科技部補助專題研究計畫成果報告
期末報告

幹細胞特性對於口腔黏膜下纖維化之角色探討及口腔黏膜下纖維化幹細胞之分選定性研究(第 3 年)

計 畫 類 別 ：個別型計畫
計 畫 編 號 ：NSC 100-2314-B-040-005-MY3
執 行 期 間 ：102 年 08 月 01 日至 103 年 07 月 31 日
執 行 單 位 ：中山醫學大學牙醫學系（所）

計 畫 主 持 人：張育超
共 同 主 持 人：余承佳
計畫參與人員：碩士級-專任助理人員：劉秀瑜

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中 華 民 國 103 年 10 月 15 日

中 文 摘 要 ： 口腔黏膜纖維化為一種口腔癌發生之癌前病變，主要與檳榔嚼食習慣相關，然而口腔黏膜纖維化症之致病分子機轉尚未清楚。幹細胞特性過度表現被認為對口腔癌腫瘤的進展、轉移、及復發扮演相當重要的角色。近年來，癌幹細胞特性的也存在對於皮膚良性腫瘤(keloid)中，因此本計畫目的欲了解幹細胞特性對於癌前病變口腔黏膜纖維化之影響，希望能對口腔黏膜纖維化症病理機轉了解有所助益。本計畫成果發現臨床口腔黏膜纖維化組織中 Stro-1 及 SSEA4 表現量較正常頰黏膜組織高。正常頰黏膜纖維母細胞在處理不同檳榔鹼處理下會增強幹細胞標記 Stro-1 及 SSEA4 的表現，初代培養之口腔黏膜纖維化細胞之 Stro-1 及 SSEA4 表現量較正常頰黏膜纖維母細胞高。利用 RNA 干擾技術抑制初代培養之口腔黏膜纖維化細胞其 Stro-1 及 SSEA4 的表現後，可降低收縮膠體能力。利用流氏細胞儀從初代培養之口腔黏膜纖維化細胞分選出 Stro-1+細胞之成骨能力、軟骨生成力、及脂肪生成力較 Stro-1-細胞強。本研究成果能了解幹細胞特性在口腔黏膜下纖維之致病機轉及訊號傳遞路徑，且希望利用幹細胞特性當作口腔黏膜下纖維化治療標靶並且作為之診斷標記。

中文關鍵詞： 口腔黏膜下纖維化； Stro-1； SSEA4

英 文 摘 要 ： Background: Oral submucous fibrosis (OSF) is regarded as a precancerous condition, and is a chronic insidious disease of oral subepithelial connective tissue. But, the pathologic mechanism(s) of OSF needs to be further clarified. Recently, the stemmess properties are also examined in benign tumor keloid and oral cancer. The purpose of this study is to investigate the stemness in the maintenance pathogenesis of OSF. Method: OSF tissues from areca quid chewers and normal buccal mucosa samples without areca quid chewing were analyzed by using immunohistochemistry for Stro-1 or SSEA4 expression in vivo. Expression of stemness marker Stro-1 and SSEA4 in buccal mucosa fibroblast cells (BMFs) with arecoline treatment was assessed by flow cytometry analysis. Collagen gel contraction capability in primary cultivated OSF cells (OSFs) with Stro-1 or SSEA4 knockdown was presented in vitro. Osteogenic/chondrogenic/adipogenic induction differentiation property in Stro-1+ and Stro-1- cells of OSFs was determined. Results: Clinically, the

expression Stro-1 and SSEA4 was significantly elevated in OSF specimens. We first observed that arecoline treatment significantly up-regulated the Stro-1 and SSEA4 positivity of BMF cells in a dose dependent manner. Compared with normal BMF cells, primary cultivated OSF cells (OSFs) displayed the higher Stro-1 positive (Stro-1+) and SSEA4 positive (SSEA4+) cells. Down-regulation of Stro-1 and SSEA4 by lentiviral infection significantly attenuated collagen gel contraction property in OSFs. MSCs properties including osteogenic/chondrogenic/adipogenic induction differentiation was increased expression in STRO-1+ OSFs. Conclusion: The enrichment of stemness property in BMF cells in vitro and in vivo induced by arecoline is critical for the development OSF.

英文關鍵詞： Oral submucous fibrosis； Stro-1； SSEA4

科技部補助專題研究計畫成果報告

（□期中進度報告/■期末報告）

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計畫主持人：張育超
共同主持人：蔡崇弘、余承佳
計畫參與人員：

本計畫除繳交成果報告外，另含下列出國報告，共 _1_ 份：
□執行國際合作與移地研究心得報告
■出席國際學術會議心得報告

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中　華　民　國 103 年 10 月 15 日

中文摘要

口腔黏膜纖維化為一種口腔癌發生之癌前病變，主要與檳榔嚼食習慣相關，然而口腔黏膜纖維化症之致病分子機轉尚未清楚。幹細胞特性過度表現被認為對口腔癌腫瘤的進展、轉移、及復發扮演相當重要的角色。近年來，癌幹細胞特性的也存在對於皮膚良性腫瘤(keloid)中，因此本計畫目的欲了解幹細胞特性對於癌前病變口腔黏膜纖維化之影響，希望能對口腔黏膜纖維化症病理機轉了解有所助益。本計畫成果發現臨床口腔黏膜纖維化組織中 Stro-1 及 SSEA4 表現量較正常頰黏膜組織高。正常頰黏膜纖維母細胞在處理不同檳榔鹼處理下會增強幹細胞標記 Stro-1 及 SSEA4 的表現，初代培養之口腔黏膜纖維化細胞之 Stro-1 及 SSEA4 表現量較正常頰黏膜纖維母細胞高。利用 RNA 干擾技術抑制初代培養之口腔黏膜纖維化細胞其 Stro-1 及 SSEA4 的表現後，可降低收縮膠體能力。利用流氏細胞儀從初代培養之口腔黏膜纖維化細胞分選出 Stro-1+細胞之成骨能力、軟骨生成力、及脂肪生成力較 Stro-1-細胞強。本研究成果能了解幹細胞特性在口腔黏膜下纖維之致病機轉及訊號傳遞路徑，且希望利用幹細胞特性當作口腔黏膜下纖維化治療標靶並且作為之診斷標記。

