Adipose-derived stem cells promote the proliferation, migration, and invasion of oral squamous cell carcinoma cells by activating the Wnt/planar cell polarity signaling pathway

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Background: Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral and maxillofacial region. Adipose-derived stem cells (ADSCs) interact with a variety of malignant tumors to promote their proliferation and metastasis. Abnormalities in Wnt/planar cell polarity (PCP) signaling and overactivation of the signaling pathway are considered to be related to the occurrence and development of various malignant tumors. In order to determine whether ADSC can promote tumorigenesis in OSCC and its molecular mechanism, we conducted a series of studies.

Methods: The effect of ADSCs on the occurrence and development of OSCC was studied in vivo and in vitro, and the molecular mechanism was investigated using Western blot and immunofluorescence (IHC) assays.

Results: The results revealed that ADSCs could promote the proliferation, invasion, and migration of OSCC cells in a dose- and time-dependent manner. With regard to the mechanism, the expression of collagen triple helix repeat-containing protein 1 (CTHRGC1) and phospho-c-Jun (p-c-Jun) increased significantly with enhancement of the interaction between ADSCs and OSCC cells, indicating that the Wnt/PCP signaling pathway was overactivated.

Conclusions: ADSCs promote the pathogenesis of OSCC by activating the Wnt/PCP signaling pathway, suggesting that proteins related to this pathway may be potential therapeutic targets for OSCC.

Keywords: Oral squamous cell carcinoma (OSCC); adipose-derived stem cells (ADSCs); Wnt/planar cell polarity pathway (Wnt/PCP pathway)

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Introduction

Head and neck cancer is the seventh most common cancer in the world, with oral squamous cell carcinoma (OSCC) accounting for more than 90% of cases (1-3). Tobacco (whether smoked or chewed), alcohol for drinking, and areca juice are potential carcinogens leading to OSCC. Surgery remains the first treatment of choice for almost all types of this disease. However, according to statistics, 30–50% of patients with OSCC develop lymph node metastasis, which is the most important factor affecting their prognosis (4). Aside from metastasizing to the lymph nodes, OSCC is also prone to being strongly invasive, having a high recurrence rate, and resulting in poor prognosis in most patients. Despite the progress made in elucidating the molecular and genetic mechanisms of OSCC, the 5-year survival rate of patients afflicted with this disease has not been significantly improved (5,6). Thus, the search for new drugs and therapeutic strategies to make up for the shortcomings of existing OSCC treatment methods is currently a hot research topic in the field of Stomatology.

Adipose tissue, a multifunctional organ, is composed mainly of mature adipocytes and adipose-derived mesenchymal stem cells/stromal vessels. The multipotent stem cells in metastatic adipose tissue have capabilities for promoting angiogenesis, antiapoptosis, and cell proliferation as well as multi-directional differentiation. Unfortunately, these regenerative properties are also thought to be associated with tumorigenesis and metastasis (7). For example, adipose-derived stem cells (ADSCs) have been reported to promote the growth, invasion, and migration of breast and ovarian cancer cells (7-9).

The Wnt/planar cell polarity (PCP) signaling pathway plays an important role in tumorigenesis and cancer development, with studies showing that the activation of this pathway is associated with a poor prognosis in patients with cancer (10). For example, the proliferation and metastasis of breast cancer cells were enhanced through the activation of Wnt/PCP signaling and the subsequent upregulated expression of proteins related to the pathway (11). Additionally, some researchers have found that the activation of this signaling pathway can increase the expression of collagen, type 1, alpha 1 (COL1A1), subsequently promoting the distant metastasis of colorectal cancer cells (12). Another study showed that collagen triple helix repeat-containing protein 1 (CTHRC1) mediated the distant metastasis of cervical cancer cells by activating the Wnt/PCP signaling pathway (13). As we know c-Jun is one of the downstream proteins of Wnt/PCP signaling pathway. It is a protein closely related to the occurrence and development of malignant tumors. Some studies have shown that the abnormal expression of c-Jun is related to the prognosis of OSCC patients (14). However, the relationship between the activation of this signaling pathway and the development of OSCC, and the interaction between ADSCs and Wnt/PCP signaling, have not been reported. In order to determine whether ADSCs can promote tumorigenesis in OSCC and its molecular mechanism, we conducted a series of studies.

