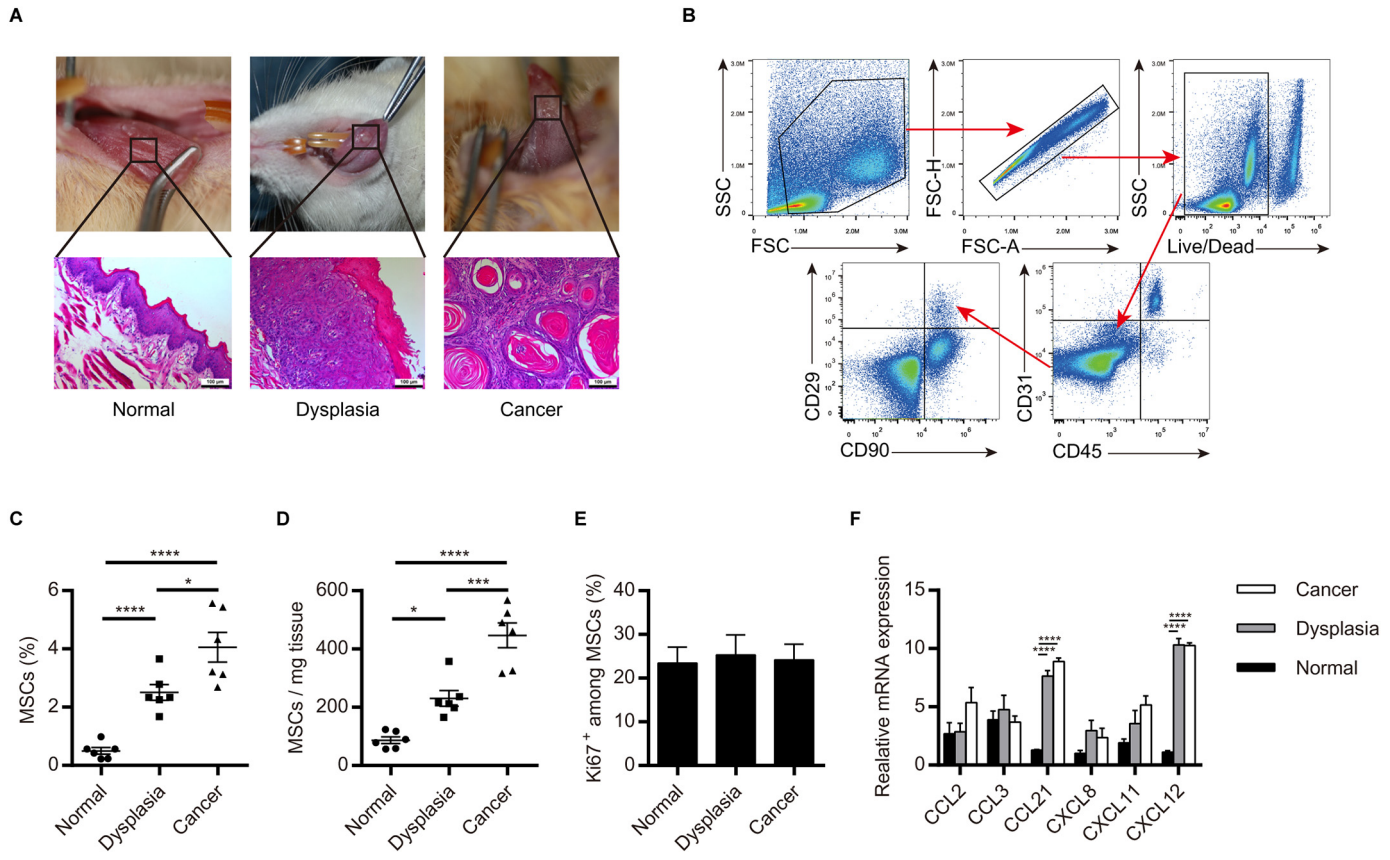

Fig. 1. Mesenchymal stem cells were enriched in carcinogen induced pre-cancerous lesions and OSCC. (A) Representative results showing the lesions on tongues of rats. The animals given 0.05 g/L 4NQO solutions for 22 weeks. Representative photographs of lesions and histopathology of lesions which indicated stained by Hematoxylin and Eosin to classified lesions as normal, dysplasia and cancer. (B) Representative FACS plot shows the gating strategy of MSCs in single cell suspension. (C–D) The frequency and number of MSCs in oral lesions were evaluated in single cell suspensions. (E) To detect proliferation capacity of MSCs in vivo. MSCs were analyzed on the basis of Ki67 expression. (F) Expression of mRNA for chemokines responded to MSCs in oral lesions and normal epithelium. Representative data were in separate two experiments are expressed as mean ± SEM. *p < .05, ***p < .001, ****p < .0001.

3.2. Different lesion-derived MSCs share the similar stemness properties

Previous study showed that normal oral tissue derived MSCs share the same stemness properties as the bone-marrow derived stem cells (BMSCs) [19]. In order to study if the microenvironment in the lesions may affect the MSCs' stemness properties, single cell suspensions were isolated from the oral lesions and cultured to obtain the MSCs (OMSC). BMSCs from each corresponding animals were also isolated as control. Over a period of 3 passages of cultures, MSCs were identified by the four MSCs-specific surface markers through immunofluorescence staining and flow cytometry (Supplemental Fig. 1B&C). Then, there were no observable gross morphological differences among normal mucosa-derived MSCs (N-OMSC), dysplasia-derived MSCS (D-OMSC), cancer-derived MSCs (C-OMSC), and the corresponding BMSCs (Fig. 2A & Supplemental Fig. 2A). Additionally, the OMSCs showed enhanced proliferative capacity compare to BMSCs in the early period (~ day 5) of cell culture, but eventually subsided as cultures continued (Fig. 2B & Supplemental Fig. 2B).