關鍵字：口腔黏膜纖維化; Stro-1; SSEA4

Abstract

Background: Oral submucous fibrosis (OSF) is regarded as a precancerous condition, and is a chronic insidious disease of oral subepithelial connective tissue. But, the pathologic mechanism(s) of OSF needs to be further clarified. Recently, the stemmess properties are also examined in benign tumor keloid and oral cancer. The purpose of this study is to investigate the stemness in the maintenance pathogenesis of OSF. **Method:** OSF tissues from areca quid chewers and normal buccal mucosa samples without areca quid chewing were analyzed by using immunohistochemistry for Stro-1 or SSEA4 expression *in vivo*. Expression of stemness marker Stro-1 and SSEA4 in buccal mucosa fibroblast cells (BMFs) with arecoline treatment was assessed by flow cytometry analysis. Collagen gel contraction capability in primary cultivated OSF cells (OSFs) with Stro-1 or SSEA4 knockdown was presented *in vitro*. Osteogenic/chondrogenic/adipogenic induction differentiation property in Stro-1+ and Stro-1- cells of OSFs was determined. **Results:** Clinically, the expression Stro-1 and SSEA4 was significantly elevated in OSF specimens. We first observed that arecoline treatment significantly up-regulated the Stro-1 and SSEA4 positivity of BMF cells in a dose dependent

manner. Compared with normal BMF cells, primary cultivated OSF cells (OSFs) displayed the higher Stro-1 positive (Stro-1+) and SSEA4 positive (SSEA4+) cells. Down-regulation of Stro-1 and SSEA4 by lentiviral infection significantly attenuated collagen gel contraction property in OSFs. MSCs properties including osteogenic/chondrogenic/adipogenic induction differentiation was increased expression in STRO-1+ OSFs. **Conclusion:** The enrichment of stemness property in BMF cells in vitro and in vivo induced by arecoline is critical for the development OSF.

Key words: Oral submucous fibrosis; Stro-1; SSEA4

Introduction

Oral submucous fibrosis (OSF) is regarded as a precancerous condition, and is a chronic insidious disease of oral subepithelial connective tissue resulting in stiffness of the oral mucosa and inability to open the mouth. OSF is characterized by epithelial atrophy and progressive accumulation of collagen fibers in the lamina propria and submucosa of the oral mucosa. The fibro-elastic changes are almost entirely due to abnormal accumulation of collagen in the subepithelial layers, resulting in dense fibrous bands in the mouth. A number of epidemiological surveys, case-series reports, large sized cross sectional surveys, case-control studies, cohort and intervention studies provide over whelming evidence that areca nut is the main etiological factor for OSF.

The role of the constituents of areca nut in the pathogenesis of OSF has been studied in detail over the last two decades. It is apparent that fibrosis and hyalinization of sub epithelial tissues account for most of the clinical features encountered in this condition. Moreover, substantial amount of research on elucidating the etiology and pathogenesis appear to have been focused on changes in the extracellular matrix (ECM). It is logical to hypothesize that the increased collagen synthesis or reduced collagen degradation as possible mechanisms in the development of the disease. There are numerous biological pathways involved in the above processes and, it is likely that the normal regulatory mechanisms are either down regulated or up regulated at different stages of the diseases.

Fibroblasts obtained from OSF subjects revealed a higher elevation for collagen synthesis than normal buccal mucosa fibroblasts (BMFs). Stabilization of collagen and prevention of collagenase degradation in oral mucosa and the attendant increase of lysyl oxidase activity lso contribute to abnormal deposition of collagen

fiber in OSF. Arecoline, a major areca nut alkaloid, was found to stimulate human BMF proliferation and collagen synthesis *in vitro*. Recently, our studies have shown that up-regulation of vimentin, cyclooxygenase-2, tissue inhibitor metalloproteinase-1, plasminogen activator inhibitor-1, interleukin-6, keratinocyte growth factor-1, insulin-like growth factor-1, nuclear factor-kappa B, cystatin, and heat shock protein 47, and heme oxygenase-1, may contribute to the extracellular components accumulation in OSF.

A crucial question in cancer biology is how tumor growth is initiated and propagated at the cellular level. Despite being monoclonal in origin, most tumors appear to contain a heterogeneous population of cancer cells. Recently, a hypothesis proposed by many investigators is a model in which every tumor contains a rare population of cells termed cancer stem cells (CSCs). The concept of CSCs first began 150 years ago when Robert Virchow, a German pathologist, observed similarities between embryonic and tumor tissues. CSCs have been demonstrated to have capacities of promoting tumor growth, tumor regeneration, metastatic progression and contributing to radio-resistance and chemo-resistance of oral cancer. Recently, the CSCs properties also examined in keloid (an exuberant fibroproliferative dermal growth unique to human skin). However, the role of stemness properties in precancerous OSF has never been determined. Based on these findings, it is worthy to investigate the importance of stemness in the maintenance of OSF pathogenesis.