At present, most studies on the interaction between ADSCs and malignant tumors are concentrated in the field of breast cancer and cervical cancer. In the interaction between ADSCs and head and neck squamous cell carcinoma, some scholars have suggested that ADSCs can accelerate the recurrence rate of HNSCC (15). Some scholars also suggest that ADSCs can promote the proliferation and migration of esophageal squamous cell carcinoma and tracheal squamous cell carcinoma (16). However, the interaction between ADSCs and OSCC and its mechanism are not clear. Therefore, in this study, we established a cell coculture system and nude mouse model to study the interaction between ADSCs and OSCC cells in vitro and in vivo and to explore the mechanism involved.

We present the following article in accordance with the ARRIVE reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-1637/rc).

Methods

Antibodies

Rabbit monoclonal phospho-c-Jun (p-c-Jun) antibody was purchased from Affinity Biosciences (Jiangsu, China; Cat. No. AF3095). Rabbit monoclonal CTHRC1 antibody was obtained from Boster Biological Technology (Pleasanton, CA, USA; Cat. No. A05203-1). Actin antibody was obtained from Wuhan Servicebio Technology Co., Ltd. (Wuhan, China; Cat. No. GB12001). Goat anti-rabbit HRP binding secondary antibody, goat anti-mouse HRP binding secondary antibody and goat anti-rat HRP binding secondary antibody was obtained from Wuhan Servicebio Technology Co., Ltd. (Wuhan, China; Cat. No. GB12001). Goat monoclonal HRP-conjugated antibody against GAPDH was obtained from Wuhan Servicebio Technology Co., Ltd. (Wuhan, China; Cat. No. GB12002). The supplier sources of the primary
and secondary antibodies were the same for both the Western blot and immunofluorescence (IHC) assays.

**Tissue specimens**

Tissue specimens of tongue squamous cell carcinoma were collected from patients at the Stomatological Hospital of Shandong University (Jinan, China). Informed consent was obtained from each patient upon their recruitment to the study, which was approved by the Institutional Research Ethics Committee of School of Stomatology, Shandong University (No. 20201002). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

**Cell culture**

The CAL 27 tongue cancer cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). CAL 27 is considered to be an experimental cell line that can replace OSCC. The identity of CAL 27 was verified by the analysis of short tandem repeats. After the verification, the cells were passaged approximately 6 times in our laboratory during a period of less than 6 months. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Hyclone, Logan, UT, USA) containing 10% (v/v) fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin at 37°C under 95% humidified air and 5% CO₂.

**Preparation of the ADSC conditioned medium (ADSC-CM)**

The human ADSCs (hADSCs) used in this study were purchased from Guangzhou SALIAI Stem Cell Science and Technology Co., Ltd. (Guangzhou, China). According to previous research, hADSCs can influence immune responses, and hence are key cell sources for tissue repair and regeneration Once the ADSCs had grown to 80% confluency (~5×10⁶ cells/10 cm well), the medium was removed and 10 mL of FBS-free DMEM was added to the cells for another 24 h. For the subsequent experiments, the culture medium was collected and centrifuged at 1,200 ×g for 12 min, and the supernatant was filtered through a 0.22 μm screen filter (Millipore, Billerica, MA, USA). To further investigate the function of the ADSC-CM, the prepared medium was diluted to 1:5 and 1:10 concentrations with phosphate-buffered saline (PBS).

**Cell proliferation test**

The Cell Counting Kit-8 (CCK-8) assay (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the proliferation of the OSCC cells every day. The growth curves of the following three groups of cells were measured: control group (CAL 27 cells without any treatment), experimental group 1 (CAL 27 cells treated with the 1:5 dilution of ADSC-CM), and experimental group 2 (CAL 27 cells treated with the 1:10 dilution of ADSC-CM) (17,18). In brief, each group of cells was seeded into 96-well plates at a density of 3,000 cells/well. Then, 10 μL of CCK-8 reagent was added to each well and the cells were cultured for 2 h. Then, colorimetric determination was performed by measuring the optical density of each well at a wavelength of 450 nm using a microplate reader.