To further evaluated whether the stemness properties (e.g. clonal formation and differentiation capacity) of OMSCs changed compared with BMSCs. Both OMSCs and corresponding BMSCs were subjected to clonogenic assay and in vitro differentiation assays. As shown in Fig. 2C & Supplemental Fig. 2C, there were no significant clonogenicity differences among the N-OMSC, D-OMSC, C-OMSC, and the corresponding BMSCs. However, the BMSCs showed the highest osteogenic and adipogenic differentiation capacity among the corresponding N-OMSC, D-OMSC, and C-OMSC (Fig. 2D–E & Supplemental Fig. 2D–E). Transcriptional changes of adipogenic and osteogenic differentiation associated genes were examined by real-time quantitative PCR. Consistent with the staining of differentiated MSCs, at day 7, the mRNA expression levels of ALP, Runx2, FABP4 and PPAR-γ were higher in BMSCs than in the OMSCs groups (Fig. 2F & Supplemental Fig. 2F). These results indicated that the pathological change in the oral mucosa did not affect the stemness of resident MSCs.

3.3. Dysplasia lesions have more infiltrating T cells than cancerous lesions

Previous studies found that OSCC was associated with inflammation [20], and MSCs play a key role in immunoregulatory of T cells and B cells [21,22]. To investigate whether MSCs were associated with potential immune-modulation in chemically-induced dysplasia lesions or cancer lesions, we performed immunohistochemical staining of $CD3^+$ cells (T cells). As shown in Fig. 3A, immunohistochemical staining showed an apparently more CD3 positive cells in dysplasia near the epithelial basal cell layer compared to cancer lesions. Due to lack of infiltrating T cells and MSCs (data not shown), normal control mucosa was not included in the analysis.