Materials and Methods

OSF patient subjects and immunohistochemistry

This research follows the tenets of the Declaration of Helsinki and all samples were obtained after informed consent from the patients. OSF patients' tissue samples with different stages of oral cancer were spotted on glass slides for immunohistochemical stainings. After deparaffinization and rehydration, the tissue sections were processed with antigen retrieval by1X Trilogy diluted in H_2O (Biogenics) and heat. The slides were immersed in 3% H_2O_2 for 10 minutes and washed with PBS 3 times. The tissue sections were then blocked with serum (Vestastain Elite ABC kit, Vector Laboratories, Burlingame, CA) for 30 minutes, and followed by incubating with the primary antibody and anti-Stro-1 or anti-SSE4 in PBS solution at room temperature for 2 hours in a container. Tissue slides were washed with PBS and incubated with biotin-labeled secondary antibody for 30 minutes and then incubated with streptavidin-horse radish peroxidase conjugates for 30 minutes and washed with PBS 3 times. Afterwards,

the tissue sections were immersed with chromogen 3-3'-diaminobenzidine plus H_2O_2 substrate solution (Vector® DBA/Ni substrate kit, SK-4100, Vector Laboratories, Burlingame, CA) for 10 minutes. Hematoxylin was applied for counter-staining (Sigma Chemical Co., USA). Finally, the tumor sections were mounted with a cover slide with Gurr® (BDH Laboratory Supplies, U.K.) and examined under a microscope. Pathologists scoring the immunohistochemistry were blinded to the clinical data. The interpretation was done in five high-power views for each slide, and 100 cells per view were counted for analysis.

Cell cultivation of BMFs

Two healthy individuals were selected from the crown lengthening procedure for this study. The normal buccal mucosa tissue samples were minced using sterile techniques and washed twice in PBS supplemented with antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml of fungizone). Explants were placed into 60 mm Petri dishes and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10 % fetal calf serum (FCS) (Gibco Laboratories, Grand Island, NY, USA) and antibiotics as described above. Cell cultures between the third and eighth passages were used in this study.

Stro-1 and SSEA4 knockdown in OSFs by Lentiviral-mediated shRNAi

The pLV-RNAi vector was purchased from Biosettia Inc. (Biosettia, San Diego, CA, USA). The method of cloning the double-stranded shRNA sequence is described in the manufacturer's protocol. Lentiviral vectors expressing short hairpin RNA (shRNA) that targets human Stro-1 or SSEA4 were synthesized and cloned into pLVRNAi to generate a lentiviral expression vector. Lentivirus production was performed by transfection of plasmid DNA mixture with lentivector plus helper plasmids (VSVG and Gag-Pol) into 293T cells using Lipofectamine 2000 (Invitrogen, Calsbad, CA, USA). Supernatants were collected 48 h after transfection and then were filtered; the viral titers were then determined by FACS at 48 h post-transduction. Subconfluent cells were infected with lentivirus in the presence of 8 μg/ml polybrene (Sigma-Aldrich, St. Louis, Missouri, USA).

Collagen gel contraction assays

The bioactivity of myofibroblast function was performed by collagen contraction assay kit (Cell BioLabs, Inc., San Diego, CA, USA). 2×10^5 cells/ml was mixed with cold collagen solution at ratio of 1:4. Cell/collagen mixture was loaded into 24-well-plate as 0.5 ml/well and covered with 1 ml of cell culture medium after polymerization of collagen. To initiate contraction, collagen gels were gently released from the sides of the culture dishes with a sterile spatula. The changes of collagen gel size (contraction index) were pictured at various times and quantified by IamgeJ software.

In vitro osteogenic differentiation

Cells were cultured in DMEM-LG (Invitrogen) supplemented with 15% FBS, 50 μg/mL ascorbate-2-phosphate, 10 nmol/L dexamethasone, and 10 mmol/L β-glycerophosphate (Sigma, St. Louis, MO) for 2 weeks. At the end of osteogenic induction, cells were washed twice with PBS, fixed for 10 min at room temperature with 3.7% paraformaldehyde, and stained with Alizarin red to assess osteogenic differentiation.

In vitro chondrogenic differentiation

Cells were cultured in DMEM High Glucose supplemented with ITS + Premix: 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 5.33 μg/ml linoleic acid, 1.25 μg/ml bovine serum albumin, 10^{-7} M dexamethasone, 50 μg/ml ascorbate-2 phosphate, 1 μM sodium pyruvate, 100 μg/mL penicillin and 10 μg/mL streptomycin) (Sigma, Saint Louis, MO, USA) in the presence of TGF-β1 (10 ng/ml) (Mylteny, Bergisch, Gladbach, Germany) for 2 weeks. At the end of chondrogenic induction, cells were washed twice with PBS, fixed for 10 min at room temperature with 3.7% paraformaldehyde, and stained with Alcian Blue to assess chondrogenic differentiation.

In vitro adipogenic differentiation

Cells were cultured in α-MEM supplemented with 16.6% FBS, 50 μg/mL ascorbate-2 phosphate, 10^{-7} M dexamethasone, 50 μM indomethacin, 0.45 mM 3-isobutyl-1-methyl-xanthine and 10μg/mL insulin for 2 weeks. At the end of adipogenic induction, cells were washed twice with PBS, fixed for 10 min at room temperature with 3.7% paraformaldehyde, and stained with Oil Red O to assess adipogenic differentiation.

Statistical analysis

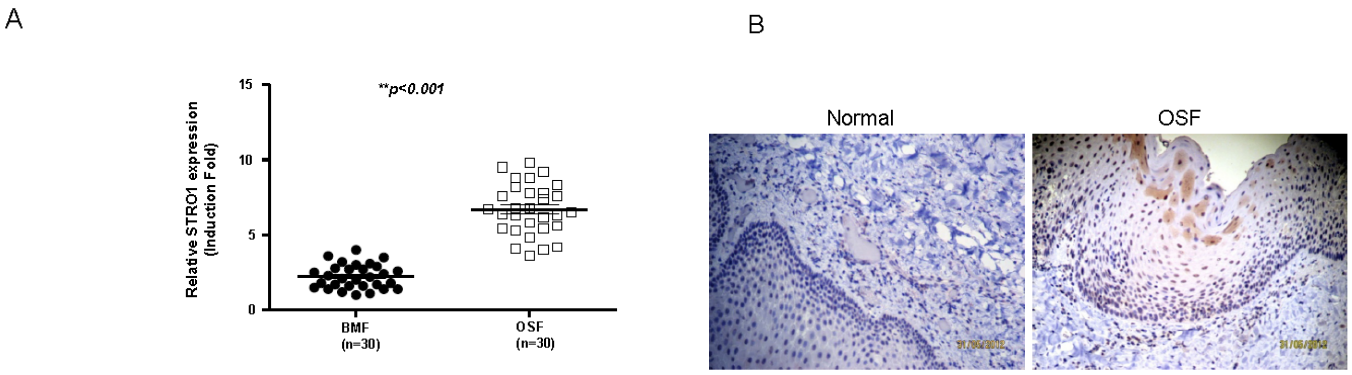
Statistical package of social sciences software (version 13.0) (SPSS, Inc., Chicago, IL, USA) was used

for statistical analysis. Student's *t* test was used to determine statistical significance of the differences between control group and experimental groups; *p* values less than 0.05 were considered statistically significant. The level of statistical significance was set at 0.05 for all tests.