**Wound healing test**

The various groups of cells (5×10⁵ cells/well) were seeded into 6-well plates and left to adhere to the bottom of the well. The monolayers were then scratched with the tip of a 200 μL pipette, following which the wells were washed twice with PBS to remove all floating cells. The cells in each well were then exposed to serum-free DMEM with or without ADSCs for 24 h. Cells were photographed at ×100 magnification under a phase-contrast microscope at each time-point. At the indicated time points (0, 6, 12, and 24 h), the cells were photographed under a phase contrast microscope at ×Plus 6.0 software.

**Transwell cell invasion assay**

CAL 27 cells (2×10⁴ cells/0.4 mL) were seeded into the upper chamber of a Transwell insert (aperture, 8 μm) containing Matrigel (Corning, New York, NY, USA) and exposed to FBS-free DMEM with or without 10 μM ADSC-CM (1:5 and 1:10). DMEM containing 10% FBS was placed in the lower chamber, and the culture environment is as described above. Thereafter, the cells were fixed with 4% formaldehyde for 15 min and stained with 0.1% crystal violet in 0.01 M PBS for 15 min. The number of cells penetrating the membrane was calculated according to a previously reported method (19), and images were taken under an optical microscope at a magnification of ×400. Images of the Transwell invasion of CAL 27 cells after different treatments were analyzed using Image-Pro.
Plus 6.0 software. The number of cells invaded was counted and expressed as the mean ± standard deviation (SD).

**Mouse model of OSCC xenotransplantation**

The 4-week-old BALB/c nude mice used in the study were purchased from Huafukang Biotechnology Co., (Beijing, China). Twenty female nude mice were equally divided into experimental group and control group. CAL 27 cells (1×10⁶ cells suspended in 200 μL of PBS) were subcutaneously injected into the peritoneum of one set of nude mice to establish the control group of the xenogeneic OSCC transplantation model. To construct the experimental groups, 1×10⁶ CAL 27 cells and 1×10⁵ ADSCs were suspended in 200 μL of PBS and injected into the peritoneal cavity of another set of nude mice. The tumor growth and volume (V) were monitored every week. The tumors were almost spherical, with a radius of L and V = 4/3πL³. The mice were sacrificed 30 days after cell injection, and the tumors were isolated, photographed, and weighed. Hematoxylin and eosin staining and IHC staining were used to analyze the pathological characteristics of the tumors. The activation of the Wnt/PCP signaling pathway was determined by detecting pathway-related proteins by Western blot assay, as described below (20).

**Western blot analysis**

The tumor cells were collected and lysed and their protein concentration was determined using the bicinchoninic acid assay. Equal amounts of protein were then isolated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the protein bands were then transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). After blocking the membrane with 5% fat-free dry milk in Tween-20 Tris-buffered saline (TBST) for 1 h, it was incubated with primary antibodies against p-c-Jun (1:500) and CTHRC1 (1:1,000) in 5% non-fat milk for 45 min at 37 °C. Thereafter, following a wash with TBST, and the membrane was after sealing with 5% fat free milk powder, at 4 °C, incubate with secondary antibody again in the dark for 1 hour with horseradish peroxidase (HRP)-conjugated secondary antibody (1:20,000). Finally, the protein bands were detected using a chemiluminescence substrate kit, captured and analyzed using Gel-Pro-Analyzer 4.0 software (Media Cybernetics, Inc.). By using gel Pro Analyzer 4.0 software to quantify the image bands of WB, measure the gray values of different bands, list the obtained gray values, and judge the amount of protein expression through histogram.