Next, we assessed quantity of $CD3^+$ (T cells) and $CD45^+$ cells (leukocytes) in isolated single cell suspensions from these lesions. Flow cytometric analysis showed that infiltrating leukocytes ($CD45^+$ cells) and T cells ($CD3^+$ cells) proportion were decreased to 1/3 of dysplasia in cancer lesions (Fig. 3B–C). Through correlating the number of OMSCs (Fig. 1C–D) with that of lesion infiltrating $CD45^+$ and $CD3^+$, we found that MSCs enriched lesions may result in fewer T cell presence (Fig. 3D).

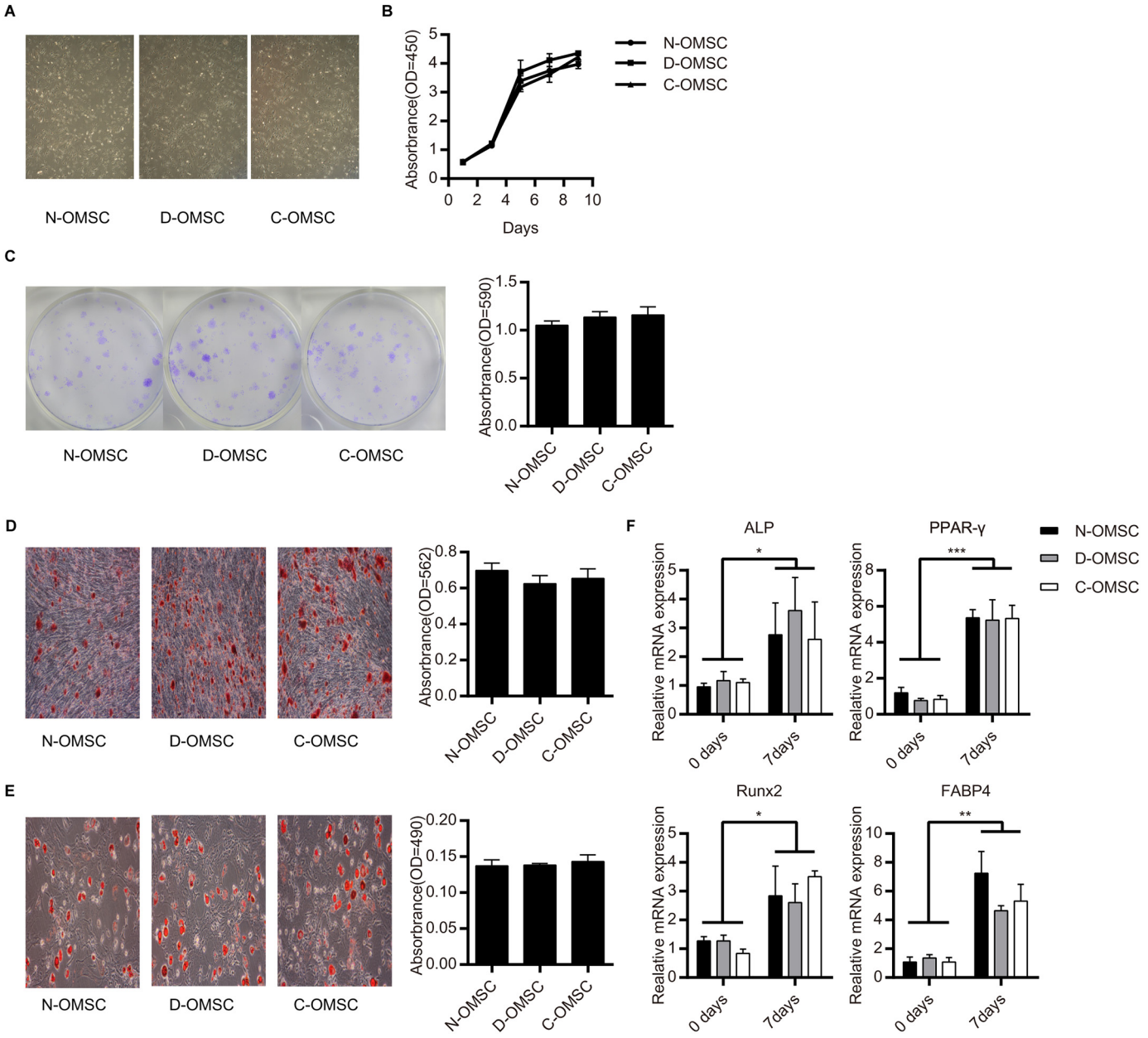

Fig. 2. Lesion derived OMSCs showed higher proliferative ability but lower differentiation capacity. (A) Morphology of cultured MSCs derived from normal mucosa (N-OMSC), dysplasia (D-OMSC) and cancer (C-OMSC). Representative images out of six per group were obtained by optic microscope with 40× magnification. (B) To detected proliferation capacity of OMSCs in vitro. The absorbance (OD = 450 nm) of cells were detected with CCK-8 assay at indicated times. (C) Representative plates for the MSCs that cultured in control medium. Total colony forming unit (CFU-F) were stained with crystal violet, staining was eluted and read at OD = 590 nm. (D-E) To determine differentiation capacity of MSCs, cells were cultured in osteogenic medium for 21 days then stained with Alizarin Red, staining was eluted and read at OD = 562 nm (D) or cultured in adipogenic medium for 14 days then stained with Oil Red O, staining was eluted and read at OD = 490 nm (E). (F) Expression of mRNA for osteoblast and adipoblast ALP, PPAR-γ, Runx2, FABP4 were evaluated at indicated time. Representative