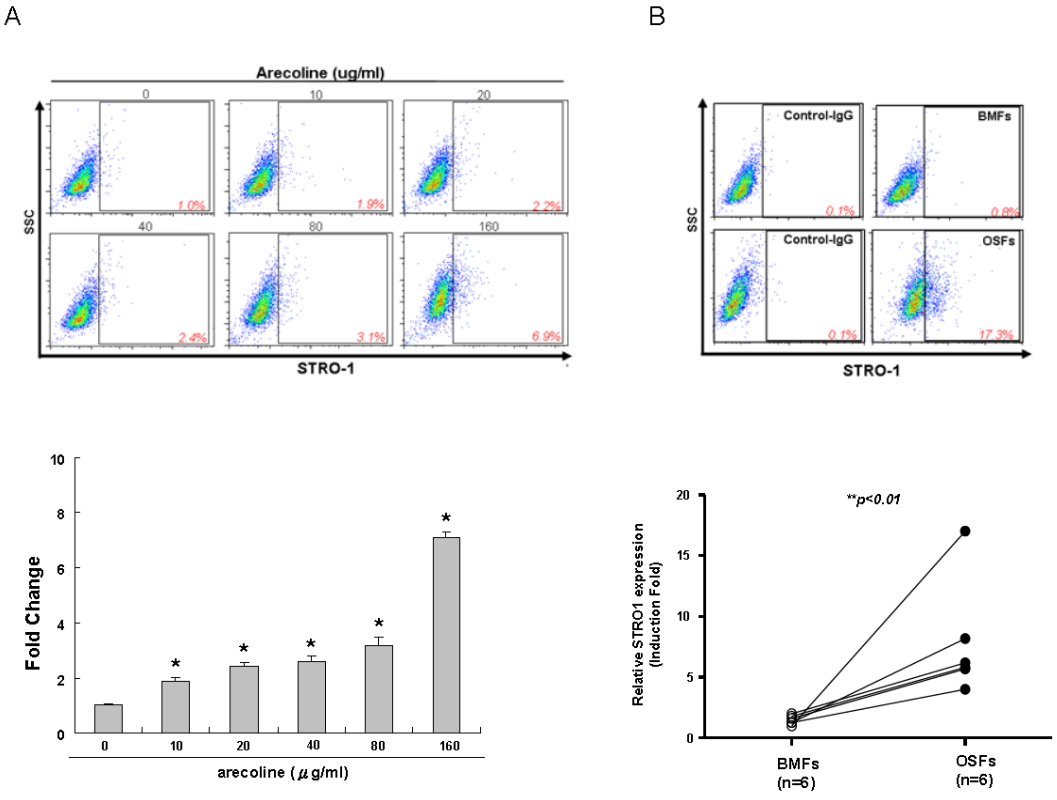
Results

Part1: Elevation of STRO-1 expression contributes the pathogenesis of Oral submucousis fibrosis

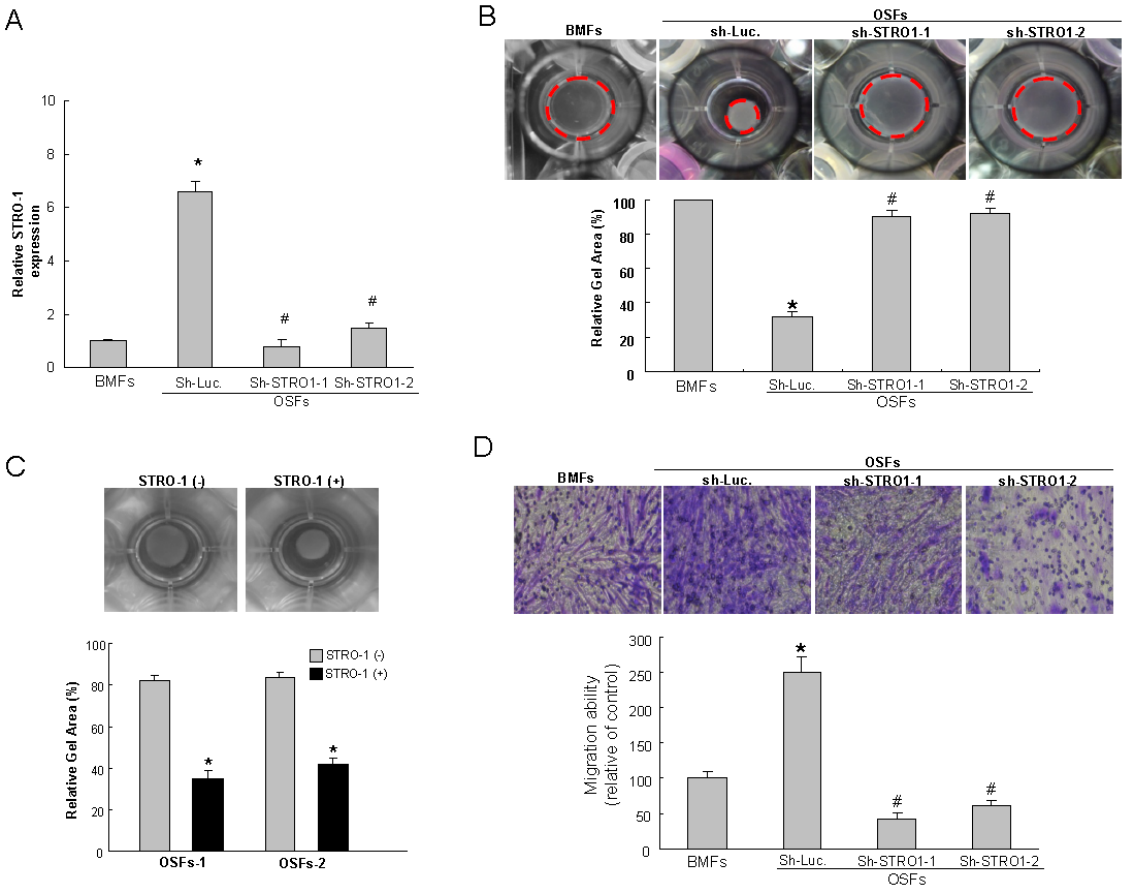
1.1. STRO-1 significantly up-regulated of in OSF specimens