**IHC**

For the IHC analysis, cells seeded on 6-well glass slides for 24–48 h, with or without ADSC-CM stimulation, were fixed with 4% paraformaldehyde for 15 min at 25 °C and then washed with PBS. After blocking with normal goat serum, the cells were incubated overnight with the primary anti-CTHRC1 antibody and the primary anti-p-c-Jun antibody (1:5,000) at 4 °C. Then, the slides were washed and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG for 1 h at ambient temperature. Thereafter, the slides were washed again with PBS, stained with 4',6-diamidino-2-phenylindole, and finally examined under a fluorescence microscope.

**Statistical analysis**

All statistical analyses were performed using SPSS version 19.0 software (SPSS, Chicago, IL, USA). Student’s t-test or analysis of variance was used to compare group distributions. All results were expressed as the mean ± SD. A value of P<0.05 was considered statistically significant.

**Ethics approval and consent to participate**

Experiments were performed under a project license (No. 20201002) granted by Institutional Ethics Board of Stomatological Hospital of Shandong University, in compliance with Chinese national or institutional guidelines for the care and use of animals.

**Results**

**ADSCs promote the proliferation of OSCC and enhance its malignancy**

OSCC cells and ADSCs at the ratio of 10:1 were injected into the abdominal cavity of a group of nude mice, and the same amount of OSCC cells was injected into the abdominal cavity of another group of nude mice to respectively establish the experimental and control groups of the mouse model of OSCC. Compared with the tumor volumes from mice in the control group, those from the experimental group were significantly larger (P<0.05) (Figure 1A). CCK-8 was used to detect the proliferation of OSCC cells. As
shown in Figure 1B, the level of OSCC cell proliferation was significantly higher for the group treated with ADSC-CM than for the control OSCC cells, and increased with the increase in time and ADSC-CM concentration, indicating that ADSCs may promote the proliferation of OSCC (n>3; P<0.05). The tumor tissues in the mice were surgically removed, sectioned, and stained, whereupon it could be seen that the tumor cells in the experimental group had more obvious atypia, more instances of mitosis, and a higher degree of malignancy than the cells in the control group (Figure 1C).

**ADSCs promote the migration and invasion of OSCC cells**

Although ADSCs play an important role in the proliferation of OSCC, it is unclear whether they can promote the invasion and migration of the cancer cells. Thus, the scratch healing test and Transwell assay were used to verify the effect of ADSCs on the invasive and metastatic abilities of CAL 27 cells. At 0, 4, 12, and 24 h after establishment

**Figure 1** ADSCs promote the proliferation of OSCC and enhance its malignancy. (A) In the tumor formation experiment, the tumor volume of the experimental group was significantly larger than that of the control group. (B) The results of CCK-8 showed that ADSC-CM could promote the proliferation of tumor cells. (C) Because of the increase of nuclear division image of cancer cells, we believe that hematoxylin-eosin staining showed that ADSC-CM treatment could increase the malignant degree of OSCC (n>3; P<0.05). ADSCs, adipose-derived stem cells; OSCC, oral squamous cell carcinoma; ADSC-CM, ADSC conditioned medium; CCK-8, Cell Counting Kit-8.
of the scratch line, the scratch healing rate of the ADSC-CM-treated cells was significantly faster than that of the control cells and increased with the increase in ADSC-CM concentration (Figure 2A). The experimental group and the control group were treated with different concentrations of ADSC-CM. After 15 hours, the number of migrating cells in the lower chamber was observed. The experiment can show that the migration number of cancer cells increases significantly with the increase of ADSC-CM concentration. This means that ADSC-CM can promote the migration of OSCC cells, and this effect increases with the increase of ADSC-CM concentration (Figure 2B).

Therefore, compared with the blank control cells, the OSCC cells treated with ADSC-CM had significantly increased migratory and invasive abilities, which increased with the increase in ADSC-CM concentration. These results suggest that ADSCs may play an important role in the migration and invasion of OSCC cells.