images of 5 random scenes were obtained by microscope with 400× magnification (E–F). Results pooled from three independent experiments are expressed as mean ± SEM. *p < .05, **p < .01, ***p < .001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Lesion derived MSCs inhibit T-cell proliferation but not migration

Since MSCs enriched lesions had less T cell infiltration, we reasoned that the immunosuppressive properties of lesion derived MSCs on T cells was enhanced with the mucosal malignancy. We thus assessed the proliferative capacity of $CD3^+$ T cells in different lesions. As a result, the proportion of Ki67-expressing $CD3^+$ T cells were decreased through malignant progress in vivo (Fig. 4A). Then, we co-cultured immobilized anti-CD3 pre-stimulated T cells with different lesion-derived MSCs in vitro. The optimal co-cultured MSC / T cell ratio was determined to be 1:10 (Supplemental Fig. 3). 96 h after co-culture, cancer-derived MSCs exhibited enhanced inhibition on $CD3^+$ T cells proliferation (~35.04 ± 1.493% of $CFSE^{low}$ cells) compare to MSCs from dysplasia (~65.25 ± 1.702% of $CFSE^{low}$ cells). However, MSCs from normal mucosa hardly inhibited T cells proliferation (~87.75 ± 1.931% of $CFSE^{low}$ cells) (Fig. 4B).

Previous studies demonstrated that MSCs suppressed T cells by promoting T cell apoptosis [21]. The total number of T cells with TCR activation were measured by Annexin V/Pi staining. 48 h after co-culture, ~26.8–40% of $CD3^+$ cells were apoptotic in the presence of N-OMSC, D-OMSC and C-OMSC (Fig. 4C), no significant variation of T cell apoptosis was founded between each group.