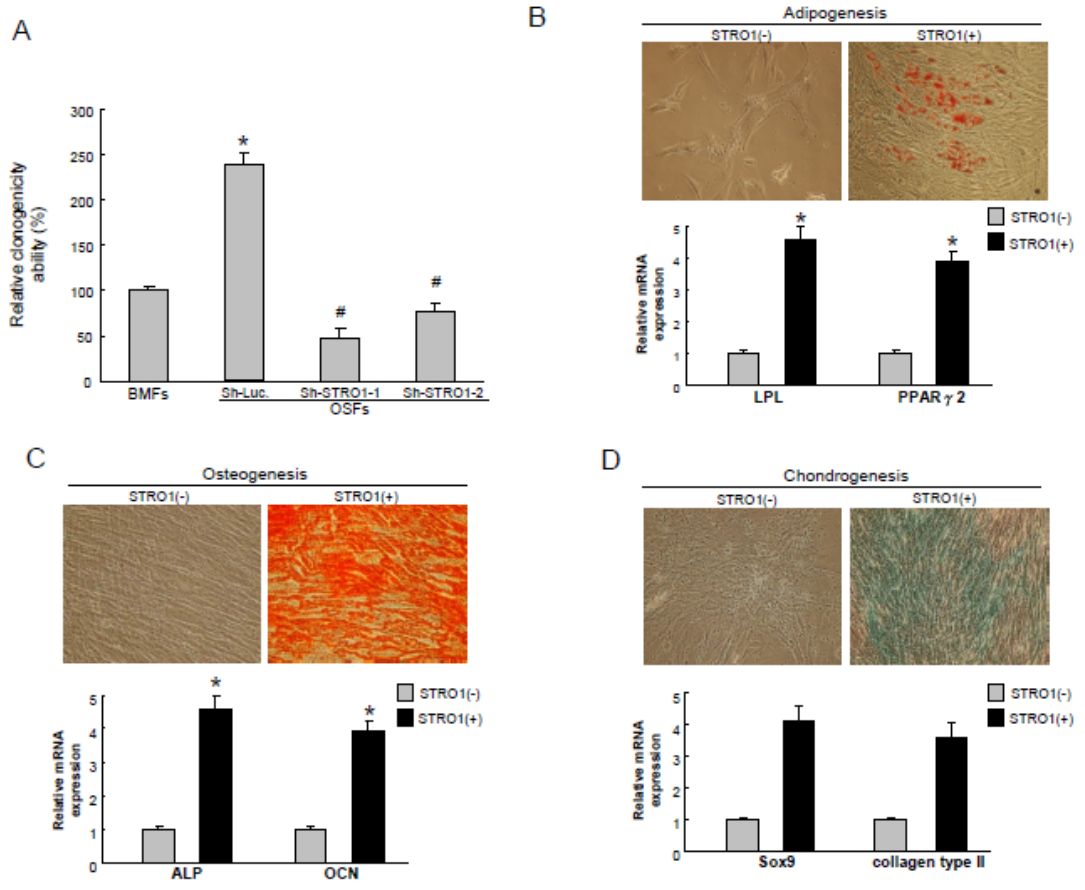
1.2. Arecoline increased STRO-1 expression in a dose-dependent manner in BMFs



1.3. Knockdown of STRO-1 repressed collagen gel contraction in OSFs

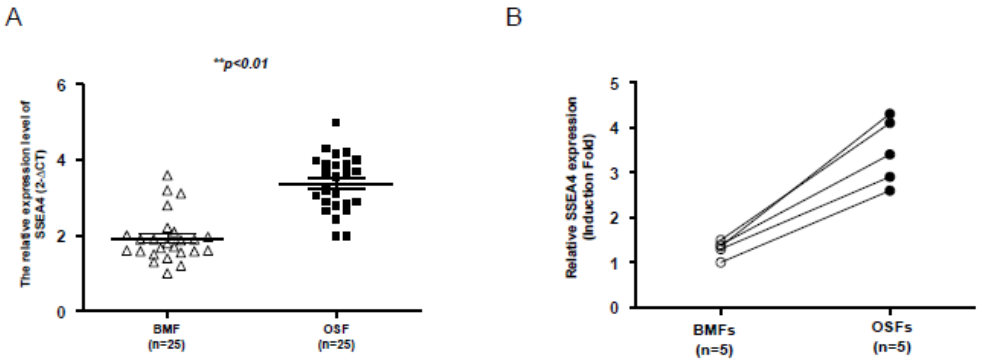


1.4. The osteogenic/chondrogenic/adipogenic induction differentiation properties STRO-1$^+$ cells were significantly higher than that of the STRO-1$^-$ cells in OSF