**ADSCs promote the growth and development of OSCC cells by activating signal proteins through the Wnt/PCP pathway**

To determine whether Wnt/PCP signaling mediates the ADSC promotion of OSCC cell growth, invasion, and migration, we examined the effect of the ADSCs on Wnt/PCP activation by using the Western blot assay to detect the expression of CTHRC1 and p-c-Jun in CAL 27 cells cultured in ADSC-CM for 24 h. CTHRC1, a downstream protein of the Wnt/PCP signaling pathway, is highly related to the development of tumor cells, whereas p-c-Jun is a signal protein upstream of the pathway. It was observed that the expression of these two proteins had increased in the ADSC-CM-treated cells, indicating that the treatment had increased Wnt/PCP activation and thereby confirming that ADSCs activated this signaling pathway in OSCC (Figure 3A).

Additionally, IHC staining was used to further analyze...
Figure 3 ADSCs promote the growth and development of OSCC cells by activating signal proteins through the Wnt/PCP signaling pathway. (A) Y-axis represents the ratio of gray value of target protein to gray value of actin. Western blot analysis showed that the expression of CTHRC1 and p-c-Jun increased in a dose-dependent manner after ADSC-CM stimulation. The same conclusion can be drawn in the gray value analysis. (B) Stained with 4’,6-diamidino-2-phenylindole, the expression and subcellular localization of CTHRC1 and p-c-Jun were observed by fluorescence microscope. After ADSC-CM, translocation of CTHRC1 and p-c-Jun (green) from cytoplasm to nucleus was observed in CAL27 cells. It was also found that the expression of CTHRC1 and p-c-Jun increased after ADSC-CM treatment, as confirmed by Western blot analysis. Scale bar: 50 μm. *P<0.05; **P<0.01. ADSCs, adipose-derived stem cells; OSCC, oral squamous cell carcinoma; PCP, planar cell polarity; CTHRC1, collagen triple helix repeat-containing protein 1; p-c-Jun, phospho-c-Jun; ADSC-CM, ADSC conditioned medium.

The interaction between tumor cells and cells in the tumor microenvironment plays an important role in tumorigenesis. Oral and maxillofacial adipose tissues are rich in ADSCs. However, whether ADSCs have tumor-promoting or antitumor effects remains a subject of debate (21,22). Previous studies have shown that ADSCs can inhibit the apoptosis of ovarian cancer cells (23) and promote the metastasis and invasion of ovarian cancer (24). It has also been reported that ADSCs can promote the metastasis and invasion of hepatocellular carcinoma (25). In this study, we found that ADSCs could promote the growth and invasion of OSCC cells in vitro in both a dose- and time-dependent manner.

The activation of the Wnt/PCP signaling pathway is considered to play an important role in the occurrence and development of cancer (11). This signaling pathway and its downstream effectors have been found to be involved in the progression of ovarian, fallopian tube, cervical, and peritoneal high-grade serous carcinomas (26,27). Ours is the first study to find that ADSCs could promote the activation of the Wnt/PCP signaling pathway in OSCC.
cells in vitro, which promoted the proliferation and invasion of the disease.

In recent years, the applicability of ADSCs in plastic surgery has attracted much attention, and they are widely used in the filling of maxillofacial defects and treatment of scars (28,29). Some researchers have recently demonstrated the therapeutic effect of ADSCs on scleroderma (30). However, based on our study, the safety of ADSCs in the maxillofacial region remains to be clarified. Some maxillofacial tumors are occult, and the potential tumor-promoting effect of ADSCs may instead have adverse effects on the health of patients.

Our study had some limitations. For example, we did not verify whether ADSCs can promote the metastasis of OSCC cells in vivo. These limitations will be the focus of our future studies. Nonetheless, our study still has some enlightening implications for subsequent research, and we will follow up with studies exploring the safety of ADSCs for maxillofacial applications. We will also continue to explore whether ADSCs can promote the occurrence and development of OSCC by activating other signaling pathways.

Conclusions

In conclusion, ADSCs can significantly promote the proliferation, invasion, and migration of OSCC, where its tumor-promoting effect is mainly achieved through the activation of the Wnt/PCP signaling pathway to produce signal proteins. However, our understanding of how ADSCs affect the development and progression of OSCC is still limited. The cell-cell interaction between stromal cells and tumor cells may play an important role in tumor progression and should be the focus of further oncological research.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-1637/rc

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