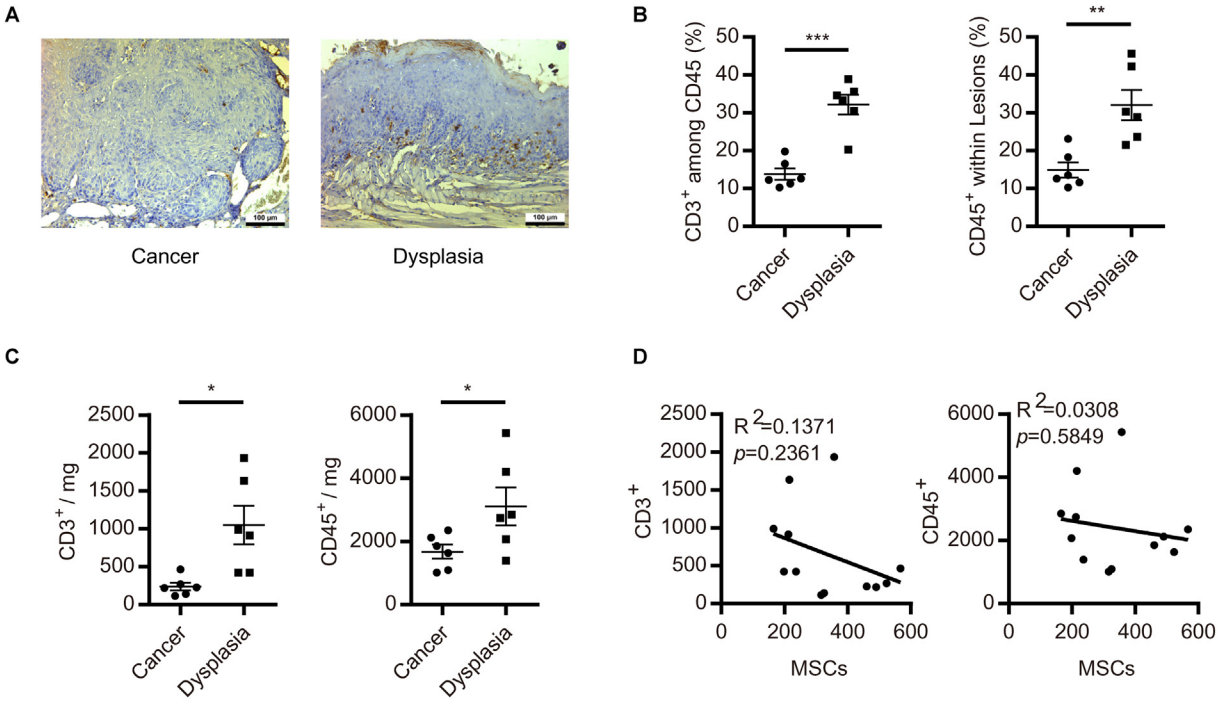

Fig. 3. More CD3 positive infiltrating lymphocytes were found in dysplasia but not in cancer. (A) Distribution of $CD3^+$ cells were examined by immunohistochemistry staining in dysplasia and cancers that classified by H&E staining in Fig. 1A. Representative images out of six per group were obtained by optical microscope with 200× magnification. (B–C) Frequency (B) and number (C) of $CD3^+$ population in $CD45^+$ lymphocytes and lesion digested single cell suspensions were analyzed by flow cytometry. (D) The number of MSCs was inversely correlated with the number of $CD3^+$ and $CD45^+$ population. Results pooled from three independent experiments are expressed as mean ± SEM. *p < .05, **p < .01, ***p < .001.

Apart from cell to cell contact that might influence the number of T cell, MSCs also attract activated T cells via production of chemokines [23]. To examine whether MSCs derived from different lessons have different chemotactic activities, we performed an in vitro transwell migration assay. As shown in Fig. 4D–E, the migration potential of T cells towards MSCs derived from different lesions was similar to that from the normal mucosa.

3.5. More lesion derived MSCs associates with higher cellular proliferation in the lesion

In order to investigate the role of lesion resident MSCs on the affected tissues, we determined the relationship between MSCs number and the expression levels of a widely used prognostic marker in both the pre-cancerous lesions and cancerous lesions [24]. Immunohistochemical staining showed that the cancer lesion harbored more Ki67-expressing cells than dysplasia (Fig. 5A). By measuring the frequency of MSCs in every lesion (Fig. 1C) and counting the average number of Ki67-positive cells in the corresponding tissue (Fig. 5A), we found that Ki67 staining was positively and highly correlated with the proportion of lesion infiltrating MSCs with an R^2 value of 0.6646 (Fig. 5B). Interestingly, the proportion of lesion infiltrating T cell was negatively correlated with the number of Ki67 positive cells in the lesions ($p = .0929$, Fig. 5C). Collectively, our results suggested that lesion resident MSCs were associated with cellular proliferative capacity in the lesions, which is a strong indicator of patient outcome.