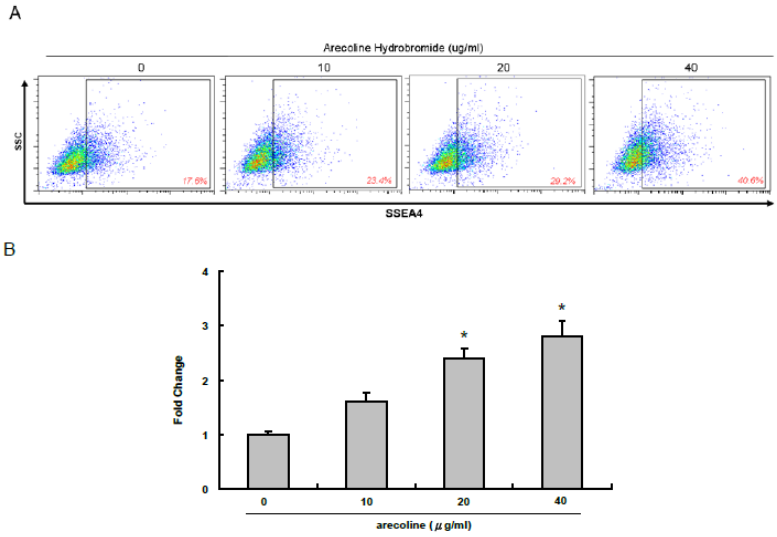


Part2: Elevation of SSEA4 expression contributes the pathogenesis of Oral submucousis fibrosis

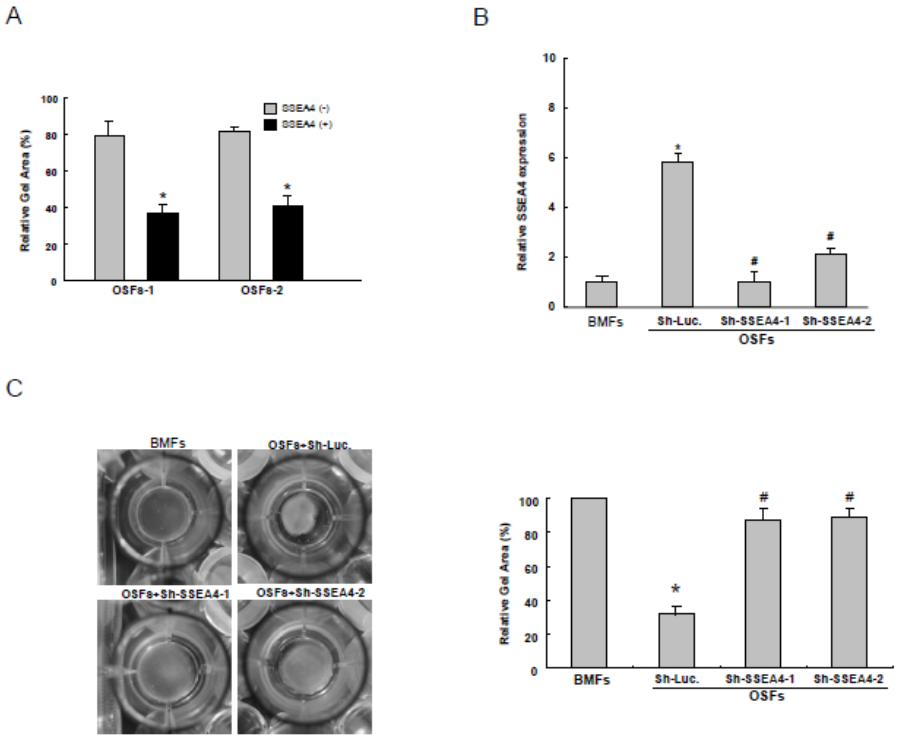
2.1. SSEA4 significantly up-regulated of in OSF specimens



2.2. Arecoline increased SSEA4 expression in a dose-dependent manner in BMFs



2.3. Knockdown of SSEA4 repressed collagen gel contraction in OSFs



科技部補助專題研究計畫出席國際學術會議心得報告

日期:102 年 9 月 1 日

計畫編號	MOST 100-2314-B-040-005-MY3		
計畫名稱	幹細胞特性對於口腔黏膜下纖維化之角色探討及口腔黏膜下纖維化幹細胞之分選定性研究		
出國人員姓名	張育超	服務機構及職稱	中山醫學大學牙醫系所 教授
會議時間	102 年 8 月 21 日至 102 年 8 月 23 日	會議地點	Bangkok, Thailand
會議名稱	(中文) 第二屆國際牙醫研究學會亞洲太平洋地區年會 (英文) 2nd Meeting of the International Association for Dental Research-Asia Pacific Region		
發表題目	(中文) 口腔黏膜下纖維化症會增加幹細胞因子 Stro-1 的表達 (英文) Increase of Stemness Marker Stro-1 in Oral Submucous Fibrosis		

一、 參加會議經過

第二屆國際牙醫研究學會亞洲太平洋地區年會，其包括了 61st Meeting of Japanese Division,53rd Meeting of Australian/New Zealand Division, 30th Meeting of Korean Division, 13th Meeting of Chinese Division, and 27th Meeting of Southeast Asian Division 等五個 Division，其年會係每四年舉辦一次，目前台灣並無單獨成立一個 Division，目前隸屬於 Southeast Asian Division，今年大會場地係在泰國曼谷 Plaza Athenee 舉行，今年共有 745 篇論文發表，本人發表的題目為 Increase of Stemness Marker Stro-1 in Oral Submucous Fibrosis 大會篇號為 620，內容是國科會多年期計畫 NSC 100-2314-B-040-005-MY3「幹細胞特性對於口腔黏膜下纖維化之角色探討及口腔黏膜下纖維化幹細胞之分選定性研究」之部分成果發表。

二、 與會心得

本次盛會收穫良多，吸取了許多寶貴的經驗及目前研究的新方向，對於往後的研究裨益良多，再此亦非常感激國科會予以經費補助參與此次第二屆國際牙醫研究學會亞洲太平洋地區年會。