4. Discussion

The oral cavity represents a tissue environment that is constantly exposed to internal and external chemical insults. The malignant transformation of oral mucosa was potentially influenced by chemicals such as tobacco, alcohol or microflora of the host. Here, we mimicked chemical carcinogenesis in the oral cavity of rats to examine the biological differences between pre-cancerous lesions and transformed cancerous lesions [25,26].

MSCs have been identified as important stromal components in tumor microenvironment, and shows dual roles in promoting and suppressing tumor growth. MSCs promotes tumor growth by regulating tumor-associated fibroblasts (TAFs) formation, immune response, angiogenesis, EMT, cancer stem cells, tumor metastasis and apoptosis [12,27]. On the other hand, MSCs also displays inhibitory function on tumor growth such as the induction of apoptosis, cell cycle arrest, inflammatory infiltrates (e.g. granulocytes and macrophage) and regulating oncogene and the associated signaling [28]. Endogenous MSCs can be recruited to the tumor site from bone marrow [29,30]. Here, we found that mesenchymal lineage cells ($CD90^+$, $CD29^+$, $CD31^-$, $CD45^-$) were significantly enriched in the lesions compared to normal mucosa (Fig. 1C–D), and increased chemokine production in the lesions (Fig. 1F). These results suggest lesion-derived MSCs play a potential role of in the oral mucosa malignancy.

Whether inflammatory cytokines promote MSCs' proliferation and multi-lineage differentiation is still unclear. Proinflammatory cytokines IL-1β, IL-6 and IL-23 treated MSCs show increased differentiation capacity [31]. On the contrary, cytokine TNF-α shows inhibition on osteogenesis of MSCs [32]. These conflicting findings could partly be explained by the differences of the cytokines investigated in different studies. In light of the apparent pathological changes during mucosa carcinogenesis and no variation of MSCs' differentiation capacity was found in this study (Fig. 2), the expression profiles of various inflammation cytokines should be evaluated to identify the related cytokines involved in the progress of malignant transformation in our future study.

MSCs are known to have unique immunoregulatory properties. It could influence T cells [33], B cells [22], Treg cells [34], dendritic cells [35] and natural killer cells [36]. T cells in oral premalignant leukoplakia have been treated as good prognosis [7]. Our results demonstrated that there were more $CD3^+$ cells in dysplasia lesions, and the

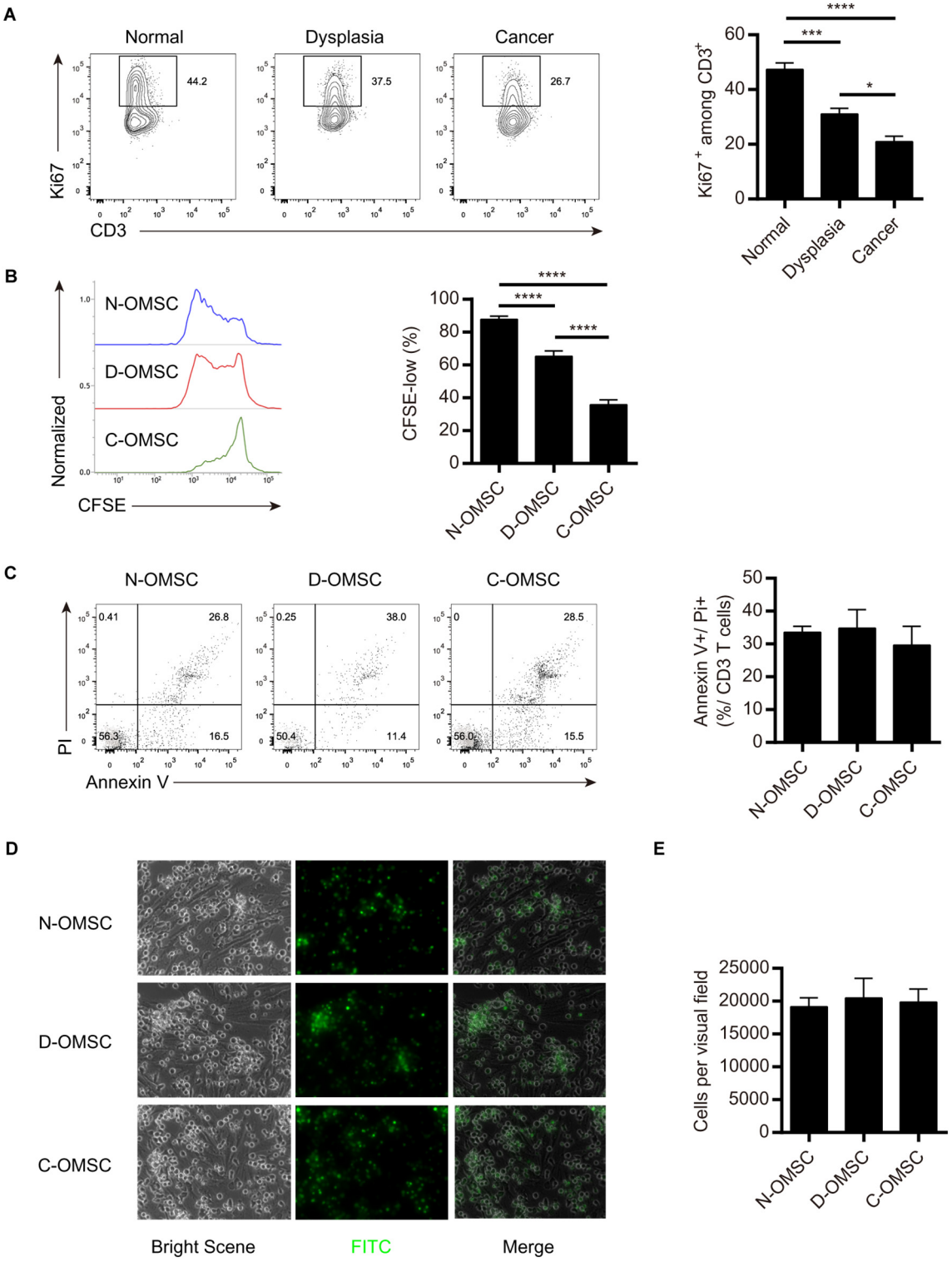

Fig. 4. Different lesions derived MSCs showed different immunomodulation capacity. (A) Lesion derived T cells population were harvested and identified by flow cytometry. Proliferation of $CD3^+$ T cells was analyzed on the basis of Ki67 expression. (B) Rat splenocytes were CFSE-labeled and stimulated with 1 μg/mL anti-rat CD3 for 48 h, then cells were harvested and directly co-cultured with different lesions derived MSCs as 1:10 for another 72 h. Cells were gated on live, singlet cells that CD3 positive, the percentage of $CFSE^{low}$ is shown. (C) Cells were prestimulated as previously, after co-cultured for 48 h, the apoptotic T cells were detected by staining with CD3 antibody, followed by Annexin V and Pi. (D–E) Labeled preactivated splenocytes (green) migrated to lesions derived MSCs were evaluated in a transwell system. Representative images of 5 random scenes were obtained by fluorescence microscope with 400× magnification (D). And migrated cells were counted (E). Data are representative of two separate experiments shown as mean ± SEM. *p < .05, ***p < .001, ****p < .0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