三、發表論文全文或摘要

Background: Oral submucous fibrosis (OSF) is regarded as a precancerous condition, and is a chronic insidious disease of oral subepithelial connective tissue. Areca quid chewing is a major risk factor of OSF. However, the pathologic mechanism(s) of OSF needs to be further clarified. Recently, the stemmess properties are also examined in benign tumor keloid and oral cancer. The purpose of this study was to investigate the stemness in the pathogenesis of OSF. **Method:** Initially, the expression of stemness marker Stro-1 in primary human buccal mucosa fibroblasts (BMFs) stimulated with arecoline, the major areca nut alkaloid, was assessed by flow cytometry analysis. Self-renewal sphere-forming ability of BMFs with different doses of arecoline was presented. Finally, the expression of Stro-1 was determined in areca quid chewing associated-OSF specimens by immunohistochemistry. **Results:** We first observed that arecoline treatment significantly up-regulated Stro-1 positivity BMFs in a dose-dependent manner ($p<0.05$). Moreover, arecoline treatment enhanced the sphere size and numbers and stemness signatures expression in BMFs. Compared with normal BMFs, primary cultivated OSF cells displayed the higher Stro-1 positive cells ($p<0.05$). OSF-derived Stro-1 positive cells displayed strong osteogenic ability under osteogenic condition. In addition, the expression Stro-1 was significantly elevated in OSF specimens compared with normal buccal mucosa specimens ($p<0.05$). **Conclusion:** Taken together, Stro-1 expression is significantly upregulated in areca quid chewing associated-OSF. The enrichment of stemness property in BMFs by arecoline may be critical for the development of OSF.

四、建議

台灣應爭取單獨成立一個 Taiwanese Division

五、攜回資料名稱及內容

Program and Abstract Book of 2nd Meeting of the International Association for Dental Research-Asia Pacific Region

六、其他

Increase of Stemness Marker Stro-1 in Oral Submucous Fibrosis

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Abstract

Background: Oral submucous fibrosis (OSF) is regarded as a precancerous condition, and is a chronic insidious disease of oral subepithelial connective tissue. Areca quid chewing is a major risk factor of OSF. However, the pathologic mechanism(s) of OSF needs to be further clarified. Recently, the stemness properties are also examined in benign tumor keloid and oral cancer. The purpose of this study was to investigate the stemness in the pathogenesis of OSF.

Method: Initially, the expression of stemness marker Stro-1 in primary human buccal mucosa fibroblasts (BMFs) stimulated with arecoline, the major areca nut alkaloid, was assessed by flow cytometry analysis. Self-renewal sphere-forming ability of BMFs with different doses of arecoline was presented. Finally, the expression of Stro-1 was determined in areca quid chewing associated-OSF specimens by immunohistochemistry.

Results: We first observed that arecoline treatment significantly up-regulated Stro-1 positivity BMFs in a dose-dependent manner ($p<0.05$). Moreover, arecoline treatment enhanced the sphere size and numbers and stemness signatures expression in BMFs. Compared with normal BMFs, primary cultivated OSF cells displayed the higher Stro-1 positive cells ($p<0.05$). OSF-derived Stro-1 positive cells displayed strong osteogenic ability under osteogenic condition. In addition, the expression Stro-1 was significantly elevated in OSF specimens compared with normal buccal mucosa specimens ($p<0.05$).

Conclusion: Taken together, Stro-1 expression is significantly upregulated in areca quid chewing associated-OSF. The enrichment of stemness property in BMFs by arecoline may be critical for the development of OSF.

Introduction

Oral submucous fibrosis (OSF) is regarded as a precancerous condition, and is a chronic insidious disease of oral subepithelial connective tissue resulting in stiffness of the oral mucosa and inability to open the mouth. OSF is characterized by epithelial atrophy and progressive accumulation of collagen fibers in the lamina propria and submucosa of the oral mucosa. The fibro-elastic changes are almost entirely due to abnormal accumulation of collagen in the subepithelial layers, resulting in dense fibrous bands in the mouth. A number of epidemiological surveys, case-series reports, large sized cross sectional surveys, case-control studies, cohort and intervention studies provide over whelming evidence that areca nut is the main etiological factor for OSF.

The role of the constituents of areca nut in the pathogenesis of OSF has been studied in detail over the last two decades. It is apparent that fibrosis and hyalinization of sub epithelial tissues account for most of the clinical features encountered in this condition. Moreover, substantial amount of research on elucidating the etiology and pathogenesis appear to have been focused on changes in the extracellular matrix (ECM). It is logical to hypothesize that the increased collagen synthesis or reduced collagen degradation as possible mechanisms in the development of the disease. There are numerous biological pathways involved in the above processes and, it is likely that the normal regulatory mechanisms are either down regulated or up regulated at different stages of the diseases.

Fibroblasts obtained from OSF subjects revealed a higher elevation for collagen synthesis than normal buccal mucosa fibroblasts (BMFs). Stabilization of collagen and prevention of collagenase degradation in oral mucosa and the attendant increase of lysyl oxidase activity lso contribute to abnormal deposition of collagen fiber in OSF. Arecoline, a major areca nut alkaloid, was found to stimulate human BMF proliferation and collagen synthesis in vitro. Recently, our studies have shown that up-regulation of vimentin, cyclooxygenase-2, tissue inhibitor metalloproteinase-1, plasminogen activator inhibitor-1, interleukin-6, keratinocyte growth factor-1, insulin-like growth factor-1, nuclear factor-kappa B, cystatin C, and heat shock protein 47, and heme oxygenase-1, may contribute to the extracellular components accumulation in OSF.