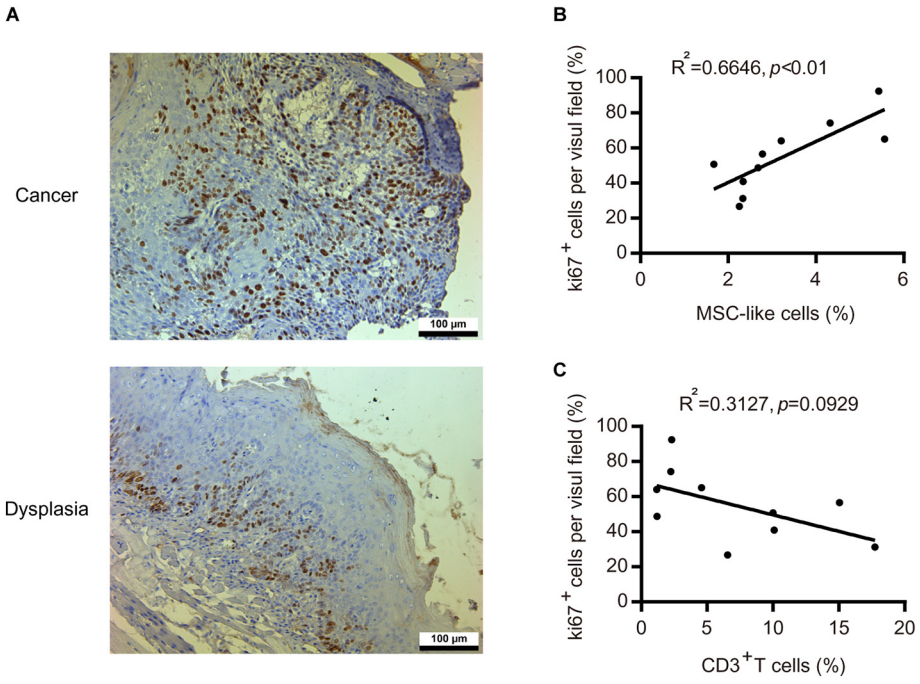

Fig. 5. MSCs was correlated with cellular proliferation in oral neoplastic lesions. (A) The expression of proliferative parameter Ki67 were evaluated by IHC staining. Representative images out of six of per group were obtained with 200× magnification. (B) The frequency of MSCs determined by flow cytometry in Fig. 1 was positively correlated w;ith the frequency of Ki67 expression. (C) The frequency of $CD3^+$ cells determined by flow cytometry in Fig. 3 was inversely correlated with the frequency of Ki67 expression. n = 10. R^2 and p values were indicated in panel.

number of $CD3^+$ cells were inversely related to MSCs (%) in the lesions (Fig. 3). Since it has been recognized that MSCs can modulate T cells through soluble factors and cell-cell contact [23], we co-cultured lesion-derived MSCs with splenocytes to evaluate the immunosuppressive function of MSCs. We found that MSCs only inhibited T cells proliferation but did not affect apoptosis nor migration. Our findings provide clues that molecular changes on MSCs, especially affecting T cell proliferation, in premalignant lesions are likely different from that of the cancerous lesions, more analysis should be conducted between these two types of lesions derived MSCs in the future.

To further investigate whether lesion resident MSCs served in malignant progress of oral mucosa, we correlated the frequency of resident MSCs and T cell with the expression of a valuable proliferative biomarker, Ki67. In accordance with others' work, the presence of more T cell appeared to be favorable (i.e. with less proliferation as indicated by less Ki67-positive cells in the lesion; Fig. 5C). In addition, lesion resident MSCs showed significantly positive correlation with cellular proliferation at the lesions which indicated by higher Ki67 positivity, a potentially adverse prognostic feature in pre-cancerous lesions and OSCC [24]. Thus, MSCs in the lesions could be explored as the potential contributor for the development of oral mucosa carcinogenesis.

In conclusion, our study demonstrated that MSCs migrated premalignant lesions prior to cancer establishment and the lesion microenvironments did not affect the stemness of MSCs. However, the immunomodulatory activities on T cell proliferation by MSCs would elevated along with lesion malignancy. Thus, the increased MSCs in the lesions provide a unique character to appraise oral lesion outcome and serve as a potential target in prevention of pre-cancerous lesion malignancy.

Conflict of interest

None declared.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2018.12.001.

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