A crucial question in cancer biology is how tumor growth is initiated and propagated at the cellular level. Despite being monoclonal in origin, most tumors appear to contain a heterogeneous population of cancer cells. Recently, a hypothesis proposed by many investigators is a model in which every tumor contains a rare population of cells termed cancer stem cells (CSCs). The concept of CSCs first began 150 years ago when Robert Virchow, a German pathologist, observed similarities between embryonic and tumor tissues. CSCs have been demonstrated to have capacities of promoting tumor growth, tumor regeneration, metastatic progression and contributing to radio-resistance and chemo-resistance of oral cancer. Recently, the CSCs properties also examined in keloid (an exuberant fibroproliferative dermal growth unique to human skin). However, the role of stemness properties in precancerous OSF has never been determined. Based on these findings, it is worthy to investigate the importance of stemness in the maintenance of OSF pathogenesis.

Materials and Methods

OSF patient subjects and immunohistochemistry

This research follows the tenets of the Declaration of Helsinki and all samples were obtained after informed consent from the patients. OSF patients' tissue samples with different stages of oral cancer were spotted on glass slides for immunohistochemical stainings. After deparaffinization and rehydration, the tissue sections were processed with antigen retrieval by1X Trilogy diluted in H_2O (Biogenics) and heat. The slides were immersed in 3% H_2O_2 for 10 minutes and washed with PBS 3 times. The tissue sections were then blocked with serum (Vestastain Elite ABC kit, Vector Laboratories, Burlingame, CA) for 30 minutes, and followed by incubating with the primary antibody and anti-Stro-1 or anti-SSE4 in PBS solution at room temperature for 2 hours in a container. Tissue slides were washed with PBS and incubated with biotin-labeled secondary antibody for 30 minutes and then incubated with streptavidin-horse radish peroxidase conjugates for 30 minutes and washed with PBS 3 times. Afterwards, the tissue sections were immersed with chromogen 3-3'-diaminobenzidine plus H_2O_2 substrate solution (Vector® DBA/Ni substrate kit, SK-4100, Vector Laboratories, Burlingame, CA) for 10 minutes. Hematoxylin was applied for counter-staining (Sigma Chemical Co., USA). Finally, the tumor sections were mounted with a cover slide with Gurr® (BDH Laboratory Supplies, U.K.) and examined under a microscope. Pathologists scoring the immunohistochemistry were blinded to the clinical data. The interpretation was done in five high-power views for each slide, and 100 cells per view were counted for analysis.

Cell culture

Ten healthy individuals, without areca quid chewing habits, attending the Oral Medicine Center (Chung Shan Medical University Hospital, Taichung, Taiwan) were enrolled with informed consents for this study. Biopsy specimens were derived from histologically normal oral mucosa at the time of surgical third molar extraction. The OSF specimens were obtained from twenty male patients with areca quid chewing habits during surgical biopsy. Clinical diagnosis was confirmed by histopathological examination of the biopsy specimens. Fibroblast cultures were grown and maintained using procedures described previously. Cell cultures between the third and eighth passages were used in this study.

Effect of arecoline on stemness mRNA and protein in BMF

BMF derived from three strains were seeded $1x10^5$ cells per well into 10 cm culture dish and incubated for 24 h. Cells arrested in G0 by serum deprivation (0.5 % FCS; 48 h) were used in the experiments. Nearly confluent monolayers of cells were washed with serum free medium and immediately thereafter exposed at the various concentrations (0, 10, 20, 40, 80, and 160 μg/ml) of arecoline (Sigma, St. Louis, MO, USA). Total RNA was isolated after 6 h incubation period for RT-PCR as described above. Cell lysates were collected at 24 h for Western blot analysis.

Results

Fig. 1
Arecoline treatment dose-dependently increased Stro-1-positive cells in BMF cells

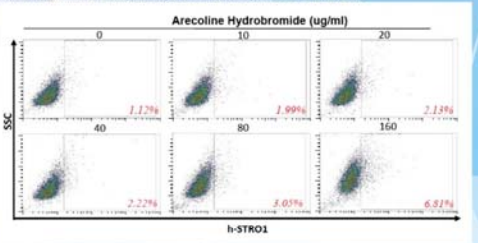


Fig. 2
Increase of stemness marker Stro-1 in primary OSF cultivated cells

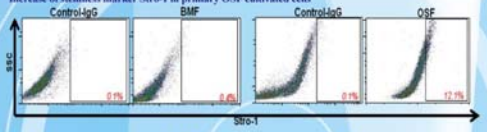
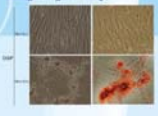


Fig. 3
Increase of stemness marker Stro-1 in oral submucous fibrosis tissue

Fig. 4
Increase of stemness marker SSEA4 in oral submucous fibrosis tissue

Fig. 5
OSF-derived Stro-1+ cells displayed strong osteogenic ability



Conclusion

Taken together, Stro-1 expression is significantly upregulated in areca quid chewing associated-OSF.

The enrichment of stemness property in BMFs by arecoline may be critical for the development of OSF.

科技部補助計畫衍生研發成果推廣資料表

日期:2014/10/12

科技部補助計畫	計畫名稱: 幹細胞特性對於口腔黏膜下纖維化之角色探討及口腔黏膜下纖維化幹細胞之分選定性研究
	計畫主持人: 張育超
	計畫編號: 100-2314-B-040-005-MY3　　學門領域: 牙醫學

無研發成果推廣資料

100 年度專題研究計畫研究成果彙整表

計畫主持人：張育超	計畫編號：100-2314-B-040-005-MY3
計畫名稱：幹細胞特性對於口腔黏膜下纖維化之角色探討及口腔黏膜下纖維化幹細胞之分選定性研究	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	1	0	100%		
國外	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	1	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 □未發表之文稿 ■撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

第二屆國際牙醫研究學會亞洲太平洋地區年會，今年大會場地係在泰國曼谷 Plaza Athenee 舉行，本人發表的題目為 Increase of Stemness Marker Stro-1 in Oral Submucous Fibrosis 大會篇號為 620，內容是部分成果發表。

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限）

有助於找到 OSF 的致病理論機轉